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Cocoa procyanidin chain length does not determine ability to protect LDL from oxidation when monomer units are controlled

Francene M. Steinberg[a,*], Roberta R. Holt[a], Harold H. Schmitz[b], Carl L. Keen[a,c]

[a]Department of Nutrition, University of California, Davis, CA, USA
[b]Analytical and Applied Sciences, Mars Inc., Hackettstown, NJ, USA
[c]Department of Internal Medicine, University of California, Davis, CA, USA

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Abstract

Cocoa flavan-3-ols (catechin, epicatechin and oligomeric procyanidins) were tested for their ability to decrease LDL oxidative susceptibility and spare α-tocopherol (α-toc) in vitro. Physiologic concentration (0.10–0.50 μM) of flavanols were used. The flavanols increased LDL conjugated diene lag times dose-dependently from 23–207% and 15–143% in response to copper and AAPH oxidation, respectively, and delayed α-toc consumption. Sparing of LDL α-toc represents a possible mechanism for flavanols to enhance the resistance of plasma and LDL to oxidative stress. Procyanidins decreased LDL oxidative susceptibility with increasing chain length. However, when based on equivalent amounts of monomeric units, they inhibited LDL oxidation to a similar extent. This suggests that antioxidant activity of procyanidins with biologic substrates is not attributable to chain length or charge delocalization through polymeric linkages, but primarily to ring structures and catechol groups. Additionally, human plasma was analyzed for the presence of oligomeric procyanidins following consumption of a flavanol-rich cocoa product. Procyanidin dimers were detected in plasma concordant with the appearance of monomeric flavanols, with a peak of 0.08 $\pm$ 0.01 μmol/L (n = 6) at two hours after consumption. Thus, this paper confirms the occurrence of procyanidins in human plasma, and extends previous structure-function observations regarding flavanoid protection of LDL. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Flavanol; Procyanidin; Cocoa; LDL; Oxidation; Alpha-tocopherol

1. Introduction

Plant flavonoids are naturally occurring polyphenolic compounds found widely distributed in foods and are ingested by humans in mg/day quantities [1]. Procyanidins are oligomeric flavonoids comprised of flavan-3-ol monomeric subunits {(−)-epicatechin and/or (+)-catechin}(Fig. 1). The oligomers vary in size, with dimers through decamers having been successfully isolated [2–4]. While several foods and beverages (such as cranberries, red wine and green tea) can contain high amounts of catechin monomers and select oligomers, the procyanidin oligomers exist in particular abundance and complexity in cocoa and apples.

Flavonoids are effective free radical scavengers and metal chelators due, in part, to the 3′4′ di-OH catechol moiety on the B ring of the basic flavonoid structure [5] (Fig. 1). This antioxidant capacity may contribute to the putative health benefits of dietary flavonoids, which include anti-inflammatory actions and a reduced risk of cardiovascular disease [6–12]. Recent evidence that cocoa procyanidins and other flavanoids are capable of directly inhibiting mammalian 15-lipoxygenase suggests another mechanism by which lipid oxidation may be modulated [13]. The oxidative susceptibility of low-density lipoprotein (LDL) and its subsequent oxidative modification in the artery wall are thought to be pivotal steps in the development of atherosclerosis [14,15]. Thus, examining *in vitro* or *ex vivo* LDL oxidation susceptibility in response to dietary flavonoids can provide useful information regarding their influence on cardiovascular health.

Others have reported the ability of plant flavanol-rich extracts, green tea and wine catechins to delay the oxidation of isolated LDL *in vitro* [16–21], and that procyanidin-rich flavanol extracts from cocoa liquor have antioxidant capacity, in numerous *in vitro* models [22–24]. While there are reports of the *in vitro* antioxidant action of purified cocoa

* Corresponding author. Tel.: +1-530-752-0160; fax: +1-530-752-8966.

E-mail address: fmsteinberg@ucdavis.edu (F.M. Steinberg).

