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Effect of almond skin polyphenolics and quercetin on human LDL and apolipoprotein B-100 oxidation and conformation☆

Chung-Yen Chen*, Paul E. Milbury, Shin-Kyo Chung[1], Jeffrey Blumberg

Antioxidants Research Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111, USA

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Abstract

Almond skin polyphenolics (ASP) and vitamin C (VC) or E (VE) inhibit the Cu^{2+}-induced generation of conjugated dienes in human low-density lipoprotein (LDL) in a synergistic manner. However, the mechanism(s) by which this synergy occurs is unknown. As modification of apolipoprotein (apo) B-100 is an early, critical step in LDL oxidation, we examined the effects of combining ASP or quercetin and antioxidant vitamins on the oxidation of this moiety as well as on the alteration of LDL conformation and electronegativity (LDL−). In a dose-dependent manner, ASP (0.12–2.0 μmol/L gallic acid equivalents) decreased tryptophan (Trp) oxidation by 6.7–75.7%, increased the generalized polarity (Gp) of LDL by 21.0–81.5% at 90 min and reduced the ratio of LDL− to total LDL (tLDL) by 38.2–83.8% at 5 h. The actions of ASP on these parameters were generally additive to those of VC and VE. However, a 10–25% synergy of ASP plus VC in protecting apo B-100 Trp against oxidation may result from their synergistic interaction in prolonging the lag time to oxidation. ASP and VE acted in synergy to reduce LDL−/tLDL by 24–43%. Quercetin's actions were similar to ASP, though more effective at inhibiting Trp oxidation. Thus, ASP and quercetin reduce the oxidative modification of apo B-100 and stabilize LDL conformation in a dose-dependent manner, acting in an additive or synergistic fashion with VC and VE.

Keywords: Almonds; Apolipoprotein B-100; Laurdan; LDL; LDL−; Tryptophan

1. Introduction

In clinical trials, almond (*Prunus dulcis*) consumption increases the intake of vitamin E (VE) and monounsaturated fats and also reduces plasma low-density lipoprotein (LDL), C-reactive protein, lipoprotein(a) and insulin resistance [1–3], actions consistent with observational data showing an inverse correlation between nut intake and risk of cardiovascular disease [4,5]. Almond constituents, including α-tocopherol, unsaturated fatty acids, plant sterols, dietary fiber and polyphenolic compounds, may each contribute to this association [6]. Almond polyphenolics include the flavonols isorhamnetin, kaempferol and quercetin, as well as the flavanone naringein [7–10], each of which possesses anti-inflammatory, vasodilatory and antioxidant activities [11].

The oxidative modification of LDL appears to play a critical role in the pathogenesis of atherosclerosis [12]. LDL is a heterogeneous molecule composed principally of phospholipids, cholesterol esters, cholesterol and the apolipoprotein (apo) B-100 [13]. In vitro experiments most commonly assess the resistance of LDL to oxidation by measuring lipid peroxidation reactions via the generation of conjugated dienes or thiobarbituric acid-reactive substances, although the sensitivity and specificity of these products are low [14,15]. Importantly, neither of these analytes indicate oxidative damage in apo B-100, although some of its amino acid constituents are susceptible to attack by reactive species, including those generated by Cu^{2+} from Fenton and Haber–Weiss reactions [13,16–18]. Characterizing the effect of antioxidant polyphenolics and vitamins on apo B-100 may provide insight into their

Abbreviations: AE-HPLC, anion exchange chromatography; apo B-100, apolipoprotein B-100; ASP, almond skin polyphenolics; Gp, generalization polarization; nLDL, native LDL; TrpF, tryptophan fluorescence; tLDL, total LDL; VC, vitamin C; VE, vitamin E.

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* Corresponding author. Tel.: +1 617 556 3136; fax: +1 617 556 3344.
E-mail address: oliver.chen@tufts.edu (C.-Y. Chen).
[1] Current address: Department of Food Science and Technology, Kyungpook National University, Daegu, Korea.

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mechanisms of action and interaction to increase the resistance of LDL to oxidation.

Oxidative modification of apo B-100 generates LDL subfractions defined by their degree of electronegativity [19,20], e.g., minimally (LDL−), moderately and extensively modified LDL [21]. While extensively modified LDL does not exist in circulation, native LDL (nLDL) and LDL− have been detected in vivo in healthy subjects, with higher concentrations observed in patients with hyperlipidemia and diabetes [22–24]. LDL− appears particularly atherogenic as it is inversely correlated with particle size and increases with the oxidation of LDL [25]. In contrast to agarose gel electrophoresis, anion exchange, high-performance liquid chromatography (AE-HPLC) can separate LDL subfractions to reveal their different degrees of oxidation.

Tryptophan (Trp) has a low resistance to oxidation, particularly to that induced by hypochlorite [26] or Cu^{2+} [16–18], and a strong fluorescence emission, so the 37 Trp residues in apo B-100 present a tool for assessing protein oxidation in LDL [16,27]. Trp oxidation occurs concurrently but independently of both VE antioxidation and early lipid peroxidation reactions and prior to the end of the lag phase of LDL oxidation [16,17]. Tryptophanyl radicals may also be involved in the initiation of lipid peroxidation in LDL [16–18,28]. Physical elements can also affect the oxidizability of LDL as estradiol, a hormone structurally related to the isoflavones, has been found to enhance the resistance of LDL to Cu^{2+}-induced oxidation by stabilizing apo B-100 conformation [27]. Changes in the conformation of LDL resulting from oxidation, including translocation of more polar, oxidized lipids to the particle surface, have been evaluated using the Laurdan membrane probe [27,29], but this method has not previously been employed to examine the action of flavonoids in this model.

