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Powerful Protective Role of 3,4-Dihydroxyphenylethanol−Elenolic Acid Dialdehyde against Erythrocyte Oxidative-Induced Hemolysis

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The present work studied and compared the capacity of four important olive oil polyphenolic compounds, oleuropein, hydroxytyrosol, and the oleuropein aglycones 3,4-dihydroxyphenylethanol−elenolic acid (3,4-DHPEA-EA) and 3,4-dihydroxyphenylethanol−elenolic acid dialdehyde (3,4-DHPEA-EDA), to protect red blood cells (RBCs) from oxidative hemolysis induced by the physiological initiator H_2O_2. The amount of hemolysis was evaluated spectrophotometrically. The compounds were also tested in the presence and absence of the naturally occurring antioxidant ascorbic acid. All compounds were revealed to significantly protect RBCs from oxidative hemolysis induced by H_2O_2 at 40 and 80 μM, with the order of activity being 3,4-DHPEA-EDA > 3,4-DHPEA-EA > hydroxytyrosol = oleuropein. At 20, 10, and 5 μM, only 3,4-DHPEA-EDA showed a significant protection against the oxidative injury. In the presence of ascorbic acid at physiological concentration, the addition of individual compounds at 40 μM increased the stability of erythrocytes. The addition of phenolic compounds at 20 and 10 μM did not produce further protection when compared with the protection given by ascorbic acid alone, except for 3,4-DHPEA-EDA. This compound was shown to produce further protection even at 5 μM. In summary, 3,4-DHPEA-EDA plays an important protective role against reactive oxygen species-induced oxidative injury in RBCs, and this effect is more potent than the one evidenced by hydroxytyrosol or oleuropein.

KEYWORDS: ***Olea europaea*; polyphenols; erythrocytes; olive oil; hydroxytyrosol; oleuropein; 3,4-DHPEA-EA; 3,4-DHPEA-EDA; ascorbic acid**

INTRODUCTION

Antioxidants have received particular attention because of their potential to modulate oxidative stress associated with chronic disease. The lower incidence of coronary heart disease and some cancers in the Mediterranean area led to the hypothesis that a diet rich in fruits, vegetables, and grains has a beneficial effect on health. The major fat component of the so-called "Mediterranean diet" is virgin olive oil (VOO) (*1*). Several studies have suggested that phenolic compounds, although considered to be among the minor constituents of VOO, may contribute to the healthy nature of this diet (*2*−*5*).

In recent years, there has been much interest in antioxidants that retard oxidative modification of low-density lipoproteins (LDL), which is believed to be a key step in the development of atherosclerosis. The stability of LDL isolated from animals and humans fed VOO is increased, and this increased stability is attributable to the minor phenolic compounds in the oil (*3*, *5*−*7*). The administration of high doses of hydroxytyrosol (10 mg/kg/day) to apo E deficient mice, however, enhanced atherosclerotic lesion development (*8*). This fact points out the importance of the matrix, the combination of all antioxidants, and the knowledge of the activity of each polyphenolic compound present in natural foods such as virgin olive oil.

Human red blood cells (RBC) are particularly useful in the evaluation of the antioxidant properties of several compounds, namely, olive oil polyphenols. RBCs are particularly susceptible to endogenous oxidative damage because of their specific role as oxygen carriers. In the normal metabolism of RBCs, around 0.3% of the oxygen molecule is shifted from its normal role with the production of superoxide anion. Moreover, during the course of inflammatory processes, superoxide radicals may be generated in large amounts. This occurrence may be due to the activation of mast cells, macrophages, eosinophils, and neutrophils (*9*). The superoxide radical is then rapidly converted into H_2O_2 by superoxide dismutase. H_2O_2 is not an inherently reactive compound, but it can easily penetrate the membranes of surrounding cells and be converted into highly reactive and deleterious products (*10*).

