Walnut Polyphenolics Inhibit In Vitro Human Plasma and LDL Oxidation[1,2]

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ABSTRACT Recent epidemiologic studies have associated nut consumption with a reduced incidence of cardiovascular mortality. However, little is known about the contribution of nut polyphenols to antioxidant and cardiovascular protection. In this investigation, polyphenol-rich extracts from English walnuts (*Juglans regia*) were studied and compared with ellagic acid for their ability to inhibit in vitro plasma and LDL oxidation, as well as their effects on LDL α-tocopherol during oxidative stress. In addition, the Trolox equivalent antioxidant activity (TEAC) was determined and liquid chromatography electrospray detection mass spectrometry (LC-ELSD/MS) analyses of the walnut extracts were performed. 2,2′-Azobis′(2-amidino propane) hydrochloride (AAPH)-induced LDL oxidation was significantly inhibited by 87 and 38% with the highest concentration (1.0 μmol/L) of ellagic acid and walnut extract, respectively. In addition, copper-mediated LDL oxidation was inhibited by 14 and 84% in the presence of ellagic acid and walnut extract, respectively, with a modest, significant LDL α-tocopherol sparing effect observed. Plasma thiobarbituric acid reacting substance (TBARS) formation was significantly inhibited by walnut extracts and ellagic acid in a dose-dependent manner, and the extracts exhibited a TEAC value greater than that of α-tocopherol. LC-ELSD/MS analysis of the walnut extracts identified ellagic acid monomers, polymeric ellagitannins and other phenolics, principally nonflavonoid compounds. These results demonstrate that walnut polyphenolics are effective inhibitors of in vitro plasma and LDL oxidation. The polyphenolic content of walnuts should be considered when evaluating their antiatherogenic potential. J. Nutr. 131: 2837–2842, 2001.

KEY WORDS: • *polyphenol* • *ellagic acid* • *LDL* • *antioxidant* • *English walnuts*

Recent epidemiologic studies have shown that consuming diets rich in plant-derived foods that are high in phenolic compounds, even while consuming high amounts of saturated fatty acids (1), is associated with a reduced incidence of cardiovascular mortality (2–5). Polyphenolics have been shown to possess free radical–scavenging and metal-chelating activity in addition to their reported anticarcinogenic properties (6). These plant-based, nonnutrient phytochemicals may have a protective effect on the susceptibility of LDL to oxidative modification and ultimately, on atherosclerosis.

The oxidative modification and inflammation hypothesis of atherogenesis is widely accepted and supported by experimental data in hypercholesterolemic animal models and human epidemiologic studies, recently reviewed by Chisolm and Steinberg (7). Atherogenesis is a multifactorial process that includes oxidatively modified LDL, which triggers pathological events through multiple pathways, leading to atherosclerosis (8). Numerous in vitro studies using a variety of oxidation methods and measurements have shown that polyphenolics from red wine (9), green tea (10) and chocolate (11) can inhibit LDL oxidation. In vivo, it is unclear whether diet-derived polyphenolics can indeed influence the atherogenic process, but it is thought that the antioxidant potential of plant-derived foods may be one factor in reducing cardiovascular risk (12).

A correlation between nut consumption and a reduced incidence of ischemic heart disease has been observed, most notably by the Adventist Health Study, the Iowa Women's Health Study and the Nurses' Health Study (13–15). Consumption of walnuts has favorable effects on human serum lipid profiles, with a decrease in total and LDL cholesterol as well as triglycerides (16–19) and an increase in HDL cholesterol and apolipoprotein A1 (20). Tree nuts are an important source of beneficial dietary lipids, and a potentially rich source of phenolic compounds that contribute to antioxidant capacity. However, there has been very little investigation into the contribution of nut polyphenols to antioxidant protection and reduction in cardiovascular risk. This study, therefore, sought to determine the antioxidant capacity of walnut polyphenolic compounds in vitro. Walnut polyphenolics are found in the highest concentration in the pellicle, the thin tan-brown "skin" that lines the meat of the nut. Thus, this paper reports on a brief characterization of the polyphenolic-rich extract of English walnuts, its relative antioxidant activity, and demon-

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strates the ability of this extract to significantly inhibit plasma and LDL oxidation.

