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Supplementation of Plasma with Olive Oil Phenols and Extracts: Influence on LDL Oxidation

RIANNE LEENEN,*,† ANNET J. C. ROODENBURG,† MAUD N. VISSERS,‡ JOHAN A. E. SCHUURBIERS,† KAREL P. A. M. VAN PUTTE,§ SHEILA A. WISEMAN,† AND FRANS H. M. M. VAN DE PUT†

Unilever Health Institute, Unilever Research & Development Vlaardingen, Olivier v. Noortlaan 120, 3133 AT Vlaardingen, The Netherlands, Division of Human Nutrition and Epidemiology, Wageningen University, Bomenweg 2, 6703 HD Wageningen, The Netherlands, and Unilever Research & Development Vlaardingen, Olivier v. Noortlaan 120, 3133 AT Vlaardingen, The Netherlands

Phenols present in olive oil may contribute to the health effects of the Mediterranean lifestyle. Olive oil antioxidants increase the resistance of low-density lipoproteins (LDL) against oxidation in vitro, but human intervention studies have failed to demonstrate similar consistent effects. To better mimic the in vivo situation, plasma was incubated with either individual olive oil phenols or olive oil extracts with different phenolic compositions, and LDL was subsequently isolated and challenged for its resistance to oxidation. The results show that the *ortho*-dihydroxy phenols (hydroxytyrosol and oleuropein-aglycone) are more efficient than their *mono*-hydroxy counterparts (tyrosol and ligstroside-aglycone) in increasing the resistance of LDL to oxidation. However, the concentration of antioxidants required to inhibit LDL oxidation when added to whole plasma was substantially higher as compared to previous data where antioxidants are directly added to isolated LDL. In conclusion, this study supports the hypothesis that extra virgin olive oil phenols protect LDL in plasma against oxidation. The explanation that in vitro studies show protective effects in contrast to the lack of effect in the majority of human studies may be that the dose of the phenols and thus their plasma concentration in humans was too low to influence ex vivo LDL oxidizability. Further studies are required to gain a better understanding of the potential health benefits that extra virgin olive oil may provide.

KEYWORDS: Olive oil; phenols; tyrosol; hydroxytyrosol; oleuropein; oleuropein-glycoside; LDL oxidation; in vitro; antioxidant

INTRODUCTION

In Mediterranean countries, where olive oil is the major source of fat, rates of coronary heart disease are relatively low as compared with more northern European societies (*1*). In addition to the favorable effects of the monounsaturated fatty acids on blood cholesterol concentrations (*2*) and on LDL oxidizability (*3*), animal and in vitro studies suggest that the high concentration of phenolic antioxidants in extra virgin olive oil also may contribute to the healthy nature of this diet (*4*−*11*). The most abundant phenols in extra virgin olive oil are the less polar oleuropein- and ligstroside-aglycones. They are formed in olives by enzymatic removal of glucose from the parent compound oleuropein-glycoside. Hydroxytyrosol and tyrosol are end products produced from the hydrolysis of oleuropein- and ligstroside-aglycones, respectively, and they are polar compounds.

Oxidation of LDL is believed to increase the risk of atherogenesis, the underlying cause of coronary heart disease. Olive oil phenols may be particularly effective in preventing oxidation of LDL. Because of their relatively nonpolar nature, they may directly associate with LDL particles. This association may lead to an increased resistance of LDL against oxidation, and this could be one of the potential mechanisms by which extra virgin olive oil may benefit health. In addition, olive oil phenols may also scavenge radicals in the aqueous surroundings. These hypotheses are supported by in vitro experiments, where it has been demonstrated that oleuropein-glycoside and hydroxytyrosol increase the resistance of LDL against copper-induced oxidation (*5*, *7*). However, in the majority of human studies (*12*−*15*), no effect of phenols present in extra virgin olive oil on ex vivo LDL oxidizability could be found in fasting or postprandial plasma samples. An explanation for this apparent discrepancy may be that the concentrations in human plasma were too low to influence ex vivo LDL susceptibility to

* To whom correspondence should be addressed (Rianne Leenen, tel: +31-10-4606467; fax: +31-10-4605726; e-mail: rianne.leenen@unilever.com).
† Unilever Health Institute.
‡ Wageningen University.
§ Unilever Research & Development Vlaardingen.

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Table 1. Relative Amount (%) of the Various Phenols in the Tested Olive Oil Extracts

description of the extract[a]	tyrosol	hydroxy-tyrosol	ligstroside-aglycones	oleuropein-aglycones
nonpolar extract 1	21	26	14	39
nonpolar extract 2	31	2	53	14
polar extract	31	45	4	20

[a] The phenol concentrations were determined by means of HPLC as described in detail under Materials and Methods.

oxidation. Alternatively, human plasma olive oil phenols may also associate with other plasma proteins than LDL, although if those phenols are directly added to the isolated LDL fraction, LDL is the major protein source to which they can bind. Finally, phenols may dissociate from LDL during isolation from plasma.