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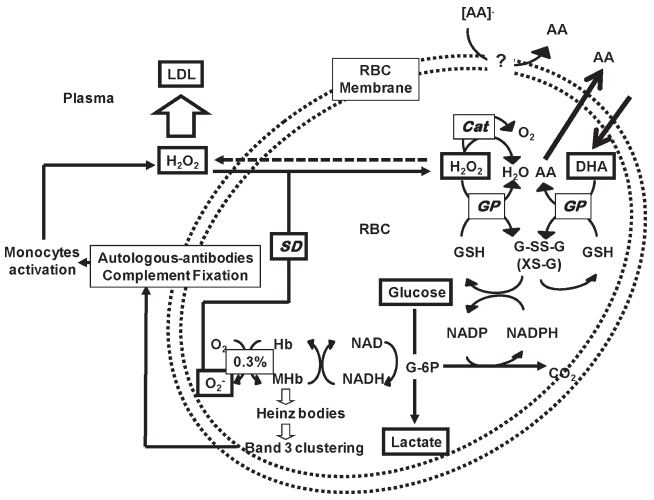

Figure 1. Erythrocyte response to oxidative stress. SD, superoxide dismutase; Cat, catalase; GP, glutathione peroxidase; GSH, glutathione; G-SS-G, oxidized glutathione; AA, ascorbic acid; DHA, dehydroascorbate; Hb, hemoglobin; G-6P, glucose-6-phosphate.

Healthy subjects are equipped with efficient RBC antioxidant endogenous systems (**Figure 1**). Ascorbate is highly susceptible to oxidation in plasma, being recycled from its oxidized form in RBCs, the most abundant blood cell (*11*) (**Figure 1**). The ascorbate redox cycling process enhances the antioxidant potential of RBCs and offers a defense against the development of oxidative stress in RBC and lipoprotein oxidation (*11*). If reactive oxygen species (ROS), such as H_2O_2 and O_2^-, are overproduced within the erythrocyte, an "oxidative stress" condition will develop, inducing oxidative damage on erythrocyte constituents, which may lead ultimately to hemolysis. Denatured hemoglobin links to the membrane at the cytoplasmic domain of band 3 protein, inducing linkage of natural antiband 3 antibodies and complementing fixation on the erythrocyte surface, marking the cell for removal by the macrophages. Whenever the hemoglobin is released from erythrocytes, it is potentially dangerous because it can be converted into oxidized forms, powerful promoters of oxidative processes in blood (*12*).

The use of antioxidants as preventive and/or as therapeutic agents in oxidative stress related diseases has generally been proposed. From this perspective, the consumption of natural antioxidants may be of extreme health importance. Actually, it is believed that olive oil consumption could reduce oxidative damage, on the one hand, due to its richness in oleic acid, and, on the other hand, due to its minor components, particularly the phenolic compounds. However, which components have a major role in this protection, is still unknown. Many studies have investigated the antioxidant properties of oleuropein, and especially of hydroxytyrosol, as well as their protective effects against cell injury and their bioavailability, but only a few studies have investigated the effects of oleuropein aglycones. A recent study from our group (*13*), evaluating the protection of olive oil polyphenolic compounds to protect RBCs from oxidative injury induced by the water-soluble radical initiator 2,2′-azobis(2-amidinopropane) dihydrochloride found that 3,4-dihydroxyphenylethanol−elenolic acid dialdehyde (3,4-DHPEA-EDA) may play a much more important protective role against ROS-induced oxidative injury in human cells than hydroxytyrosol or oleuropein. However, the protection provided by oleuropein aglycones against oxidative damage induced by H_2O_2, a physiological and biologically relevant oxidizing agent in erythrocytes (**Figure 2**), is not, as far as we know, currently known.

In this work, the capacity of four olive oil polyphenolic compounds (**Figure 2**), hydroxytyrosol, oleuropein, and its aglycones 3,4-dihydroxyphenylethanol−elenolic acid (3,4-DHPEA-EA, compound **1**) and 3,4-DHPEA-EDA (compound **2**), to protect RBCs from oxidative injury induced by H_2O_2 was evaluated in the presence and absence of the naturally occurring antioxidant ascorbic acid.

MATERIALS AND METHODS

Phenolic Compounds. Hydroxytyrosol was synthesized from 3,4-dihydroxyphenylacetic acid (Sigma-Aldrich Quimica-S.A., Madrid, Spain) according to the procedure of Baraldi et al. (*14*). Oleuropein was extracted from olive leaves according to the procedure of Gariboldi et al. (*15*). The aglycone **1** was obtained from oleuropein by enzymatic reaction using β-glycosidase (Fluka, Buchs, Switzerland), according to the procedure of Limirioli et al. (*16*). The olive oil component **2** (3,4-DHPEA-EDA) was obtained from olive leaves, according to the procedure of Paiva-Martins and Gordon (*17*). Purity of compounds was assessed first by HPLC and then by NMR. All of the phenolic compounds were solubilized in distilled water followed by 15 min under ultrasound in the dark.

