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Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages

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Hydroxytyrosol, the major representative phenolic compound of virgin olive oil, is a dietary component. Its possible protective effect on hydrogen peroxide (H_2O_2)-induced oxidative alterations was investigated in human erythrocytes. Cells were pretreated with micromolar hydroxytyrosol concentrations and then exposed to H_2O_2 over different time intervals. Subsequently, erythrocytes were analyzed for oxidative hemolysis and lipid peroxidation. Our data demonstrate that hydroxytyrosol prevents both oxidative alterations, therefore, providing protection against peroxide-induced cytotoxicity in erythrocytes. The effect of oxidative stress on erythrocyte membrane transport systems, as well as the protective role of hydroxytyrosol, also were investigated in conditions of nonhemolytic mild H_2O_2 treatment. Under these experimental conditions, a marked decrease in the energy-dependent methionine and leucine transport is observable; this alteration is quantitatively prevented by hydroxytyrosol pretreatment. On the other hand, the energy-independent glucose transport is not affected by the oxidative treatment. The reported data give new experimental support to the hypothesis of a protective role played by nonvitamin antioxidant components of virgin olive oil on oxidative stress in human systems. (J. Nutr. Biochem. 10:159–165, 1999)

Keywords: antioxidants; oxidative hemolysis; lipid peroxidation; erythrocyte; hydroxytyrosol; olive oil

Introduction

Polyphenols are bioactive substances that are widely distributed in the vegetable kingdom and present in high concentrations in typical components of the Mediterranean diet,[1] including olive oil. Converging evidence clearly indicates that the well-known benefits of olive oil intake on human health should be ascribed not only to the elevated oleic acid content but also to the antioxidant properties of its minor components, including polyphenols.[2] Among them, particular attention has been focused on the specific olive oil polyphenol hydroxytyrosol (3,4-dihydroxyphenyl)ethanol (DPE)[3–5] (*Figure 1*), which is present in a particularly high concentration in extra virgin olive oil either in a free or esterified form, where it accounts for 70 to 80% of total phenolic fraction. Indeed, DPE concentration varies greatly in different olive oils depending on agronomic and technological factors, such as fruit variety and extraction procedures.[6] DPE, an efficient scavenger of peroxyl radicals,[7] is mainly responsible for the intrinsic defense of the oil against the autoxidation of unsaturated fatty acids[8] and has been proved to act as a powerful inhibitor of peroxidation of human lipoproteins on in vitro studies.[9] Based on these observations, a possible role of DPE in preventing the oxidative damages in living cells has generally been proposed. In fact, in a previous work[10] we directly demonstrated that DPE counteracts free radical-induced cytotoxicity in human intestinal epithelial cells in culture (Caco-2 cells).

To further elucidate the antioxidant properties of olive oil DPE in human cells and to identify the molecular mechanisms responsible for its cytoprotective effect, human erythrocytes [(red blood cells (RBCs)] were selected as a metabolically simplified model system. In these cells, which lack transcriptional and translational machinery, the defense mechanisms mediated by the induction of protein synthesis are not operative.

RBCs are particularly exposed to oxidative hazard because of their specific role as oxygen carriers.[11] In fact, the spontaneous autoxidation of hemoglobin (Hb) continously

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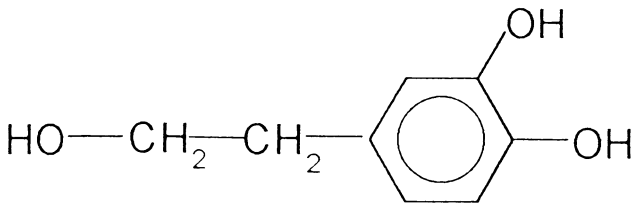

Figure 1 Chemical structure of hydroxytyrosol (DPE)

produces radical anion superoxides,[12] which dismutate to hydrogen peroxide (H_2O_2). In the presence of reduced metal ions, particularly iron, these compounds in turn form the highly reactive hydroxyl radical, which can damage both plasma membrane and cytosolic components, eventually leading to oxidative hemolysis.[13,14] Under physiologic conditions there is a steady-state balance between the production of reactive oxygen species (ROS) and their destruction by the endogenous defense system, which includes enzymatic and nonenzymatic antioxidants, such as vitamin E.[15] However, if ROS are overproduced or if the endogenous defenses are impaired, the situation referred to as "oxidative stress" will result. In this respect, considerable evidence supports the hypothesis that chronic oxidative stress is an important factor in the etiology of some hereditary anemias, characterized either by an impaired antioxidant defense system (glucose-6-P dehydrogenase deficiency)[16] or by the increased production of ROS, which overwhelm the endogenous defenses (β-thalassemia and sickle cell anemia).[17–19] Based on these findings, the use of antioxidants as therapeutic agents to counteract oxidative alterations in such pathologies has generally been proposed.[11,20]

