Cocoa antioxidants and cardiovascular health¹–⁴

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ABSTRACT
An increasing body of epidemiologic evidence supports the concept that diets rich in fruits and vegetables promote health and attenuate, or delay, the onset of various diseases. Epidemiologic data support the idea that these health benefits are causally linked to the consumption of certain flavonoids present in fruit and vegetables. In the context of cardiovascular health, a particular group of flavonoids, namely, the flavan-3-ols (flavanols), has received attention. Flavanol-rich, plant-derived foods and beverages include wine, tea, and various fruits and berries, as well as cocoa and cocoa products. Numerous dietary intervention studies in humans and animals indicate that flavanol-rich foods and beverages might exert cardioprotective effects with respect to vascular function and platelet reactivity. This review discusses the bioactivity of flavanols in the context of cardiovascular health, with respect to their bioavailability, their antioxidant properties, and their vascular effects. Am J Clin Nutr 2005;81(suppl):298S–303S.

KEY WORDS Antioxidants, cardiovascular health, chocolate, cocoa, flavanols, platelets, procyanidins

INTRODUCTION
An increasing body of epidemiologic evidence supports the concept that diets rich in fruits and vegetables promote health and attenuate, or delay, the onset of various diseases, including cardiovascular disease, cancer, and certain neurodegenerative disorders (ref 1 and references therein). Furthermore, the epidemiologic data support the idea that these health benefits can be linked, in part, to the presence of certain flavonoids in fruits and vegetables (ref 2 and references therein). In the context of cardiovascular health, a particular group of flavonoids, namely, the flavan-3-ols (flavanols), has recently received attention. Flavanol-rich, plant-derived foods and beverages include wine, tea, and various fruits and berries, as well as cocoa and cocoa products, in which flavanols can be present as either the monomers (−)-epicatechin and (+)-catechin or oligomers of epicatechin and/or catechin (procyanidins) (3). Numerous dietary intervention studies with humans and experimental animals indicate that flavanol-rich foods and beverages can exert cardioprotective effects with respect to vascular function and platelet reactivity (4–8). Although data from the aforementioned studies are well correlated with epidemiologic findings and thus strongly support flavanols as cardioprotective agents, the mechanisms underlying these flavanol-mediated biological effects in vivo are not well defined. On the basis of several in vitro studies, however, it can be speculated that mechanisms contributing to the biological effects of flavanols may include their antioxidant effects, their ability to modulate certain cell signaling pathways and gene expression, and their ability to influence cell membrane properties and receptor function. This review discusses the bioactivity of flavanols in the context of their ability to affect cardiovascular health. Attention is focused on flavanol bioavailability, the antioxidant properties of flavanols, and the potential direct vascular effects of these nutrients.

FLAVANOL AND PROCYANIDIN BIOAVAILABILITY
In discussions of the biological activity of flavanols or procyanidins, several factors must be considered, such as whether they can be absorbed, their tissue and cellular distributions after absorption, and their bioactive forms. Flavanols are distinct from other flavonoid classes; instead of appearing as glycosides, flavanols are present in the aglycone form, as oligomers, or esterified with gallic acid (9). Although it was initially thought that procyanidins were degraded under the acidic conditions of the stomach (10), data from human subjects showed that flavanols and procyanidins are stable during gastric transit (11). Once in the mesenteric circulation, flavanols exist predominately in a conjugated form. The flavanols are absorbed from the jejunal lumen into the epithelial cell layer, where they are methylated and glucuronidated (12, 13). In the liver, additional glucuronidation and methylation, as well as sulfation, can take place (12, 13). In addition to being present in the plasma and urine of experimental animals and human subjects (14–16), flavanol conjugates have been found in rat bile (16) and brain (17). It has been reported that colonic microflora can degrade the flavan structure of flavonoids to form simple phenolic and ring-fission metabolites that may be pharmacologically relevant (18, 19). Among human subjects, increased urinary excretion of 4 phenolic acids was found 9–48 h after cocoa consumption (20). These phenolic acids (m-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, m-hydroxybenzoic acid, and m-hydroxyphenylacetic acid) are possible products of colonic microbial degradation of the procyanidins (20); therefore, products of colonic metabolism must be considered in examinations of the potential chronic biological effects of cocoa and chocolate, as well as other flavanol- and procyanidin-rich foods.