Preparation of RBC Suspensions. Blood was obtained from healthy nonsmoker volunteers (two women and two men aged 20−40 years), by venipuncture, and collected into tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Blood samples were centrifuged at 400*g* for 10 min; plasma and buffy coat were carefully removed and discarded. RBCs were washed three times with phosphate-buffered saline (PBS; 125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) and finally resuspended in PBS to obtain 10 and 2% hematocrits.

To evaluate the capacity of oleuropein, hydroxytyrosol, 3,4-DHPEA-EA, and 3,4-DHPEA-EDA to protect RBCs from oxidative injury induced by H_2O_2, we evaluated, by in vitro studies, the RBC lysis, membrane-bound hemoglobin, and membrane protein profile, using the following assay conditions.

Protective Effect of Phenolic Compounds against H_2O_2-Induced Hemolysis. The assays were performed using H_2O_2 solutions at a final concentration of 100 mM, when using RBC suspensions at 10% hematocrit, or at 7.5 mM, when using RBC suspensions at 2% hematocrit. The incubations were carried out at 37 °C for 4 h, under gentle shaking of the RBC suspensions. The cells were pretreated at 37 °C for 15 min in the presence of the chosen concentrations of the polyphenols (80, 40, and 20 μM for all and 10 and 5 μM only for EDA) with or without ascorbic acid (Sigma-Aldrich Quimica-S.A.) at its physiological concentration (in accordance with the hematocrit used, 3 μM), and then H_2O_2 solution was added. Four assays (n = 4) were performed for each tested antioxidant system, all tests and controls being run in duplicate. The amount of hemolysis was determined spectrophotometrically, according to the method of Ko et al. (*18*). In all sets of experiments, a negative control (RBCs in PBS) was used. Besides the control assay (RBCs in PBS), we also tested the hemolytic effect of each compound upon RBCs, and we did not observe hemolysis (data not shown). After 4 h of incubation, an aliquot of the RBC suspensions was taken out, diluted with 20 volumes of saline, and centrifuged (1180*g*, for 10 min). The absorption (A) of the supernatant was read at 540 nm. To yield the absorption (B) of a complete hemolysis, an aliquot of the RBC suspension was treated with 20 volumes of ice-cold distilled water and, after centrifugation, the absorption was measured at the same wavelength. The percentage of hemolysis was then calculated: $(A/B) \times 100$.

Protective Effect of Phenolic Compounds against H_2O_2-Induced Erythrocyte Membrane Changes. To study the effect of the phenolic compounds to protect hemoglobin and the RBC membrane from oxidative injury initiated by H_2O_2, we needed to choose the most suitable concentration of this compound. From that perspective, we evaluated the changes induced in membrane-bound hemoglobin (MBH) and in membrane protein profile, by increasing concentrations of H_2O_2 (**Figure 5**). Because a higher number of RBC membranes were needed to perform these studies, RBC suspensions at 10% hematocrit were used. RBC suspensions were incubated at 37 °C for 3 h, under gentle shaking. Afterward, RBCs were washed in a saline solution and immediately lysed,

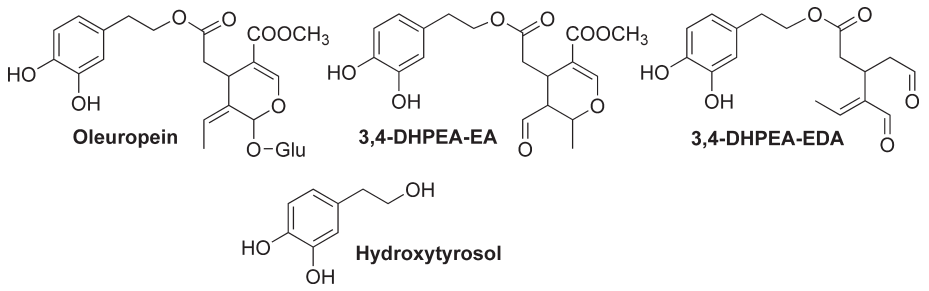

Figure 2. Structures of olive oil phenolics.

