Cocoa polyphenols and inflammatory mediators[1–4]

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ABSTRACT

Cocoa products are sources of flavan-3-ols, which have attracted interest regarding cardiovascular health. This review provides a survey of our research on the effects of cocoa polyphenols on leukotriene and nitric oxide (NO) metabolism and on myeloperoxidase-induced modification of LDL. Because intake of flavonoid-rich chocolate by human subjects was reported to decrease the plasma concentrations of proinflammatory cysteinyl leukotrienes, we assessed whether cocoa polyphenols inhibited human 5-lipoxygenase, the key enzyme of leukotriene synthesis. (−)-Epicatechin and other cocoa flavan-3-ols proved to be inhibitory at the enzyme level. This action may confer antileukotriene action in vivo. In a double-blind crossover study, 20 individuals at risk for cardiovascular diseases received cocoa beverages with high or low contents of flavan-3-ols. NO-dependent, flow-mediated dilation of the brachial artery and concentrations of nitroso compounds in plasma were measured, and it was shown that ingestion of the high-flavanol coca drink but not the low-flavanol cocoa drink significantly increased plasma concentrations of nitroso compounds and flow-mediated dilation of the brachial artery. Therefore, ingested flavonoids may reverse endothelial dysfunction through enhancement of NO bioactivity. Oxidative modification of LDL appears to be crucial for atherogenesis, and one of the mediators is the proinflammatory proatherogenic enzyme myeloperoxidase. Micromolar concentrations of (−)-epicatechin or other flavonoids were found to suppress lipid peroxidation in LDL induced by myeloperoxidase in the presence of physiologically relevant concentrations of nitrite, an NO metabolite. Adverse effects of NO metabolites, such as nitrite and peroxynitrite, were thus attenuated. *Am J Clin Nutr* 2005;81(suppl):304S–12S.

KEY WORDS Antioxidants, arachidonic acid, atherogenesis, chocolate, cocoa, flavan-3-ols, flavonoids, flow-mediated dilation, inflammation, leukotrienes, lipoxygenase, LDL, myeloperoxidase, nitrite, nitrosothiols, peroxynitrite, procyanidins

INTRODUCTION

Flavonoids constitute a group of natural compounds that occur in fruits and vegetables, wine and tea, and also chocolate and other cocoa products. Recently, flavonoids have attracted increasing interest from nutritional biochemists. First, these polyphenols exert potent antioxidant actions in numerous in vitro systems (1–3). Second, the daily dietary intake of flavonoids and similar polyphenols exceeds that of antioxidative vitamins and provitamins. In epidemiologic studies, increased intake of flavonoids was associated with reduced risk of major cardiac events (4–7). The presumed beneficial effects of flavonoids are mainly ascribed to their inherent capacity to scavenge reactive oxygen and nitrogen species, thus counteracting conditions of oxidative stress that accompany disorders such as coronary artery disease and other vascular diseases, stroke, inflammatory diseases, and cancer. However, several nonantioxidant effects of flavonoids are also being considered (1–3).

For a fuller understanding of the protective actions of flavonoids against oxidative stress, the effects of flavonoids on oxidant enzymes such as lipoxygenases and myeloperoxidase (MPO) must be elucidated. Lipoxygenases are involved in arachidonic acid metabolism leading to several inflammatory mediators, including (among others) leukotrienes, conjugated hydroxyeicosatetraenoic acids (HETEs) (predominately 5-HETE, 12-HETE, and 15-HETE), hepoxilins, and lipoxins (8–10), in addition to cyclooxygenase-mediated eicosanoids such as prostaglandins, prostacyclin, and thromboxanes. Non–heme iron-containing lipoxygenases and heme-containing cyclooxygenases are dioxygenases that primarily attack one of the bis-allylic methylenes present in arachidonic acid or other polyenoic fatty acids. This initial reaction step is shared with nonenzymatic lipid peroxidation and yields analogous compounds (hydroperoxyeicosatetraenoic acids and isoprostanes) but, unlike in the nonenzymatic route, with positional and stereochemical specificity. Oxygenating enzymes involved in eicosanoid syntheses may also generate deleterious, fatty acid-derived, free radicals and reactive oxygen species through side reactions, which could be blunted by flavonoids and other dietary polyphenols.

The interactions of dietary flavonoids with arachidonic acid metabolism have not been fully elucidated. It was reported that intake of flavonoid-rich chocolate by volunteers caused a significant decrease in the ratio of the concentrations of the plasma metabolites of cysteinyl leukotrienes and prostacyclin (prostaglandin I_2) (11). These changes were paralleled by significant increases in the plasma concentrations of (−)-epicatechin and its metabolites, which suggested that the observed beneficial effects of chocolate on arachidonic acid metabolism were attributable to

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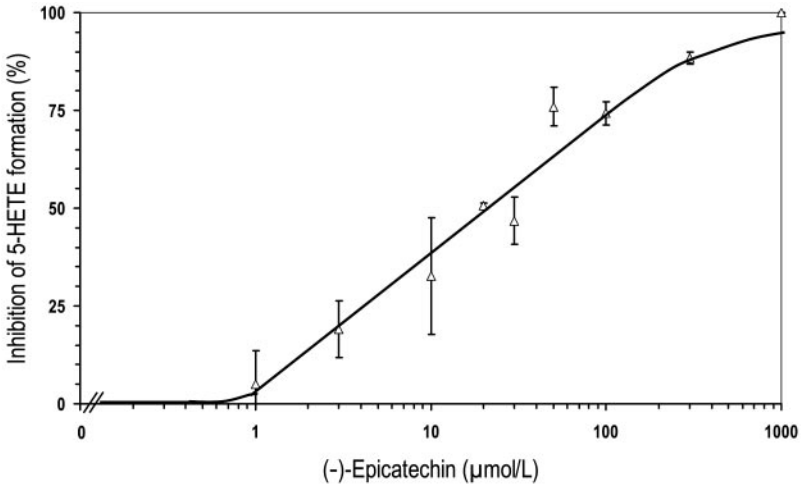