The aim of the present in vitro study was to evaluate the effect of olive oil phenols on the resistance of LDL to oxidation. To mimic the in vivo situation as closely as possible, plasma was incubated with olive oil phenols. LDL was subsequently isolated and the resistance of LDL to oxidation was evaluated by monitoring copper-induced lipid peroxidation. In addition to pure olive oil compounds, we also examined the effects of structurally similar compounds, other dietary antioxidants such as vitamin E and tea catechins, and three olive oil extracts with different phenolic compositions to determine whether not only phenol concentration but also the antioxidant properties influence the resistance of LDL to oxidation.

MATERIALS AND METHODS

Tested Antioxidants. The following antioxidants or olive oil extracts were investigated for their ability to protect LDL against copper-induced oxidation and their antioxidant properties: tyrosol (Acros, New Jersey, USA); oleuropein-glycoside (Roth, Karlsruhe, Germany); DL-α-tocopherol (Merck, Darmstadt, Germany); green tea extract (Lipton, New Jersey, US). Gallic acid, 3,4-di-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, and catechin were obtained from Sigma, Saint Louis, USA. Hydroxytyrosol, two nonpolar extracts and a polar extract derived from extra-virgin olive oil, were prepared at Unilever Research & Development Vlaardingen, The Netherlands.

Two batches of hydroxytyrosol were prepared by lithium aluminum hydride reduction of 3,4-dihydroxyphenyl acetic acid according to the method described by Baraldi et al. (*16*). The purity of the two batches was found to be at least 93%. Oleuropein- and ligstroside-aglycones and their derivatives were not available in the pure form. Therefore, we prepared several olive oil extracts, in which the various phenols were present in varying amounts (**Table 1**; method of analysis is described below).

The polar phenols were extracted from olive oil using water. Demineralized water was added to olive oil at a ratio of 1:2, and the fluids were mixed vigorously with a motor-driven ribbon stirrer for 5 min at room temperature. The two phases were allowed to separate, and the water phase was collected. The nonpolar extracts (1 and 2) were extracted from two different sources of extra virgin olive oil with ethanol, which was later removed by rotary evaporation under vacuum at 40 °C.

Analysis of the Phenolic Composition of Polar and Nonpolar Olive Oil Extracts. The phenolic composition of the extracts was analyzed by means of HPLC, and the method is based on the method described by Montedoro et al. (*17*). To this end, a Waters 600 S liquid chromotograph was used. Injection of the samples was carried out by a 10 μL Rheodyne sample loop. A Chrompack 25 cm × 4.6 mm × 1/4 in. Intersil 5 ODS column was applied using a flow rate of 1 mL/min. The elution system consisted of solvent A (2% acetic acid in water) and solvent B (methanol). Gradient 0−20 min: A/B 85/15%; in 30 min 15−75 B in A; in 5 min A/B 25/75; in 1 min 75−100 B in A; in 10 min, 100% B. Olive oil phenols were detected at 280 nm, and the traces of three analyzed extracts are shown in **Figure 1**.

HPLC was combined to mass spectrometry (MS) to determine the molecular weights of the compounds. The mass spectrometer ionization mode that was used was negative atmospheric pressure chemical ionization (APCI) as well as positive and negative thermospray (TSP). The HPLC settings for merely the same for both ionization modes: RP C18 (Chrompack Inertsil 5 ODS-2) (250 × 4.6 mm i.d., dp = 5 μm), temperature 25 °C and an injection volume was 20 μL. Solvent A consisted of 85% water, 15% methanol + 0.25% formic acid, and solvent B consisted of 100% methanol + 0.25% formic acid. A gradient was used with the following flow program: 20 min A, in 30 min to A/B 29/71, in 1 min to 100%B, 15 min 100% B, in 5 min to 100% A. The APCI experiments were carried out at a flow rate of 1 mL/min and a postcolumn splitting down to 300 μL. The TSP experiments were carried out at a flow rate of 0.8 mL/min. For the positive TSP a postcolumn addition of 0.1 mL/min of a 1 M ammoniumacetate in methanol/water (50/50) was used. The MS conditions for the APCI experiments, carried out on a Micromass Quatro II (Micromass, Manchester, UK), were scan range *m*/*z* 70−500, flow probe 300 μL/min, source temperature 110 °C, Corona discharge needle −1.8 kV, counter electrode 0 kV, cone voltage −28 V, scan speed 3 s/scan. The MS conditions for the TSP experiments, carried out on a Trio 3 (Micromass, Manchester, UK), were scan range *m*/*z* 100−600, source temperature 200 °C, temperature capillary 180 °C, scan speed 3 s/scan. Because of the low ionization efficiency, the intensity of the peaks was low when negative TSP or APCI were used. The positive thermospray mode is the best way to identify the mol masses, sometimes with the help of negative APCI. In this mode, the $[M + NH_4]^+$ was used for the determination of the mole masses.

In addition, HPLC-MS-MS was used to further elucidate the identity of the olive oil phenols. The HPLC experimental settings were as described above for the positive TSP experiments. The injection volume was 50 μL. The MS settings were as described above, the collision energy was 5 eV, the gas (Xe) pressure was 5×10^{-6} mb. This method confirms the presence or absence of a hydroxytyrosol or tyrosol moiety and identifies other characteristic fragments.