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Methylated epicatechin forms, such as 3'-O-methylepicatechin, can also be found in the plasma in the micromolar range and, like the rest of the metabolites, are rapidly excreted (14). There is some evidence that certain flavanols are better absorbed than others. Lee et al (23) reported that, whereas epigallocatechin and epigallocatechin-3-gallate were present at similar concentrations in a green tea drink, the plasma epigallocatechin concentration was \(\sim 2–3\) times greater than the epigallocatechin-3-gallate concentration after tea consumption. Similar results have been reported for cocoa. After human subjects were given a cocoa beverage containing epicatechin and catechin in a 1:1 ratio, peak plasma catechin concentrations were < 10% of the peak epicatechin concentrations (0.16 and 5.93 \(\mu\)mol/L, respectively) (21). Part of this difference in plasma concentrations between epicatechin and catechin may be attributable to dimer cleavage (15, 24). Although several research groups have examined the bioavailability of the monomeric flavanols, there is limited information on the metabolism of the procyanidins. Dimer B2 [epicatechin-(4\(\beta\)-8)-epicatechin] and dimer B5 [epicatechin-(4\(\beta\)-6)-epicatechin] have been detected in the nanomolar range in the plasma of humans and rats (15, 21, 22, 25), whereas other procyanidins, such as dimer B3 [catechin-(4\(\alpha\)-8)-catechin] (26) and trimer C2 [catechin-(4\(\alpha\)-8)-catechin-(4\(\alpha\)-8)-catechin] (26), could not be detected. It is important to note that the oligomers found in the plasma are those consisting of epicatechin and not catechin subunits. Therefore, the observed differences in plasma concentrations between epicatechin and catechin, and their oligomers, might be attributable to stereochemical differences, which result in differences in hydrophobicity and some biological properties, such as antioxidant activity (27–29).

Cocoa and chocolate represent food and beverage products that have varying concentrations of flavonoids, as a result of many different factors. As with most plants, genetic and agronomic factors can markedly influence the contents of phytochemicals available at the time of harvest. Postharvest handling plays a critical role at this juncture, because most cocas undergo some fermentation steps, which subject flavonoids in the cocoa to heat and acidic conditions (29). Subsequent processing steps, such as roasting and alkali treatment, can also reduce the flavonoid contents. Finally, the recipe for the finished food or beverage product determines the amount of a given cocoa (and flavonoid) added. Depending on harvesting and processing procedures, as much as 90% of the flavonoids can be lost during processing (30, 31).

This illustrates the potential effects of food processing on the flavonoid contents of foods. Similarly, there can be significant food matrix effects on the bioavailability and potential biological activity of cocoa flavanols and procyanidins. A recent study suggested that milk proteins reduced the antioxidant activity of milk chocolate, compared with dark chocolate (32). Although the protein-binding ability of the flavanols and procyanidins is well known (33, 34), studies of flavanol consumption from tea showed large (35, 36) or no (37, 38) effects of milk addition on antioxidant activity and flavanol bioavailability. In a recent study, no differences in either antioxidant capacity or epicatechin bioavailability were observed when cocoa was provided with or without milk, under isocaloric and islipidemic conditions, to healthy human subjects (39). Therefore, observed differences in antioxidant activity and bioavailability between dark chocolate and milk chocolate (32) were the result of the food matrix altering the kinetics of absorption and were likely not attributable to flavanol-milk protein interactions.

**FLAVANOLS AND PROCYANIDINS FROM COCOA AS ANTIOXIDANTS**

Cocoa, cocoa extracts, and purified cocoa flavanols and procyanidins exert strong antioxidant effects in vivo. The antioxidant properties of flavanols are based in part on their structural characteristics, including the hydroxylation of the basic flavan ring system, especially 3’,4’-dihydroxylation of the B-ring (catechol structure), the oligomer chain length, and the stereochemical features of the molecule (40). These structural characteristics of flavanols represent the molecular basis for both their hydrogendonating (radical-scavenging) properties and their metal-chelating antioxidant properties. For example, cocoa and purified cocoa flavanols and procyanidins have been reported to attenuate the copper-mediated and endothelial cell-mediated oxidation of LDL (41, 42), to reduce the production of reactive oxygen species by activated leukocytes (43), to protect against erythrocyte hemolysis (25, 44), and to inhibit ultraviolet C-induced DNA oxidation (45). In the latter case, the cocoa flavanoids that were tested were as effective (on a molar basis) as ascorbate, \(\alpha\)-tocopherol, and glutathione. Interestingly, cocoa powder and cocoa extracts have been shown to exhibit greater antioxidant capacity than many other flavanol-rich foods and food extracts, such as green and black tea, red wine, blueberry, garlic, and strawberry (41, 46).