FIGURE 1. Dose-dependent inhibition by (−)-epicatechin of the formation of 5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (analyzed as 5-HETE) by recombinant human 5-lipoxygenase. Recombinant human 5-lipoxygenase was prepared from transfected *Escherichia coli* strain HB101 as described elsewhere (29). Bacterial lysate supernatant containing 5-lipoxygenase was added to 215 μL of 50 mmol/L tris buffer (pH 7.4) and preincubated for 10 min in the presence or absence of flavonoid (dissolved in 2-methoxyethanol; final concentration: 2%, by vol). An assay mixture (25 μL) containing 1 mmol/L ATP, 4 mmol/L $CaCl_2$, 1 mmol/L EDTA, and 13 mg/L dipalmitoyl phosphatidylcholine was then added. The reaction was started after 5 min with the addition of 0.75 μL of 33 mmol/L arachidonic acid in methanol and was stopped after 15 min with the addition of 250 μL of cold methanol. For reduction of hydroperoxy fatty acids and acidification, 5 μL of sodium borohydride (saturated solution in cold ethanol) and 25 μL of glacial acetic acid were added. After centrifugation, the supernatants were directly subjected to reverse-phase HPLC, which was performed with a Nucleosil C-18 column (KS system, 250 mm × 4 mm, 5-μm particle size; Macherey-Nagel, Düren, Germany) coupled with an appropriate guard column (30 mm × 4 mm, 5-μm particle size). A Shimadzu HPLC system connected to a Hewlett-Packard diode-array detector (model 1040; Hewlett-Packard, Palo Alto, CA) was used. For separation of the oxygenated products of arachidonic acid, a solvent system of methanol/water/acetic acid (75:25:0.1, by vol), at a flow rate of 1 mL/min, was used. Absorption was monitored at 235 nm (conjugated dienes and dioxygenase products) and 270 nm (conjugated trienes and hydrolysis products of leukotriene A_4). The reaction products [eg, 5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE)] were quantified according to peak area. The activity of the control samples amounted to 0.70 ± 0.12 nmol 5-HETE/mg bacterial lysate supernatant protein (n = 7). Data from reference 28.

its high concentrations of (−)-epicatechin and its oligomers, the procyanidins (12). The molecular targets of these flavanols remain to be addressed, however.

We studied the interactions of the flavonoids of cocoa with selected enzymes of arachidonic acid metabolism. 5-Lipoxygenase, the key enzyme in the synthesis of proinflammatory leukotrienes (9), was of particular interest. Other mammalian lipoxygenases also merit some attention. For example, the subfamily of reticulocyte-type 12/15-lipoxygenases (eg, human and rabbit 15-lipoxygenase-1) has been proposed to be involved in early stages of atherogenesis and may be a potential target for compounds that are protective for the cardiovascular system (13, 14). Flavonoids were reported to ameliorate progression of atherosclerosis in apolipoprotein E–deficient mice (15). For the same reason, we also studied the interactions of cocoa flavonoids with MPO, another prooxidant enzyme that is thought to be involved in atherosclerosis and inflammation (16, 17).

Vasculoprotective actions of dietary flavonoids, as evidenced by improvement of endothelial function, were noted after intake of flavonoid-containing beverages such as black tea or red wine (18, 19). These effects are apparently attributable not only to scavenging of free radicals and suppression of proinflammatory mediators but also to enhancement of bioactive nitric oxide (NO), an antiinflammatory mediator and vasodilator with concomitant antiplatelet activity (20–22). The bioavailability of NO is determined by both synthesis via various isoforms of NO synthase and conversion to nitrite, nitrate, or other products. The various NO metabolites differ considerably with respect to their biological activities (23–25). Although the bioactivity of NO is preserved in *S*-nitrosothiols, *N*-nitroso compounds, and nitrosohemoglobin [collectively referred to as organic nitroso compounds (RNO)] (24), which contribute to the circulating NO pool in human blood (25), nitrite and peroxynitrite may exert predominantly adverse effects. For example, nitrite is a known substrate of MPO (26), and the MPO/nitrite system is involved in the modification of LDL, rendering it atherogenic (27). To examine the improvement of NO bioactivity by cocoa polyphenols in vivo, we compared the effects of high-flavanol and low-flavanol cocoa beverages on plasma nitroso compounds and on flow-mediated dilation (FMD) of the brachial artery among individuals at risk for cardiovascular diseases. Our data from both in vitro and in vivo studies substantiate the purported beneficial actions of cocoa polyphenols on the human cardiovascular system.

INHIBITION OF MAMMALIAN LIPOXYGENASES

To elucidate whether the reported decrease in the plasma concentrations of cysteinyl leukotrienes with intake of procyanidin-rich chocolate (11) could be attributable to direct inhibition of arachidonate 5-lipoxygenase by flavan-3-ols at the enzyme level, we investigated the effects of flavonoids occurring in cocoa on the reaction of isolated recombinant human 5-lipoxygenase with arachidonic acid (28). As shown in **Figure 1**, the formation of the major primary product, 5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, was inhibited by (−)-epicatechin to completeness in a dose-dependent manner, with a concentration of half-inhibition (IC_{50}) of 22 μmol/L. The formation of hydrolysis

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products of 5,6-leukotriene A_4 (diastereomers of 5,12- and 5,6-dihydroxyeicosatetraenoic acids) was inhibited with comparable potency (28). It follows from these observations that (−)-epicatechin inhibits both the dioxygenase and leukotriene A_4 synthase activities of 5-lipoxygenase, ie, the first 2 consecutive steps of the conversion of arachidonic acid into various proinflammatory leukotrienes, which are catalyzed by the same enzyme (9, 10). The dual effects of the flavan-3-ol on the 5-lipoxygenase-catalyzed reactions indicate direct blockage of the active site of the enzyme. An involvement of 5-lipoxygenase-activating protein in the inhibitory action appears to be ruled out, because the activity of 5-lipoxygenase is dependent on 5-lipoxygenase-activating protein only in intact cells or in reconstituted systems (30) and not in a membrane-free system, as used here.