Analysis of the phenolic fractions in a variety of olive oils in our laboratory has led to the identification of 12 major peaks that can be related to either hydrotyrosol or tyrosol or the aglycones of oleuropein or ligstroside and the peaks are described below. Some other minor peaks have also been detected, but no hydroxytyrosol or tyrosol could be detected by means of LC-MS-MS and are not numbered in the HPLC traces. The intensity of the peaks differs between the different varieties of extra virgin olive oils that have been analyzed in our laboratory (results not shown).

The identified oleuropein- and ligstroside-aglycone derivates differ in that the elenolic acid ring structure is either open or closed, the number of aldehydes, and the presence or absence of a carboxy-group. The determined molecular masses together with the information of the MS-MS analysis lead to the assignment of the various oleuropein- and ligstroside-algycones that have been described in the literature (*17*−*20*). Peak 1 is hydroxytyrosol; peak 2 is tyrosol; peaks 3, 4, 5, 7, 8, 9, and 10 are oleuropein aglycones; peak 3 is a dialdehydic form lacking a carboxymethyl group (MW 320; open ring structure); peaks 4 and 5 represent other oleuropein aglycones with a molecular weight identical to that of 8 and 10 (i.e., MW 378); and peak 7 has a molecular weight of 376; the structures of peaks 4, 5, and 7 remain to be identified; peak 8 is a dialdehydic form (MW 378; open ring structure); the identity of peak 9 remains elusive (MW 194); peak 10 is an aldehydic form of oleuropein-aglycone (MW 378, closed ring structure). Peaks 6, 11, and 12 are ligstroside aglycones; peak 6 is a dialdehydic form lacking a carboxy group (MW 304, open ring structure); peak 11 is a dialdehydic form (MW 362, open ring structure); and peak 12 is an aldehydic form (MW 362, closed ring structure).

The formation of the various aglycones from their glycosylated parent compounds was confirmed by enzymic hydrolysis of pure oleuropein-glycoside. Briefly, 132 mg of oleuropein-glycone was incubated in the presence of 60 units of β-glucosidase in 10 mL of acetate buffer according to the method described by Limiroli et al. (*18*). The obtained aglycones were extracted with 20 mL of CH_2Cl_2/methanol (2/1, v/v).