Synergistic antioxidant interactions between polyphenolics and vitamin C (VC) and VE during LDL oxidation have been reported by several laboratories [30–34]. The mechanism(s) by which this synergy occurs is not clear, though chelation, sparing, recycling and other reactions have been suggested. Also, independent actions of these compounds on the oxidation of lipid and protein constituents in the LDL particle might contribute to this relationship. In addition, polyphenolics may stabilize the LDL particle structure via an interaction with the apo-B domain while the antioxidant vitamins act to quench reactive species. To determine the potential contribution of some of these factors to this interaction, we examined the combination of polyphenolics extracted from almond skin (ASP) and a principal ASP flavonoid, quercetin, together with VC or VE on changes in LDL protein oxidation, electronegativity and conformation induced by Cu^{2+} in vitro. While the relevance of Cu^{2+}-induced oxidation to an in vivo milieu is limited [35–37], its characteristics are very well characterized and thus provide a useful model to test our hypothesis [38–40].

2. Materials and methods

2.1. Chemicals and reagents

The following reagents were obtained from Sigma Co. (St. Louis, MO, USA): $CuSO_4$, VC (ascorbic acid), VE (α-tocopherol), NaCl, quercetin, sodium phosphate monobasic, sodium phosphate dibasic, butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA) and Folin Ciocalteu's phenol reagent. The Laurdan probe (6-dodecanoyl-2-dimethylaminonaphthalene) was purchased from Invitrogen (Carlsbad, CA, USA). All organic solvents, glacial acetic acid and KBr were purchased from Fisher Co. (Fair Lawn, NJ, USA). High-grade NaCl, Tris-HCl and Tris-base used for AE-HPLC were obtained from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Extraction of polyphenolics from almond skins

ASP were extracted with glacial acetic acid/H_2O/methanol (3.7:46.3:50, v/v/v) by sequential extraction over 16 h at 4°C followed by concentration via evaporation under N_2 [30]. Aliquots of dried ASP residue were stored at −20°C and used within 30 days. Total phenols were assessed by the Folin-Ciocalteu's reaction according to Singleton et al. [41] and expressed as micromoles per liter of gallic acid equivalents.

2.3. Collection of human LDL

Venous blood was collection into vacutainers containing EDTA from 10 nonfasted healthy volunteers at 1400 hours with a procedure approved by the Tufts/New England Medical Center Human Investigations Review Committee. Samples were centrifuged at 1000×*g* for 20 min at 4°C, pooled and mixed with sucrose (0.6% final concentration), aliquoted and stored at −80°C, and then used within 60 days [42]. On the day before use, LDL (1.019–1.063 g/ml) was collected from the frozen plasma according to Chung et al. [43] using a Beckman NVT-90 rotor in a Beckman L8-M centrifuge (Palo Alto, CA, USA). Harvested LDL was stored at 4°C in the dark under N_2 and used within 2 days. Prior to assay, NaCl and EDTA were removed using a PD-10 column (Amersham Pharmacia Biotech, Sweden). The concentration of LDL was calculated from the protein content of the sample, determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

2.4. LDL lag time conjugated diene assay

LDL oxidation induced by Cu^{2+} was performed in triplicate according to a slight modification of the method described by Esterbauer et al. [15]. Briefly, 100 μg LDL (182 nmol) was oxidized by 10 μmol/L $CuSO_4$ in a total volume of 1.0 ml phosphate buffered saline (PBS: 7.79 mmol/L Na_2HPO_4, 2.59 mmol/L NaH_2PO_4 and 150 mmol/L NaCl, pH 7.4). Formation of conjugated dienes was monitored by absorbance at 234 nm at 37°C over 6 h using a Shimadzu UV1601 spectrophotometer (Japan) equipped with a six-position automated sample changer. The results

of assay are expressed as lag time, the intercept at the abscissa in the diene-time plot [19].

2.5. Antioxidant addition to LDL

An aliquot of ASP residue was redissolved in PBS and added to the assays in concentrations reflecting those that can be obtained in vivo from dietary intake. VE was dissolved in methanol (final concentration 0.5%) and diluted with PBS. ASP and VE were incubated with LDL at 37°C for 30 min before initiation of oxidation. VC was dissolved in PBS and added to the mixture immediately prior to initiation of oxidation by Cu^{2+}.

2.6. nLDL and LDL− separation by AE-HPLC

Separation of nLDL and LDL− was accomplished using AE-HPLC with a multistep gradient of Tris saline buffer as described by Vedie et al. [21]. Briefly, Cu^{2+}-induced LDL oxidation was terminated by addition of 50 μmol/L ETDA and 20 μmol/L BHT (final concentrations). Subsequently, the LDL was dialyzed against 10 mmol/L Tris buffer containing 10 μmol/L EDTA (pH 7.4) at 4°C in the dark for ≥18 h. After filtration with a 0.45-μm PVDF filter (Millipore, Bedford, MA, USA), 100 μg LDL was manually injected into the HPLC and nLDL and LDL− subfraction monitored at 280 nm. The proportion of LDL−, as the ratio of LDL−/total LDL (tLDL) expressed as a percent value, where tLDL=nLDL+LDL−, was calculated using the area under curve of the trace derived from each peak. Fig. 1 shows the ratio of LDL−/tLDL increasing from 0–300 min of the oxidation reaction. The small peak present at a retention time of 22.5 min was not an electronegative form of LDL because it is also present in the blank. The ratio of LDL−/tLDL after 5 h of Cu^{2+}-induced oxidation at 95.9±0.6% was designated to reveal the effect of in vitro antioxidant addition in this study.