Mammalian lipoxygenases constitute a family of closely related enzymes that share a common basic catalytic mechanism and similar active site structures but differ genetically and with respect to the positional specificity of the dioxygenation of arachidonic acid and other enzymatic characteristics (31–33). Therefore, the question of whether flavan-3-ols are general inhibitors of mammalian lipoxygenases, irrespective of genetic subfamily, reaction specificity, and biological function, arose. We demonstrated that purified 15-lipoxygenase-1 from rabbit reticulocytes was inhibited by (−)-epicatechin and related flavan-3-ols, as well as by the flavonol quercetin (34) and even more effectively by the flavone luteolin (35). The inhibitory effects occurred in every case, irrespective of whether arachidonic acid or linoleic acid was used as substrate or whether conjugated diene formation, oxygen consumption, or formation of a specific reaction product (eg, 15*S*-hydro(pero)xy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid from arachidonic acid) was measured. The mode of the inhibitory action of flavonoids on this enzyme and the structure-activity relationships were studied in more detail and revealed complexity involving both reversible and irreversible processes (35). The inhibitory effects of (−)-epicatechin and procyanidins were also observed with human 15-lipoxygenase-1 and porcine leukocyte 12-lipoxygenase (34), which belong to the same subfamily (reticulocyte-type 12/15-lipoxygenases).

Recombinant human platelet 12-lipoxygenase, a representative of another subfamily of mammalian lipoxygenases, also was inhibited by (−)-epicatechin (**Figure 2**). Therefore, we conclude that this compound and related flavan-3-ols are general inhibitors of mammalian lipoxygenases (**Table 1**).

A large proportion of the flavonoids in cocoa products are present as oligomeric procyanidins. Therefore, we also tested the effects of procyanidin fractions, isolated from the seeds of the cocoa tree *Theobroma cacao* according to oligomer size, on both recombinant human 5-lipoxygenase and rabbit reticulocyte 15-lipoxygenase-1 (**Figure 3**). It should be emphasized that in these studies we applied identical amounts by weight, so that the molar concentrations decreased with increasing oligomer size, on the basis of the assumption that each epicatechin subunit of the procyanidin molecule might contain the relevant structural features required for inhibitory capacity (eg, the 5 hydroxyl groups). With 5-lipoxygenase, only the fractions of small procyanidins (dimer through pentamer) revealed inhibitory potencies similar to those of the monomer, which continuously decreased with

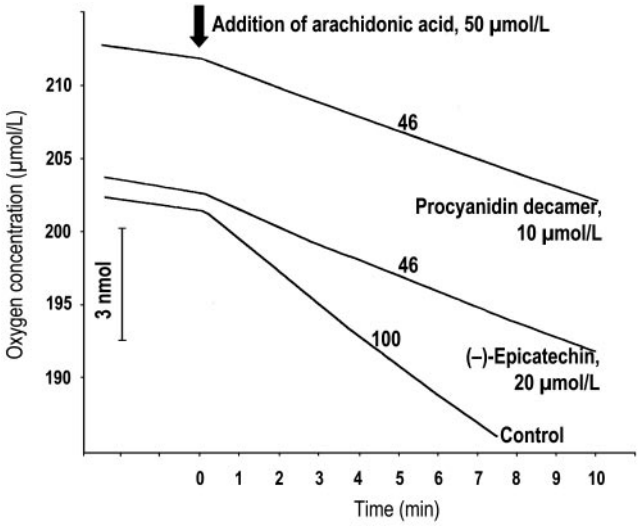

FIGURE 2. Effects of (−)-epicatechin and the procyanidin decamer fraction isolated from the seeds of *Theobroma cacao* (36) on the reaction of recombinant human platelet 12-lipoxygenase with arachidonic acid. Oxygen consumption was measured oxygraphically (Oxygenmeter 781; Strathkelvin Instruments, Glasgow, United Kingdom), in 0.1 mol/L air-equilibrated potassium phosphate (pH 7.4) containing 0.1 mmol/L diethylenetriamine pentaacetic acid, at 20 °C. Recombinant human 12-lipoxygenase (Calbiochem, Bad Soden, Germany) was preincubated with polyphenol for 3 min before the reaction was started with the addition of potassium arachidonate. The numbers at the traces denote residual activities, in percentage of control values, corrected for baseline drift. Modified from reference 34, with permission.

increasing molecular mass, whereas the larger procyanidin fractions produced only weak effects (if any). A quite different pattern was noted for 15-lipoxygenase-1. The inhibitory potency first decreased from monomer to tetramer, reaching a minimum, but then increased with increasing oligomer size, with the decamer fraction being most potent (IC_{50}: 0.8 μmol/L). Unlike (−)-epicatechin, the procyanidin fractions from cocoa also inhibited the activity of soybean lipoxygenase L-1, with potencies that continuously increased with increasing oligomer size (34).