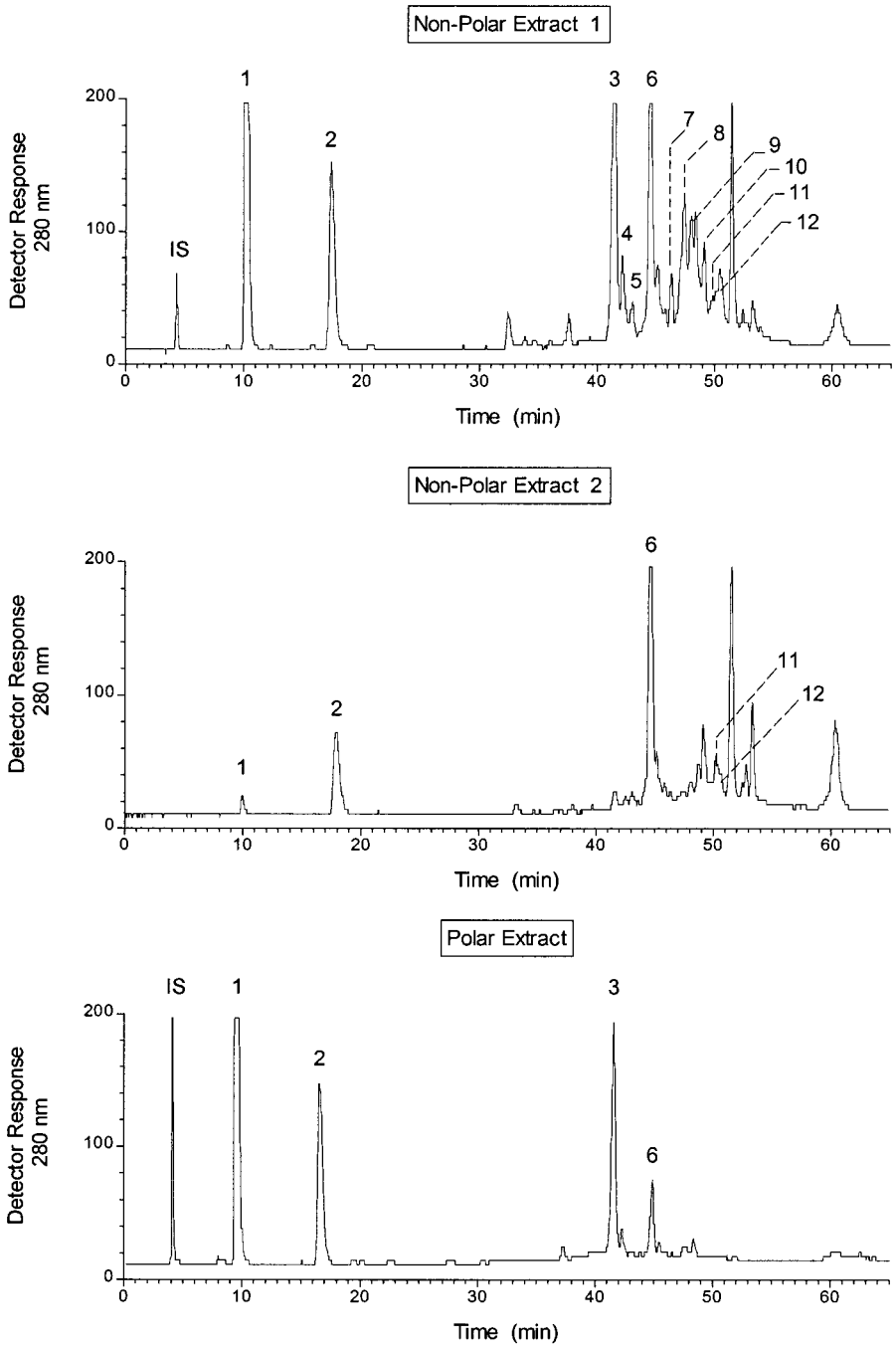

Figure 1. HPLC chromatograms of the nonpolar and polar extracts prepared from olive oil. Nonpolar extract 1, nonpolar extract 2, and polar extract were prepared and submitted to HPLC. LC-MS and HPLC-MS-MS were used to further identify the peaks. (1) Hydroxytyrosol; (2) tyrosol; (3), (4), (5), (7), (8), (9), and (10) are oleuropein-aglycones; (6), (11), and (12) are ligstroside-aglycones. The molecular weights and the identity of the peaks are described in further detail in the materials and method section. Gallic acid was used as an internal standard (IS). The detector response on the *Y*-axis is limited to 200 a.u. for all three traces for reasons of comparison between the extracts and to visualize the presence of the minor peaks; the response of some of the major peaks is actually higher than depicted. Nonpolar extract 1 is relative rich in hydroxytyrosol (1) and oleuropein-aglycones (predominantly (3)); nonpolar extract 2 is relative rich in tyrosol (2) and ligstroside-aglycones (predominantly (6)). The polar extract is relatively rich in hydroxytyrosol (1) and tyrosol (2), although some aglycones could be detected (predominantly (3) and (6)). Each extract was analyzed in duplicate and gave reproducible results.

After evaporation of the solvent, a mixture of 40 mg was obtained. Spectral analysis of the mixture showed that the mixture has a similar UV spectrum to that of oleuropein. HPLC analysis demonstrated the formation of several aglycones having similar properties to the oleuropein-aglycones endogenously present in olive oil (results not shown).

The following reference compounds were used to quantify the olive oil phenols: hydroxytyrosol and tyrosol. The amounts of secoiridoids present in the extracts were determined according to Owen et al. (*20*), i.e., the various secoiridoids present in olive oil were quantified against a standard curve of hydroxytyrosol. HPLC analysis of the two nonpolar extracts and the polar extract clearly demonstrate the difference in hydroxytyrosol/tyrosol and oleuropein-/ligstroside-aglycone composition (**Figure 1**; **Table 1**). The polar extract contains predominantly hydroxytyrosol and tyrosol. Oleuropein- and ligstroside-aglycones are more abundantly present in the nonpolar extracts. Nonpolar extract 1 contains relatively more oleuropein-aglycone (but also hydroxytyrosol). The majority of the olive oil phenols in nonpolar extract 2 can be

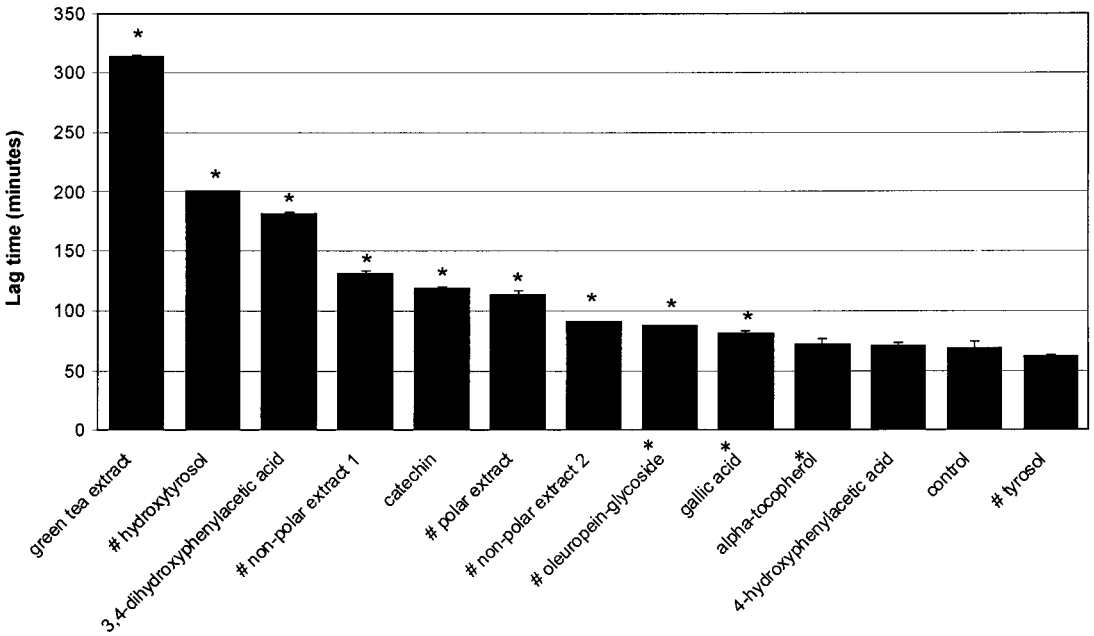

Figure 2. Effect of all compounds and extracts tested on the lag time of copper-mediated LDL oxidation. Plasma was incubated with the compounds at a concentration of 1000 μM. LDL was isolated by means of density gradient ultracentrifugation and submitted to oxidation by the addition of cupric ions. The formation of conjugated dienes was monitored spectrophotometrically and the lag time of the oxidation process was determined as described in detail in Materials and Methods. The results presented are the mean of two independent determinations of the lag time. Compounds indicated with # are present in olives or olive oil. * = Significant increase in lag time as compared to control samples.