MATERIALS AND METHODS

Materials. 1,1,3,3-Tetramethoxypropane [malondialdehyde (MDA)[4]], 2,2′-azobis′(2-amidino propane) hydrochloride (AAPH), HPLC grade ethyl alcohol and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Hoffman La-Roche) were purchased from Aldrich (Milwaukee, WI). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO). The English walnuts (a mixture of Hartley and Chandler cultivars) were purchased from the Mariani company (Winters, CA). For the oxidation experiments, ellagic acid and gallic acid standards were dissolved in a 10% ethanol solution and subsequent dilutions made in deionized H_2O. Catechin was dissolved in water, adjusted with 11.6 mol/L HCl (Fisher, Pittsburg, PA) to a pH of 3.5, and vortexed for at least 10 min. The catechin was used as a positive control due to its previously described ability to inhibit LDL oxidation (21,22). For the antioxidant activity assay, the compounds and extracts were diluted in 100% HPLC grade ethyl alcohol.

Preparation of walnut polyphenolic extracts and Folin assay. The walnuts were frozen for 24 h; the shelled kernel was then immersed in a solution of 75% acetone + 526 μmol/L sodium metabisulfite and the headspace purged with N_2 (nitrogen) to guard against oxidation. After incubation at 4°C for 24 h, solutions were decanted, resulting in a cold extract. To obtain a heat extract, an additional step of gentle refluxing of the decanted solution under N_2 gas for 24 h was added. To remove all lipids, the extraction solutions were evaporated under reduced pressure to remove all acetone, and methanol (50% aqueous, v/v) was added; the solutions were then extracted three times with hexanes. To prevent oxidation, the extract solutions were lyophilized to a dry powder and stored at −20°C. Before use, the lyophilized powder was dissolved into a 10% dimethyl sulfoxide solution and ultrapure water. The concentration of the dissolved extracts was determined using the Folin-Ciocalteu assay, a colorimetric assay for total phenolics (23). Briefly, the Folin-Ciocalteu reagent was mixed with serial dilutions of walnut extract and gallic acid standards. After a short incubation, 1.9 mol/L sodium carbonate was added followed by a 1-h incubation. The absorbance at 765 nm was obtained and compared with that from gallic acid standards, with results expressed in gallic acid equivalents (GAE).

Antioxidant assay. The antioxidant activity of the test compounds and extracts was determined using a 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) cation decolorization assay as previously described (24). Briefly, Trolox, a water-soluble vitamin E analog, was used as a standard and thus the results are expressed as Trolox equivalent activity capacity (TEAC). The ABTS radical cation solution was produced by the addition of potassium persulfate to a final concentration of 2.45mmol/L in 7mmol/L ABTS and incubated for 12–16 h, in which time the radical cation stabilizes. Absorbance at 734nm was obtained 4 min after the addition of the compounds and extracts. This assay was carried out in triplicate on three separate days.