Other monomeric flavan-3-ols also inhibited rabbit 15-lipoxygenase-1. (+)-Catechin did not reveal any significant difference, compared with its (−)-epimer. In contrast, the tea flavan-3-ol (−)-epigallocatechin gallate was a much more potent inhibitor of both 15-lipoxygenase-1 [IC_{50}: ~4 μmol/L (34)] and 5-lipoxygenase [IC_{50}: ~3 μmol/L (28)]. These higher potencies are presumably attributable to the gallic acid moiety, because aliphatic alkyl gallates were reported also to be strong 5- and 15-lipoxygenase inhibitors (38, 39). Greater inhibitory potency, compared with (−)-epicatechin, was observed also for the flavonol quercetin, with IC_{50} values of ~4 μmol/L for 15-lipoxygenase-1 (34) and ~0.6 μmol/L for 5-lipoxygenase (28).

SUPPRESSION OF MPO- AND PEROXYNITRITE-MEDIATED MODIFICATIONS OF LDL

Another oxidant enzyme with pronounced effects on inflammation and atherogenesis is MPO (17, 40). Unlike lipoxygenases, MPO is a hemoprotein dimer. MPO occurs in azurophilic granules of neutrophils, macrophages, and other phagocytes and is released into the extracellular space after activation of these cells by inflammatory stimuli. This process apparently also occurs in atherosclerotic lesions, because MPO-derived products

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TABLE 1
Inhibitory efficiency of (−)-epicatechin toward some lipoxygenases[1]

Enzyme, species	Substrate used	Activity	Assay method	IC_{50}	Ref
				μmol/L	
5-Lipoxygenase, human	Arachidonic acid	Dioxygenase	5-H(p)ETE formation, HPLC	22	28
	5-HpETE	Leukotriene A_4 synthase	5,12-Dihydroxy-HETE formation, HPLC	50	28
12-Lipoxygenase					
Platelet-type, human	Arachidonic acid	Dioxygenase	Oxygen uptake	15	34
Leukocyte-type, porcine	Arachidonic acid	Dioxygenase	12-H(p)ETE formation, HPLC	ND	34
15-Lipoxygenase-1					
Rabbit reticulocytes	Linoleic acid	Dioxygenase	Oxygen uptake, conjugated dienes	60	34
Human	Arachidonic acid	Dioxygenase	15-H(p)ETE formation, HPLC	ND	34
Lipoxygenase L-1, soybean	Linoleic acid	Dioxygenase	Oxygen uptake	Inactive at 1 mmol/L	34

[1] HETE, hydroxyeicosatetraenoic acid; 5-HpETE, 5*S*-hydroxyperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; 5-H(p)ETE, 5-HpETE measured after reduction as the most stable corresponding hydroxy derivative; 12-H(p)ETE, 12*S*-hydro(pero)xy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; 15-H(p)ETE, 15*S*-hydro(pero)xy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; ND, not determined, but pronounced inhibitory effect was qualitatively observed.

were found in human atherosclerotic tissue (41). The biological role of MPO was long regarded solely with respect to its reaction with hydrogen peroxide and chloride, forming hypochlorous acid, as part of the organism's defense system against foreign microbes. However, the discovery that nitrite is also a MPO substrate (26, 42) opened a new era of contemporary knowledge regarding the biochemical features of MPO. A close connection to the metabolism of NO is obvious and was supported by the report that MPO also acts as a NO oxidase (43).

In vitro, MPO is capable of binding to LDL, catalyzing oxidative modification and rendering it atherogenic (44, 45). Therefore, study of the interaction of MPO with LDL with and without flavonoids is a promising approach to elucidate putative beneficial effects of dietary flavonoids in atherosclerosis.

MPO has been reported to cause lipid peroxidation of LDL in the presence of nitrite (27). We studied this reaction in more detail (46). Surprisingly, chloride was not able to support MPO-mediated lipid peroxidation of LDL, and its presence was not required for the nitrite-supported, lipid peroxidation of LDL. A moderate level of MPO-mediated lipid peroxidation, apparently accounted for by LDL-bound tyrosines, was strongly stimulated by low concentrations of nitrite. The effect of nitrite was significant at 5 μmol/L, and half-maximal stimulation of conjugated diene formation in LDL in the presence of chloride was achieved with 7 μmol/L nitrite (46). This concentration of nitrite is within the range found in arterial tissue (47), which is the putative site of oxidative modification of LDL during atherogenesis in vivo. Therefore, a biological role of the MPO/nitrite system in atherogenesis may be considered.

The MPO/nitrite-mediated lipid peroxidation of LDL was effectively blocked by the cocoa polyphenol (−)-epicatechin, the corresponding procyanidins, and other flavonoids (46, 48). The