The aforementioned in vitro data have been shown to translate, at least in part, into vivo model systems. Among healthy human subjects, inhibition of LDL oxidation was reported within 2 h after the consumption of a flavanol-rich cocoa product (47). After rats were provided an oral dose of cocoa powder, the rates of both copper and 2,2’-azo-bis-(2-amidopropane) dihydrochloride (AAPH)-induced LDL oxidation were significantly reduced (48). Similarly, long-term feeding studies with a flavanol-rich cocoa showed an increase in total plasma antioxidant capacity (49) and a reduction in the susceptibility of LDL to ex vivo oxidation (49, 50). In the aforementioned studies, no differences were observed between the control and cocoa-fed groups with respect to total plasma cholesterol, triacylglycerol, LDL, or HDL concentrations. In rats, the chronic consumption of diets containing 2% cocoa powder, providing 1.57 mg/g diet of flavanols and procyanidins, was associated with reduced DNA and glutathione oxidation (51). Diabetes mellitus-induced cataracts and ex vivo lipid peroxide formation were decreased in rats given cocoa liquor (52). Among human subjects, the consumption of a flavonoid-rich chocolate increased plasma antioxidant capacity and reduced the amounts of plasma 2-thiobarbituric acid-reactive substances, in a dose-dependent manner (53). Although the precise mechanisms underlying the antioxidant effects of flavonoids have yet to be identified, the research reported to date suggests that consumption of flavanols and procyanidins in the diet can significantly augment the oxidative defense system.

What are the possible mechanisms that contribute to the antioxidant protection of flavanols? For the flavanols present in cocoa (epicatechin and catechin), antioxidant activity is attributed to the presence of a catechol group on the B ring, which can trap free radicals and chelate redox-active metals (9, 40). Therefore, a reasonable hypothesis would be that antioxidant activity...
increases with increasing oligomer chain length. Consistent with this, Lotito et al (54) observed that, in a model of liposome oxidation, the antioxidant capacity of cocoa procyanidins was influenced by oligomer chain length and the nature of the oxidant. When AAPH (a water-soluble radical generator) or ultraviolet C light was used as a radical initiator, dose-dependent protection was observed; however, this protection was not dependent on oligomer chain length according to monomeric equivalents, which allows comparison of equivalent numbers of catechol groups. In contrast, when 2,2’-azo-bis(2,4-dimethylvaleronitrile) (a lipid-soluble radical generator) was used as an initiator, an increase in chain length was associated with increased protection. Finally, an inverse association of chain length with protection was observed with the use of ferrous ascorbate as the initiator (54). Consistent with this, Steinberg et al (22) observed no differences in the capacity of monomers, dimers, pentamers, and hexamers to inhibit LDL oxidation when AAPH or copper was used as an oxidizing agent. However, Verstraeten et al (55) observed that procyanidin chain length was a determinant of the capacity to protect liposomes against 2,2’-azo-bis(2,4-dimethylvaleronitrile)-induced oxidation and against membrane disruption induced by the detergent Triton X-100. The accumulation of procyanidins at the liposome surface, through hydrogen bonding with the polar head group of phospholipids, prevented access of deleterious molecules to the hydrophobic core of the bilayer (55).

Taken together, these studies suggest that the flavanols and procyanidins have different interactions with biological membranes. Schroeder et al (28) demonstrated that epicatechin is amphiphilic in nature, with an observed partition coefficient in an octanol-buffer system of 1.45. In the same study, catechin was more lipophilic (partition coefficient of 2.92) than epicatechin, and compounds containing gallic acid and glucosyl residues, such as epigallocatechin-3-gallate, rutin, and α-glucosyl-rutin, were hydrophilic in nature. Similar partition coefficient values were observed for catechin, morin, and taxifolin (partition coefficients of 2.92, 2.53, and 2.02, respectively), which suggests that it is the difference in stereochemistry at C3 between epicatechin and catechin that affects hydrophobicity. The authors also observed uptake of epicatechin in cell lysates of murine aortic endothelial cells after 30 min of incubation (28). In a separate study, catechin and epicatechin dose-dependently accumulated to similar extents in the whole-cell fraction of Jurkat cells, and epicatechin, but not catechin, could be detected in the nuclear fraction. Dimer B2 was also found in both the whole-cell and nuclear fractions, although to a lesser extent than epicatechin (56). Therefore, in contrast to longer-chain oligomers, the flavanol monomers and dimers (particularly those with epicatechin subunits) appear to be able to diffuse across the membrane and into the cell. As discussed below, the ability of the smaller oligomers to diffuse into the cell is important, because cocoa products and isolated flavanols and procyanidins have been observed to affect enzymes and signaling cascades.