2.7. Intrinsic Trp fluorescence

Intrinsic Trp fluorescence (TrpF) was determined by the method of Brunelli et al. [27]. Briefly, 50 μg LDL was oxidized with 5 μmol/L Cu^{2+} in a total volume of 1 ml and the reaction terminated at selected time points by addition of EDTA and BHT as described above. TrpF emission spectra were equilibrated after 15 min at room temperature and then recorded at 295 nm excitation and 330 nm emission with an LS-5 fluorescence spectrophotometer (Perkin-Elmer, Wellesley, MA, USA). The addition of ASP, quercetin, and VC and VE to LDL without Cu^{2+}-induced oxidation did not alter TrpF intensity. Results are presented as the percent of maximum TrpF obtained from LDL without Cu^{2+}-induced oxidation.

2.8. Generalized polarization of Laurdan

The generalized polarization (Gp) value of Laurdan was determined according to the methods of Parasassi et al. [44] and Dousset et al. [45]. Briefly, the Laurdan probe was dissolved in dimethylsulfoxide (DMSO) and stored at −20°C. The protocol for LDL oxidation was the same as described above for TrpF. Following addition of ETDA and BHT for termination of oxidation, LDL (50 μg protein/ml) at room temperature was labeled with Laurdan in DMSO at a final concentration of 0.375 μmol/L. The mixture then was incubated for 50 min at room temperature in the dark and the fluorescence emission spectra recorded between 400 and 500 nm with excitation at 360 nm using an LS-50B fluorescence spectrophotometer (Perkin Elmer, Wellesley, MA, USA). The Gp value was calculated according to Parasassi et al. [44] as (I440−I490)/(I440+I490), where I440 and I490 are the emission intensities at 440 and 490 nm, respectively.

2.9. Statistical analysis

All results were reported as mean±S.E. The Tukey–Kramer honestly significant difference test was used in experiments testing the ASP dose on lag time, LDL−/tLDL, TrpF and Gp after significant differences were obtained by

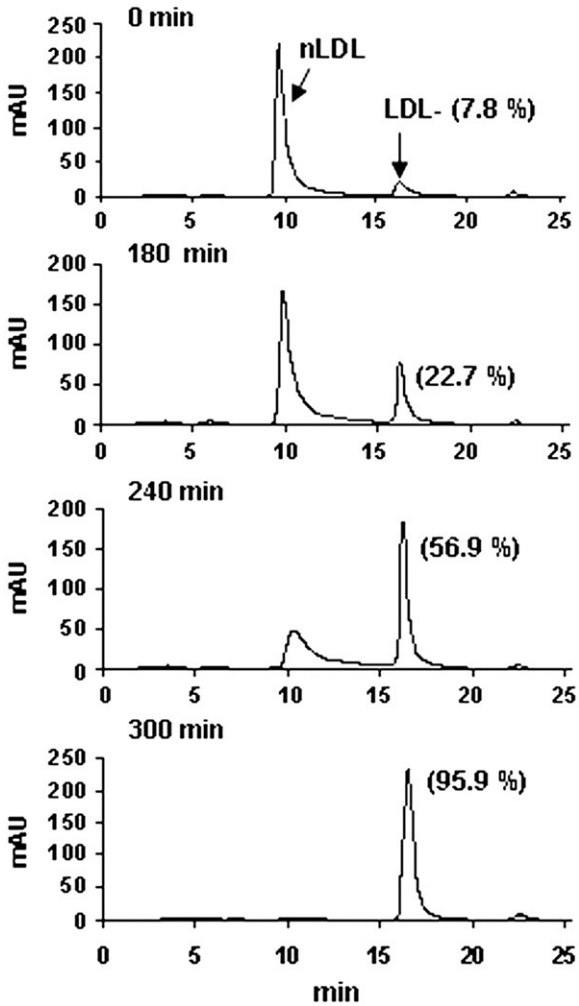

Fig. 1. The time course of LDL−/tLDL. nLDL and LDL− subfractions were eluted on an anion exchange column and monitored at 280 nm. Numbers in parentheses represent the ratio of LDL−/tLDL where tLDL=nLDL+LDL−.

Table 1
The effect of combined polyphenolics and vitamin C or E on lag time to Cu^{2+}-induced LDL oxidation[1]

Treatment (μmol/L)	Control	ASP		Quercetin	
		0.25	0.5	0.25	0.5
	Lag time, min				
None	56.4±0.44	62.5±0.5	69.5±0.3	62.8±0.3	66.7±1.7
VC, 0.5	61.8±0.3	70.5±2.5 (1.04)	78.3±1.5* (1.05)	75.8±0.9 (1.11[a])	86.0±1.5 (1.19[a])
VC, 2	72.7±1.2	81.3±0.7† (1.03)	93.8±0.7* (1.09[a])	89.8±1.6 (1.14[a])	101.0±3.3 (1.22[a])
VE, 0.5	58.5±0.3	67.5±1.4 (1.04)	73.3±1.6 (1.02)	64.5±0.3 (0.99)	76.0±1.3 (1.10[a])
VE, 2	78.2±1.3	87±2.5 (1.03)	102.2±4.3 (1.12[a])	90.0±1.3 (1.06[a])	100.5±3.9 (1.14[a])