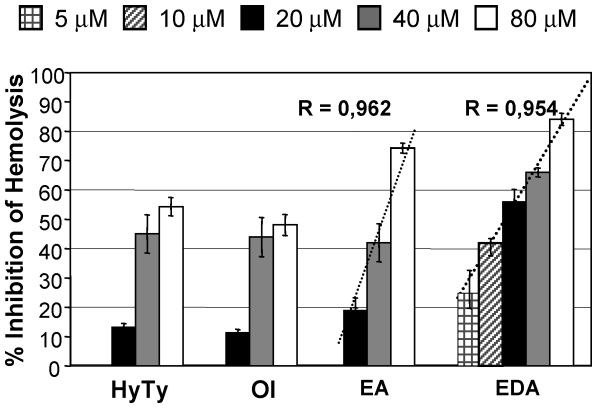

Figure 3. Inhibition of hemolysis of RBCs (mean ± SE) at 2% hematocrit, incubated for 4 h with phenolic compounds at 80, 40, and 20 μM and with 7.5 mM H_2O_2. DHPEA-EDA was also tested at 10 μM. HyTy, hydroxytyrosol; Ol, oleuropein; EA, 3,4-DHPEA-EA; EDA, 3,4-DHPEA-EDA.

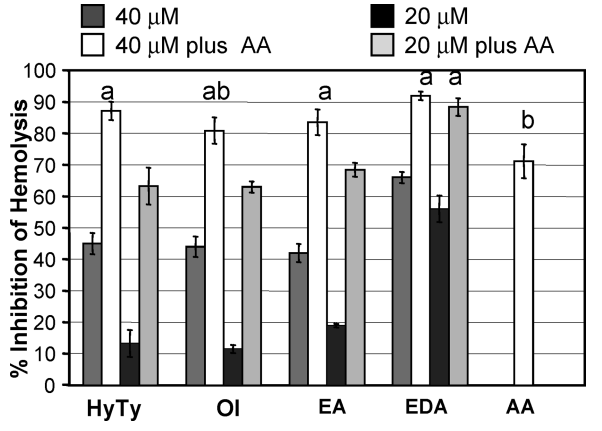

Figure 4. Inhibition of hemolysis of RBCs (mean ± SE) at 2% hematocrit, incubated for 4 h with phenolic compounds at 20 and 40 μM with and without ascorbic acid (3 μM) and in the presence of 7.5 mM H_2O_2. HyTy, hydroxytyrosol; Ol, oleuropein; EA, 3,4-DHPEA-EA; EDA, 3,4-DHPEA-EDA; AA, ascorbic acid. Mean values with different letters are significantly different ($P < 0.05$).

by hypotonic lyses according to the method of Dodge et al. (*19*). The obtained membranes were washed in Dodge buffer, adding in the first two washes phenylmethanesulfonyl fluoride, as a protease inhibitor, with a final concentration of 0.1 mM. The protein concentration of the RBC membrane suspensions was determined by using Bradford's method (*20*).

MBH was measured spectrophotometrically after protein dissociation of membrane components with Triton X-100 (5% in Dodge buffer) at 415 nm; the absorbance at this wavelength was corrected by subtracting the absorbance of the background at 700 nm. This value and membrane protein concentration were then used to calculate the percent MBH.

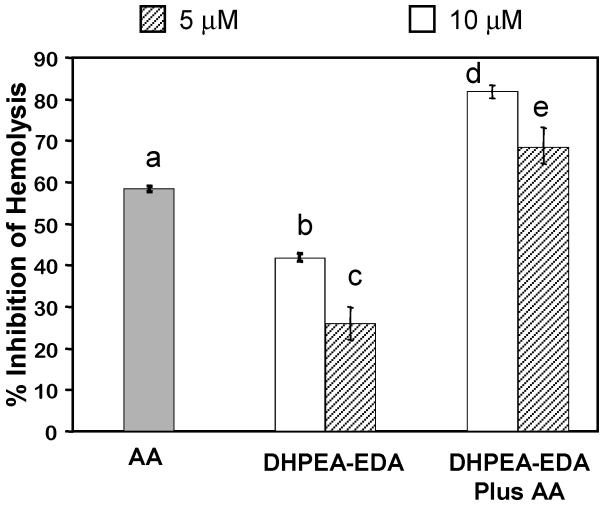

Figure 5. Inhibition of hemolysis of RBCs (mean ± SE) at 2% hematocrit, incubated for 4 h with DHPEA-EDA at 10 and 5 μM with and without 3 μM ascorbic acid and 7.5 mM H_2O_2. AA, ascorbic acid. Mean values with different letters are significantly different ($P < 0.05$).