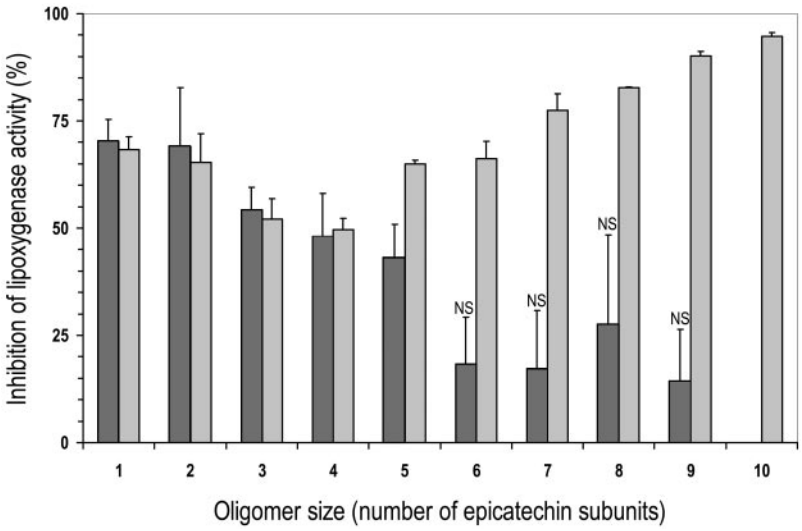

FIGURE 3. Inhibition of human 5-lipoxygenase (dark shaded columns) and rabbit 15-lipoxygenase-1 (light shaded columns) activities by procyanidin fractions isolated from seeds of *Theobroma cacao*. 5-Lipoxygenase activity (formation of 5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, analyzed as 5-HETE) was assayed as for Figure 1. The activity of rabbit reticulocyte 15-lipoxygenase (prepared as described in reference 37) was measured as for Figure 2, with the exception that the reaction was started with the addition of 20 μL of 5.3 mmol/L potassium linoleate in 0.1 mol/L potassium phosphate (pH 7.4) containing 4% (wt:vol) sodium cholate, with a final volume of 400 μL. The final concentrations of cocoa procyanidins (isolated as described in reference 36) were 50 μEq/L and 100 μEq/L for the 5-lipoxygenase and 15-lipoxygenase assays, respectively. Values are mean ± SD (n = 3–5). NS, not significant, compared with the vehicle control (2% 2-methoxyethanol). Modified from reference 34, with permission, including data from reference 28.

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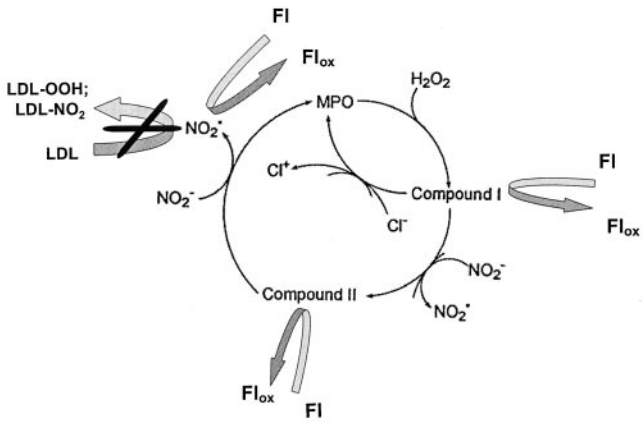

FIGURE 4. Catalytic cycle of MPO and the putative sites of action of flavonoids. Flavonoids (Fl) have been demonstrated to be substrates of MPO, being oxidized to still undefined species (Fl_{ox}), and are also thought to scavenge the oxidant and nitrating species $NO_2 \cdot$, formed from nitrite by MPO in the presence of H_2O_2. In this way, the reaction of $NO_2 \cdot$ with LDL, causing both peroxidation of the lipid moiety (LDL-OOH) and tyrosine nitration of the apoprotein (LDL-NO_2), is prevented.

inhibitory effects of flavonoids occurred through 2 different actions, ie, dose-dependent prolongation of the lag phase of conjugated diene formation at concentrations of ~1 μmol/L and lowering of the reaction rate of the propagation phase of lipid peroxidation at somewhat higher concentrations (~2 μmol/L). The mode of action of flavonoids with MPO was quite different from that with lipoxygenases. Flavonoids are not MPO inhibitors per se; they even serve as MPO substrates. For example, during the reaction of MPO with (−)-epicatechin in the presence or absence of LDL, a new chromophore with an absorption maximum at 435 nm was formed (48), the structure of which remains to be defined. It is tempting to speculate that MPO-mediated dehydrogenation of (−)-epicatechin or other flavonoids leads to several types of semiquinones, quinones, and quinone methides, which may be less functional as antioxidants than the parent flavonoids. The overlap of antioxidant actions of flavonoids on the MPO/nitrite reaction system with MPO-mediated conversion of flavonoids may explain the lag-phase phenomenon at low concentrations of flavonoids.

The MPO/nitrite system also caused protein tyrosine nitration in LDL; however, this reaction required higher concentrations of nitrite (≥ 100 μmol/L) than did formation of conjugated dienes in the lipid moiety of LDL. This reaction was suppressed by (−)-epicatechin at concentrations as low as 0.1 μmol/L (46).

The diagram in **Figure 4** depicts the interactions of nitrite and flavonoids with the catalytic cycle of MPO, as well as the putative mode of protection of LDL by flavonoids against oxidative and nitrating modification. The involvement of $NO_2 \cdot$ radicals in the MPO/nitrite reaction system and the in vivo role of this system appear to be well substantiated by recent studies by Hazen and coworkers (49, 50).

Peroxynitrite is another deleterious product of NO. Bolus addition of this compound to LDL caused only minute lipid peroxidation but strong tyrosine nitration of the apoprotein, with the latter being independent of the presence of CO_2 (46). Again, 0.1 μmol/L (−)-epicatechin strongly suppressed this reaction. In other systems, this major cocoa flavanol selectively attenuated nitrating reactions of peroxynitrite at low concentrations, whereas suppression of oxidation reactions by this compound required higher concentrations of (−)-epicatechin (51).

Some protective actions of (−)-epicatechin against several kinds of oxidative and nitrating modifications of human LDL we observed are compiled in **Table 2**. These data demonstrate that cocoa flavanols are universal protectors of human LDL against various types of proatherogenic modifications. This conclusion was corroborated by a recent report that intake of flavan-3-ol–rich cocoa products decreased significantly the oxidative susceptibility of LDL ex vivo, although the total antioxidative capacity of plasma was not changed under those conditions (52).