Human RBCs can be oxidized in vitro by several drugs[21] and peroxides, including H_2O_2. Because of its biologically relevant oxidizing role in RBCs, H_2O_2 is frequently used to initiate radical formation in intact cells.[22–25] Principal among H_2O_2 molecular targets is Hb, which is converted to the oxidized forms metHb and ferrylHb.[22] In addition, the exposure of RBCs to H_2O_2 also results in side chain protein alterations, such as carbonyl[14] and tyrosine dimer formation,[23] and in lipoperoxidation.[24] As a consequence of such oxidative modifications, drastic changes in RBC shape do occur, resulting in echinocytic transformation and, eventually, hemolysis.[25]

In the present study, intact human RBCs exposed in vitro to H_2O_2 were used to test the ability of the olive oil DPE to prevent oxidative hemolysis and membrane lipid peroxidation. Moreover, we investigated the effect of a nonhemolytic mild H_2O_2 treatment, along with the protection operated by DPE, on RBC glucose and amino acid transport systems. Previous reports on isolated RBC membranes[26] suggest that ion transport is affected by conditions promoting ROS generation.

Methods and materials

Chemicals

H_2O_2 was purchased from Sigma (St. Louis, MO USA). L-(methyl-^{3}H)methionine (specific radioactivity 80 Ci/mmol), L-(4,5-^{3}H)leucine (specific radioactivity 120 Ci/mmol), and D-(2-^{3}H)glucose (specific radioactivity 20 Ci/mmol) were obtained from Amersham International plc (Little Chalfont, UK). All other chemicals used in this study were of research highest purity grade.

Synthesis of DPE

DPE was synthesized by reduction of 2(3,4-dihydroxyphenyl)acetic acid with $LiAlH_4$ in boiling tetrahydrofuran and purified according to Baraldi et al.[27]; the compound was 97 to 98% pure as verified by gas chromatography and nuclear magnetic resonance.

Preparation of RBCs and induction of oxidative stress

Blood samples were obtained from healthy volunteers by venipuncture, after obtaining informed consent. The citrated blood was centrifuged at 1,100 g for 10 minutes. The pellet was resuspended in phosphate buffered saline (PBS; 125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) containing 5 mM glucose. Leukocytes and platelets were removed by filtration through a nylon net. RBCs were then washed three times and finally suspended in PBS to 2% hematocrit.

In order to induce oxidative stress, aliquots of a 30% H_2O_2 solution were added to the RBC suspension, and incubation was carried out at 37°C for different times. The reaction mixture was gently shaken while being incubated.

Assay system for hemolysis

The extent of hemolysis was determined spectrophotometrically, according to Ko et al.[20] At the end of incubation, aliquots of the reaction mixture were centrifuged at 1,100 g for 10 minutes and the absorption (A) of the supernatant (S1) at 540 nm was measured. Packed RBCs were hemolyzed with 40 volumes of ice-cold distilled water and the lysate centrifuged at 1,500 g for 10 minutes. The supernatant (S2) was then added to S1 and absorption (B) of the combined supernatant (S1 + S2) was measured at 540 nm; percentage hemolysis was calculated from the ratio of the readings $(A/B) \times 100$.

Evaluation of lipid peroxidation

Malondialdehyde (MDA) formation was determined as a measure of lipid peroxidation, according to Gilbert et al.[28] After incubation with H_2O_2, trichloroacetic acid was added to 2 mL of cell suspension, to a 10% final concentration, and the precipitate was removed by centrifugation. The supernatant was then heated with 1 mL of thiobarbituric acid (TBA) reagent (1% TBA in 0.05 M NaOH) for 15 minutes, and cooled and extracted with 1 mL n-butanol. The upper layer, which contained the TBA-reactive substances, was read at both 453 and 532 nm. The MDA concentration, expressed as nmoles per milligram of total proteins, was obtained by subtracting 20% of the absorbance at 453 nm from the absorbance at 532 nm, and using the value of 153,000 (mol/L)/cm as the extinction coefficient of MDA-TBA adduct.

