Red Wine Antioxidants Bind to Human Lipoproteins and Protect Them from Metal Ion-Dependent and -Independent Oxidation

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Plant-derived polyphenols may exert beneficial effects on atherosclerosis and cardiovascular diseases, in part, because of their antioxidant properties. In this study we compared the effects of unbound (free) and lipoprotein-associated red wine components on in vitro antioxidant protection of human low-density lipoprotein (LDL). Preincubation of LDL (1 mg protein/mL) with 0−2.5% (v/v) red wine for 3 h at 37 °C followed by gel filtration to remove unbound red wine components resulted in a dose-dependent, up to 4-fold increase in LDL-associated antioxidant capacity (measured as Trolox equivalents). Similar results were obtained with high-density lipoprotein (HDL) and bovine serum albumin (BSA). Furthermore, LDL was subjected to oxidation by copper and aqueous peroxyl radicals (2,2′-azobis[2-amidinopropane] dihydrochloride, AAPH). Under both types of oxidative stress, LDL-associated and free red wine components significantly decreased oxidation of the lipoprotein's protein moiety (assessed by tryptophan fluorescence) and lipid moiety (assessed by thiobarbituric acid-reactive substances and conjugated dienes). Similar protective effects of red wine components were observed against HDL oxidation. In contrast, red wine exerted a pro-oxidant effect on copper-induced oxidation of BSA tryptophan residues, while protecting them from AAPH-induced oxidation. Ascorbate strongly enhanced the protective effect of red wine against copper-induced LDL oxidation, and had an additive effect against AAPH-induced oxidation. Our data indicate that red wine components bind to LDL and HDL and protect these lipoproteins from metal ion-dependent and -independent protein and lipid oxidation.

Keywords: *Albumin; copper; high-density lipoprotein; low-density lipoprotein; oxidation; peroxyl radicals; red wine*

INTRODUCTION

Increased consumption of plant-derived polyphenols has been associated with a reduced risk of cardiovascular diseases, cancer, and other chronic diseases (*1*−*3*). Red wine is a significant natural source of polyphenols in Mediterranean-type diets, contributing as much as one gram of polyphenols per day from moderate wine consumption (*4*). Red wine polyphenols as a natural mixture and individual red wine polyphenols, such as quercetin and resveratrol, exert a range of biological activities in different experimental systems (*5*−*7*).

Several in vitro studies have shown that red wine components can prevent free radical-induced oxidation of low-density lipoprotein (LDL), an important step in the development of atherosclerotic lesions (*8*−*10*). Dietary supplementation of experimental animals and humans with red wine increases the total plasma antioxidant status and the resistance of plasma-derived LDL to ex vivo oxidation (*11*−*13*). These results indicate that red wine components are absorbed into the blood stream and interact with plasma lipoproteins.

Despite the large number of studies investigating the antioxidant effects of plant-derived polyphenols on LDL oxidation, the underlying mechanism(s) remain incompletely understood. Polyphenols may act as free radical scavengers in the aqueous phase or metal ion chelators, thereby preventing the formation of free radicals (*8*, *10*). Alternatively, polyphenols may be able to protect LDL from oxidative damage by modification of the lipoprotein particle. Although different polyphenols have been demonstrated to associate with proteins and liposomes through hydrophobic interactions and hydrogen bonding (*14*, *15*), the data are limited and inconsistent. Red wine polyphenols and resveratrol also can associate with LDL in vitro (*16*). However, it is unclear whether polyphenols bind to the protein or lipid moiety of LDL, and whether their association can provide site-specific protection of LDL components from oxidation induced by free radicals.

To address these questions, we investigated the effects of both LDL-associated and unbound (free) red wine components on LDL oxidation by transition metal ion-dependent and -independent mechanisms. We found that both preincubation and co-incubation of LDL with red wine decreases oxidative modification of the lipid and protein moiety of LDL. These effects were observed in both the copper- and peroxyl radical-dependent models of oxidative stress. The effects of red wine were not specific to LDL, but were also observed for high-density lipoprotein (HDL).

MATERIALS AND METHODS

Materials. The red wine used in this study was a California Cabernet-Sauvignon (Turning Leaf, 1996, 12% alcohol). After a bottle was opened, the wine was divided into 1-mL aliquots and stored under nitrogen at 4 °C for up to two weeks. The total phenol content was 1.87 g/L as determined by the method of Singleton et al. (*17*), using quercetin as a standard. 2,2′-

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Azobis(2-amidinopropane) dihydrochloride (AAPH) was from Wako Chemical Company (Richmond, VA). (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was supplied by Fluka (Milwaukee, WI). All other chemicals were from Sigma (St. Louis, MO). Deionized water and buffers were stored over 2 g/L of the chelating resin Chelex 100 (Bio-Rad, Richmond, CA) to remove metal contaminants. Phosphate-buffered saline (PBS) was made up of 10 mM phosphate and 0.15 M NaCl and had a pH of 7.4.

Lipoprotein Preparation. LDL and HDL were prepared from blood plasma of healthy human volunteers by two-step ultracentrifugation as described previously (*18*). Lipoproteins and lipoprotein-deficient plasma (LDP, bottom fraction after first ultracentrifugation step) were transferred to PBS containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) by two sequential filtration steps using PD-10 columns (Pharmacia Biotech, Uppsala, Sweden). The samples were stored at 4 °C under nitrogen for up to one week. Protein content was assayed by the Lowry Micro Method kit (Sigma P5656). Prior to experiments, DTPA was removed from the LDL and HDL samples by passing them through Bio-Rad EconoPack P6 desalting columns (Bio-Rad Laboratories, Hercules, CA) equilibrated with PBS.

Antioxidant Capacity. The antioxidant capacity of lipoproteins and red wine was assayed with a luminometer Photochem system (F. A. T. GmbH, Berlin, Germany) as described by Popov and Lewin (*19*). Briefly, the experimental mixture consisted of 0.1 M carbonate buffer, pH 10.5, 0.05 mM EDTA, and 0.03 mM luminol. UV irradiation of luminol results in the generation of free radicals, predominantly superoxide anions, which in turn induce luminol chemiluminescence. Light emission was detected, and its intensity was recorded for 60 s at one-second intervals. Addition of antioxidants causes a concentration-dependent decrease in the slope and maximum intensity of the kinetic light emission curve, which was described by the area-under-the-curve ("integral") parameter using Poplab 2.0 software (F. A. T. GmbH). The antioxidant capacity of duplicate experimental samples was calibrated against a Trolox standard (20−100 nM) and expressed as Trolox-equivalent antioxidant capacity (TEAC). The red wine used in this study had a TEAC of 4.2 mM.