explained by the presence of ligstroside-aglycone (and tyrosol). The total phenol concentration was diluted to the appropriate concentrations indicated in the text.

LDL Isolation and Measurement of Resistance of LDL to Copper-Induced Oxidation. Plasma was prepared by centrifugation at room temperature for 10 min at 2500*g* and 10 μL of a 600 g/L sucrose solution was added per mL of plasma (*21*). A single batch of pooled plasma was prepared to avoid donor variation, and plasma was stored at −80 °C until use. Plasma was mixed with the compounds, and extracts were tested for 30 min at room temperature. A control plasma was treated in the same way. The olive oil extracts were directly added, whereas α-tocopherol was dissolved in ethanol, and all other antioxidants were dissolved in water prior to addition to plasma. The final concentration of all compounds and extracts tested were 1000 μM. For a selection of these compounds (the two nonpolar extracts, the polar extract, hydroxytyrosol, tyrosol, oleuropein-glycoside, and DL-α-tocopherol) the minimal concentration required to increase LDL resistance to oxidation was determined by incubating plasma with a concentration range: 0, 50, 100, 250, 500, and 1000 μM. For the green tea extract, the concentrations were 0, 80, 160, 320, 640, and 960 μM.

LDL was subsequently isolated by density gradient ultracentrifugation using an SW 41Ti rotor (Beckman Instruments, Palo Alto, CA), 36000*g* for 24 h at 10 °C (*22*). All density gradients solutions contained 0.1 mM Na_2EDTA to prevent metal ion catalyzed LDL oxidation during the isolation procedure. The LDL fraction was isolated in a density range of 1.019−1.063 g/mL.

The protein concentration of the LDL fraction was determined as approximately 500 μg/mL (*23*). This fraction was initially diluted to a concentration of 200 μg/mL with gradient solution containing 0.1 mM Na_2EDTA (density was 1.019 g/mL). This solution was further diluted with PBS to a concentration of 50 μg/mL in a cuvette thermostated to a temperature of 30 °C. The oxidation of LDL was initiated by the addition of 10 μL of 5 mM $CuCl_2$ in a total volume of 1 mL. This method was essentially the same as described by Princen et al. (*24*). The formation of conjugated dienes was monitored spectrophotometrically at a wavelength of 234 nm at 2 min intervals using a UV−Vis spectrophotometer equipped with a thermostat controlled multicuvette holder for a period of 400 min or until oxidation was complete (Cary 50 Spectrophotometer, Varian Inc., Mulgrave, Victoria, Australia). The oxidation profile of each LDL sample was plotted against time. The lag-phase and the maximum rate of oxidation were determined as described by Esterbauer et al. (*25*). Each condition was tested in duplicate.

Determination of α-Tocopherol Levels in LDL. A small aliquot of the LDL fraction that had been incubated with DL-α-tocopherol was stored at −80 °C until analysis of its content in α-tocopherol. Butylated hydroxy toluene (BHT) was added to prevent oxidation. α-Tocopherol in LDL was determined by HPLC on a 5-μm column (Lichrospher RP-18; Merck, Darmstadt, Germany). α-Tocopheryl acetate (Merck) was used as an internal standard. The mobile phase consisted of methanol/2-propanol/water (50:50:8, by vol) and the flow rate was 0.6 mL/min. α-Tocopherol was detected by UV−Vis spectrometry at 292 nm, and α-tocopheryl acetate was detected at 284 nm.

Statistical Analyses. The duplicate values were averaged before data analysis. We applied the least square means of the general linear models (GLM) of the statistical analyses system (*26*) to analyze whether the various tested phenols significantly affected the absolute values of the lag time and maximum rate of LDL oxidation as compared to control-treated plasma (one-tailed, $p < 0.05$). Control-treated plasma was included in each experiment as either a monoplo or duplo, and the observed lag time and maximum rate were adjusted for run as an independent variable. The Dunnett multiple comparison test was used to adjust for multiple comparisons.

For the concentration-dependent oxidizability of LDL, we compared each concentration with the control-treated plasma by using ANOVA, with dose and run as independent variables.

RESULTS

Effect of Olive Oil Phenols, Extracts, and Other Dietary Antioxidants on LDL Oxidizability. The effects of preincubation of plasma with antioxidants at a concentration of 1000 μM on the lag time of copper-induced LDL oxidation are presented in **Figure 2**.