Evaluation of amino acid and glucose transport in RBCs

The amino acid transport in RBCs was evaluated as described by Galletti et al.[29] with minor modification. After incubation with H_2O_2, the RBC samples were centrifuged and resuspended in fresh PBS, containing either L-(methyl-^{3}H)methionine or ^{3}H-leucine (1.0×10^5 dpm). After a 30-minute incubation, the RBCs were washed once with PBS and hemolyzed in 20 volumes of distilled water. Proteins were precipitated by adding trichloroacetic acid at 10% final concentration, and the supernatant radioactivity was measured. The glucose transport in RBCs was evaluated as

reported for amino acid transport, except that a PBS solution without glucose was used, which contained 1.0×10^5 dpm of D-(2-^{3}H)glucose. The amino acid and glucose transport was expressed as dpm of labeled compound incorporated per milligram of total proteins.

Effect of the olive oil polyphenol DPE

To assay the capacity of DPE to protect RBCs from H_2O_2-induced oxidative injury, the cells were pretreated at 37°C for 15 minutes in the presence of the chosen concentration of the polyphenol, and the medium was changed to remove DPE before the addition of H_2O_2. Longer preincubation times did not improve the antioxidant effect. At the end of incubation, the above mentioned markers were evaluated.

Protein concentration

Protein concentration was estimated by the method of Bradford[30] using a Bio-Rad protein assay kit I (Bio-Rod, Hercules, CA USA).

Statistical analysis

Results are reported as means ±SEM. One-way analysis of variance was used to analyze data from *Figure 2* and *Figure 5*. Student's *t*-test was utilized when only two treatment groups are compared (*Figure 2* and *Figure 3*; *Table 1*). All statistical analyses were considered significant at $P < 0.05$.

Results

Mammalian RBCs are a unique and interesting cellular model for research on ROS-induced oxidative stress, as well as for studies on the molecular mechanisms underlying the protective effect of antioxidant molecules. To investigate the protective effect of the olive oil polyphenol DPE on the ROS-induced cytotoxicity in human cells, RBCs were preincubated for 15 minutes with increasing concentrations of DPE, and oxidative stress was induced by the addition of H_2O_2. To avoid a direct interaction between the polyphenol and the oxidant source, DPE was removed before the oxidative treatment. At the end of incubation, cellular and molecular alterations were evaluated.

Effect of DPE on oxidative hemolysis of human erythrocytes

The capability of DPE to protect RBCs from ROS-induced cytotoxicity was tested in conditions of moderate and extensive oxidative lysis, induced by 200 μM and 300 μM H_20_2, respectively. It should be pointed out that, according to our data and that found in the literature, the concentration-dependence of the H_20_2-induced hemolytic process is not linear but it follows a sigmoid kinetic. As shown in *Figure 2*, which is consistent with previous reports, the incubation of RBCs over different time intervals in the presence of 300 μM H_2O_2 results in extensive hemolysis, in a time-dependent manner. Indeed, up to 40% of hemolysis occurs after 2 hours of treatment. Moreover, a reduced hemolysis is observable in cells pretreated with micromolar concentrations of DPE before they are challenged with H_2O_2, indicating that this polyphenol effectively protects RBCs against ROS-induced cytotoxicity. In the experimental condition (200 μM H_2O_2) inducing a moderate hemolysis (approximately 20%), a DPE concentration as little as 50 μM shows a significant effect, whereas 100 μM polyphenol completely prevents oxidative damage (*Figure 3*). DPE itself, up to a concentration of 1 mM, does not cause lysis in RBCs (data not shown).