[a] Interaction between ASP or quercetin and antioxidant vitamin is synergistic at $P \leq .05$.
* $P \leq .05$ and † $P \leq .01$ for means differing between ASP and quercetin at the same combined doses.
[1] Numbers in parentheses indicate ratio of actual observation to calculated sum of each antioxidant alone. Numbers >1.0 indicate synergy.

one-way ANOVA. A Student *t*-test was performed to determine the difference in measured parameters between ASP and quercetin at the same concentrations, as well as their combination with VC or VE. A Student *t*-test also was employed to determine the significance of the synergistic or antagonistic interactions between flavonoids and VC and VE by comparing the observed (actual) values with the expected (calculated) sums from polyphenolics and vitamins alone. The ratios between actual and calculated values are presented in parentheses in Tables 1–4 to indicate an additive, synergistic or antagonistic effect. The overall difference in magnitude of synergism generated between all the concentrations of ASP and quercetin and vitamins was evaluated with the Student *t*-test. Means of LDL−/tLDL and lag time obtained from ASP, quercetin, vitamins and their combinations were included in a Pearson's correlation test. Differences with $P \leq .05$ were considered significant. The JMP IN 4 statistical software package (SAS Institute Inc., Cary, NC, USA) was used to perform all statistical analyses.

3. Results

3.1. Effects of ASP on lag time of LDL oxidation

ASP at 0.25 to 2 μmol/L reduced the susceptibility of LDL to Cu^{2+}-induced oxidation in a dose-dependent and linear manner ($R^2=0.97$, $P \leq .0001$) (Fig. 2A). Lag times of LDL oxidation with both ASP and the aglycone quercetin at 0.25 and 0.50 μmol/L were comparable (Table 1). ASP at 0.5 μmol/L plus VC or VE at 2 μmol/L synergistically increased lag time by 9% and 12%, respectively, over the calculated sums of lag time of each antioxidant alone ($P \leq .05$). Quercetin at 0.25 and 0.5 μmol/L plus VC or VE at 0.5 and 2 μmol/L also synergistically increased lag time by 6–22% over the calculated sums of lag time of each antioxidant alone ($P \leq .05$). ASP at 0.25 and 0.5 μmol/L plus 2 μmol/L VC had significantly shorter lag time than quercetin plus VC at the same combined doses (81.3±0.7 vs. 89.8±1.6 and 93.8±0.7 vs. 101.0±3.3 min, respectively, $P \leq .05$). Combination of ASP or quercetin with VE had comparable lag times.

3.2. Effect of ASP on LDL−/tLDL

nLDL and LDL− subfractions were separated using an AE-HPLC. The amount of LDL− subfraction was 7.8% of tLDL obtained from LDL samples without in vitro oxidant challenges. With Cu^{2+}-induced oxidation, the amount of LDL− subfraction was enhanced from 7.8% to 95.9% in a time-dependent fashion (Fig. 1). With addition of ASP at concentrations of 0.25 to 2 μmol/L, LDL− formation was diminished in a dose-dependent manner (Fig. 2A). There was a negative association between lag time increase and the ratio of LDL−/tLDL ($R^2=0.51$, $P \leq .0001$) (Fig. 2B). At concentrations of 0.25 and 0.5 μmol/L, ASP treatment yielded smaller ratios of LDL−/tLDL than quercetin did at the same doses ($P \leq .005$) (Table 2).

Table 2
The effect of combined polyphenolics and vitamin C or E on LDL−/tLDL ratio after 300 min of Cu^{2+}-induced oxidation[1]

Treatment, μmol/L	Control	ASP		Quercetin	
		0.25	0.5	0.25	0.5
	LDL−/tLDL, %				
None	95.9±0.6	59.2±1.3*	46.9±2.6*	79.5±3.0	60.3±0.3 %
VC, 0.5	77.3±2.6	29.6±0.5* (0.74[a])	27.5±0.4* (0.97)	45.1±0.1 (0.74[a])	33.5±0.6 (0.80[a])
VC, 2	59.8±1.0	26.9±0.5* (1.27)	26.3±0.3* (2.90[a])	33.2±0.9 (0.78[a])	32.7±0.4 (1.36[a])
VE, 0.5	87.0±1.4	38.4±1.5 (0.76[a])	26.9±0.6* (0.71[a])	40.9±0.2 (0.58[a])	32.2±0.9 (0.65[a])
VE, 2	79.0±1.1	24.9±0.5* (0.57[a])	23.1±0.1 (0.75[a])	33.3±0.3 (0.5[a])	22.8±1.4 (0.5[a])

[a] Interaction between ASP or quercetin and antioxidant vitamin is synergistic at $P \leq .05$.
* $P \leq 005$ for means differing between ASP and quercetin at the same combined doses.
[1] Numbers in parentheses indicate ratio of actual observation to calculated sum of each antioxidant alone. Numbers <1.0 indicate synergy.