Hydroxytyrosol, 3,4-dihydroxyphenylacetic acid, and catechin all increased the lag time of the LDL oxidation process as compared to LDL derived from control treated plasma, indicating that an *ortho*-dihydroxy structure on the phenol ring is important in determining antioxidant function. The *mono*-

Table 2. Effect of Antioxidant Concentration on the Lag-Time of Copper-Induced LDL Oxidation[a]

	antioxidant concentration (μM)					
	0	50	100	250	500	1000
green tea extract[b]	62 ± 6	65 ± 4	78 ± 5**	98 ± 1***	166 ± 1***	264 ± 3***
hydroxytyrosol	63 ± 1	70 ± 6	72 ± 5*	81 ± 1**	116 ± 8***	191 ± 11***
nonpolar extract 1	66 ± 1	69 ± 3	76 ± 1*	86 ± 4***	102 ± 1***	161 ± 4***
polar extract	63 ± 1	68 ± 0	73 ± 1*	80 ± 3**	99 ± 0***	145 ± 8***
nonpolar extract 2	59 ± 1	63 ± 4*	67 ± 1**	73 ± 4***	77 ± 0***	96 ± 3***
oleuropein-glycoside	65 ± 2	66 ± 4	72 ± 6**	76 ± 5***	73 ± 4**	80 ± 4***
α-tocopherol	63 ± 0	65 ± 1	64 ± 0	67 ± 0**	68 ± 1**	78 ± 3***
tyrosol	65 ± 3	73 ± 0***	66 ± 1	63 ± 1	57 ± 3***	60 ± 2**

[a] Plasma was incubated with the ingredients in concentrations varying between 0 and 1000 μM. LDL was isolated and its resistance to oxidation was determined as described in Materials and Methods. The lag-times (min) presented are the mean (± SD) of two independent determinations of the lag time. *p < 0.05, **p <0.01, ***p < 0.001 indicate a significant change in lag-time compared to control incubated samples. [b] For green tea extract, the following concentration range was used: 0, 80, 160, 320, 640, and 960 μM.

hydroxy phenols (tyrosol and 4-hydroxyphenyl acetic acid) were, however, ineffective in affording protection. This was also the case for α-tocopherol, whereas gallic acid slightly increased the lag time. Green tea extract was most efficient in delaying the lag time as compared to all other ingredients examined in this study. All three olive oil extracts, normalized to a concentration of 1000 μmol of phenols/L, were shown to increase LDL resistance to in vitro oxidation.

The effect of all compounds or extracts tested on the maximum rate of oxidation during the propagation phase at a concentration of 1000 μM were also determined. Green tea extract and 3,4 dihydroxyphenyl acetic acid slightly reduced, albeit significantly, the oxidation rate as compared to control plasma [18.6 nmol of conjugated dienes min^{-1} (mg of LDL protein)$^{-1}$ for the green tea extract ($p = 0.004$) and 19.3 nmol of conjugated dienes min^{-1} (mg of LDL protein)$^{-1}$ for 3,4-dihydroxy phenylacetic acid ($p = 0.02$) versus 22.5 nmol of conjugated dienes min^{-1} (mg of LDL protein)$^{-1}$ for the control plasma]. All other components tested did not significantly alter the oxidation rate (data not shown).

For some of the compounds investigated, the minimum concentration required to increase LDL resistance to oxidation was determined by incubating plasma with concentrations ranging from 0 to 1000 μM. The results are shown in **Table 2**.

For most olive oil components, a minimum concentration of 100 μM was required to significantly increase the lag time, and at this concentration the increases in lag time were comparable for these components. However, when higher concentrations were used, the increase in lag time varied considerably between the ingredients. Green tea extract, hydroxytyrosol, nonpolar extract 1, and the polar extract all progressively increased the lag time with increasing concentrations. In contrast, increasing doses of nonpolar extract 2 and oleuropein-glycoside only moderately influenced the lag time.

The maximum rates of LDL oxidation during the propagation phase were not studied for the various concentrations of the compounds and extracts tested as this was the case for the lag time (**Table 2**), because preincubation of plasma with those compounds at a concentration of 1000 μM had no major effect on the maximum rate of LDL oxidation.

Effect of Incubation of Plasma with α-Tocopherol on Its Levels in LDL. Because of its lipophilicity, vitamin E is incorporated in lipoproteins such as LDL. The effect of incubating plasma with α-tocopherol on its partitioning in LDL was evaluated. The results in **Figure 3** show that α-tocopherol was incorporated dose-dependently in LDL. Despite the nearly 5-fold increase in vitamin E content of the LDL particles following an incubation of plasma with 1000 μM α-tocopherol, only a marginal increased protection of LDL against oxidation was observed (**Figure 2** and **Table 2**).

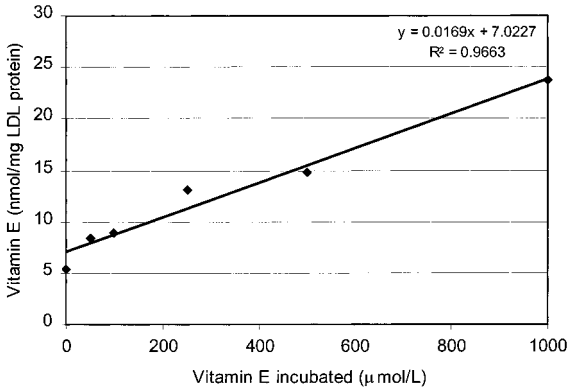

Figure 3. Vitamin E content in LDL isolated from plasma incubated with vitamin E. Plasma was incubated with vitamin E concentrations ranging from 0 to 1000 μM. LDL was isolated by means of density centrifugation and the vitamin E content in LDL was determined by HPLC as described in Materials and Methods and is expressed in nanomoles per milligram of LDL.