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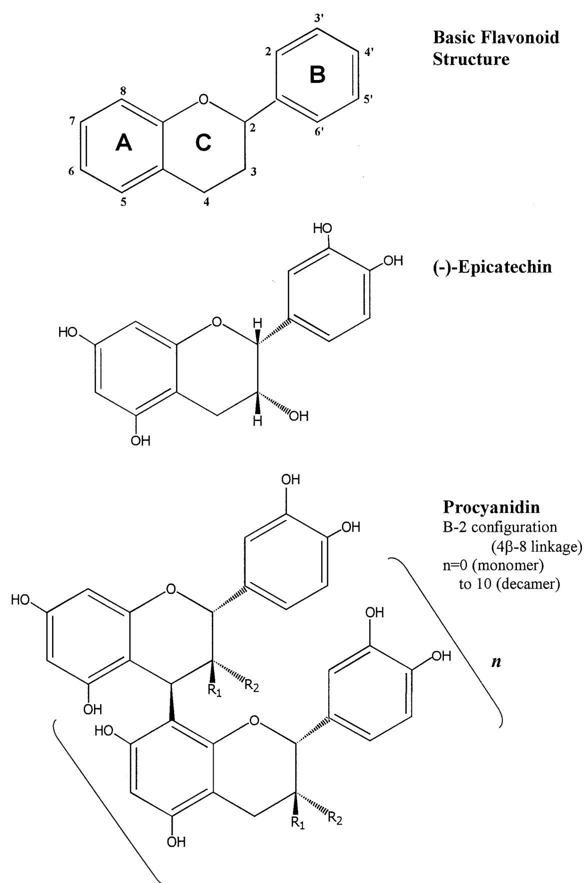


Fig. 1. Basic flavonoid structure with $C_6C_3C_6$ skeleton; monomer ((−)-epicatechin); and oligomer (B_2 procyanidin) epicatechin-(4β-8)-epicatechin, with possible linkages of 1–10 subunits.

procyanidin oligomers using synthetic liposomes and LDL [25,26], and some information exists on the effectiveness of cocoa procyanidin consumption to delay *ex vivo* human LDL oxidation susceptibility [27–29], the question of whether the monomers and procyanidin compounds of varying chain length differ in their ability to effectively protect has not been fully addressed. Recent investigations of procyanidin from grape seeds (*Vitis vinifera)* suggest that the oligomers are more effective as *in vitro* radical scavengers than the monomers [30,31]. Procyanidins derived from cocoa, especially the tetramers, were reported to be more effective than monomers at protecting against oxidation and nitration reactions [32]. The question of the antioxidant efficacy of different oligomeric procyanidins has biologic relevance, as experiments using cell culture and perfused isolated small intestine show that dimeric and trimeric procyanidins are transported across enterocytes as well as flavanol monomers [1,33], and that B_2 dimers occur in human plasma following the consumption of procyanidin-rich foods [34].

In the present work we extended observations regarding flavanol structure-function relationships by examining the ability of catechin, epicatechin, cocoa flavan-3-ol monomers and procyanidin oligomers of varying chain length to delay human LDL oxidation *in vitro.* The oxidation was catalyzed by metal dependent and independent means, using copper and 2,2′Azobis(2-methylpropionamidine) dihydrochloride (AAPH). The ability of the procyanidin fractions to protect endogenous LDL α-tocopherol was examined. Additionally, we confirmed the observation of procyanidin dimers in human plasma.

2. Methods and materials

2.1. Subjects

Six non-smoking adult volunteers (2 males and 4 females, age range 23–39, body mass index 23.1 ± 0.7) with no apparent disease participated in the study. The participants' health status was evaluated via questionnaire, and written informed consent was given prior to participation in the study. The study protocol was approved and conducted according to guidelines set by the Human Subjects Institutional Review Board of the University of California, Davis.

Subjects were asked to abstain from alcohol, analgesics and flavonoid-rich foods on the day prior to the experiment. After a twelve-hour evening fast, the subjects consumed 37.5 g of cocoa (Cocoapro™, Mars Incorporated, Hackettstown, NJ) in 400 ml of water. One gram of the cocoa provided 12.2 mg of monomers, 9.7 mg of dimers and 20.2 mg of larger procyanidins (trimers through decamers). In addition to the beverage, the subjects were given a white bread product, and a low flavonoid lunch was provided later in the day. The study was repeated on the following day.

Blood samples were drawn via venipuncture into tubes containing sodium heparin at baseline (0 h), and at 1, 2, and 6 h post cocoa consumption. Blood was centrifuged and the plasma was separated and frozen at −80°C until analysis.