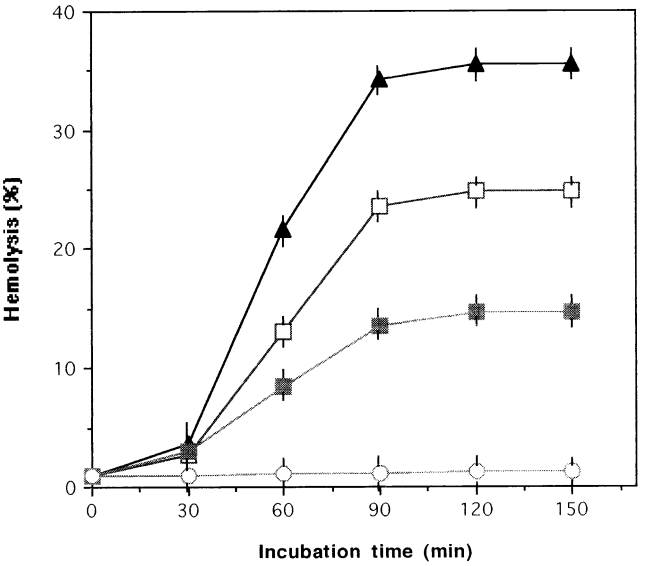

Figure 2 Time course of the effect of hydroxytyrosol (DPE) on hydrogen peroxide (H_2O_2)-induced hemolysis of red blood cells (RBCs). The cells were pretreated for 15 minutes at 37°C with 50 μM (□) and 100 μM (■) DPE and then incubated over different time intervals in the presence of 300 μM H_2O_2. Parallel sets of samples received only H_2O_2 treatment (▲) or no treatment at all (○). At the end of incubation, hemolysis was measured as described in Methods and materials. Values are means ±SEM of four experiments. The data were analyzed by analysis of variance; starting from 1 hour incubation time, all hemolysis values of DPE pretreated samples were significantly lower than H_2O_2 treated RBCs ($P < 0.05$).

Effect of DPE on human erythrocyte membrane lipoperoxidation

In order to verify the effect of DPE on the specific molecular alterations that ultimately result in oxidative hemolysis, the level of lipid peroxidation was measured. Membrane phospholipids constitute a major target for the cytotoxic effect of ROS in that their polyunsaturated fatty acids are particularly prone to peroxidation, leading to the formation of hydroperoxides, which are subsequently degraded to MDA.[31] As shown in *Figure 4*, when RBCs were treated with 200 μM H_2O_2, a dramatic increase in MDA concentration is observable, indicating severe peroxidative damage of the RBC membrane. Pretreatment of cells with increasing micromolar DPE concentrations, however, prevents the phospholipid oxidative alteration in a concentration-dependent manner. As little as 50 μM DPE exerts effective protection against lipid peroxidation, and no increase in MDA is observable upon preincubation with 100 μM DPE. MDA concentrations in RBCs incubated only in the presence of DPE are similar to control cells (*Figure 4*).

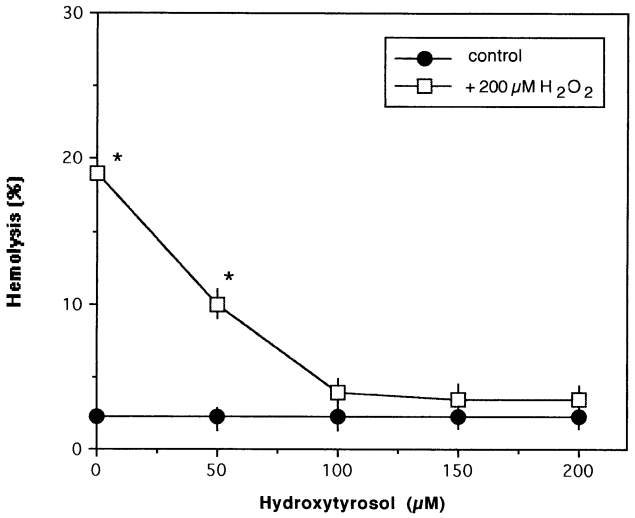

Figure 3 Dose-dependence of the effect of hydroxytyrosol (DPE) on hydrogen peroxide (H_2O_2)-induced hemolysis of red blood cells (RBCs). The cells were pretreated for 15 minutes at 37°C with increasing amounts of DPE and then incubated for 2 hours in the presence or absence of 200 μM H_2O_2. At the end of incubation, hemolysis was measured as described in Methods and materials. Values are means ± SEM of six experiments. The data were analyzed by Student's *t*-test; values with asterisks are significantly different from the control ($P < 0.05$).