**VASCULAR EFFECTS OF FLAVANOL-RICH COCOA AND CHOCOLATE**

**Other mechanisms**

Although flavanol-rich cocoa and chocolate have the potential to augment an individual’s antioxidant defense system, there are other cellular mechanisms through which these flavanol-rich foods can affect cardiovascular health. Inflammation, platelet aggregation, and nitric oxide (NO)-mediated endothelial changes are additional factors that can be influenced by flavanols.

As a broad concept that applies to this discussion, it is important to recognize the interaction between reactive oxygen species and reactive nitrogen species that occurs in vivo, which results in the formation of peroxynitrite (a powerful oxidant) and the consumption of NO (decreasing vasorelaxation) (57). As discussed above, flavanols and procyanidins can trap reactive oxygen species, thus acting as effective protectors against peroxynitrite-dependent oxidation and nitration reactions (58).

**Inflammation**

Atherosclerosis and heart failure, as well as risk factors such as hypertension and hypercholesterolemia, can activate several proinflammatory enzyme systems, such as xanthine oxidase, NADH/NADPH oxidase, and myeloperoxidase (59). Once activated, these enzymes produce reactive oxygen species and other radicals that, as indicated above, can modify NO availability and LDL and contribute to endothelial dysfunction (59). Flavanol-rich cocoa liquor has been shown to stimulate NO production and to significantly reduce the activities of xanthine oxidase and myeloperoxidase after ethanol-induced oxidative stress (60). In addition, cocoa flavanols and procyanidins may modulate other mediators of inflammation. For example, there is emerging evidence that flavanols and procyanidins can suppress the production of the proinflammatory cytokines interleukin (IL)-1β and IL-2 (43, 61) in peripheral blood mononuclear cells, enhance the production of the antiinflammatory cytokine IL-4 (62), suppress 15-lipoxygenase activity (63), and beneficially modulate transforming growth factor-β, and tumor necrosis factor-α concentrations (64) in peripheral blood mononuclear cells. Catechins have also been reported to suppress microvascular endothelial cell production of IL-8 (65, 66), a potent chemoattractant in the initiation and progression of atherosclerosis (67).

Rel/NF-κB transcription factors are activated by multiple signals and regulate the expression of numerous genes involved in inflammation, cell proliferation and survival, and stress and immune responses (68). NF-κB regulates gene transcription of cytokines and adhesion molecules involved in the onset and progression of atherosclerosis. Activated NF-κB is present in macrophages, vascular smooth muscle cells, and endothelial cells of atherosclerotic lesions (69, 70). In rats, NF-κB is also activated in vascular smooth muscle cells after arterial injury (71). Significantly, the use of a cis-element decoy for NF-κB, a synthetic double-stranded DNA that binds NF-κB with high affinity and inhibits NF-κB-driven gene expression, prevents the vascular hyperplasia that occurs after rat carotid artery injury (72).

Epicatechin, catechin, and an isolated fraction of B-type dimers (B2 and B5) were recently shown to regulate NF-κB (56). This regulation occurs at multiple cell levels, in the early cytosolic events in the NF-κB activation cascade (ie, modulation of oxidant concentrations, IκB kinase activation, and subsequent IκB phosphorylation) and at later stages (inhibition of NF-κB binding to its consensus DNA sequence). Procyanidins have been observed to modulate the expression of the NF-κB-dependent IL-2 and IL-1β (43, 61). As evidence that epicatechin, catechin, and the B-type dimers isolated from cocoa can inhibit NF-κB-driven gene transcription, Mackenzie et al (56) demonstrated decreased IL-2 expression in Jurkat cells.
Some of the effects of epicatechin, catechin, and the B-type dimers on NF-kB can be attributed to the antioxidant capacity of these compounds. There is evidence indicating that NF-κB is a transcription factor sensitive to oxidant stimuli and to changes in the cellular thiol redox state (73, 74). In Jurkat cells, phorbol-12-myristate 13-acetate induced an increase in cell oxidant concentrations that was inhibited by epicatechin, catechin, and the B-type dimers (56). Similarly, in RAW 264.7 macrophages stimu-
lation with IFN-γ, the flavanols and dimers inhibited NO production and NF-κB-dependent transcriptional activity (75).