LC-ELSD-MS of walnut extracts. A HPLC method coupled to electrospray ionization mass spectrometry (LC-ELSD/MS) was used for characterization of the walnut phenolics. A Waters (Milford, MA) 600 binary pump fed a Gilson (Middleton, WI) 215 multiple injection autosampler with an injection volume of 20 μL onto a Betabasic C18 column (4.6 × 50 mm, 5-μm particle size) from Western Analytical (Temecula, CA). The mobile phase was a water (A)/acetonitrile (B) gradient both containing 0.1% acetic acid, starting with 5% B and progressing via linear gradients to these amounts at these times, 1 min 5%; 8.5 min 75%; 9 min 95%. After 1 min at 95% B, the solvent was equilibrated back to 5% B. The flow was 1.2 mL/min, and of this, 0.1 mL/min was delivered to the mass detector. The MS data were acquired on a LCT orthogonal TOF spectrometer (Micromass; Beverly, MA) fitted with an eight-way multiplexed electrospray interface (MUX). In these analyses, each liquid stream was sampled for 0.1 s with mass spectra acquired from 200-1000 Da into eight simultaneously open data files synchronized with the spray being sampled. The time taken to move to the adjacent sampling position was 0.05 s. This cycle produced a data point for each spray every 0.15 s. The LCT and MUX were operated under MassLynx V3.4. The ionization conditions and energy levels for both electrospray negative and positive modes were as follows: desolvation temperature, 380°C; source temperature, 120°C; ion energy, 35 eV; extraction cone, 2 eV; desolvation gas flow, 1200 L/h; and cone gas flow, 11 L/h. Additionally, the energy conditions that differed for electrospray negative and positive modes were capillary 3000 and 3500eV, sample cone 45 and 50eV, and RF lens 500 and 350ev, respectively. The instrument was calibrated in both ionization modes over a 150- to 1000-Da mass range using a 0.02 g/L solution of poly-DL-alanine dissolved in methanol.

Plasma and LDL isolation. Blood from five healthy normocholesterolemic adult volunteers was collected into vacutainer tubes containing EDTA and centrifuged (833 × *g* for 10 min) to isolate plasma. The plasma was pooled and stored at −70°C in 2-mL aliquots and used for all subsequent experiments. LDL was isolated by density centrifugation in a Sorvall RC-120GX Micro-Ultracentrifuge using a Sorvall (Newtown, CT) S120-AT2 rotor, based on the method of Brousseau et al. (25). The LDL layer was removed and dialyzed against 4 L of PBS, pH 7.4, and purged with nitrogen for 12–18 h. The PBS was treated with 10–20 g/L of Chelex (Bio-Rad, Hercules, CA), which was removed before use in dialysis. After dialysis, the cholesterol concentration in the pooled LDL sample was determined using a Cholesterol/HP assay kit (Roche Molecular Biochemicals, Indianapolis, IN) as described by the manufacturer. The LDL fraction was used immediately or purged with nitrogen and stored at 4°C for no >48 h.

Copper-mediated LDL oxidation. To evaluate the ability of the test compounds and extracts to inhibit copper (Cu)-mediated LDL oxidation, conjugated dienes (CD) were continually monitored (5-min intervals) at 37°C by obtaining UV absorption at 234 nm (26). LDL oxidation was initiated by adding copper sulfate at a final concentration of 5 μmol/L to 75 μg of LDL cholesterol in a volume of 1 mL. The purified test compounds (ellagic acid and catechin obtained from Sigma Chemical) and walnut extracts were added immediately before the addition of the oxidant, using the extracts at a final concentration of 0.1–1.0 μmol/L GAE and the purified compounds at a final concentration of 0.5–1.0 μmol/L. Concentrations of 0.1 and 0.5 μmol/L represent physiologic plasma levels of phenolic compounds achievable after ingestion of phenolic-rich foods, and 1.0 μmol/L was chosen to determine whether a dose-response effect might be seen with higher levels of the phenolic compounds. All LDL experiments were performed twice in triplicate. The lag time in the presence or absence of the test compounds was determined to be the intercept of the slopes for the lag and propagation phases, and was compared with the control oxidized LDL to determine the percentage of LDL oxidation inhibition.

HPLC determination of α-tocopherol. The LDL was oxidized in the same manner as described above with extracts and ellagic acid, final concentration of 0.5 and 1.0 μmol/L, respectively, added immediately before or exactly 10 min after the addition of Cu. This mixture was incubated in a 37°C water bath, aliquots removed at various time points and oxidation stopped with the addition of BHT. The LDL α-tocopherol was extracted with hexane, the organic phase removed and evaporated under a low pressure nitrogen stream. The resulting sample was reconstituted in 200 μL of HPLC mobile phase (50:50 acetonitrile/ethanol) and transferred to amber injection vials for direct injection and separation using a C-18 Ultramex 150 × 4.6 mm, 5-μm particle size reverse phase column (Phenomenex, Torrence CA), with α-tocopherol detected at 293 nm wavelength as previously described (27).