Table 3
The effect of combined polyphenolics and vitamin C or E on reduction of LDL TrpF after 90 min of Cu^{2+}-induced oxidation[1]

Treatment, μmol/L	Control	ASP		Quercetin	
		0.12	0.24	0.12	0.24
	% Reduction of initial TrpF				
None	50.6±0.7	54.0±0.9	62.6±0.9*	57.3±1.6	66.8±0.6
VC, 0.5	55.7 ±1.4	73.8±2.0† (1.25[a])	76.0±1.2 (1.12[a])	63.5±0.8 (1.02)	75.5±0.5 (1.05)
VC, 2	61.6±2.1	78.0±0.9† (1. 20[a])	81.2±0.9 (1.10[a])	68.2±1.0 (1.00)	79.3±1.1 (1.02)
VE, 0.5	55.0±1.4	61.9±0.4* (1.06)	66.8±0.4† (1.00)	65.5±0.8 (1.06)	71.7±0.1 (1.01)
VE, 2	62.4±1.1	69.6±1.4 (1.06)	74.6±0.5 (1.00)	71.3±0.4 (1.03)	75.1±0.7 (0.96)

[a] Interaction between ASP or quercetin and antioxidant vitamin is synergistic at $P \leq .05$.
* $P \leq .05$ and † $P \leq .01$ for means differing between ASP and quercetin at the same combined doses.
[1] Numbers in parentheses indicate ratio of actual observation to calculated sum of each antioxidant alone. Numbers >1.0 indicate synergy.

VC and VE, each at 0.5 and 2.0 μmol/L, attenuated LDL−/tLDL (Table 2). ASP at 0.5 μmol/L plus 0.5 μmol/L VC significantly decreased LDL−/tLDL by 26% over the expected ratio calculated from each antioxidant alone ($P \leq .05$). However, ASP at 0.5 μmol/L plus 2 μmol/L VC enhanced LDL−/tLDL by 190% ($P \leq .05$). A synergism between the polyphenolics and vitamins was apparent when the ratio of actual measurement of LDL−/tLDL and calculated sum of each treatment alone was significantly smaller than 1.0. ASP at 0.25 and 0.5 μmol/L plus VE at 0.5 or 2 μmol/L reduced LDL−/tLDL by 24–43% over the expected ratio calculated from each antioxidant alone ($P \leq .05$). Quercetin at 0.25 and 0.5 μmol/L plus VC or VE at 0.5 or 2 μmol/L significantly reduced LDL−/tLDL by 35–50% over the expected ratio calculated from each antioxidant alone ($P \leq .05$). Combined ASP at 0.25 and 0.5 μmol/L and VC at 0.5 or 2 μmol/L generated smaller ratios of LDL−/tLDL than quercetin plus VC at the same combined doses ($P \leq .005$). ASP plus VE at two combined concentrations (0.5±0.5 and 0.25±2 μmol/L) had smaller ratios of LDL−/tLDL than quercetin plus VE at the same combined doses ($P \leq .005$).

3.3. Tryptophan fluorescence decay during LDL oxidation

There were two phases of TrpF reduction during 120 min of Cu^{2+}-induced LDL oxidation (Fig. 3). TrpF was not influenced by added antioxidants. VE or VC did not affect the first phase of reduction although they did decelerate the rate of the second phase (data not shown). ASP at concentrations of 0.12 to 0.96 μmol/L diminished the rate of TrpF reduction in a dose-dependent manner (Fig. 3). While ASP at 0.12 to 0.48 μmol/L did not protect Trp from oxidation, ASP at 0.96 μmol/L did delay the first phase of TrpF attenuation. As low doses of these antioxidants did not protect Trp during the initial phase of LDL oxidation, we compared the effect of ASP and quercetin alone and in combination with VC or VE on TrpF decay 90 min after initiation of oxidation. Although quercetin is a principal component of ASP, 0.24 μmol/L quercetin provided a greater protection of Trp residues than ASP at the same concentration (66.8±0.6% vs. 62.6±0.9%, respectively, $P \leq .05$), while no difference was observed at a concentration of 0.12 μmol/L (57.3±1.6% vs. 54.0±0.9%) (Table 3). ASP at 0.12 and 0.24 μmol/L plus VC at 0.5 or 2 μmol/L had a larger synergistic protection of Trp by 10–25% than expected protection calculated from each antioxidant alone ($P \leq .05$), while only an additive effect was noticed between ASP and VE. Quercetin plus VE or VC showed an additive protection of Trp against oxidation. ASP at 0.12 μmol/L plus VC 0.5 or 2 μmol/L had a larger protection of Trp than quercetin plus VC at the same combined doses ($P \leq .01$). However, ASP at 0.12 or 0.24 μmol/L plus 0.5 μmol/L VE had a smaller protection of Trp than quercetin plus VE at the same combined doses ($P \leq .05$).

3.4. Changes in LDL polarity and molecular order during oxidation

The Gp value was increased from 0.607±0.001 at 0 min to 0.653±0.002 after 120 min of Cu^{2+}-induced oxidation

Table 4
The effect of combined polyphenolics and vitamin C or E on LDL Gp values after 90 min of Cu^{2+}-induced oxidation[1]

Treatment, μmol/L	Control	ASP		Quercetin	
		0.12	0.24	0.12	0.24
	Gp				
None	0.653±0.002	0.637±0.003	0.627±0.0003	0.636±0.003	0.622±0.003
VC, 0.5	0.632±0.006	0.616±0.003* (0.99)	0.602±0.004 (0.99)	0.623±0.000 (1.00)	0.609±0.002 (1.00)
VC, 2	0.623±0.003	0.607±0.001† (0.99)	0.609±0.003 (1.00)	0.619±0.002 (1.00)	0.611±0.001 (1.02[a])
VE, 0.5	0.640±0.001	0.628±0.001† (0.99)	0.623±0.002* (0.98)	0.612±0.003 (0.97[a])	0.609±0.004 (0.99)
VE, 2	0.633±0.004	0.622±0.005* (0.99)	0.611±0.003 (1.01)	0.607±0.002 (0.97[a])	0.605±0.004 (0.99)

[a] Interaction between ASP or quercetin and antioxidant vitamin is synergistic at $P \leq .05$.
* $P \leq .05$ and † $P \leq .01$ for means differing between ASP and quercetin at the same combined doses.
[1] Numbers in parentheses indicate ratio of actual observation to calculated sum of each antioxidant alone. Numbers <1.0 indicate synergy.