Membranes of RBCs were treated with a solubilization buffer, heat-denatured, and submitted to electrophoresis (8 μg of protein/lane). The electrophoresis was carried out on a discontinuous system of polyacrylamide in the presence of sodium dodecyl sulfate (SDS-PAGE), using a 5−15% linear acrylamide gradient gel and a 3.5−17% exponential acrylamide gradient gel, according to the Laemmli and Fairbanks methods, respectively (*21*, *22*). The proteins were stained with Coomassie brilliant blue, and, finally, the gel was scanned (Darkroom CN UV/wl, BioCaptMW version 99, Vilber Lourmat, France).

The concentration of 1 mM of H_2O_2 (**Figure 6**) was found to be suitable for the assays, as it was capable of producing important modifications in RBC membrane proteins (visible after gel staining with Coomassie blue) and, therefore, would allow us to study the capacity of the phenolic compounds to inhibit oxidative changes.

In all sets of experiments ($n = 4$), controls (RBCs in PBS and RBCs in PBS with H_2O_2) were used. Controls and tests, using a 200 μM (final concentration) for phenolic compounds, were run in duplicate. The assay conditions were those described above for the H_2O_2 assays (1 mM H_2O_2, 4 h of incubation at 37 °C, under gentle shaking).

Statistical Analysis. The results obtained for the four independent hemolysis experiments, performed in duplicate, are expressed as means ± SEM. Statistical differences between groups of experiments with different antioxidant compounds were analyzed by two-way analysis of variance with post hoc testing using Tukey's test. A P value of < 0.05 was accepted as being statistically significant.

RESULTS AND DISCUSSION

In recent years, increasing evidence has supported the hypothesis that a number of nutrients or nonnutrient dietary components, labeled as "antioxidants", might have a beneficial role regarding the course of chronic degenerative diseases. In

particular, it has been claimed that polyphenolic components may play a major role in the protective effects of olive oil against oxidative damage. However, few studies have investigated the protective effects of oleuropein aglycones, namely, 3,4-DHPEA-EA and 3,4-DHPEA-EDA, the most important phenolic compounds found in olive oil.

To further elucidate the antioxidant properties of olive oil polyphenols in human cells and to identify the molecular mechanisms responsible for its cytoprotective effect, human RBCs were selected as a metabolically simplified model system. Human RBCs can be oxidized in vitro by several drugs and peroxides, including H_2O_2. Because of its biologically relevant oxidizing role in erythrocytes, H_2O_2 was preferentially used to initiate radical formation in intact cells to test the ability of olive oil phenolics to prevent hemolysis. At 2% hematocrit and in the presence of H_2O_2 at 7.5 mM, all compounds were shown to significantly protect RBCs from oxidative-induced hemolysis, with the order of activity (percent of hemolysis inhibition in parentheses) being 3,4-DHPEA-EDA (56%) > 3,4-DHPEA-EA (19%) > hydroxytyrosol (14%) = oleuropein (13%) (**Figure 3**). Moreover, the inhibitory effect was particularly striking for 3,4-DHPEA-EDA, which strongly protected RBCs in a concentration-dependent manner from hemolysis, even when its concentration was as low as 5 μM (**Figure 3**). The data reported in this paper on the ability of 3,4-DHPEA-EDA to counteract H_2O_2-induced oxidative stress in intact human RBCs confirm our previous results (*13*) obtained for this phenol in a similar biological system using AAPH as a radical initiator. However, a much different protection capacity was found for hydroxytyrosol and 3,4-DHPEA-EA. Apparently, hydroxytyrosol was much better in protecting RBC from oxidative injury when the free radicals were generated outside the cell than when free radicals were generated on both sides or within the membrane. Moreover, 3,4-DHPEA-EA, which showed the worst activity against the AAPH-induced injury, has shown now better activity than hydroxytyrosol or oleuropein (*13*). The lower protection capacity found for hydroxytyrosol and oleuropein when compared with the two oleuropein aglycones suggests an important role for their lipophilicity. Oleuropein aglycones, having higher log *P* values (*23*), may more easily penetrate the membranes and therefore reach the radicals formed within them.