DISCUSSION

The results of the present study show that LDL isolated from plasma that has been incubated with the *ortho*-dihydroxy phenol hydroxytyrosol and with extracts rich in hydroxytyrosol have an increased resistance of LDL to copper-induced oxidation. However, the *mono*-hydroxy counterparts, tyrosol and tyrosol-rich olive oil extracts, appeared to have little effect on the susceptibility of LDL to oxidation.

We compared the effects of the various phenols to determine whether structural features influence antioxidant activity. The importance of the *ortho*-dihydroxy feature in antioxidant function is confirmed in this study, since hydroxytyrosol, 3,4-dihydroxy-phenylacetic acid, and catechin all efficiently increased the lag time of the LDL oxidation process. From the literature, it is known that mono-hydroxy polyphenols have weak antioxidant properties (*27*), which was also the case for tyrosol and 4-hydroxyphenylacetic acid in the present study. In addition to pure individual phenols, olive oil extracts and green tea extract were also tested. The polar and nonpolar olive oil extracts as well as green tea extract all contained phenols, including *ortho*-dihydroxy phenols. Indeed, all extracts increased LDL resistance. A positive relationship between the concentrations of hydroxytyrosol and oleuropein-aglycone in the extract and the lag time could be observed: nonpolar extract 1 and the polar extract

both with a relatively high content of oleuropein-aglycone and hydroxytyrosol were more effective in protecting LDL from oxidation than nonpolar extract 2 with a relatively low oleuropein-aglycone and hydroxytyrosol content (**Tables 1** and **2**).

Only green tea extract and 3,4-dihydroxyphenyl acetic acid slightly reduced the oxidation rate of the propagation phase of the LDL oxidation process, whereas all other antioxidants or antioxidant-rich extracts did not influence this oxidation rate. This indicates that, similar to the loss of antioxidants endogenously present in LDL, the majority of supplementary antioxidants are destroyed during the lag-phase and that after complete consumption of the antioxidants present in LDL, the other constituents such as lipids become prone to copper-mediated oxidation (*25*).

Of all antioxidants evaluated, α-tocopherol was the most lipophilic. α-Tocopherol levels in isolated LDL increased approximately 5-fold following incubation of plasma with 1000 μM of this antioxidant, but only marginally increased LDL resistance. In contrast, human studies have reported vitamin E (DL-α-tocopherol) supplementation protects LDL from oxidation (*28*, *29*). The concentrations of vitamin E in isolated LDL in the current in vitro study were similar to the concentrations of vitamin E in isolated LDL in a human study by Princen et al. (*28*) (5.3−23.7 nmol/mg of LDL protein and 13.2−29.6 nmol/mg of LDL protein, respectively). However, the amounts added to plasma in the current in vitro study were much higher (0−1000 μmol/L) as compared to the plasma concentrations of the subjects in the human study of Princen et al. (23.6−61.2 μmol/L) who consumed vitamin E supplements for minimally two weeks. This indicates that under these in vitro conditions, the plasma α-tocopherol concentration needs to be much higher to end up with similar concentrations in LDL after isolation as compared to the in vivo situation. A likely explanation for this difference may be that after ingestion vitamin E is incorporated in LDL through the α-tocopherol transfer protein (*30*), which is not present in an in vitro situation. In addition, the location of vitamin E within the LDL particle after incubation may differ from vitamin E, which is incorporated via the metabolic route. This might influence its effectiveness in protecting lipids in the LDL particles against oxidation. Comparison with other in vitro studies also reveals several discrepancies. Some studies showed no or limited protecting effects of vitamin E (*31*), whereas in another in vitro study (*32*) a protective effect of vitamin E could be observed. Differences in experimental conditions such as incubation time and higher vitamin E concentrations in LDL make it difficult to compare these in vitro studies.

The amounts of antioxidants required to inhibit LDL oxidation when added to whole plasma were substantially higher as compared to experiments where antioxidants are directly added to isolated LDL (*5*, *7*). An approximately 100-fold higher concentration of antioxidants needs to be added to plasma to achieve a similar inhibitory effect as compared to the condition where antioxidants are directly added to isolated LDL. When phenols are directly incubated with isolated LDL they can directly trap radicals generated in the system. In plasma, olive oil phenols might bind to a number of other proteins present. In fact, the majority of the polyphenol quercetin indeed binds to plasma proteins of which serum albumin is the major binding site (*33*). Moreover, other lines of evidence suggest that flavonoids and phenols bind to the proteins present in LDL (*34*, *35*). Phenols are less lipophilic than tocopherol and carotenoids; therefore, integration within the core of the LDL particle is less likely. A loss of phenols during the isolation procedure is expected to reduce its concentration in the final experimental conditions (*36*). Fito and co-workers (*37*) compared the effect of spiking plasma with antioxidants on the resistance of LDL oxidation following isolation with that of incubating isolated LDL with phenols. In that study, it was also shown that plasma needs to be spiked with substantially higher amounts of olive oil phenols to achieve an increase in LDL resistance to oxidation. They found that 160 mg of caffeic acid equivalents per liter of plasma, which equals to about 900 μmol of olive oil phenols per liter of plasma, extended the lag time by about 110%, which is comparable to the increases in lag time observed in the current study. To summarize, our observations are in line with the idea that the majority of the phenols present in plasma are lost during the isolation of LDL.