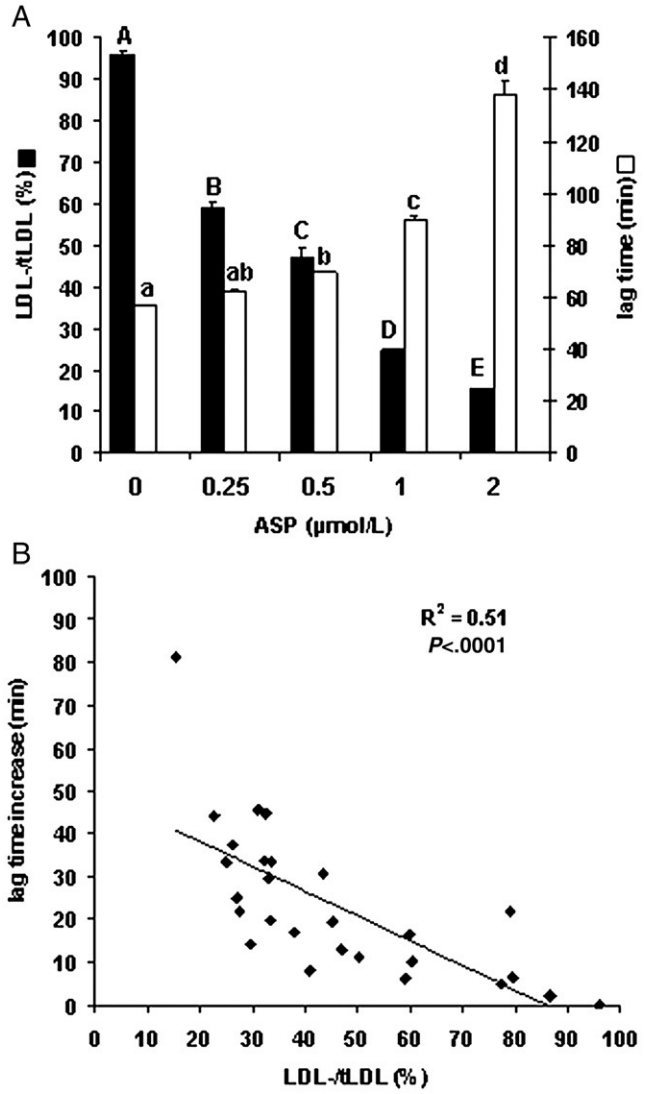

Fig. 2. (A) Effect of ASP on LDL−/tLDL and LDL lag time after Cu^{2+}-induced oxidation of LDL. Black and white bars illustrate LDL−/tLDL and lag time, respectively. Values are mean±S.E. ($n=3$). Means in each category without a common letter differ ($P\leq.0001$). (B) Correlation between lag time and LDL−/tLDL.

(Fig. 4). While ASP alone did not affect the Gp value of LDL in the absence of oxidation, it significantly reduced the increase in Gp at 90 and 120 min of oxidation in a dose-dependent manner (Fig. 4). ASP at 0.96 μmol/L maintained Gp constant in spite of an oxidant challenge. VC and VE at 0.5 and 2 μmol/L reduced Gp values at 90 min of LDL oxidation (Table 4). The combination of ASP with antioxidant vitamins only had an additive effect on Gp values. Quercetin at 0.12 μmol/L plus VE at 0.5 or 2 μmol/L synergistically reduced Gp increase by 3% over the expected values calculated from each antioxidant alone ($P\leq.05$). ASP at 0.12 μmol/L plus VC at 0.5 or 2 μmol/L had smaller Gp values than quercetin plus VC at the same combined doses ($P\leq.05$ and $P\leq.01$, respectively). ASP

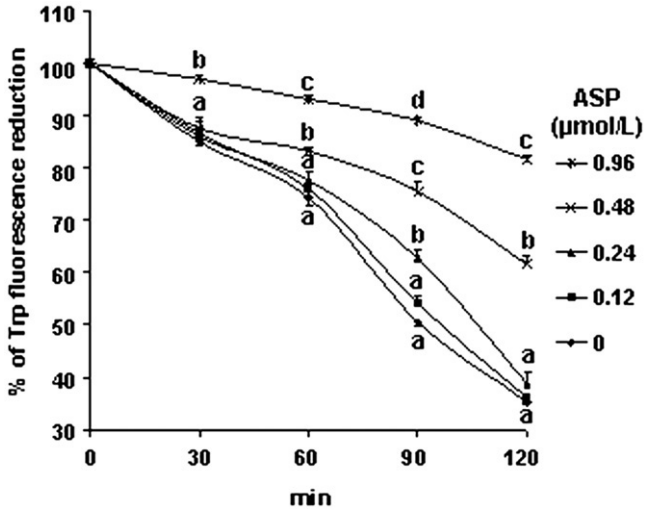

Fig. 3. Effect of ASP on reduction in LDL TrpF after 30, 60, 90 and 120 min of Cu^{2+}-induced oxidation. Values are mean±S.E. ($n=3$). Means at each time point without a common letter differ ($P\leq.05$).

plus VE at all tested combined doses, except 0.24 plus 2 μmol/L, had larger Gp than quercetin plus VE ($P\leq.05$).