2.2. Plasma catechin, epicatechin and procyanidin dimers

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Plasma samples were extracted as described by Holt et al. [34]. Fifty μL of the resulting solution was analyzed for catechin, epicatechin and procyanidin. Chromatography was carried out using an HP 1100 HPLC system with Chemstation software (Hewlett Packard, Wilmington, DE), in series with an ESA CoulArray 5600 detector (Chelmsford, MA). Separation was achieved using a reversed phase Alltima C18 column (150 × 4.6 mm, 5 μm particle size; Alltech Associates, Deerfield, IL). Epicatechin and catechin peak identification at +150 mV and procyanidin dimer B_2 peak identification at +700 mV were based on co-elution with authentic standards and quantified using external standards from cocoa (Cocoapro™, Mars Incorporated, Hackettstown, NJ) [2,35].

2.3. Flavanol monomers and procyanidin oligomers

Catechin and epicatechin were purchased from Sigma Chemicals Co. (St. Louis, MO). Procyanidins were purified from Cocoapro™ cocoa [2], and were provided by Mars, Inc. (Hackettstown, NJ). The purities of the cocoa flavanol fractions were 95% monomer, 98% dimers, 93% pentamers and 89% hexamers. The impurities in the fractions reflect small contributions of higher molecular weight compounds. Stock procyanidin solutions were made in water at 2 mM concentrations (based on MW of the primary oligomer) and stored at −80°C. Stock solutions were diluted to yield final concentrations of 0.1 μM, 0.25 μM, and 0.5 μM.

Antioxidant activity of procyanidin extracts and oligomers was determined using a 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) cation decolorization assay as described by Re et al. [36]. Trolox, a water-soluble vitamin E analog, was used as a standard. Results are expressed as Trolox equivalent activity capacity (TEAC).

2.4. Plasma LDL isolation

Whole blood was obtained from 12 healthy volunteers. Pooled EDTA plasma was isolated by centrifugation, aliquoted, and stored at −80°C until needed. LDL was isolated from plasma using micro-ultracentrifugation (model RC-M120GX, Sorvall, Newtown, CT) [37]. EDTA was removed from the LDL by overnight dialysis in PBS (pH 7.4) that had been pretreated with Chelex-100 resin to remove free metals and purged with nitrogen.

2.5. Conjugated dienes

LDL cholesterol concentration was assayed spectrophotometrically using a Cholesterol/HP kit (Boehringher Mannheim, Indianapolis, IN) [38]. LDL conjugated diene (CD) formation was measured with a modification of the Esterbauer method [39], using dialyzed LDL (75 μg total cholesterol) and $CuSO_4$ or AAPH solution (5 or 10 μM respectively). Incubations with 0.1, 0.25, or 0.5 μM flavanols were compared to controls lacking flavanols. CD formation was monitored every 5 min for 200 min or until a maximum absorbance at 234 nm occurred.

LDL α-Tocopherol (α-toc) concentration was measured using HPLC on a reverse phase C-18 column (Ultramex 150 × 4.6 mm, 5 μm particle size; Phenomenex, Torrence, CA) with absorbance monitored at 300 nm [40]. Oxidation of LDL for the α-toc experiments was similar to that described above for the CD assay, with the exception that 150 μg LDL cholesterol was used. The procyanidin was 0.5 μM monomer or 0.1 μM pentamers added either concomitant with, or 10 and 20 min past the initiation of the oxidant stress. Controls lacked procyanidin. Aliquots were taken at time 0, 10, 20, 30, 45 and 60 min, the reaction was stopped with BHT, and the samples were extracted for HPLC analysis.

Table 1
Plasma flavanol and B_2 Dimer concentrations after consumption of flavanoid-rich beverage

Compound	Time	Day 1	Day 2
Epicatechin	0h	0.00 ± 0.00*	0.03 ± 0.01
	1	3.46 ± 0.33$^+$	3.86 ± 0.52$^+$
	2	4.11 ± 0.81$^+$	3.83 ± 0.96$^+$
	6	1.13 ± 0.42	0.67 ± 0.18
Catechin	0	0.00 ± 0.0	0.00 ± 0.0
	1	0.40 ± 0.12*	0.36 ± 0.15
	2	0.40 ± 0.17*	0.32 ± 0.16
	6	0.23 ± 0.13	0.18 ± 0.15
B_2 Dimer	0	0.02 ± 0.01	0.03 ± 0.01
	1	0.07 ± 0.02*	0.08 ± 0.02
	2	0.08 ± 0.01*	0.09 ± 0.03
	6	0.04 ± 0.01	0.04 ± 0.02

*mean ± SEM.
$n = 6$ subjects Day 1, $n = 4$ subjects Day 2.
Significant difference vs. time 0 h (baseline) $^*p < 0.05$, $^{**}p < 0.01$, $^+p < 0.001$.