Azo-initiated LDL and plasma oxidation. LDL oxidation (75 μg of LDL cholesterol in a 1 mL volume) was initiated by the addition of AAPH, a free radical generator, at a final concentration of 1

[4] Abbreviations used: AAPH, 2,2′-azobis′(2-amidino propane) hydrochloride; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; CD, conjugated diene; GAE, gallic acid equivalents; LC-ELSD/MS, liquid chromatography electrospray detection mass spectrometry; MDA, malondialdehyde (1,1,3,3-tetramethoxypropane); TBARS, thiobarbituric acid reacting substances; TEAC, Trolox equivalent antioxidant capacity.

mmol/L and CD formation measured as described above. The test compounds and walnut extracts were used at the same concentrations as described for the Cu-mediated experiments. In another experiment, plasma oxidation products were measured by means of thiobarbituric acid reacting substance (TBARS) formation as previously described and carried out with slight modifications (28). Plasma (100 μL) oxidation was initiated with the addition of AAPH at a final concentration of 50 mmol/L in the presence of the walnut extracts or ellagic acid. Samples were incubated for 4 h at 37°C and the oxidation subsequently stopped with the addition of BHT. TBARS concentration was determined by UV absorption at 532 nm, compared with a MDA standard curve, and results expressed as micromolar MDA equivalents.

Statistical analysis. All values are expressed as means ± SD. A parametric one-way ANOVA was performed on oxidation data using Systat 9 (Chicago, IL). To determine significant difference from control values, a Tukey multiple comparison post-hoc test was performed. Differences with $P < 0.05$ were considered significant.

RESULTS

Total phenols in 50 g of walnuts (~8–9 shelled walnuts) as determined by the Folin-Ciocalteu assay, contained 802 mg GAE. Mass spectrometry (LC-ELSD/MS) screening analysis of the walnut phenolic extracts (**Fig. 1**) identified ellagic acid as was previously reported (29). In addition, the closely related ellagitannins, valoneic acid dilactone (negative electrospray ionization: mass 469.4) and pedunculagin (mass 783.7) were observed. There were also three different compounds that appeared to be ellagitannins on the basis of mass (mass 907.8, 951.9, 1104.0), but their specific identity was not clear. One flavonol, a quercetin pentoside (mass 433.4) was also observed. These screening data show that the phenolics in walnut are principally of the nonflavonoid type and fall into the category of ellagitannins. However, the specific identities of all of the major phenolic components remain to be established.

The walnut extracts had TEAC values of 1.46 ± 0.06 and

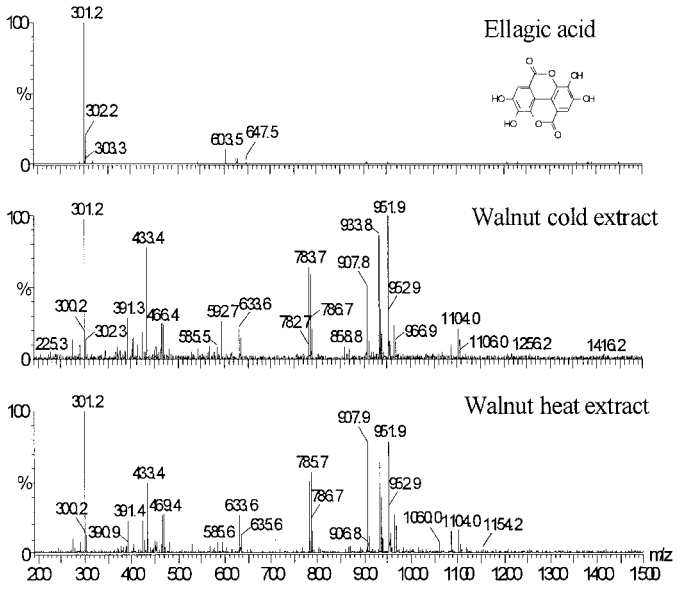

FIGURE 1 Liquid chromatography electrospray detection mass spectrometry (LC-ELSD/MS) characterization of walnut extracts (heat and cold) compared with ellagic acid standard (negative electrospray ionization mass 301.2). The presence of ellagic acid and other related ellagitannins (mass 469.4, 783.3, 907.8, 951.9, 1104.0) was observed in both heat and cold walnut extracts, along with one flavonol (quercetin pentoside, mass 433.4). The remaining phenolic compounds await complete identification.