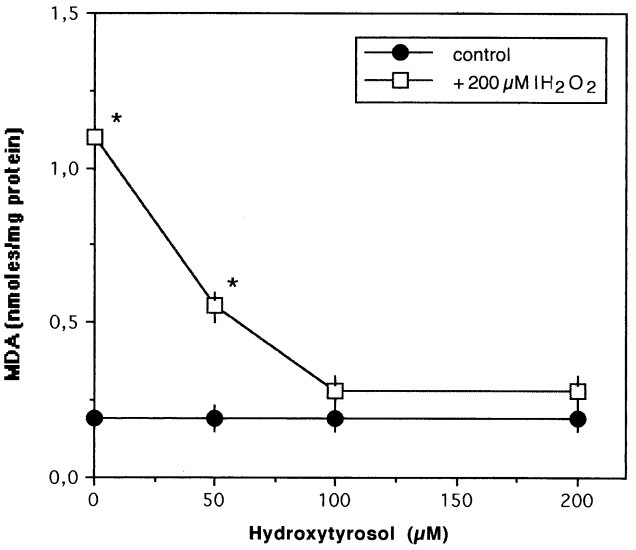

Figure 4 Effect of hydroxytyrosol (DPE) on malondialdehyde (MDA) levels of hydrogen peroxide (H_2O_2)-treated red blood cells (RBCs). The cells were pretreated for 15 minutes at 37°C with increasing amounts of DPE and then incubated for 2 hours in the presence or absence of 200 μM H_2O_2. At the end of incubation, MDA was measured as described in Methods and materials. Values are means ±SEM of six experiments. The data were analyzed by Student's *t*-test; values with asterisks are significantly different from the control ($P < 0.05$).

Amino acid and glucose transport in H_2O_2-treated human erythrocytes

The majority of the studies concerning ROS-induced oxidative stress have focused on the molecular alterations of cellular components. In our opinion, however, a critical issue that also must be investigated is the cell membrane structure–function relationship following oxidative treatment. In this respect, previous observations on isolated RBC membranes by Rohn et al.[26] have demonstrated that ion transport ATPases are a major target for oxidative injury to the RBC membrane, suggesting that in vivo ion gradients can be disrupted by conditions promoting ROS formation. Therefore, to further investigate the effect of H_2O_2-induced oxidative alterations on the functional integrity of the RBC membrane, both amino acid and glucose transport were examined in intact RBCs exposed to mild oxidative treatment (150 μM H_2O_2), a condition that does not result in hemolysis.[25] As shown in *Table 1*, when RBCs pretreated with H_2O_2 as previously described are incubated in the presence of either [^{3}H]methionine or [^{3}H]leucine, a marked decrease in the uptake of both amino acids is observable compared with control cells. Conversely, unlike the amino acid transport, the H_2O_2 treatment does not appear to modify [^{3}H]glucose uptake (*Table 1*).

Effect of DPE on the H_2O_2-induced decrease in amino acid transport in human erythrocytes

We then examined whether H_2O_2-induced functional alteration of amino acid transport in intact RBCs was prevented by DPE. As shown in *Figure 5*, pretreatment of cells with 100 μM DPE almost completely counteracts the impairment in the transport of both amino acids tested, methionine and leucine, a significant effect being observable at 50 μM DPE.

Discussion

During the last decade, a large body of evidence from both epidemiologic and biochemical studies clearly demonstrated that dietary intake of antioxidant vitamins plays a protective role in human health, decreasing the incidence of several diseases in different populations. More recently, a number of studies also focused attention on the role of nonvitamin dietary antioxidants, such as polyphenols, whose role in human nutrition could be crucial. Converging data confirm that the elevated phenolic antioxidant content of the components of the Mediterranean diet, together with

Table 1 Effect of H_2O_2 treatment on amino acid and glucose uptake in RBCs

RBC treatment	^{3}H-methionine uptake (dpm/mg protein)	^{3}H-leucine uptake (dpm/mg protein)	^{3}H-glucose uptake (dpm/mg protein)
—	1195 ± 19.4	382 ± 9.3	874 ± 22.7
H_2O_2	802 ± 13.8*	304 ± 6.7*	860 ± 18.0

Note: Red blood cells (RBCs) were incubated for 2 hours at 37°C in the presence or absence of 150 μM hydrogen peroxide (H_2O_2) and then the amino acid and glucose uptakes were measured as described in Methods and materials. Values are means ± SEM of five experiments. The data were analyzed by Student's *t*-test, and the values with asterisks are signficantly different from the control ($P < 0.05$).