To evaluate the effects of red wine components on the lipoprotein-associated antioxidant capacity of LDL, HDL, LDP, and bovine serum albumin (BSA, fraction V, Sigma), triplicate samples were incubated in PBS for 3 h at 37 °C at a concentration of 1 mg protein/mL (for LDL and HDL) or 2 mg protein/mL (for LDP and BSA) with the indicated dilutions (v/v) of red wine. The highest corresponding concentration of ethanol was added to control samples. Incubations were terminated by passing 50 μL of the sample through Bio-Rad Micro Bio-Spin P-6 columns equilibrated with PBS, in accordance with the manufacturer's instructions. The desalted samples were then assayed for antioxidant capacity as described above. Addition of ethanol to control samples at concentrations corresponding to those in samples containing wine, followed by incubation for 3 h at 37 °C, did not alter the lipoprotein-associated antioxidant capacity, as compared to ethanol-free lipoprotein samples kept at 4 °C. The experimental results were corrected for "contamination" with red wine antioxidants not associated with the lipoproteins, as determined by passing an appropriately diluted wine sample, in the absence of lipoproteins, through a Micro Bio-Spin P-6 column, followed by determination of the eluate's TEAC value (detection limit, 20 nM Trolox activity). In all experiments, "contamination" with unbound red wine antioxidants was less than 1% of the initial wine concentration.

Oxidation of Lipoproteins with Copper and AAPH. To evaluate the effects of red wine on the resistance of lipoproteins to oxidative stress, triplicate samples of LDL or HDL were incubated for 3 h at 37 °C at a concentration of 1 mg protein/mL with the indicated dilutions of wine (v/v). At the end of the incubation period, a 500-μL aliquot was applied to a Bio-Rad EconoPack P6 desalting column preequilibrated with PBS, and eluted with 4 mL of PBS. The first 2 mL eluting from the column was discarded; the subsequent 2 mL of eluate was collected and diluted with about 2.5 mL of PBS to a final protein concentration of 0.1 mg/mL. This procedure resulted in negligible contamination with unbound (free) red wine components, and protein recovery was 90−95%. The resulting samples henceforth will be referred to as "pre-incubated." Another 500-μL aliquot not treated by gel filtration was diluted with 4.5 mL of PBS and henceforth will be referred to as "co-incubated." BSA samples at a concentration of 2 mg/mL were incubated with red wine and then left untreated or desalted with Bio-Rad Micro Bio-Spin P-6 columns as described for lipoproteins.

The oxidation of preincubated or co-incubated lipoprotein samples was initiated by addition of the indicated concentrations of $CuSO_4$ or AAPH dissolved in water. Absorbance at 234 nm was measured at the indicated time points to assess conjugated diene formation (in $CuSO_4$-containing lipoprotein samples only). Formation of conjugated dienes was not assessed in samples containing AAPH, because its strong absorbance at 234 nm interferes with the assay. Aliquots were withdrawn from the incubations at the indicated times to measure tryptophan fluorescence (280 nm excitation/331 nm emission) (*20*) and thiobarbituric acid-reactive substances (TBARS, lipoproteins only) (*21*).

Results for BSA oxidation are expressed as means ± SD from three independent experiments. Because of significant variations in the response to oxidation of lipoprotein samples isolated from different donors, the results for LDL and HDL oxidation (triplicate incubations) represent one of at least three experiments using different lipoprotein preparations. Despite individual variations in absolute changes, all tested lipoprotein preparations showed the same relative responses to treatment with red wine.

Statistical Analysis. A significant difference between the samples treated with red wine and controls was accepted at the $p < 0.05$ level, using the two-tailed *t*-test (Microsoft Excel) or ANOVA (StatView, SAS Institute Inc., Cary, NC).

RESULTS

Red Wine Increases the Lipoprotein-Associated Antioxidant Capacity. To determine whether antioxidant components of red wine bind to plasma lipoproteins, the antioxidant capacities of LDL, HDL, LDP, and BSA were measured following incubation with increasing concentrations of red wine (0−2.5%, v/v) and subsequent removal of unbound wine components by gel filtration. Such treatment resulted in a dose-dependent increase in TEAC of LDL, HDL (Figure 1A), LDP, and BSA (Figure 1B). These data indicate that red wine components bind to lipoproteins, and thus enhance their total antioxidant capacity. The antioxidant effect of red wine on lipoproteins appeared nonsaturable up to a concentration of 2.5% (Figures 1A and B). Higher red wine concentrations could not be tested because they caused LDL to precipitate out of solution.

Red Wine Protects LDL from Copper- and Peroxyl Radical-Induced Oxidation. Red wine co-incubated with LDL inhibited copper-induced oxidation in a dose-dependent manner (Figures 2A and C). Both conjugated diene formation (Figure 2A) and loss of tryptophan fluorescence (Figure 2C), markers of lipid and protein oxidation, respectively, were significantly ($p < 0.01$, ANOVA) delayed in the presence of 0.2% red wine (i.e., 1:500 dilution). Higher concentrations of red wine, i.e., 0.4% (data not shown) and 0.8% (Figures 2A and C), further enhanced protection from LDL oxidation, whereas 0.1% red wine had little effect (data not shown). Similar results were obtained when unbound red wine components were removed from LDL by gel filtration prior to incubation with copper (Figures 2B and D). These data are in agreement with those in