Our results are also in accordance with the ones reported by Manna et al. (*24*) for hydroxytyrosol, also tested in the protection of RBC from oxidative injury induced by H_2O_2. In this study, hydroxytyrosol showed a protective effect for concentrations between 50 and 200 μM.

When RBC are prepared, their content in tocopherol and other endogenous antioxidant defenses remains intact in the erythrocyte structure, but the exogenous antioxidant defenses are washed out during the manipulation. Therefore, to understand the effective protection of compounds against oxidative-induced injury in blood cells, their activity was also tested in the presence of the most important extracelullar antioxidant, ascorbic acid. It is important to keep in mind that antioxidant substances work synergically in vivo, not alone. In the presence of a physiological concentration of ascorbic acid (3 μM, proportional to the hematocrit used, 2%), the effect of compounds at 40 μM significantly diminished the hemolysis of RBCs when compared with the activity of compounds tested alone (**Figure 4**). However, at the concentration of 20 μM none of the phenolic compounds increased the protection given by ascorbic acid to the RBCs, except 3,4-DHPEA-EDA. Indeed, in the presence of ascorbic acid (3 μM), the association with 3,4-DHPEA-EDA (at a concentration of 20 μM) showed a significant improvement in the protection against oxidative injury (**Figure 4**) when compared to the protection given by only ascorbic acid. Even at concentrations of 10 and 5 μM, 3,4-DHPEA-EDA still significantly protected RBCs from oxidative-induced hemolysis, and its association with ascorbic acid also showed a significant improvement in the protection against oxidative injury (**Figure 5**).

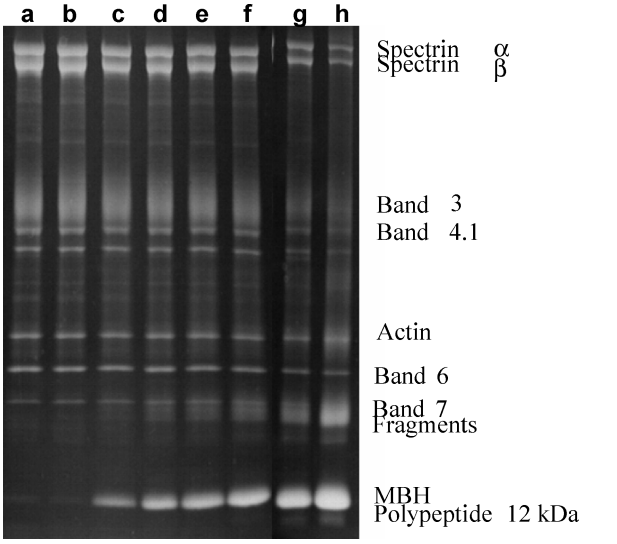

Figure 6. SDS−polyacrylamide gel electrophoresis (linear gradient gel) of RBC membrane proteins after incubation of RBCs for 3 h with increasing concentrations of H_2O_2 (0.1, 0.2, 0.3, 0.4, 0.5, 1, and 5 mM). The gel was stained with Coomassie blue. Lanes: a and b, control; c, 0.1 mM; d, 0.2 mM; e, 0.3 mM; f, 0.5 mM; g, 1 mM; h, 5 mM.

To study the capacity of the phenolic compounds to inhibit RBC membrane oxidative changes, we chose the concentration of 1 mM of H_2O_2, as it was capable of producing important modifications in RBC membrane proteins. Actually, at lower concentrations slight changes were observed, and at higher concentrations they were too strong (visible after gel staining with Coomassie blue) (**Figure 6**).

For all assays performed with H_2O_2, a reduction in α- and β-spectrin and in band 3 protein (**Figure 6**) was observed. In the absence of H_2O_2, protein analysis showed interactions between 3,4-DHPEA-EDA and RBC membrane proteins, as shown by the appearance of a new protein band (**Figure 7**, in the upper right dotted square). This band was already described in a previous work and identified by mass spectrometry as being α-spectrin plus band 3 protein (*13*). An additional band was also observed for EDA without H_2O_2 and for all of the phenolic compounds after incubation with H_2O_2 (**Figure 7**, lower dotted square). This band has also been previously identified by mass spectrometry as being a hemoglobin β chain (*13*).