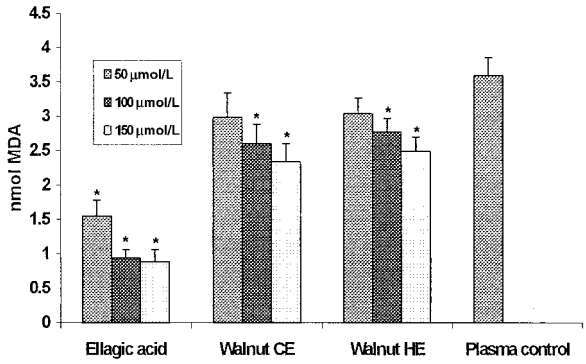

FIGURE 2 Thiobarbituric acid reacting substance (TBARS) production in 2,2′-azobis′(2-amidino propane) hydrochloride (AAPH)-mediated plasma oxidation is reduced in the presence of walnut phenolic-rich extract and ellagic acid. Ellagic acid and the walnut extracts [heat (HE) and cold (CE)] were tested at 50, 100 and 150 μmol/L compared with the control. Values are malondialdehyde (MDA) equivalents ± SD, $n = 6$. *Significantly different from control, $P < 0.001$.

1.10 ± 0.14 for heat and cold extracts, respectively. Purified ellagic and gallic acids, monomeric phenolics in walnut pellicles as reported by LC-MS analysis, had TEAC values of 3.12 ± 0.07 and 2.61 ± 0.10, respectively. Catechin TEAC was determined to be 2.51 ± 0.05.

All three concentrations of ellagic acid (50, 100 and 150 μmol/L) significantly inhibited AAPH-mediated oxidation in plasma by 57, 74 and 75% respectively, compared with control plasma (**Fig. 2**). The two higher concentrations (100 and 150 μmol/L GAE) of walnut extracts significantly inhibited oxidation by 23 and 31% for heat extract, and 28 and 35% for cold extract, respectively. These whole-plasma results correspond well with the AAPH-mediated LDL oxidation data.

Oxidation of LDL was initiated by AAPH or Cu ions and CD formation was measured. Both walnut extracts at concentrations of 0.1, 0.5 and 1.0 μmol/L GAE inhibited AAPH-mediated LDL oxidation in a dose-dependent manner because lag time was lengthened by an average of 4.5, 20 and 38%, respectively, compared with control LDL (**Fig. 3**A). The effect was significantly greater in the Cu-mediated oxidation system, with lag time increasing an average of 0, 39 and 82% (Fig. 3*B*). Purified ellagic acid and catechin were used as control phenolic compounds and compared with control LDL. The highest concentration of ellagic acid resulted in 87 and 14% increases in lag time with AAPH- and Cu-mediated oxidation systems, respectively, and catechin also significantly increased lag time by 82 and 43%, respectively. Figure 3C depicts CD formation during copper-mediated LDL oxidation using a representative concentration (1 μmol/L) of ellagic acid, heat and cold walnut extracts and compared with control LDL.

Ellagic acid significantly spared α-tocopherol up to 20 min, regardless of whether it was added immediately before or 10 min after the initiation of oxidation (**Fig. 4**A). The walnut heat extract significantly spared the α-tocopherol at the 20-min time point when added immediately before oxidation, but not when added 10 min after initiation (Fig. 4*B*). No α-tocopherol was detected in the walnut phenolic extracts (data not shown).