2.6. Statistical analysis

Data are expressed as mean ± SEM. Treatments were compared using single factor ANOVA. Post-hoc analysis for significant ($p < 0.05$) mean differences between control LDL and procyanidin treatments was done using the Student Newman Keuls test for multiple comparisons and Dunnett's test where appropriate. Each point represents replicates of 3–6 samples.

3. Results

3.1. Plasma flavonoid concentrations

One h after consuming the cocoa, plasma epicatechin and catechin levels increased above baseline levels ($p < 0.001$) (Table 1). Plasma concentrations continued to increase at 2 h after consumption, but had declined by 6 h. The B_2 dimer increased in the plasma in a manner similar to the monomers, being elevated at 1 and 2 h ($p < 0.05$) relative to baseline. Similar concentrations were observed on both experiment days.

3.2. TEAC antioxidant activity assay of procyanidin oligomers

TEAC values obtained for purified oligomeric fractions, as well as a crude cocoa extract, were similar, and all were greater than the reference value of Trolox. The results for crude extract, monomer, dimers and pentamers were 1.87 ± 0.24, 1.68 ± 0.11, 1.70 ± 0.29, and 1.62 ± 0.10, respectively.

Table 2
Dose response effect of procyanidin oligomers on LDL CD lag time

	LDL Conjugated Diene Lag Time, min			
	Monomer	Dimer	Pentamer	Hexamer
Cu-oxidation				
LDL Control	65.06 ± 3.41[a]	65.06 ± 3.41[a]	65.06 ± 3.41[a]	65.06 ± 3.41[a]
0.10 μM	80.30 ± 3.41[*b]	80.13 ± 2.17[*b]	112.29 ± 9.84[+b]	100.93 ± 2.68[+b]
0.25 μM	82.81 ± 4.07[**b]	105.48 ± 5.31[+c]	155.10 ± 7.47[+c]	200.00[+c]
0.50 μM	99.54 ± 4.03[+c]	136.03 ± 5.21[+d]	200.00[+d]	200.00[+c]
AAPH-oxidation				
LDL Control	82.44 ± 1.48[a]	82.44 ± 1.48[a]	82.44 ± 1.48[a]	82.44 ± 1.48[a]
0.10 μM	94.43 ± 2.80[*b]	104.54 ± 3.82[**b]	118.62 ± 1.19[+b]	107.49 ± 2.35[+b]
0.25 μM	107.94 ± 2.57[+c]	120.91 ± 2.05[+c]	166.06 ± 3.75[+c]	154.94 ± 4.67[+c]
0.50 μM	115.65 ± 2.82[+d]	145.50 ± 6.21[+d]	200.00[+d]	200.00[+d]

**mean ± SEM, $n = 5$–6 per condition.
Significant difference vs. control LDL *p < 0.05, **p < 0.01, [+]p < 0.001.
[a–d]Values with different letters within a column are significantly different from each other. Values of 200 min indicate that oxidation was completely inhibited up to 200 min, at which time the reaction was terminated.

3.3. Effect of flavanol monomers and procyanidin oligomers on LDL oxidation

Purified catechin and epicatechin significantly ($p < 0.01$) and progressively lengthened the lag time for LDL CD formation at all concentrations with Cu-mediated oxidation. The percentage increase over control ranged from 17 to 48%. All of the procyanidins increased the Cu-mediated LDL oxidation lag time in a dose-dependent manner (Table 2). The protective effect of lengthening CD formation lag time was significant ($p < 0.05$–0.001) at all concentrations, and the percentage increase over control ranged from 23 to 207%. Higher concentrations of the pentamers and hexamers resulted in complete inhibition of oxidation to 200 min, at which time the reaction was stopped. There were no significant differences in the propagation rate or the level of maximum CD formation between controls and the LDL to which flavanols had been added. Similar results were observed with AAPH-mediated LDL oxidation. The addition of procyanidin oligomers significantly ($p < 0.05$–0.001) increased the lag time in a dose-dependent manner, with the percentage increase over control varying from 15 to 143% (Table 2).