COCOA FLAVAN-3-OLS PROMOTE BIOACTIVITY OF NO IN VIVO

NO is a pivotal endothelial mediator. Unless produced in excessive amounts under conditions of oxidative stress, NO predominately exerts several antiinflammatory, antioxidant, antiatherosclerotic, and vasodilatory actions. However, NO is also a precursor of potent prooxidant and nitrating compounds, such as peroxynitrite and nitrogen dioxide (53). Therefore, the aim of any dietary or pharmacologic intervention regarding NO metabolism is to improve the beneficial bioactivity of NO without enhancement of or even with attenuation of the formation of NO-derived deleterious compounds. For this reason, the bioactivity of NO, rather than the total amount formed, is critical for protection of the cardiovascular system.

To elucidate the effects of cocoa flavanols on NO bioactivity in vivo, we compared the effects of cocoa beverages containing different contents of flavanols among selected volunteers (54). As shown in **Figure 5**, ingestion of flavanol-rich cocoa by individuals with diminished endothelial function led to an increase in the plasma RNO concentration from 22 ± 2.7 nmol/L to 36 ± 5.3 nmol/L ($P < 0.001$), approaching the mean concentrations of

TABLE 2
Efficacies of (−)-epicatechin against oxidative and nitrating modification of human LDL

Agent	Reaction/criterion	EC_{50} [1]
		μmol/L
Myeloperoxidase (nitrite)	Lipid peroxidation, prolongation of lag phase	~2
	Lipid peroxidation, decrease of reaction rate	~5
	Tyrosine nitration	<0.1
Myeloperoxidase (tyrosine)	Lipid peroxidation, extent	~5
Cu^{2+}	Lipid peroxidation, prolongation of lag phase	0.6
Peroxynitrite	Tyrosine nitration	<0.1

[1] Effective concentration to achieve either 50% inhibition or doubling of lag phase.

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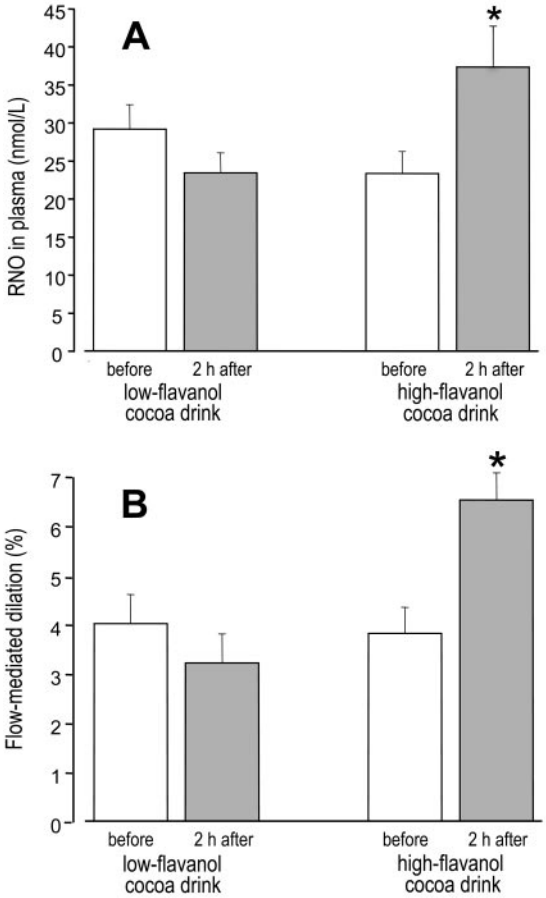

FIGURE 5. Effects of high-flavanol and low-flavanol cocoa drinks on RNO concentrations in plasma (A) and FMD (B) among individuals at risk for cardiovascular diseases. The test subjects (11 male and 9 female subjects) had at least one major cardiovascular risk factor (hypertension, hypercholesterolemia, diabetes mellitus, and/or smoking) (55). Exclusion criteria were elevated C-reactive protein concentrations (> 0.005 g/L), atrial fibrillation, acute coronary syndrome, or heart failure (New York Heart Association class III or IV). The individuals refrained from smoking and drinking caffeine-containing beverages and fasted for at least 12 h before the study. Analyses were performed 2 h after ingestion of 100 mL of either a high-flavanol cocoa drink (containing 176 mg of total flavanols) or a low-flavanol cocoa drink (containing < 10 mg of flavanols), in a randomized, double-blind, crossover manner. Plasma RNO concentrations were measured after treatment of plasma with 0.1 volume of 5% sulfanilic acid in 1 mol/L HCl and subsequent reductive cleavage, with a reaction mixture containing 45 mmol/L iodide and 10 mmol/L triiodide, to NO, which was assayed on the basis of its chemiluminescent reaction with ozone (24). Endothelial function was assessed noninvasively by measuring FMD of the brachial artery with high-resolution ultrasonography, after 5 min of lower arm occlusion (56). Values are mean ± SEM ($n = 20$). *Significant differences from baseline values before ingestion of the drink ($P < 0.001$, paired t test) and significant differences with the high-flavanol drink, compared with the low-flavanol control drink ($P = 0.005$ in A and $P < 0.001$ in B, paired t test). Statistical analyses were performed with SPSS for Windows, version 11.0.1 (SPSS Inc, Chicago, IL). Data from reference 54.

healthy control subjects, and doubled FMD from 3.4 ± 0.5% to 6.3 ± 0.6% ($P < 0.001$) after 2 h. Plasma concentrations of RNO were correlated with FMD ($r = 0.42$, $P = 0.02$), which suggests that the improvement of endothelial function might be attributable to increased bioavailability of NO. The changes occurred transiently 2 h after ingestion, as determined in a separate pilot study addressing the time dependence (54). In contrast, these changes did not occur among individuals who received either water or a low-flavanol cocoa drink, which excluded effects of beverage constituents other than flavanols. The changes in RNO and FMD caused by the high-flavanol cocoa beverage proved to be highly selective, because resting diameter and endothelium-independent dilation of the brachial artery (12.9 ± 1.8%), forearm blood flow at rest and reactive hyperemia, blood pressure, heart rate, and plasma nitrite and nitrate concentrations did not change under these conditions.