DISCUSSION

Walnuts are unique among the edible tree nuts because of their relatively high content of polyunsaturated linoleic and linolenic fatty acids. It is this favorable lipid profile of nuts that

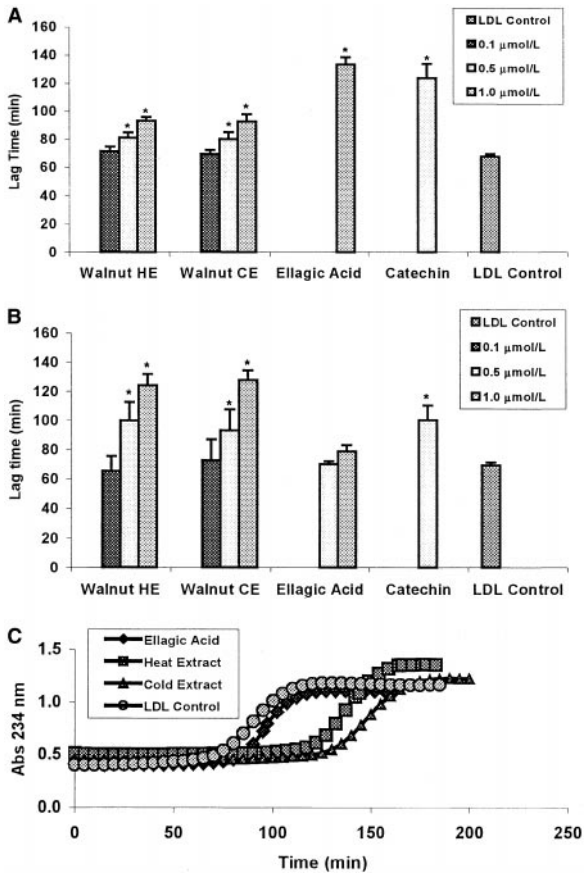

FIGURE 3 Effects of walnut polyphenol-rich extract and purified phenolic compounds on conjugated diene (CD) formation lag time. Three concentrations (1.0, 0.5 and 0.1 μmol/L) of the walnut extracts [heat (HE) and cold (CE)] were tested and compared with control LDL. Ellagic acid and catechin standards were included for comparison. (*A*) 2,2′-azobis′(2-amidino propane) hydrochloride (AAPH)-mediated LDL oxidation; (*B*) Cu-mediated LDL oxidation. Values are means ± SD, $n = 6$. (*C*) Representative plot of CD formation produced during Cu-mediated LDL oxidation, using walnut extracts (1.0 μmol/L, heat and cold), ellagic acid and control LDL. *Significantly different from control, $P < 0.001$.

has previously been proposed as the mechanism of their apparent antiatherogenic effect in humans. The fatty acids of walnuts are prone to oxidation (rancidity) and thus, it is of interest that the pellicle surrounding the kernel is naturally rich in antioxidant polyphenolics, in addition to the high content of tocopherols in the kernel itself (30,31). Walnut pellicles contain a number of nonflavonoid phenolics, based on early work using liquid and paper chromatography (32). At least 10 polyphenols have been reported to be present in English walnut pellicle extract, including the monomers ellagic acid, gallic acid and methyl gallate, which when present as polymers and bound to sugars are known as hydrolyzable tannins, and comprise the majority of the polyphenolics present (29,32,33). The LC-ELSD/MS data presented here confirm that the hydrolyzable tannins, and specifically ellagitannins, are the most abundant phenolic constituents. Minor differences were identified between the heat and cold extracts with respect to relative peak size. However, the antioxidant capacities of the two walnut extracts were very similar. The activity of these substances warrants a more thorough analysis to determine whether the activity is dependent on just a few or many of the components, and then to establish their complete chemical identity.