Hemoglobin when denatured links to the RBC membrane via the cytoplasmic domain of band 3 protein, inducing its clustering and the linkage of anti-band 3 antibodies that mark the cell for death. In our study, we observed an increase in MBH in the assays performed with H_2O_2 (**Table 1**; **Figure 7**), but the amount of MBH was lower in the presence of antioxidants as compared with the control, with the lowest value observed with 3,4-DHPEA-EDA and the highest value with hydroxytyrosol (**Table 1**). The higher value of MBH in the presence of 3,4-DHPEA-EDA (even in the absence of the oxidant) was already observed in a previous work from our group, which led us to investigate the nature of hemoglobin that is linked to the RBC membrane (*13*). By performing spectral scans (450−650 nm) of lysed RBC suspensions in the presence of this phenolic compound (lysis performed after 3 h of incubation, without oxidant), we demonstrated that most of the hemoglobin linked

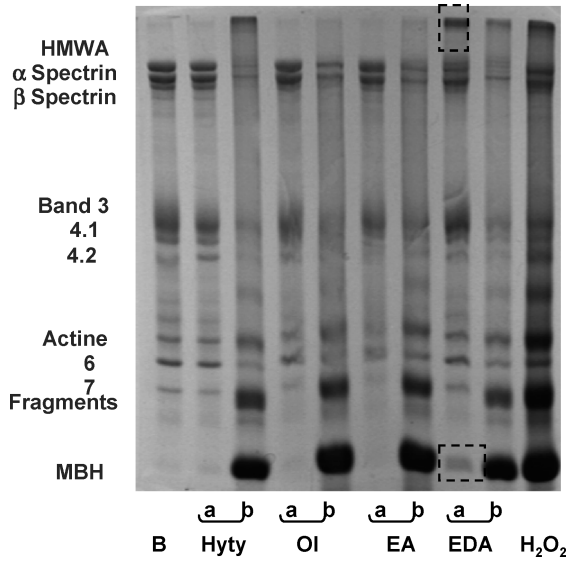

Figure 7. SDS−polyacrylamide gel electrophoresis (exponential gradient gel) of human red blood cell membrane proteins incubated with phenolic compounds (200 μM). The gel was stained with Coomassie blue. Lanes: B, control; a, phenolic compounds without H_2O_2; b, phenolic compounds with 1 mM H_2O_2; HyTy, hydroxytyrosol; Ol, oleuropein; EA, 3,4-DHPEA-EA; EDA, 3,4-DHPEA-EDA; H_2O_2, hydrogen peroxide.

Table 1. Erythrocyte Membrane-Bound Hemoglobin (MBH) after Incubation of RBCs at 10% Hematocrit for 3 h with Phenolic Compounds (200 μM) with and without H_2O_2 (1 mM)[a]

		% MBH × 10^{-3}				
		HyTy	Ol	EA	EDA	control
with H_2O_2	mean	23.8	14.3	12.5	9.0	25.4
	SD	1.5	1.7	0.7	0.8	1.7
without H_2O_2	mean	0.5	0.5	0.7	3.1	0.4
	SD	0.2	0.3	0.1	0.4	0.1

[a] HyTy, hydroxytyrosol; Ol, oleuropein, EA, 3,4-DHPEA-EA; EDA, 3,4-DHPEA-EDA.

to the RBC membrane induced by 3,4-DHPEA-EDA was not in the oxidized form (*13*).

According to these results, all compounds seem to confer antioxidant protection, especially the oleuropein aglycone 3,4-DHPEA-EDA, even at low concentration, either alone or in the presence of ascorbic acid. In our experimental system, an important protection was observed in RBCs treated with 5 μM (final concentration in the buffer containing the RBCs) of DHPEA-EDA. This observation strongly indicates that olive oil polyphenols can exert their beneficial action also in vivo. Indeed, even though oleuropein aglycone concentrations achieved in vivo by the dietary intake of olive oil have not been evaluated, their higher lipophilicity (log $P_{(3,4\text{-DHPEA-EDA})}$ = 1.02; log $P_{(\text{oleuropein})}$ = 0.13) (*29*) suggests that these compounds may also be absorbed into the bloodstream. In fact, according to the "Lipinski rule of 5" (*25*), oleuropein aglycones are likely to be absorbed. Moreover, a preliminary absorption study performed by Corona et al. (*26*) for an olive oil phenolic extract using a rat segment of jejunum and ileum model reported a concentration of hydroxytyrosol and its metabolites in the absorbed fluid higher than expected for the initial content of hydroxytyrosol in the extract. Other compounds, probably hydroxytyrosl secoiridoid derivatives, are contributing to these high concentrations because hydroxytyrosol is one of the metabolites expected for these secoiridoids after absorption. However, the initial concentration of this phenolic extract in the perfusate was too low (18 mg/L) to be able to detect secoiridoids in the absorbed fluid.