A comparison of CD lag time was made at the same molar concentration for each of the procyanidins. When a concentration of 0.5 μM procyanidin based on the MW of each fraction was used, all oligomers increased lag time compared to control ($p < 0.001$). The lag time was lengthened progressively with increasing oligomer size such that the activity was ranked as hexamers = pentamers > dimers > monomer in both Cu- and AAPH-catalyzed oxidations. The percentage increase in CD lag time versus control in the Cu-mediated system was 53%, 109%, >200% and >200%, and in the AAPH-mediated system 40%, 77%, >142% and >142% for the monomer, dimers, pentamers and hexamers, respectively. A comparison of the different oligomers was also made at a concentration of 0.5 μM as monomer unit equivalents, in order to standardize the antioxidant contribution of the additional phenolic groups and hydroxyl groups in the polymeric compounds (Table 3). When the adjustment was made to equivalent monomer units, all oligomers were equally effective in lengthening CD lag time compared to control in both Cu- and AAPH-mediated oxidation conditions ($p < 0.01$–0.001). The percentage increase in CD lag time versus control in the Cu-mediated system was 53%, 62%, 73%, 57%, and 40%, 47%, 44%, 33% in the AAPH-mediated system for the monomer, dimers, pentamers and hexamers, respectively.

3.4. Effect of procyanidin oligomers on LDL α-tocopherol concentration

Addition of 0.5 μM monomer simultaneous with initiation of oxidation spared the LDL α–toc at the 20 min time point ($p < 0.01$) when compared to control conditions (Fig. 2A). Addition of the monomer at 10 or 20 min following initiation of the oxidative stress, when the α-toc was partially depleted, resulted in α–toc levels that were higher than control at subsequent time points ($p < 0.05$–0.001). The use of pentamers at 0.1 μM (equivalent to 0.5 μM based on monomer units) resulted in a similar, but slightly more distinct, pattern to the monomer (Fig. 2B). The CD results measured at the same time points as α-toc were consistent with the expected results, i.e. an increase in CD formation corresponding to the consumption of the endogenous α-toc (data not shown).

4. Discussion

The present studies demonstrate the ability of purified monomeric flavanols and oligomeric cocoa procyanidin fractions to delay the consumption of endogenous LDL α-tocopherol, and to decrease the susceptibility of LDL to

Table 3
Effect of oligomer chain length on LDL CD formation

	LDL Conjugated Diene Lag Time, min			
	0.5 μM, molecular weight		0.5 μM, monomer unit equivalent	
	Cu-ox	AAPH-ox	Cu-ox	AAPH-ox
LDL control	65.06 ± 0.96^{a}	82.44 ± 1.48^{a}	65.06 ± 0.96^{a}	82.44 ± 1.48^{a}
Monomer	99.54 ± 4.03^{+b}	115.65 ± 2.82^{+b}	99.54 ± 4.03^{**b}	115.65 ± 2.82^{+b}
(% ↑ vs. control)	(53.0)	(40.3)	(53.0)	(40.3)
Dimer	136.03 ± 5.21^{+c}	145.50 ± 6.21^{+c}	105.48 ± 5.31^{+b}	120.91 ± 2.05^{+b}
(% ↑ vs. control)	(109.0)	(76.5)	(62.1)	(46.7)
Pentamer	200.00^{+d}	200.00^{+d}	112.29 ± 9.84^{+b}	118.62 ± 1.19^{+b}
(% ↑ vs. control)	(≥207)	(≥142)	(72.6)	(43.9)
Hexamer	200.00^{+d}	200.00^{+d}	101.87 ± 2.98^{+b}	109.61 ± 2.40^{+b}
(% ↑ vs. control)	(≥207)	(≥142)	(56.6)	(33.0)

mean ± SEM, n = 5–6 per condition.
Significant difference vs. control LDL $^{*}p < 0.05$, $^{**}p < 0.01$, $^{+}p < 0.001$.
$^{a-e}$Values with different letters within a column are significantly different from each other.
Values of 200 min indicate that oxidation was completely inhibited up to 200 min, at which time the reaction was terminated.

metal ion-dependent and metal ion-independent oxidation *in vitro*. The results illustrate that when the contribution of monomeric units is controlled for, the oligomer chain length does not substantially influence antioxidant activity with regard to LDL protection.