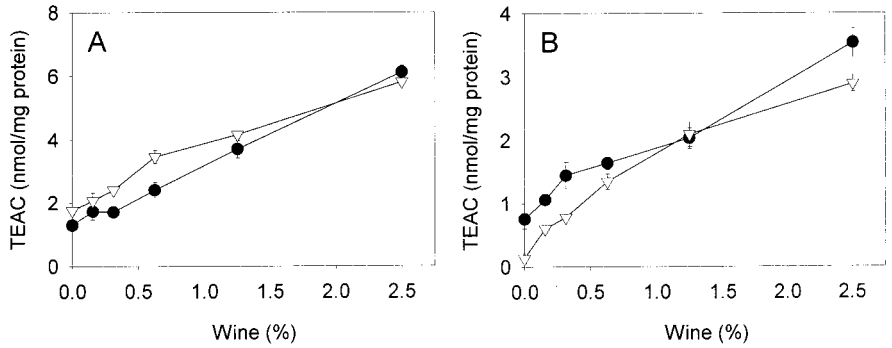

Figure 1. Red wine increases the lipoprotein-associated antioxidant capacity. (A) LDL (●) or HDL (▽) (1 mg protein/mL), and (B) lipoprotein-deficient plasma (●) or bovine serum albumin (▽) (2 mg protein/mL) were incubated with the indicated concentrations of red wine for 3 h at 37 °C. Lipoprotein-associated antioxidant capacity was measured after desalting over a Micro Bio-Spin P-6 column as described in Materials and Methods.

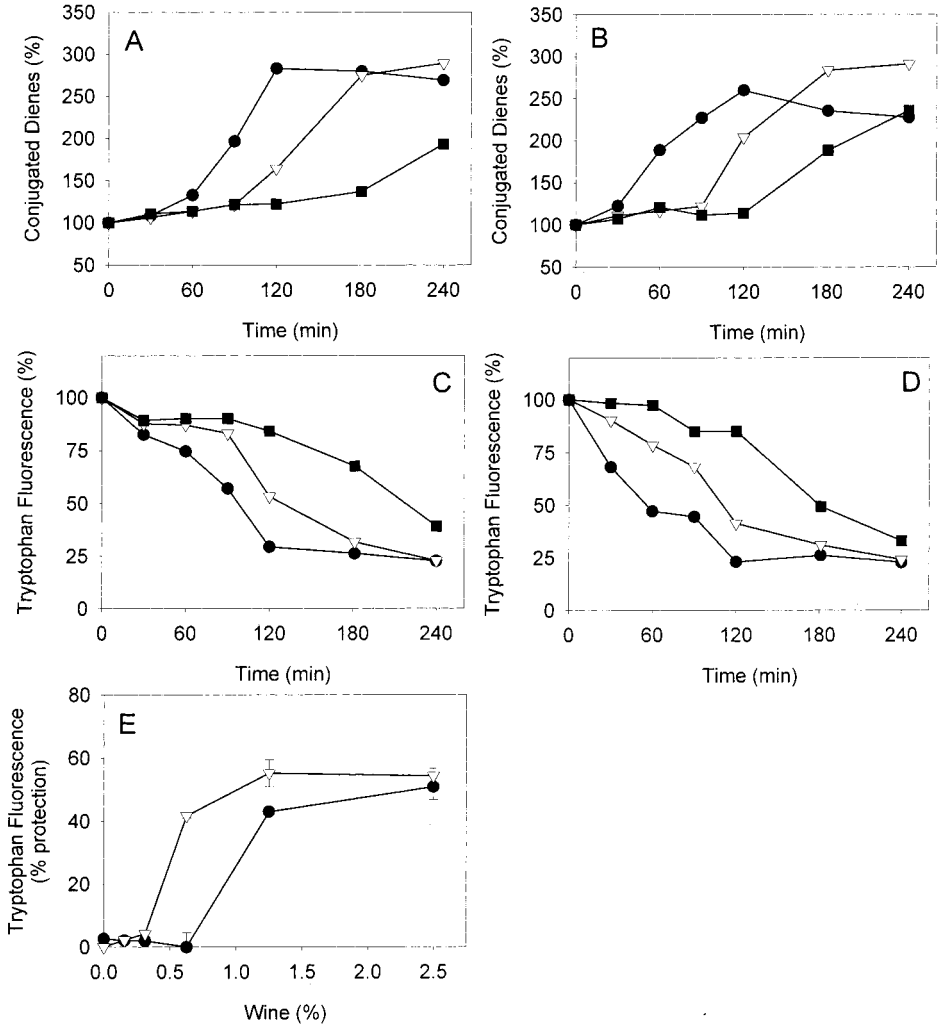

Figure 2. Red wine protects LDL from copper-induced oxidation. (A−D) LDL (1 mg protein/mL) was incubated with 0.08% ethanol (●) or 0.2% (▽) or 0.8% (■) red wine for 3 h at 37 °C, desalted by gel filtration (preincubation: B, D) or left untreated (co-incubation: A, C), and then diluted to 0.1 mg/mL with PBS. (E) LDL (1 mg/mL) was incubated with the indicated concentrations of red wine for 3 h at 37 °C, desalted (●) or left untreated (▽), and then diluted to 0.05 mg/mL with PBS. Oxidation was initiated by addition of $CuSO_4$ (3 μM final concentration and incubation at 37 °C). Conjugated dienes (A, B) and tryptophan fluorescence (C, D, E) were measured at the indicated times (A−D) or at 3 h (E) and are expressed as % control (no copper addition) or % protection (for E).

Figure 1A, and indicate that a substantial portion of the antioxidants in red wine bind to LDL and protect it from metal ion-dependent oxidation. Comparison of the antioxidant efficacy of LDL-associated (preincubated) and total (co-incubated) red wine components revealed that the concentration required for 50% protection (IC_{50}) of LDL tryptophan residues was about 2.4% and 1.0% red wine, respectively (Figure 2E).