4. Discussion

Polyphenolics can exert a direct antioxidant action via scavenging reactive species or chelating transition minerals, although it is important to recognize their ability to act indirectly by affecting signal transduction and gene expression pathways [9]. Almond skins are particularly rich in polyphenolics, including a variety of flavonoids that have been demonstrated to enhance the resistance of LDL to lipid peroxidation [9,30]. Their apparent antioxidant synergy with VC and VE led us to test their actions, as well as that of pure

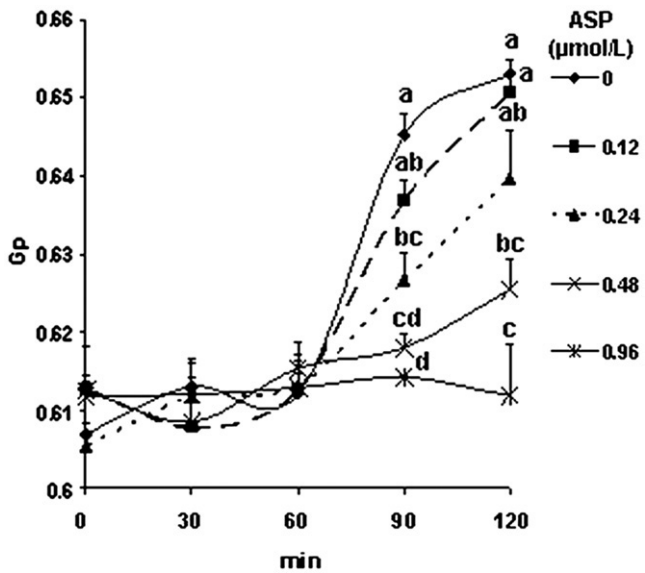

Fig. 4. Effect of ASP on increases of LDL Gp value after 30, 60, 90 and 120 min of Cu^{2+}-induced oxidation. Values are mean±S.E. ($n=3$). Means at each time point without a common letter differ ($P\leq.05$).

quercetin, on LDL protein oxidation, electronegativity and conformation induced by Cu^{2+} in vitro. We did not examine chelation as a potential mechanism of action in this model as the ASP and quercetin concentrations were negligible compared to the added Cu^{2+}.

Confirming earlier reports [9,30], ASP reduced the susceptibility of LDL to Cu^{2+}-induced oxidation in a dose-dependent and linear manner. Flavonoid aglycones appear to possess greater antioxidant potency than their corresponding glycones [46,47]. Nonetheless, we noted a comparable lag time of LDL with both ASP and the aglycone quercetin at 0.25 and 0.50 μmol/L, although majority of ASP are glycones [10]. Interestingly, Yeomans et al. [48] found that combining two different types of flavonoid aglycones increased LDL lag time beyond the calculated sum of the individual compounds.

ASP interact in a synergistic fashion with VC or VE to extend the lag time of LDL oxidation [30], which was confirmed by the results of this study. Although ASP and quercetin each produced comparable lag times of LDL, quercetin plus VC provided a 10% longer lag time than ASP plus VC. Furthermore, the synergy observed between quercetin and VC at 11–22% was greater than that found between ASP and VC at 3–9%. However, there was no difference in lag time between combining ASP or quercetin with VE. Thus, within physiologically relevant concentrations, these results suggest the presence and magnitude of synergy are dependent on the type of polyphenolics or specific flavonoid and antioxidant vitamin [48].

Byproducts of lipid peroxidation can modify apo B-100, especially on positively charged lysine residues, and generate more electronegative LDL subfractions. Comina-cini et al. [49] suggested that such derivatization of apo B-100 is likely initiated only following the lag phase of LDL oxidation. Indeed, with a 56.4-min lag time, new LDL− generation after the first hour of Cu^{2+}-induced oxidation was not evident in our experiments, although the LDL−/tLDL ratio increased from 7.8% to 22.7% after 180 min of Cu^{2+}-induced oxidation. Consistent with findings by Vedie et al. [21], the amount of LDL− formed during oxidation was enhanced in a time-dependent fashion. Furthermore, ASP between 0.25 and 2.0 μmol/L diminished generation of LDL− in a dose-dependent manner. Although ASP and quercetin at the same concentrations resulted in a comparable lag time, ASP treatment yielded smaller ratios of LDL−/tLDL than quercetin at the same concentration. A simple antioxidant action on lipid peroxidation does not appear to explain the difference between ASP and quercetin on LDL−/tLDL, although there was an inverse correlation between this ratio and lag time (R^2=0.51). Of the change in LDL−/tLDL, 49% is unexplained by inhibition of lipid peroxidation and, thus, may result from an influence of these polyphenolics on protein oxidation and LDL conformation.

The combination of ASP or quercetin and these vitamins further decreased LDL−/tLDL compared to their individual actions. While greater reductions in LDL−/tLDL were observed with ASP than with quercetin, ASP plus VC tended to have smaller ratios than quercetin plus VC. Combined with VE, ASP still tended to be more potent in lowering LDL−/tLDL than quercetin. However, these results are in contrast to the respective results with the determination of lag time, where quercetin plus VC or VE had a larger impact than ASP combined with these vitamins. These results suggest the total polyphenolics in almond skin possess less potent antioxidant capacity than pure quercetin, but are more effective in inhibiting modification of apo B-100.