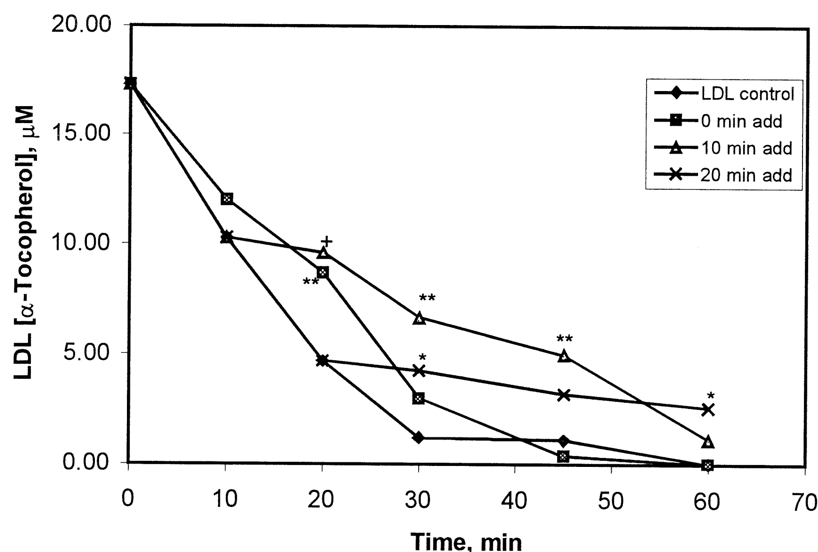
In vitro studies suggest that the acidic gastric environment results in the hydrolysis of most procyanidin oligomers to mixtures of epicatechin and catechin monomers and dimers [41]. Spencer et al. reported that perfusion of rat intestine with procyanidin B_2 and B_5 dimers results primarily in their cleavage to monomers within the enterocyte, as well as transfer of limited amounts of dimer and O-methylated dimer to the serosal side [33]. The finding of low nanomolar amounts of dimers in the plasma of human subjects following consumption of procyanidin rich cocoa is consistent with the *in vitro* model. The absorption and occurance of the dimers in plasma was rapid and mirrored the pattern seen for the monomers, consistent with the previous report [34]. Epicatechin was the most abundant monomer in plasma following the consumption of the cocoa beverage, being present in a 10:1 ratio to catechin. This observation is of interest, as the epicatechin:catechin in the beverage was approximately 3:1, suggesting preferential absorption of epicatechin and or *in vivo* modification of catechin.

Previous literature has variously reported that epicatechin has the same antioxidant capacity as, or is slightly more effective than, catechin, possibly due to greater area for charge localization [5,30,42]. However, a differential effect of these monomeric flavanols was not observed in the present *in vitro* studies, as catechin and epicatechin were equally effective as radical scavengers and in delaying Cu-mediated LDL oxidation. The biologic effects and *in vivo* antioxidant capacities of epicatechin vs. catechin are not completely understood at this time. It is possible that differences may occur *in vivo* at the cellular level with respect to membrane interactions and intracellular trafficking.

Potential mechanisms by which oligomeric procyanidin are hypothesized to have greater antioxidant activity than the respective monomers relate to having the catechol moieties close together in the B_2 configuration and the ability of the C_4-C_8 linkage to increase the electron dislocation capacity of the phenoxyl radical which is formed [30,31,42, 43]. Consistent with other studies, in the present work, we noted that the antioxidant capacity of the procyanidins with regard to LDL protection *in vitro* tended to increase with oligomer chain length. The only previously reported exception to this was that the hexamer tended to be slightly, but non-significantly, less effective than the pentamer [25]. However, when equivalent amounts of purified fractions were used based on monomer units instead of total procyanidin concentrations, we did not see significant differences in antioxidant protection. This allows for comparison of equivalent numbers of catechol groups. No significant advantage in LDL protection was noted between the different procyanidin fractions that could be attributed to charge delocalization through the polymeric linkages. This suggests that the antioxidant activity of procyanidins with biologic substrates is not dictated by oligomer chain length. This concept is strengthened by the results of the TEAC assay on the procyanidin fractions, where the oligomers had similar antioxidant capacities. The increase in CD lag time was slightly greater in the Cu-mediated system as opposed to the AAPH-mediated oxidant stress. This may be explained by the ability of the procyanidins with multiple catechol groups to chelate metal ions in addition to scavenging free radicals, resulting in a greater apparent antioxidant potential.