LDL-associated red wine antioxidants also effectively protected the lipoprotein from oxidation by aqueous peroxyl radicals generated by AAPH (Figure 3). Preincubation of LDL with 1% red wine resulted in the

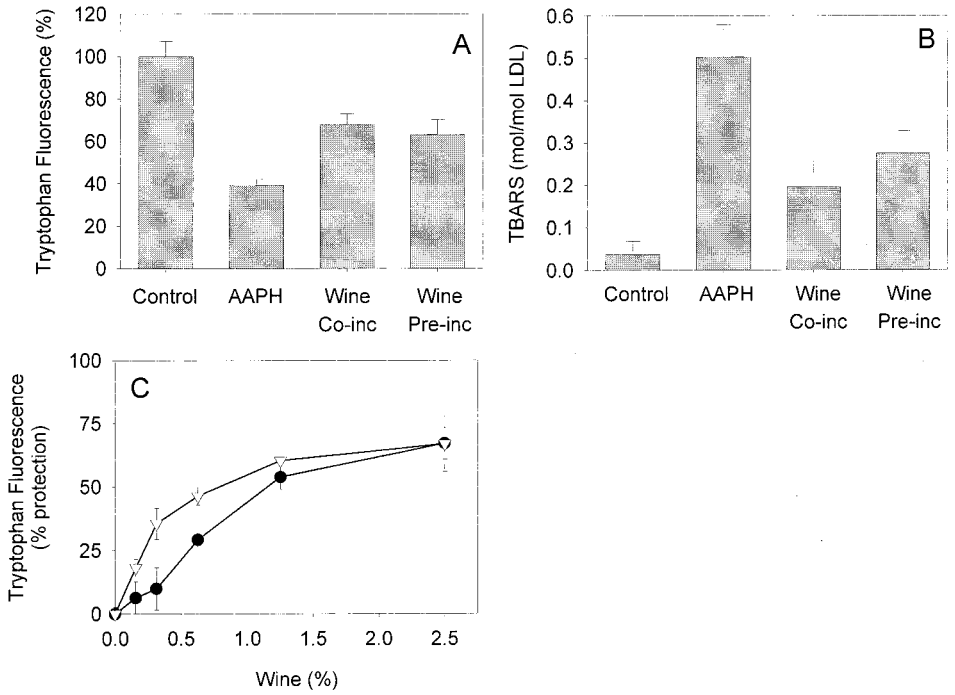

Figure 3. Red wine protects LDL from AAPH-induced oxidation. (A, B) LDL (1 mg protein/mL) was incubated with 0.08% ethanol or 1% red wine for 3 h at 37 °C, desalted by gel filtration (pre-incubation), and then diluted to 0.125 mg/mL with PBS. Alternatively, LDL (0.125 mg protein/mL) was incubated with 0.03% red wine for 3 h at 37 °C (co-incubation). (C) LDL (1 mg/mL) was incubated with the indicated concentrations of red wine for 3 h at 37 °C, desalted (●) or left untreated (▽), and then diluted to 0.125 mg/mL of PBS. Oxidation was initiated by addition of AAPH (10 mM final concentration). Tryptophan fluorescence (A, C) and TBARS (B) were measured after incubation for 1 h at 37 °C. Tryptophan fluorescence is expressed as % control (no AAPH addition) or % protection (for C).

protection of both its protein and lipid moiety, as assessed by tryptophan oxidation (Figure 3A) and TBARS formation (Figure 3B), respectively. LDL-bound red wine components were somewhat less effective at preventing AAPH-induced tryptophan oxidation than total (bound and free) red wine components (IC_{50} = 1.2% and 0.8% red wine, respectively, Figure 3C).

Red Wine Protects HDL from Copper- and Peroxyl Radical-Induced Oxidation. To investigate the specificity of the interaction of red wine antioxidants with LDL, experiments similar to those described above were performed replacing LDL with HDL (Figures 4 and 5) or with albumin (Figure 6). Red wine co-incubated or preincubated with HDL significantly decreased copper-induced oxidation of both the protein and lipid moiety of HDL, respectively, as assessed either by the decline in tryptophan fluorescence (Figure 4A) or the formation of TBARS (Figure 4B) and conjugated dienes (Figure 4C). Similar protective effects of free and HDL-associated red wine components were observed against AAPH-induced oxidation (Figures 5A and B). As with LDL, preincubation of HDL with red wine was less effective at protecting the lipoprotein against loss of tryptophan residues than co-incubation, in the presence of both copper (IC_{50} = 0.3% and 0.2% red wine, respectively, Figure 4D) and AAPH (IC_{50} = 1.9% and 0.6% red wine, respectively, Figure 5C). Thus, the protective effects of red wine against HDL oxidation were similar to those observed with LDL.

Effect of Red Wine on BSA Oxidation by Copper and Peroxyl Radicals. To estimate the relative contribution of the interaction of red wine antioxidants with the lipid and protein moiety of lipoproteins to their antioxidant protection, experiments were performed using BSA (Figure 6). Incubation of BSA with copper did not cause significant oxidation of BSA tryptophan residues (Figure 6A). However, red wine co-incubated or preincubated with BSA caused a significant dose-dependent decrease in tryptophan fluorescence. This pro-oxidant effect of red wine was saturated at 4% concentration, resulting in 20−30% loss of tryptophan fluorescence. The difference between the samples co-incubated and preincubated with red wine was not statistically significant (Figure 6A), suggesting that the BSA-associated, not free, red wine components are responsible for the observed pro-oxidant effect (Figure 6A).

In contrast, co-incubation with red wine dose-dependently inhibited BSA tryptophan oxidation by AAPH (Figure 6B). The protective effect of the red wine reached its maximum (80−90% of control) at a concentration of 1%. Preincubation with red wine also resulted in effective protection of the tryptophan residues of BSA from AAPH-induced oxidation, reaching saturation (60−70% of control) at 2% red wine. These data indicate that only about two-thirds of BSA tryptophan residues are protected by protein-bound red wine components. The effects of red wine on AAPH-induced tryptophan oxidation in BSA were similar to those observed for LDL and HDL (compare Figure 6B with Figures 3C and 5C).