The maximal effects of the high-flavanol cocoa beverage coincided with the peak of (−)-epicatechin metabolites in plasma (57), both occurring ~2 h after intake. This observation defines them as rapid actions. Therefore, they cannot be attributed to time-dependent increases in the expression of endothelial NO synthase, which was recently reported for red wine, another important source of dietary flavanols (22, 58). Rather, the observed actions may occur either at the level of cell signaling or at the level of endothelial NO synthase activity. Together with the data reported in the literature, these data indicate that the NO-promoting actions of dietary flavanols are attributable to more than one mechanism.

DISCUSSION

General implications regarding flavonoid actions in vivo

We provided evidence that flavan-3-ols and their procyanidins are general inhibitors of mammalian lipoxygenases at the enzyme level. The lipoxygenase-inhibiting potencies of (−)-epicatechin and related flavonoids may therefore be considered to contribute to the antioxidative potential of these polyphenols. Whether this is actually the case for 5-lipoxygenase in vivo remains to be ascertained in future studies, which may include measurement of urinary excretion of leukotriene E_4 and estimation of the 5-lipoxygenase-mediated formation of arachidonic acid products (mainly 5-HETE and leukotriene B_4) from ionophore-stimulated neutrophils ex vivo. A finding supporting this assumption is that intake of procyanidin-rich chocolate among human subjects led to significant decreases in the plasma concentrations of cysteinyl leukotrienes, which are metabolites of the 5-lipoxygenase pathway of arachidonic acid metabolism, in parallel with increases in the plasma concentrations of (−)-epicatechin metabolites (11). Although the IC_{50} values for the various isolated lipoxygenases proved to be higher than the achievable plasma concentrations (57, 59) and most plasma (−)-epicatechin is present as conjugates (eg, glucuronides), the 5-lipoxygenase inhibition appears to be of biological relevance, inasmuch as no information is available regarding the distribution of flavonoids and their metabolites in compartments of the organism other than blood plasma. We demonstrated that (−)-epicatechin is accumulated in murine aortic endothelial cells and thus exerts intracellular protective actions (60). This observation suggests that the plasma concentrations of flavanol metabolites are different from the actual intracellular concentrations.

The pertinent literature shows the tendency to overrate the biological role of the plasma metabolites of (−)-epicatechin and other flavonoids, which are thought by many authors to be both final metabolites and bioactive forms. Several reports are not in line with this suggestion. Flavonoid glucuronides were found to be deconjugated by various human cells and thus taken up as aglycones (61). In a study with [4-^{14}C]quercetin among human

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subjects, the urinary recovery of radioactivity after oral ingestion of this isotope was as low as 3.3–5.7%, despite a high absorption rate of ~50%, whereas a large percentage was found as $^{14}CO_2$ in expired air. Furthermore, the half-life of the labeled flavonoid was rather long, ranging from 20 to 72 h (62). Together, these observations argue that flavonoid glucuronides may serve as transport forms in plasma, rather than as final metabolites.

Biological consequences of the inhibition of lipoxygenase activities

The biological role of 5-lipoxygenase is closely connected with the biosynthesis of leukotrienes, and the inhibition of human 5-lipoxygenase by cocoa flavonoids suggests antileukotriene actions of these compounds, which may confer some antiinflammatory, vasoprotective, and antibronchoconstrictory capacity. Leukotriene B_4 causes adherence of neutrophils to endothelial cells and is a potent chemotactic agent for these and other inflammatory cells. It also stimulates release of lysosomal enzymes and generation of superoxide anion in neutrophils. The cysteinyl leukotrienes increase vascular permeability and contract airway smooth muscle (8–10).

The subfamily of reticulocyte-type 12/15-lipoxygenases (including human and rabbit 15-lipoxygenase-1) differ from other mammalian lipoxygenases. This refers to the capability of directly dioxygenating polyenoic fatty acids in esterified form, such as those present in membrane phospholipids and in cholesterol esters of plasma lipoproteins, without requiring a lipid-cleaving enzyme. Unlike 5-lipoxygenase and platelet-type 12-lipoxygenase, the reticulocyte-type lipoxygenases are thus catalysts of enzymatic lipid peroxidation (for details, see reference 33 and references cited therein). In addition to involvement in the biologically programmed maturational breakdown of mitochondria in reticulocytes (63), a role in oxidative modification of LDL has been discussed (13, 14). Oxidative modification of LDL by 15-lipoxygenase-1, MPO, peroxynitrite, thiols, or transition metals renders it atherogenic (16). According to the oxidation hypothesis of atherosclerosis (16), this process is thought to play a fundamental role in early stages of the development of atherosclerotic lesions. If so, then any agent that interferes with oxidative modification of LDL should be protective for vascular endothelium, including that of coronary arteries. Most of the agents proposed to be involved in oxidative modification of LDL have been found by us to be counteracted by dietary polyphenols, in particular by (−)-epicatechin.