The procyanidin monomer and pentamer fractions delayed the consumption of LDL α-tocopherol in a manner similar to the effect of catechin on plasma α-tocopherol levels [44]. This supports the thesis that oligomer chain length does not uniquely influence the ability of procyanidin to protect endogenous LDL antioxidants against oxidation.

2A Cocoa Flavanol Monomer Fraction



2B Cocoa Flavanol Pentamer Fraction

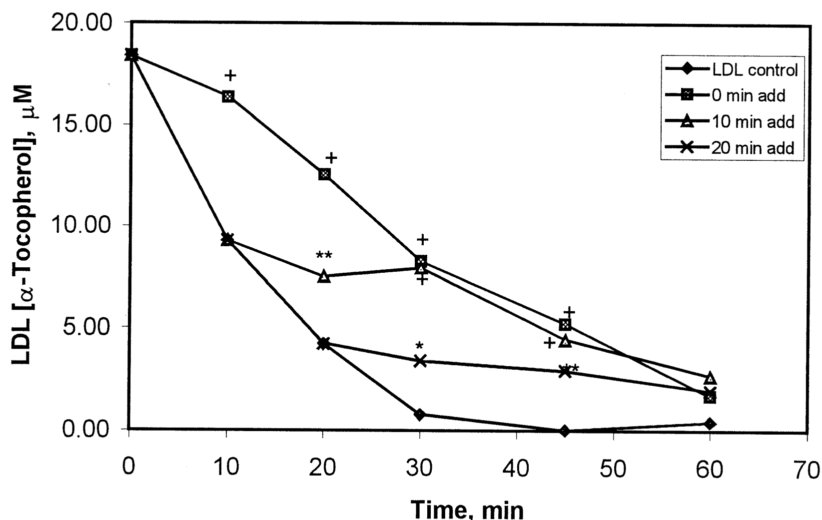


Fig. 2. LDL α-tocopherol concentration during oxidation with and without flavanol oligomers. A) Cocoa flavanol monomer fraction (0.5 μM monomer equivalent units) or B) Cocoa flavanol pentamer fraction (0.5 μM monomer equivalent units) was added to LDL either at initiation of oxidation (0 min), or at 10 or 20 min after the initiation of oxidation. Aliquots were taken at multiple times for HPLC analysis of α-toc. Significant difference vs. LDL control $*p < 0.05$, $**p < 0.01$, $^{+}p < 0.001$.

The presumed mechanisms of protection (radical scavenging and metal chelation) contribute to the delayed consumption of α-tocopherol, but it is doubtful that the hydrophilic procyanidin would recycle α-tocopheryl quinone back to α-tocopherol in the lipid core of the lipoprotein. It is generally postulated that flavonoids and their conjugated metabolites circulate in plasma, possibly bound to albumin, and can be adsorbed to the lipoprotein particle at the lipid-water interface and exert protective effects by limiting radical transfer into the lipoprotein [45]. However, it has been reported that some flavonoids and isoflavonoids can partition into, and interact with, lipid bilayers, thereby stabilizing and decreasing membrane fluidity which may contribute to slowing the mobility of free radicals and thus inhibit lipid peroxidation [46].

The concentrations of flavanols and procyanidins used in this study (0.1 to 0.5 μM) were within the physiologic range of plasma epicatechin following the consumption of fla-

vonoid-rich foods [47,48]. The observations reported herein support the concept that plasma levels of catechin, epicatechin, and procyanidins after consumption of flavonoid-rich foods can provide significant antioxidant protection *in vivo.* Consistent with this, Wang et al. have reported increased plasma antioxidant capacity and decreased plasma TBARS levels following the consumption of procyanidin-rich foods [48].

In summary, we have provided confirmation of the presence of procyanidin dimers in human plasma following consumption of a flavanol-rich beverage, lending physiologic relevance to *in vitro* determinations of oligomeric procyanidin antioxidant capacity using the biologic substrate LDL. When the different fractions were normalized to the number of catechol groups, the different oligomers were similar with respect to their ability to inhibit both Cu- and AAPH-mediated LDL oxidation. LDL α-tocopherol consumption was delayed in the presence of the procyanidins, and represents a possible mechanism for the ability of these compounds to enhance the resistance of plasma and LDL to oxidative stress.

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