Effect of Ascorbate on Red-Wine-Dependent Protection from LDL Oxidation. To assess whether ascorbate, an important plasma antioxidant (*22*), can enhance the protective effects of red wine against LDL oxidation, LDL was preincubated with a concentration of ascorbate (54 μM) possessing the same antioxidant capacity as 1% red wine (based on TEAC values). Preincubation of LDL with ascorbate alone did not inhibit copper-induced oxidation of the protein and lipid moiety of LDL (no addition vs ascorbate: 23 ± 0 vs 19

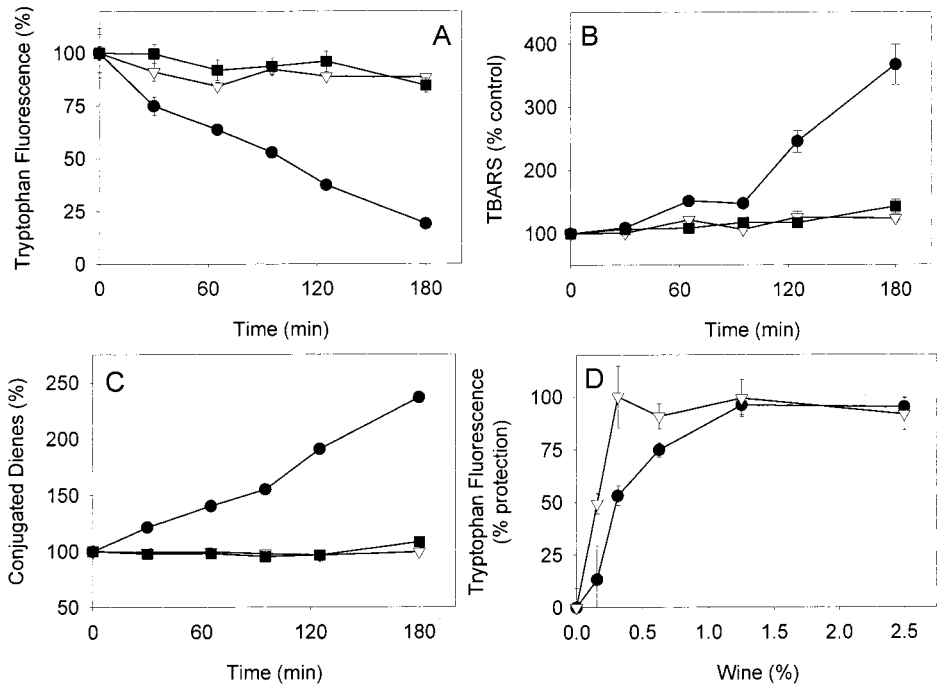

Figure 4. Red wine protects HDL from copper-induced oxidation. (A−C) HDL (1 mg protein/mL) was incubated with 0.08% ethanol (●) or 1% red wine (■) for 3 h at 37 °C, and desalted by gel filtration (preincubation). Alternatively, HDL (mg/mL) was incubated with 0.1% red wine for 3 h at 37 °C (co-incubation, ▽). (D) HDL (1 mg/mL) was incubated with the indicated concentrations of red wine for 3 h at 37 °C, and desalted (●) or left untreated (▽). All samples were diluted to 0.25 mg/mL with PBS. Oxidation was initiated by addition of $CuSO_4$ (3 μM final concentration) and incubation at 37 °C. Tryptophan fluorescence (A, D), TBARS (B), and conjugated dienes (C) were measured at the indicated times (A−C) or at 3 h (D) and are expressed as % control (no copper addition) or % protection (for D).

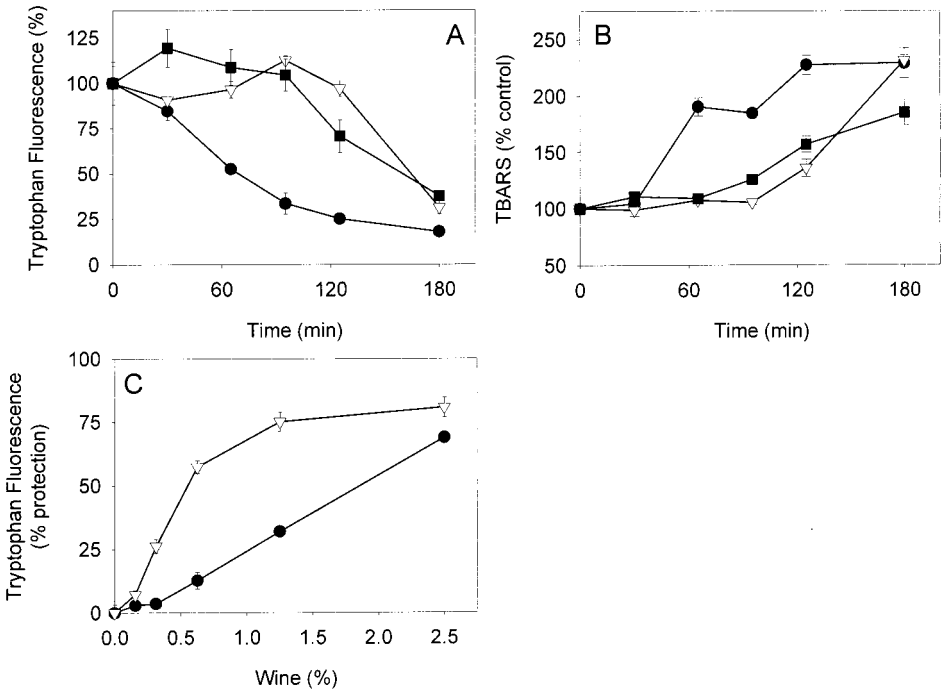

Figure 5. Red wine protects HDL from AAPH-induced oxidation. (A, B) HDL (1 mg protein/mL) was incubated with 0.08% ethanol (●) or 1% red wine (■) for 3 h at 37 °C, and desalted by gel filtration (preincubation). Alternatively, HDL (1 mg/mL) was incubated with 0.1% red wine for 3 h at 37 °C (co-incubation, ▽). (C) HDL (1 mg/mL) was incubated with the indicated concentrations of red wine for 3 h at 37 °C, and desalted (●) or left untreated (▽). All samples were diluted to 0.25 mg/mL with PBS. Oxidation was initiated by addition of AAPH (10 mM final concentration) and incubation at 37 °C. Tryptophan fluorescence (A, C) and TBARS (B) were measured at the indicated times (A, B) or at 1 h (C), and are expressed as % control (no AAPH addition) or % protection (for C).