The extract from a handful of walnuts, one serving of 50 g or ~8–9 shelled walnuts, contained 802 mg GAE of total phenols, thus explaining the astringency often associated with eating raw walnuts. Pecans have a total phenol content of ~500 mg GAE in 50 g of nuts (34). Unfortunately, no direct comparison of polyphenolic content can be made to other nuts because of the lack of existing data. Walnuts have favorable levels of total phenols relative to other foods reported to have high phenolic and antioxidant levels, when compared on a per serving basis. A handful of walnuts has significantly more phenolics than a glass of apple juice (117 mg in 240 mL or 8-oz glass) (35), a milk chocolate bar (205 mg in a 1.5 oz or 43-g chocolate bar) (11), or even a glass of red wine (372 mg GAE of total phenols in 150 mL or 5-oz glass) (36). The number of servings of these other foods that would have to be consumed to equal the total phenolics in a serving of walnuts is as follows: 1 serving of walnuts = 2.2 servings red wine = 3.9 servings of milk chocolate = 4.6 servings of apple juice. However, the energy content of the foods should also be taken into account when consideration is given to substituting foods low in polyphenolics with higher energy polyphenol-rich foods. Expressed in terms of phenolic nutrient density (in this case GAE/kJ in one serving), the foods can be ranked in the following order: red wine 3.0 > walnuts 2.5 > apple juice 1.0 ≅ milk chocolate 0.9. In summary, the overall nutritional quality of walnuts as a whole food is such that it represents one of the best dietary sources of phenolic antioxidants, α-tocopherol and unsaturated fatty acids, in addition to other components, that can be easily incorporated into individual diets.

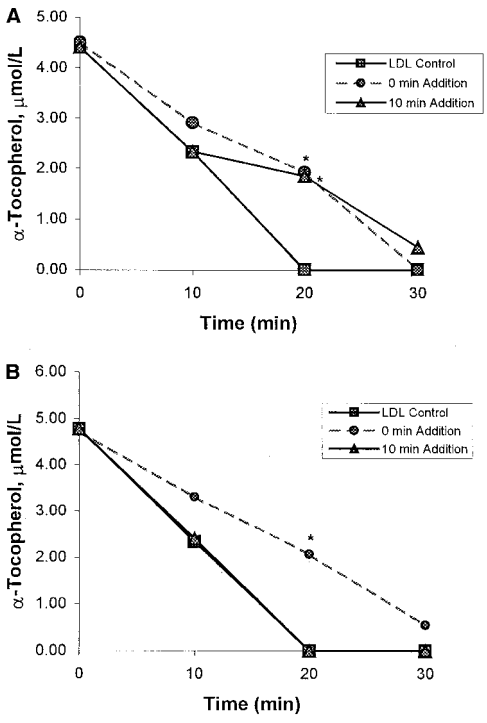

FIGURE 4 Effect of oxidation on endogenous LDL α-tocopherol concentration in the absence or presence of test compounds. LDL α-tocopherol concentration with addition of (*A*) 1 μmol/L ellagic acid, (*B*) 0.5 μmol/L walnut heat extract, at varying time points during Cu-mediated oxidation. Ellagic acid and the walnut extracts were added to the LDL at the time oxidation was initiated or 10 min postinitiation. Values are means ± SD, $n = 3$. *Significantly different from control, $P < 0.001$.

Antioxidant activities of the extracts and purified compounds were in close agreement with previous reports of phenolic compounds (37–39). The TEAC data demonstrate that walnut extracts have more antioxidant activity than α-tocopherol and that purified ellagic and gallic acid (monomeric components of walnuts) have even greater potency than α-tocopherol, as assessed by this method. A possible explanation for the discrepancy between TEAC values for walnut extracts and the purified compounds is that the presence of phenolic compounds as larger complexes of hydrolyzable tannins, very good metal complexing agents compared with simple ellagic acid, in the extract may result in a lower overall antioxidant activity in this assay. Additionally, the complex mixture of phenolics found in the extract may contain compounds that inherently do not have a TEAC value as high as the purified ellagic acid, or it is possible that some of the antioxidant activity is lost due to the extraction methods.