The ability of olive oil phenols to associate with LDL through binding to the proteins in LDL may be an important factor determining LDL resistance (*34*, *35*). Support for this idea was found when comparing the effects of oleuropein-glycoside and its aglycone. Oleuropein-glycoside is, just as its aglycone, equipped with a hydroxytyrosol moiety but is considerably more hydrophilic due to its glycosylation and is, therefore, expected to poorly associate with LDL. Indeed, oleuropein-glycoside only slightly increased the LDL resistance, whereas the oleuropein-aglycone rich extract resulted in a considerable protection of LDL against oxidation.

Feeding trials in rabbits and rats demonstrated that phenolic antioxidants from olive oil showed a favorable effect on the susceptibility of LDL to oxidation (*4*, *8*, *11*). During these feeding trials, the animals were fed with considerable amounts of olive oil for a prolonged period. In the study reported by Wiseman and co-workers (*8*), rabbits were fed for a 6-week period with an experimental diet rich in extra virgin olive oil. This probably leads to much higher serum levels of olive oil phenols leading to the accumulation of sufficient amounts of phenols in LDL that provide protection in the ex vivo determination of LDL resistance.

In contrast, most human intervention studies (*12*−*15*) have failed to demonstrate a protective effect of olive oil consumption on ex vivo LDL oxidizability in either fasting and postprandial plasma samples. Only the study of Ramirez-Tortosa et al. (*38*) showed a decrease in LDL oxidizability after 3 months of consumption of extra virgin olive oil as compared to refined olive oil. However, the method for measurement of LDL oxidation described in this study was completely different and cannot be compared with our own study and the other published studies. One possible explanation for the lack of effect in the majority of the human studies is that the dose of the phenols was too low to reduce LDL oxidizability. We do not know the plasma concentrations of the various phenols that were reached in these human studies, but, based on results of a study with quercetin, a dose of maximally 100 mg (about 275 μmol) probably did not result in a plasma concentration higher than 1 μmol/L (*39*). Daily intake of olive oil phenols in the Mediterranean region is estimated to be 10−20 mg (*7*), which implies that the level of phenols in olive oil is probably too low to produce meaningful effects on LDL oxidizability within the normal range of consumption. Thus, it is likely that the plasma concentrations in humans were too low to produce an effect on the ex vivo oxidation of LDL.

Data concerning intestinal absorption of phenols from olive oil are scarce. Furthermore, it is not known whether sufficient phenols accumulate in LDL particles to provide protection from oxidation. Until now, only Bonanome et al. (*13*) recovered hydroxytyrosol and tyrosol in LDL, albeit with a high variability among subjects. Phenols are probably present in the conjugated

or metabolized form in plasma or LDL. Recently, it has been shown that tyrosol and hydroxytyrosol were mainly excreted in urine in the conjugated form (*40*) and that the metabolite *O*-methylated hydroxytyrosol was excreted in high amounts after phenol-rich olive oil had been consumed (*41*). Conjugation, such as glucuronidation and sulfation, renders phenols more hydrophilic, and this might reduce their antioxidant capacity (*42*, *43*). The antioxidant capacity of the metabolite *O*-methylated hydroxytyrosol is not known, but for quercetin it has been demonstrated that the magnitude of prolonging the lag phase for 3′-*O*-methylquercetin is about half of that of its aglycone (*43*). Future research should therefore focus more on the kinetics and metabolism of phenols in humans.

In conclusion, the present in vitro study shows that olive oil phenols and olive oil extracts rich in phenols increase the resistance of LDL against oxidation. The *ortho*-dihydroxyphenol and oleuropein-aglycone are more efficient than their *mono*-hydroxy counterparts in protecting LDL. The ability of phenols to partition in LDL is also expected to be a relevant factor for the antioxidant efficacy. Glycosylated phenols, such as oleuropein-glycoside, are very hydrophilic and only marginally increase LDL resistance, whereas a mixture rich in the aglycone counterparts display more antioxidant activity in this model system. The explanation that in vitro studies show protective effects whereas the majority of human studies do not, may be because the dose of the phenols and thus their plasma concentration in humans was too low to reduce LDL oxidizability. This implies that the level of phenols in olive oil might be too low to produce meaningful effects on ex vivo LDL oxidizability within the normal range of olive oil consumption. However, more in vivo studies are needed to confirm these protective effects. In addition, it is plausible that olive oil antioxidants may play a role in other mechanisms of actions related to coronary heart disease. Thus, we need to increase our knowledge on the bioavailability, metabolism, and other mechanisms of action of olive oil phenols for a better understanding of the potential health benefits that extra virgin olive oil may provide.

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