Detailed studies of the 15-lipoxygenase-1–mediated oxidation of LDL (64) revealed that this process involves 2 steps, ie, direct enzymatic attack on cholesteryl linoleate in LDL, leading to predominately specific oxygenation products, and lipoxygenase-mediated nonenzymatic lipid peroxidation, leading to nonspecific secondary reaction products. The longer the reaction continues, the greater is the share of nonspecific lipid peroxidation products, which can eventually predominate, although this process is enzymatically initiated. Dietary flavonoids appear to suppress both steps of this process; they inhibit 15-lipoxygenase-1 activity (34, 35) and they are known to effectively scavenge lipid-derived free radicals, which are intermittently formed during lipoxygenase-mediated, nonenzymatic, lipid peroxidation. Because the flavonoids may also scavenge secondary free radicals during oxidative modification of LDL evoked by endogenous oxidants other than 15-lipoxygenase-1, their antioxidative protection of LDL and, consequently, of the cardiovascular system appears to be universal. On the basis of these data taken together, we propose that inhibition of 15-lipoxygenase-1 activity and of the complex reactions with LDL contributes to the effects of cocoa flavonoids on the vascular endothelium.

The biological role of platelet 12-lipoxygenase is not well understood (65). This enzyme appears to be not involved in platelet aggregation and adhesion; therefore, the suppression of these phenomena by cocoa and wine flavonoids (66–68) cannot be attributed to inhibition of 12-lipoxygenase but rather is attributable to enhanced bioactivity of NO (20–22). Two types of biologically active eicosanoids are formed via the 12-lipoxygenase pathway of arachidonic acid metabolism, ie, 12*S*-hydro(pero)xy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid and hepoxilin A_3 and B_3 (69). In most cells possessing platelet-type 12-lipoxygenase, the formation of 12-HETE prevails. Inhibition of glutathione peroxidases causes a shift toward hepoxilin synthesis (70). For 12-HETE, a role in several processes related to tumor metastasis has been proposed (71), but other biological actions have also been described (72). The main biological actions of hepoxilin A_3 are related to its ability to release Ca^{2+} from intracellular stores in human neutrophils and other cells (69). Whether intervention in the 12-lipoxygenase pathway contributes to the potential beneficial effects of dietary flavonoids remains to be clarified.

Lipoxygenases and free radicals

It should be stressed that the inhibition of lipoxygenases by flavonoids and other polyphenols is not necessarily a consequence of the free radical-scavenging properties of the polyphenols. Although intermediate free radicals are formed during the catalytic cycles of lipoxygenase-catalyzed reactions (33), these radicals remain tightly bound at the active site of the enzyme and are not accessible to such well-known lipophilic radical scavengers as 2,6-di-*tert*-butyl-4-methylphenol and probucol (73), as well as α-tocopherol. However, a variety of polyphenols are concomitantly free radical scavengers and lipoxygenase inhibitors. The most well-characterized example is nordihydroguaiaretic acid. For soybean lipoxygenase L-1, the inhibitory effect of nordihydroguaiaretic acid is obviously attributable to reduction of the active ferric form of the enzyme to the silent ferrous form, with interruption of the catalytic cycle of the dioxygenase reaction (74).

Intermediate enzyme-bound free radicals formed during lipoxygenase catalysis can be released from the active site under certain circumstances, to initiate secondary lipid peroxidation or other deleterious consecutive reactions. The extent of these side processes depends on the reaction conditions, particularly the type of substrate for the lipoxygenase. Lipophilic free radical scavengers selectively suppress such side reactions, although they are not true lipoxygenase inhibitors (73, 75). Dietary flavonoids and other polyphenols obviously combine the 2 properties, however, and suppress complex lipoxygenase-catalyzed processes in a dual manner.

Possible role of quercetin in the dietary value of cocoa products

Although (−)-epicatechin and related procyanidins contribute most of the total polyphenol content of cocoa products, the possible role of the quercetin content should not be overlooked. The

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total amount of quercetin and related flavonols in cocoa has been reported to be 0.03% by wt (76), which corresponds to approximately one-tenth of the content of flavan-3-ols. We observed 37-fold and 15-fold higher inhibitory potencies of quercetin, compared with (−)-epicatechin, toward 5-lipoxygenase and 15-lipoxygenase-1, respectively; therefore, the lipoxygenase-inhibiting activity of the total mixture of cocoa flavonoids may be partly attributable to quercetin.

Cocoa polyphenols and NO metabolism

The data from our human study (54) indicated that the bioactivity of NO is markedly improved after ingestion of cocoa polyphenols and that the vascular endothelium is a major target of dietary polyphenols. These data are in line with those from other studies of human subjects who received other flavanol-containing beverages (18, 19) or of rats (21), as well as with studies with endothelial cells (22). The NO-promoting effects of cocoa polyphenols may also contribute to the recently reported decreases in blood pressure among elderly individuals after intake of dark chocolate (77).

In vitro investigations at the cellular and subcellular levels are needed to define how the NO-promoting action of cocoa polyphenols is brought about. The bioactivity of NO is expected to be the result of both synthesis of NO and its conversion to bioactive storage forms and other metabolites, including those exerting deleterious actions. It is tempting to speculate that dietary polyphenols interfere with NO metabolism at several levels. They may favor the synthesis of NO and concomitantly suppress effects of deleterious metabolites such as superoxide, peroxynitrite, and nitrogen dioxide. Another possibility could involve inhibition of the oxidation of NO to nitrite, a process that is not well understood. Suppression of the modifications of LDL by MPO/nitrite or peroxynitrite, as we observed, might also contribute to the complex effects of dietary polyphenols on NO metabolism.

CONCLUSIONS

Inhibitory effects on mammalian lipoxygenases, particularly 5-lipoxygenase and 15-lipoxygenase-1, may contribute to the potentially beneficial actions of cocoa flavonoids, although clear in vivo evidence is still lacking. Another target of these polyphenols is MPO, with the NO metabolite nitrite as substrate. Finally, our human study and other studies with human subjects and laboratory animals suggest that improvement of NO bioactivity is a pivotal action of dietary flavonoids.

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