Walnut polyphenolic extracts, at concentrations within the range of expected physiologic plasma levels of dietary phenolics, (40–43), were compared with ellagic acid in view of its large contribution to the total polyphenolic content in English walnuts (Fig. 1). Ellagic acid has been studied previously in lipid oxidation assays by a variety of methods, yielding conflicting results (9,22,44,45). Formation of CD during LDL oxidation was used in the present study because clinical studies report the lag time of CD to be a measure of LDL oxidation that is associated with coronary heart disease risk (46,47). Ellagic acid significantly inhibited AAPH- but not Cu-mediated LDL and plasma oxidation (Fig. 3). In contrast, both walnut extracts demonstrated significantly greater ability to inhibit Cu-mediated LDL oxidation than ellagic acid. Additionally, the extracts significantly inhibited AAPH-mediated plasma and LDL oxidation, although to a lesser degree than purified ellagic acid. This suggests that other phenolics present in the extracts contribute markedly to the inhibition of Cu-mediated oxidation, probably due in large part to metal chelation by the tannins. Thus, walnut extracts containing ellagic acid monomers, polymeric tannins and other phenolics effectively inhibited in vitro plasma and LDL oxidation induced by generation of hydroxyl, peroxyl and superoxide radicals in both metal ion–dependent and –independent oxidation systems.

The walnut polyphenolics and purified ellagic acid had similar modest significant in vitro effects on maintaining LDL α-tocopherol during oxidative stress. It can be speculated that ellagic acid may be all or partly responsible for the observed in vitro effect on LDL α-tocopherol, but this is not confirmed by the present experiments. Previous in vitro studies have demonstrated the ability of other selected phenolics to maintain and extend LDL α-tocopherol levels and in some cases regenerate α-tocopherol (48–51). The concentration of LDL α-tocopherol, despite being quantitatively the predominant antioxidant, contributes to, but is not the primary determinant that predicts the resistance of LDL to oxidative stress (51–54). The complex milieu of endogenous antioxidants in the plasma and LDL, including dietary derived polyphenols, all contribute to the protection of LDL in vivo.

A recent in vivo study reported that daily consumption of walnuts significantly lowered total and LDL cholesterol but did not significantly change LDL α-tocopherol levels or the ability to resist oxidation when initiated ex vivo (17). However, the ability of LDL to resist oxidation was preserved by the walnut diet despite a 14 and 83% enrichment in LDL lipids of polyunsaturated linoleic acid and α-linolenic acid, respectively, which are prone to oxidation. Even though there was no effect on oxidation susceptibility of isolated LDL after daily consumption of walnuts, it cannot be ruled out that in vivo, plasma and LDL lipids have acquired an increased resistance to free radical damage.

It is likely that polyphenolic compounds are localized in the plasma and are loosely associated with the LDL surface, rather than partitioned into the LDL lipid core, due to their combined lipophilic and hydrophilic properties (55,56). Therefore, measurement of both plasma and LDL oxidation susceptibility and specific markers of oxidative damage ex vivo will more closely reflect the physiologic action of dietary polyphenols and their circulating metabolites (57). In vitro results for polyphenolic compounds generally show protection against LDL oxidation and depletion of α-tocopherol, yet in vivo investigations have produced mixed results, possibly due to variations in design, methodology and the variability of polyphenolic content in food sources. However, protective effects of polyphenols have been observed more consistently in studies in which the antioxidant capacity of whole plasma or plasma lipid peroxidation products was measured ex vivo (55,58–64).

The current in vitro data on polyphenolic-rich walnut extracts used at expected physiologic concentrations, demonstrate their potent antioxidant capacity with plasma and LDL lipids and contribute to the overall understanding of how dietary-derived polyphenolics may participate in the complex antioxidant network. The brief LC-ELSD/MS characterization of the predominant walnut phenolic compounds adds to current knowledge of polyphenolic distribution in foods. In addition to the favorable lipid profile of nuts, their phenolic content must now be considered as a potential contributor to the apparent antiatherogenic effect of nuts. These dual benefits of walnuts can be derived only from a whole food. Thus, the role of nuts as antiatherogenic is strengthened by the addition of in vitro data to existing feeding and epidemiologic studies. Further research is required to examine the effects of whole-walnut consumption on plasma polyphenol levels, plasma and LDL antioxidant capacity and ex vivo resistance to oxidative stress.

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