Platelets

Given the prominent role of platelets in the development and manifestation of acute myocardial infarction, stroke, and venous thromboembolism, several antiplatelet strategies have been developed to prevent secondary events. Several studies suggest that, in addition to providing antioxidant vitamins, certain fruits and vegetables may provide protection against thrombosis because of their flavanol contents. Cocoa was shown to reduce ADP/collagen- and adrenaline/collagen-activated, platelet-related, primary hemostasis within hours after subjects consumed high (897 mg) or moderate (220 mg) amounts of cocoa flavanols (76–78). These antiaggregatory effects observed with cocoa were shown to be attributable, in part, to a reduction in the ADP- and adrenaline-induced expression of the activated conformation of the GPIIa/IIIb surface protein (76, 78). In addition, cocoa was able to reduce GPIIa/IIIb expression to an extent that was only slightly less than that achieved with low-dose aspirin (81 mg) (78). The effects observed in the aforementioned short-term studies could also be extended to a 4-wk study, during which subjects consumed moderate amounts of cocoa flavanols. As a result, decreases in P-selectin expression, ADP-induced platelet aggregation, and platelet volume (marker of lowered activation status) were observed (5).

It is possible that flavanols may mediate their activity through antioxidant and NO-related mechanisms, and such mechanisms can be implicated in platelet function. Superoxide anion is known to enhance platelet aggregation and can bind to NO to form peroxynitrite. Freedman et al (8) observed that purple grape juice consumption reduced platelet superoxide release, with a corresponding increase in platelet NO production and a reduction in platelet aggregation. In addition, catechin was shown to reduce platelet aggregation and hydrogen peroxide production in vitro (79). These studies suggest that flavanols may exert their antioxidant effects through their well-described antioxidant activities, although it should be emphasized that flavanols may act through nonantioxidant mechanisms. For example, purple grape juice has been reported to reduce platelet protein kinase C activity (8), whereas dimer B2 has been shown to inhibit platelet thrombaxone production (80). In addition, cocoa flavanols and procyanidins have been reported to inhibit human platelet 12-lipoxygenase (63) and 5-lipoxygenase (81). Inhibition of 5- and 12-lipoxygenase could partly explain the observations of Schramm et al (82) and Holt et al (77) that the ratio of plasma prostacyclin and leukotriene concentrations increases among human subjects after the consumption of flavanol-rich chocolate.

Endothelium

The vascular endothelium regulates hemostasis through maintenance of vasomotor tone and through its influence on platelet function and leukocyte adherence. Under normal physiologic conditions, several mediators are released from the endothelium, such as endothelin, prostacyclin, leukotrienes, NO, and adenosine (83). Shear stress, ischemia and reperfusion, inflammation, and disease states, such as atherosclerosis, diabetes mellitus, and hypertension, can disrupt endothelial function, which is associated with alterations in endothelium-derived regulatory mediators, an inability to regulate vascular tone, and an overall shift toward the prothrombotic state. It is possible that flavanols, by functioning as antioxidants in addition to modulating prostacyclin and leukotriene concentrations (82), can improve endothelial function through the prevention and possible reduction of oxidative damage. However, other mechanisms that have yet to be elucidated may also be involved.

CONCLUSIONS

There is now a large body of information that supports the idea that cocoa flavanols and procyanidins have the ability to act as in vivo antioxidants. These nutrients have been shown to affect numerous intracellular signaling cascades and to influence the cardiovascular system by enhancing vascular function and decreasing platelet reactivity. Several in vivo studies have provided strong support for the hypothesis that the consumption of flavanol-rich foods, such as certain cocaos and chocolates, may be associated with reduced risk for vascular disease. Significantly, in vitro studies with highly purified flavanols and procyanidins support the hypothesis that many of the biologic effects observed with flavonoid-rich foods can be directly attributed to the flavonoids. A number of important questions remain, however. For example, there is little information on the extent to which flavonoids interact with other nutrients in the diet before and after absorption. There is also limited information on the intracellular metabolism of these compounds and on the bioactivity of the different metabolites. Another area in which we have a dearth of information concerns the acute and chronic effects of dietary flavonoids. Although several short-term clinical trials have been reported, the health effects of these nutrients will best be determined from long-term, randomized, clinical trials.

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