The intake of olive polyphenols in some Mediterranean countries has been estimated as being around 13 μmol per day for a consumption of a VOO with an average of 180 ppm in total polyphenols content (*27*). Therefore, it is not considered possible to achieve concentrations higher than 3−4 μM in plasma. Nevertheless, this value can be much higher if a high polyphenol content oil is consumed or if it would be possible to supplement these oils with these compounds. In some olive oils, the compound 3,4-DHPEA-EDA may represent 50% of the phenolic fraction, and it can be found in a concentration of up to 780 mg/kg (*28*, *29*). Therfore, if 50 g of extra virgin olive oil with such a high concentration of 3,4-DHPEA-EDA is ingested per day, of which 30−90% could be absorbed, then this would correspond to an intake of up to 32 mg per day. This dose would still be relatively low (plasma concentration of up to 5−10 μM), but it is known that regular low doses, for example, of aspirin can confer cardiovascular health benefits (*30*). Moreover, the present results, together with our previous results, suggest that oleuropein aglycones, and in particular 3,4-DHPEA-EDA, strongly interact with the RBC membrane. Depending on the strength of this interaction, this aglycon may be present longer in the blood and reach a higher net concentration than expected in subjects with a daily consumption of extra virgin olive oil. In fact, other olive oil phenolic compounds, such as hydroxytyrosl and its metabolites, have been found not only in the plasma but also attached to lipoproteins (*5*). Therefore, it is possible that the regular low lifetime intake of olive oil results in an overall protective effect. This phenomenon was demonstrated by clinical trials showing that short-term consumption of olive oil in humans (50 mL/day) can change several oxidative stress markers (*31*, *32*), although the concentrations of phenols are lower than those required to show biological activity in vitro.

Combining the results of hemolysis with those of RBC membrane protein analysis, we may say that the enhanced capacity of 3,4-DHPEA-EDA to prevent H_2O_2-induced damage may be mediated by interactions of this phenolic compound with RBC proteins (spectrin, band 3, and hemoglobin), improving the stability of RBCs. In a recent study, similar results were found by Fabianni et al. (*33*) for 3,4-DHPEA-EDA at 10 μM in the protection of HL60 and human blood mononuclear cells from H_2O_2-induced DNA damage. In these cell systems, both 3,4-DHPEA-EDA and hydroxytyrosol showed the best activity (range of protection = 89−93%). In this study, protective activity was also shown by 3,4-DHPEA-EA, oleuropein, tyrosol, 4-HPEA-EDA (the dialdehydic form of elenoic acid linked to tyrosol), and caffeic acid, although with a lower efficacy (range of protection = 25−75%).

Several human and animal studies have demonstrated that the degree of oxLDL in vivo decreases as the phenolic content in the administered olive oil increases (*4*, *5*, *7*). However, three human studies (*32*) have demonstrated that phenol-rich virgin olive oil does not lower oxLDL. These different results have been justified by the short study period. However, only the total phenolic intake and not the composition of the phenolic extracts in these studies has been taken into account to explain these different results. According to our results, the phenolic constituents of extracts in these trials should have been strictly controlled because the composition of different extra virgins olive oils can vary widely.

Results in this paper represent further evidence that DHPEA-EDA, one of the main phenolic compounds found in olive oil, may play a much more important protective role against

ROS-induced oxidative injury in human cells than hydroxytyrosol or oleuropein and therefore contribute to the protective role of olive oil in the Mediterranean diet. In addition, our results yield basic knowledge about the interactions of phytochemicals with biological systems and may thus be useful for the design of "functional foods". Therefore, studies on the bioavailability of these compounds are needed to address some of the cardiovascular benefits of olive oil to these molecules.

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