± 1% for tryptophan oxidation, 290 ± 3 vs 251 ± 18% for conjugated dienes, and 573 ± 3 vs 655 ± 70% for TBARS). Likewise, the presence of ascorbate during preincubation of LDL with red wine did not enhance the red wine-dependent protection of LDL from oxidation (red wine vs ascorbate plus red wine: 57 ± 6 vs 64 ± 10% for tryptophan oxidation, 102 ± 1 vs 113 ± 4% for conjugated dienes, and 117 ± 10 vs 167 ± 44% for

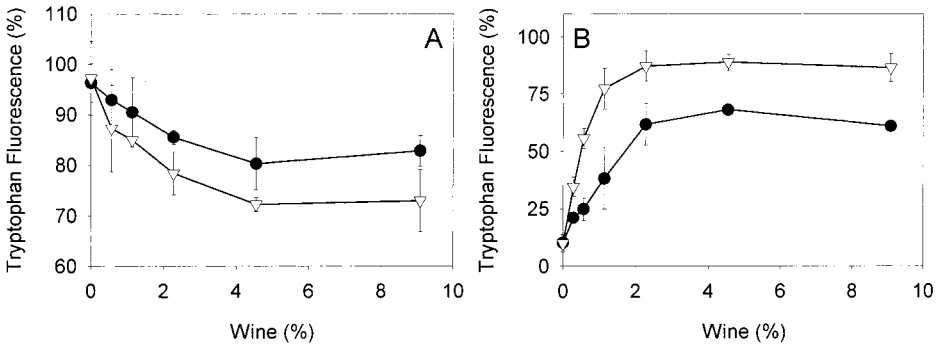

Figure 6. Effect of red wine on BSA oxidation by copper and AAPH. BSA (2 mg/mL) was incubated with the indicated concentrations of red wine for 3 h at 37 °C, and desalted by gel filtration (preincubation, ●) or left untreated (co-incubation, ▽). (A) The samples were diluted to 0.5 mg/mL with PBS and incubated with 50 μM $CuSO_4$ for 2 h at 37 °C. (B) The samples were diluted to 1.5 mg/mL with PBS and incubated with 30 mM AAPH for 1 h at 37 °C. Tryptophan fluorescence was measured at the end of the incubation, and is expressed as % control (no copper or AAPH addition).

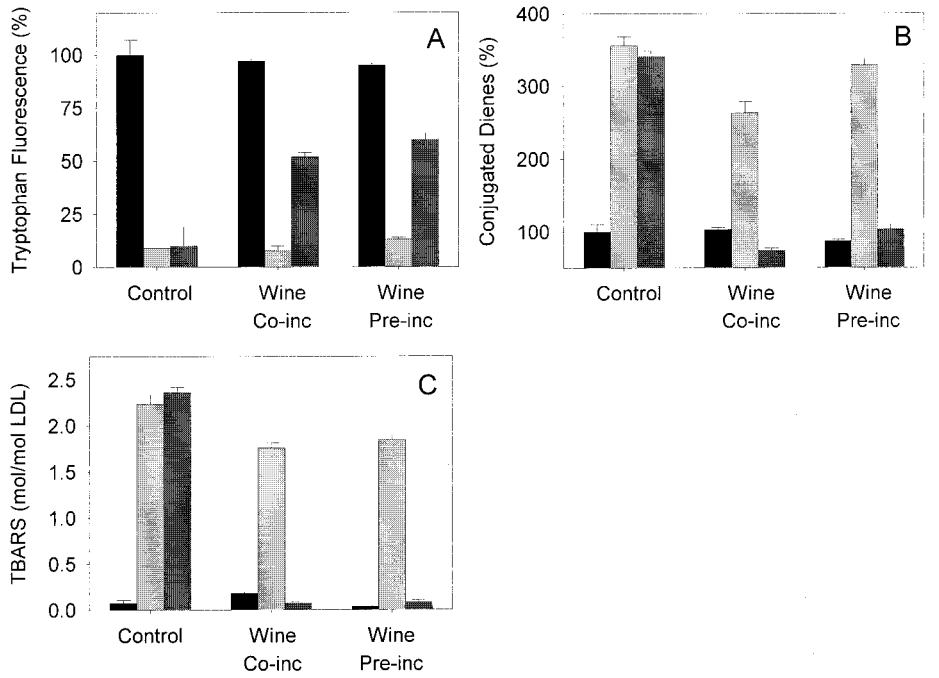

Figure 7. Ascorbate potentiates red wine-dependent protection of LDL from copper-induced oxidation. LDL (1 mg protein/mL) was incubated with 0.08% ethanol (control) or 1% red wine for 3 h at 37 °C, desalted by gel filtration (pre-incubation) or left untreated (co-incubation), and then diluted to 0.125 mg/mL with PBS. The samples were incubated for 4 h at 37 °C (black bars) or with 10 μM $CuSO_4$ in the absence (light gray bars) or presence of 5 μM ascorbate (dark gray bars). Tryptophan fluorescence (A), conjugated dienes (B), and TBARS (C) were measured at the end of the incubation.

TBARS). These data confirm that ascorbate does not associate with LDL particles.

In contrast, addition of ascorbate to LDL preincubated or co-incubated with red wine strongly inhibited copper-induced protein (Figure 7A) and lipid oxidation (Figures 7B and C). The concentrations of red wine and ascorbate used in these experiments were chosen to provide little protection when used separately. However, their combination gave strong antioxidant protection, which was significantly greater than an additive effect under the experimental conditions used. In contrast, ascorbate and red wine showed a strictly additive effect against AAPH-induced oxidation of LDL (data not shown).

DISCUSSION

Although many different purified polyphenols have been shown to protect LDL in vitro against various types of oxidative stress, the underlying mechanism(s) are incompletely understood. It has been speculated that the protective effects occur at different levels. Thus, polyphenols can directly scavenge water-soluble free radicals forming less reactive products (*23*). In addition, at least some polyphenols can chelate transition metal ions, such as iron and copper, decreasing their participation in free radical generation by Fenton-type reactions (*24*). Although different polyphenols have been shown to bind to or associate with proteins and liposomes (*14*, *15*), this property has not been studied in detail with respect to free radical-induced oxidation of LDL.