A synergism between the polyphenolics and antioxidant vitamins was also apparent on LDL− generation during LDL oxidation. The synergy between quercetin and VE overall was larger than that between ASP and VE, but no significant differences were observed with the combination of polyphenolics and VC. Interestingly, an antagonistic effect on LDL−/tLDL was observed between the ASP or quercetin treatment (at 0.5 μmol/L) and VC (at 2.0 μmol/L). Nonetheless, these ratios were still smaller than those obtained from polyphenolics or VC alone. While further investigations are required to elucidate the mechanism for this apparent antagonism between polyphenolics and VC, our results demonstrate that ASP and quercetin at physiological concentrations protect nLDL from becoming more electronegative.

Oxidation of apo B-100 by Cu^{2+} yields derivatized products of oxidized amino acids such as Trp hydroperoxides that are capable of initiating lipid peroxidation [16–18]. Consistent with the report by Giessauf et al. [16], we found two phases of TrpF reduction during 120 min of Cu^{2+}-induced LDL oxidation. Flavonoids have been found to inhibit Trp oxidation and repair Trp radicals during LDL oxidation [50,51]. Although we did not determine the capacity of ASP to restore oxidized Trp, 0.96 μmol/L ASP did delay the first phase of TrpF attenuation, suggesting that ASP and antioxidant vitamins may protect Trp from oxidation via different mechanisms, e.g., with the greater potency of polyphenolics due to their binding in close proximity to Trp [50]. Protection of ASP against Trp oxidation was dose dependent. Although quercetin is a principal component of ASP [10], 0.24 μmol/L quercetin provided greater protection of Trp residues than ASP at the same concentration. These results suggest that flavonoids as glycones may be less effective in protecting Trp than as aglycones.

The antioxidant synergy between polyphenolics and VC or VE on lag time of LDL oxidation might arise from an action on apo B-100 centered Trp radicals initiating lipid peroxidation reactions. Milde et al. [31] found a synergistic protection of Trp against Cu^{2+}-induced oxidation by rutin plus VC. However, we observed additive effects only between quercetin and VC. Quercetin (0.12 and 0.24 μmol/L) plus VE (0.5 μmol/L) was more potent in preventing Trp oxidation than ASP plus VE, but neither

reaction was synergistic in nature. These results suggest that ASP constituents other than quercetin are providing the synergy found with VC on the protection of Trp from oxidation. However, it is unclear why such synergy on TrpF oxidation was not obtained with ASP and VE. Further investigation is required to determine whether this synergy is a function of the compounds themselves and/or the doses in which they are tested. While no synergism between the polyphenolics and vitamins was observed at the highest concentrations tested of each, the lower concentrations may prove to be more physiologically relevant.

The conformation and integrity of lipoproteins can influence their oxidizability as measured by conjugated diene formation [13,27,52]. The Laurdan probe has been used to monitor molecular order and polarity in the external lipid phase of LDL by anchoring its lauric tail into the hydrophobic core and leaving a polar naphthalene in residence at the level of the phospholipid glycerol backbone [27,44]. Ferretti et al. [53] reported the isoflavone genistein, in addition to serving as a radical scavenger, acted to maintain the integrity of HDL during oxidation. We found that Gp value increased along with length of LDL oxidation, reflecting movement of the probe deeper into the hydrophobic core [27]. While ASP did not affect the Gp of LDL in the absence of oxidation, it significantly reduced the increase in Gp at 90 and 120 min of oxidation in a dose-dependent manner, consistent with experiments testing estradiol [27].

Consistent with their comparable effects on LDL lag time, ASP and quercetin generated similar Gp values after 90 min of LDL oxidation. However, a lower Gp value was associated with ASP (0.12 μmol/L) plus VC (0.5–2.0 μmol/L) than quercetin plus VC at the same concentrations, indicating the former combination to be more effective in stabilizing LDL conformation during oxidation. However, this difference in Gp was lost when ASP or quercetin was added at 0.24 μmol/L with VC, possibly due to maintenance of LDL conformation such that a substantial oxidation and molecular rearrangement in the particle was not achieved. In contrast to the synergy observed in the other parameters described above, only additive interactions between polyphenolics and VC were noted with regard to Gp. The absence of synergy on measures of Gp suggests that structural stabilization by the combination of ASP and antioxidant vitamins is not a significant factor in the observed synergism of their extending LDL lag time.

In summary, at physiologically relevant concentrations and in a dose-dependent manner, ASP protect LDL against oxidative attack by Cu^{2+} by quenching free radicals, preventing apo B-100 modification and maintaining the integrity of the molecular order and polarity of its lipid phase. Quercetin, a principal flavonoid component of ASP, provided similar activity, though the natural mixture of polyphenolics in almond skins appears more potent in preventing apo B-100 modification (LDL− formation), while quercetin is more effective at inhibiting protein (Trp) oxidation. We have reported a synergism between ASP and antioxidant vitamins with regard to LDL lag time and extend this observation here to suggest an underlying mechanism may include interactions that delay Trp oxidation. Accessibility of antioxidants with different binding or partition characteristics to individual constituents in LDL, e.g., specific phospholipids and amino acids, may enhance the overall antioxidant capacity of the mixture to achieve synergy.

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