In this study we investigated how association of red wine antioxidants with LDL particles might affect their resistance to oxidative modification. Red wine was chosen because it represents a natural mixture of polyphenols with apparent biological activity (*4*−*7*). In our study, only one type of red wine was used (Cabernet-Sauvignon), but the results most likely can be generalized to many other types of red wine, as their polyphenol

compositions and contents are comparable. The association of red wine components with LDL and other plasma lipoproteins was determined indirectly by the change in lipoprotein-associated antioxidant activity, using a sensitive method based on the inhibition of photoinduced chemiluminescence of luminol (*19*). The results showed that LDL and HDL had similar initial levels of antioxidant capacity (about 2 nmol TEAC units per mg protein), which was about twice that of lipoprotein-deficient plasma. This difference may be attributable to lipoprotein-associated antioxidants, especially α-tocopherol. LDL and other plasma lipoproteins preincubated with increasing concentrations of red wine exhibited increased antioxidant capacity. These data indicate that red wine polyphenols bind to lipoproteins, and that their antioxidant activity is retained following binding. Our experiments did not identify the nature of the bound red wine components, nor did they determine whether the composition of the red wine components bound to different lipoproteins was the same. Analysis of the binding properties of different purified components of red wine is an area of future investigation.

The association of red wine components with LDL was further confirmed by the increased resistance of LDL protein and lipid to in vitro oxidation by copper and AAPH. Thus, LDL-associated red wine polyphenols may chelate metal ions and scavenge aqueous peroxyl radicals. It is not known whether the same red wine components are responsible for the antioxidant protection from copper- and AAPH-induced oxidation. Coincubation, as opposed to preincubation, with red wine resulted in substantially increased antioxidant protection of LDL, indicating that some polyphenols bind to LDL, while others remain in solution. The amount of antioxidant capacity that remained associated with the lipoprotein after desalting accounted for less than five percent of its initial value in wine.

The antioxidant effects of red wine were not restricted to LDL, as substitution with HDL gave similar results. To estimate the relative importance of the lipid and protein moieties of lipoproteins in binding red wine components and conferring antioxidant protection, we used serum albumin as a model protein. Although BSA-associated red wine components protected against AAPH-induced oxidation, they promoted copper-induced oxidation, i.e., exerted a pro-oxidant effect. These data suggest different mechanisms for the interaction of red wine components with proteins compared to that with lipoproteins. There are several possible explanations for this discrepancy. The composition of the red wine components that bind to BSA may be different from the composition of LDL- and HDL-associated components because of the absence of lipids in albumin. In addition, BSA-associated red wine components may chelate copper in a redox-active manner, increasing its local concentration and thereby catalyzing site-specific production of free radicals by Fenton chemistry on the surface of the protein. Furthermore, the protective effects of LDL- and HDL-associated red wine components against copper-induced oxidation may occur through breaking the lipid peroxidation chain reaction and/or by sparing or regenerating lipoprotein-associated α-tocopherol (see below), which is absent from albumin.

Ascorbate and plant-derived polyphenols have been demonstrated previously to interact in different biological systems with either antagonistic or synergistic effects (*25*−*27*). In our experimental model, addition of ascorbate to LDL samples during preincubation with red wine did not affect subsequent oxidation of the lipoprotein with copper or AAPH. These data show that, in contrast to red wine components, ascorbate does not associate with LDL. However, the combined presence of ascorbate and red wine during LDL oxidation by copper, or addition of ascorbate to LDL samples containing preassociated red wine components, resulted in enhanced, most likely synergistic, protection against LDL lipid and protein oxidation. These findings suggest that red wine polyphenols and ascorbate inhibit LDL oxidation by different, mutually enhancing mechanisms, e.g., copper chelation and regeneration of LDL-associated α-tocopherol, respectively, or regeneration of LDL-associated polyphenols by ascorbate. It could also be that red wine components enhance the antioxidant interaction between ascorbate and lipoprotein-associated α-tocopherol. In contrast, no synergistic interaction between ascorbate and red wine polyphenols was observed with respect to peroxyl radical-induced LDL oxidation, indicating different mechanisms of LDL oxidation by copper and AAPH. It is likely that ascorbate directly scavenges aqueous peroxyl radicals, thereby inhibiting AAPH-induced LDL oxidation (*28*), whereas the protection by ascorbate against copper-induced oxidation may occur primarily through regeneration of α-tocopherol (*29*).

The relatively low bioavailability of plant-derived polyphenols has been discussed (*30*). In our experiments, significant protection from oxidation was observed following preincubation of lipoproteins with 0.2% red wine (Figures 2−5). Assuming a dose of 300 mL (10.6 oz.) of red wine in an adult person and only 1% bioavailability of the wine's polyphenols, the corresponding plasma concentration would be about 0.1%, which is comparable to that which was used in our study. Furthermore, direct association of polyphenols with lipoproteins may increase their local concentration on the surface of the lipoprotein particles and increase their antioxidant efficacy. Lipoprotein-associated polyphenols may provide continued protection against oxidative damage once the lipoproteins have crossed the endothelium and reside in the subendothelial space of the arterial wall. In agreement with this notion, several studies have demonstrated increased resistance of plasma-derived LDL to ex vivo oxidation following dietary supplementation with red wine in animal models and human volunteers (*11*−*13*). These data also confirm that red wine polyphenols can bind to LDL in vivo, because unbound compounds would be separated during the LDL isolation procedure. Furthermore, relatively low amounts of LDL-associated polyphenols can produce significant effects on LDL oxidation when combined with plasma antioxidants, as demonstrated in the present study for ascorbate.

In conclusion, red wine components bind to human lipoproteins and protect them from metal ion-dependent and -independent protein and lipid oxidation. These data may explain, in part, the beneficial effects of red wine consumption on cardiovascular disease risk.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; BSA, bovine serum albumin; DTPA, diethylenetriaminepentaacetic acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDP, lipoprotein-deficient plasma; PBS, phosphate-buffered saline; TBARS, thiobar-

bituric acid-reactive substances; TEAC, Trolox equivalent of antioxidant capacity.

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LITERATURE CITED

(1) Lin, J. K.; Liang, Y. C.; Lin-Shiau, S. Y. Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade. *Biochem. Pharmacol.* **1999**, *58*, 911−915.

(2) Lampe, J. W. Health effects of vegetables and fruit: assessing mechanisms of action in human experimental studies. *Am. J. Clin. Nutr.* **1999**, *70*, 475S−490S.

(3) Kushi, L. H.; Meyer, K. A.; Jacobs, D. R., Jr. Cereals, legumes, and chronic disease risk reduction: evidence from epidemiologic studies. *Am. J. Clin. Nutr.* **1999**, *70*, 451S−458S.

(4) Soleas, G. J.; Diamandis, E. P.; Goldberg, D. M. Wine as a biological fluid: history, production, and role in disease prevention. *J. Clin. Lab. Anal.* **1997**, *11*, 287−313.

(5) Johansen, K. M.; Skorpe, S.; Olsen, J. O.; Osterud, B. The effect of red wine on the fibrinolytic system and the cellular activation reactions before and after exercise. *Thromb. Res.* **1999**, *96*, 355−363.

(6) Feng, A. N.; Chen, Y. L.; Chen, Y. T.; Ding, Y. Z.; Lin, S. J. Red wine inhibits monocyte chemotactic protein-1 expression and modestly reduces neointimal hyperplasia after balloon injury in cholesterol-fed rabbits. *Circulation* **1999**, *100*, 2254−2259.

(7) Casalini, C.; Lodovici, M.; Briani, C.; Paganelli, G.; Remy, S.; Cheynier, V.; Dolara, P. Effect of complex polyphenols and tannins from red wine (WCPT) on chemically induced oxidative DNA damage in the rat. *Eur. J. Nutr.* **1999**, *38*, 190−195.

(8) Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E.; Kinsella, J. E. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **1993**, *341*, 454−457.

(9) Rifici, V. A.; Stephan, E. M.; Schneider, S. H.; Khachadurian, A. K. Red wine inhibits the cell-mediated oxidation of LDL and HDL. *J. Am. Coll. Nutr.* **1999**, *18*, 137−143.

(10) Zou, J. G.; Huang, Y. Z.; Chen, Q.; Wei, E. H.; Hsieh, T. C.; Wu, J. M. Resveratrol inhibits copper ion-induced and azo compound-initiated oxidative modification of human low-density lipoprotein. *Biochem. Mol. Biol. Int.* **1999**, *47*, 1089−1096.

(11) Nigdikar, S. V.; Williams, N. R.; Griffin, B. A.; Howard, A. N. Consumption of red wine polyphenols reduces the susceptibility of low- density lipoproteins to oxidation in vivo. *Am. J. Clin. Nutr.* **1998**, *68*, 258−265.

(12) Hayek, T.; Fuhrman, B.; Vaya, J.; Rosenblat, M.; Belinky, P.; Coleman, R.; Elis, A.; Aviram, M. Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler. Thromb. Vasc. Biol.* **1997**, *17*, 2744−2752.

(13) Aviram, M.; Fuhrman, B. Polyphenolic flavonoids inhibit macrophage-mediated oxidation of LDL and attenuate atherogenesis. *Atherosclerosis* **1998**, *137*, S45−S50.

(14) Feldman, K. S.; Sambandam, A.; Lemon, S. T.; Nicewonger, R. B.; Long, G. S.; Battaglia, D. F.; Ensel, S. M.; Laci, M. A. Binding affinities of gallotannin analogues with bovine serum albumin: ramifications for polyphenol−protein molecular recognition. *Phytochemistry* **1999**, *51*, 867−872.

(15) Nakayama, T.; Ono, K.; Hashimoto, K. Affinity of antioxidative polyphenols for lipid bilayers evaluated with a liposome system. *Biosci., Biotechnol., Biochem.* **1998**, *62*, 1005−1007.

(16) Belguendouz, L.; Fremont, L.; Gozzelino, M. T. Interaction of transresveratrol with plasma lipoproteins. *Biochem. Pharmacol.* **1998**, *55*, 811−816.

(17) Singleton, V. L.; Rossi, J. A. Colorimetry of total polyphenols with phosphomolibdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.

(18) Shwaery, G. T.; Mowri, H.; Keaney, J. F., Jr.; Frei, B. Preparation of lipid hydroperoxide-free low-density lipoproteins. *Methods Enzymol.* **1999**, *300*, 17−23.

(19) Popov, I.; Lewin, G. Antioxidative homeostasis: characterization by means of chemiluminescent technique. *Methods Enzymol.* **1999**, *300*, 437−456.

(20) Hazell, L. J.; Stocker, R. Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages. *Biochem. J.* **1993**, *290*, 165−172.

(21) Schuh, J.; Fairclough, G. F., Jr.; Haschemeyer, R. H. Oxygen-mediated heterogeneity of apo-low-density lipoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3173−3177.

(22) Frei, B.; England, L.; Ames, B. N. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6377−6381.

(23) Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. Mechanistic studies of catechins as antioxidants against radical oxidation. *Arch. Biochem. Biophys.* **1999**, *362*, 79−86.

(24) Brown, J. E.; Khodr, H.; Hider, R. C.; Rice-Evans, C. A. Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochem. J.* **1998**, *330*, 1173−1178.

(25) Kuo, S. M.; Morehouse, H. F., Jr.; Lin, C. P. Effect of antiproliferative flavonoids on ascorbic acid accumulation in human colon adenocarcinoma cells. *Cancer Lett.* **1997**, *116*, 131−137.

(26) Noroozi, M.; Angerson, W. J.; Lean, M. E. J. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. *Am. J. Clin. Nutr.* **1998**, *67*, 1210−1218.

(27) Vieira, O.; Laranjinha, J.; Madeira, V.; Almeida, L. Cholesterol ester hydroperoxide formation in myoglobin-catalyzed low-density lipoprotein oxidation: concerted antioxidant activity of caffeic acid and *p*-coumaric acids with ascorbate. *Biochem. Pharmacol.* **1998**, *55*, 333−340.

(28) Frei, B.; Stocker, R.; Ames, B. N. Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 9748−9752.

(29) Kontush, A.; Meyer, S.; Finckh, B.; Kohlschutter, A.; Beisiegel, U. α-Tocopherol as a reductant for Cu(II) in human lipoproteins. Triggering role in the initiation of lipoprotein oxidation. *J. Biol. Chem* **1996**, *271*, 11106−11112.

(30) Wiseman, H. The bioavailability of nonnutrient plant factors: dietary flavonoids and phyto-estrogens. *Proc. Nutr. Soc.* **1999**, *58*, 139−146.

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