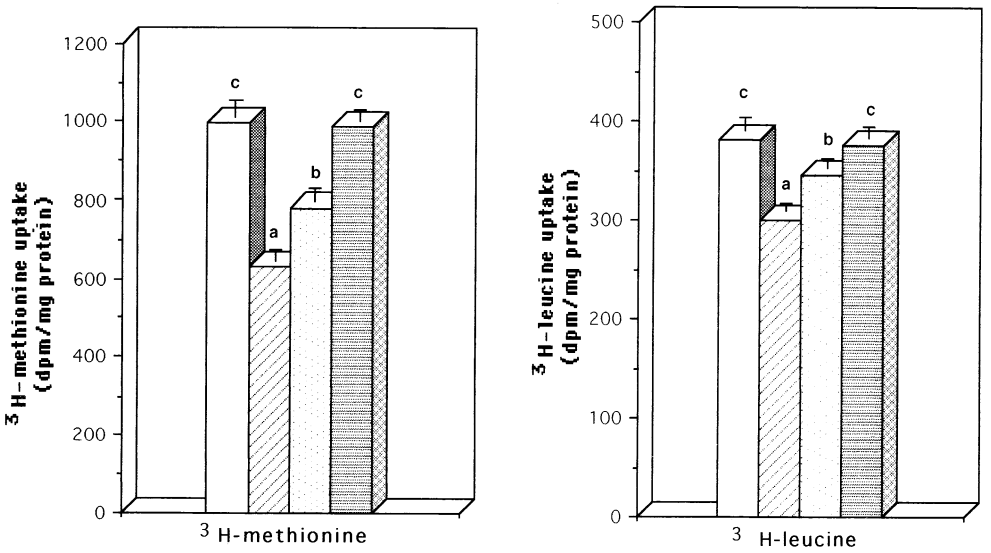

Figure 5 Effect of hydroxytyrosol (DPE) on hydrogen peroxide (H_2O_2)-induced decrease in ^{3}H-methionine and ^{3}H-leucine transport in red blood cells (RBCs). The cells were pretreated for 15 minutes at 37°C with either 50 μM (▨) or 100 μM (▤) DPE and then incubated for 2 hours with 150 μM H_2O_2. Parallel sets of samples received only H_2O_2 treatment (▨) or no treatment at all (□). At the end of incubation, amino acid transport was measured as described in Methods and materials. Values are means ± SEM of five experiments. The data were analyzed by analysis of variance; values that do not share the same letter are significantly different ($P < 0.05$).

antioxidant vitamins, greatly contributes to the health beneficial effects of this dietary habit.[2,32]

The data reported in this paper on the ability of DPE to counteract H_2O_2-induced oxidative stress in intact human RBCs confirm our previous observation of the antioxidant role of this phenol in biological systems. When RBCs are pretreated with micromolar amounts of DPE, before the cells are challenged with H_2O_2, a protective effect against the oxidative hemolysis is observed. Similarly, pretreatment with DPE inhibited membrane lipid peroxidation, as indicated by complete prevention of MDA increase after exposure of RBCs to the oxidant source. Our data also provide new insight on the physiologic implications of the oxidative alterations of membrane structure on incubation of the intact cells under conditions of nonhemolytic mild H_2O_2 treatment. We demonstrated that both methionine and leucine transport is significantly reduced under these experimental conditions and that the decrease in amino acid transport can be prevented by DPE. This finding is consistent with previous observations on functional alterations of RBC isolated membrane following incubation in vitro in the presence of ferrous sulfate and ethylenediamine tetraacetic acid.[26] The authors report that ion transport ATPases are a major target for oxidative injury in RBC membrane, suggesting that in vivo ion gradients are disrupted by conditions that promote ROS formation.

ROS-induced damages to membrane phospholipids likely underlie the reported alterations in amino acid transport. In this respect, Mason et al.[33] directly characterized changes in the molecular structure of the membrane lipid bilayer resulting from oxidative stress. These authors reported that even a small increase of phospholipid hydroperoxides in the membrane produces marked changes in the molecular organization of the lipid bilayer, as monitored by small-angle X-ray diffraction analysis. Furthermore, ROS-induced covalent crosslinking of oxidized Hb with cytoskeletal proteins results in condensation of the inner lipid monolayer area.[25] On the basis of these observations, our finding that oxidative damages to the RBC membrane do not affect the glucose transport appears contradictory. However, it should be noted that amino acid trasport is an energy-dependent mechanism, whereas glucose uptake occurs via an energy-independent permease. Therefore, it is conceivable that the well-known oxidative stress-induced reduction in cellular energy charge[34] affects the RBC transport systems differently. On the other hand, the observed inhibition of amino acid transport can be due, at least in part, to a direct effect of ROS on the specific carrier proteins.

The reported decrease of methionine transport is particularly relevant from a metabolic point of view. In RBCs this amino acid, which is not utilized in protein biosynthesis, is actively converted into the biological methyl donor S-adenosylmethionine (AdoMet).[35] The only AdoMet-dependent reaction virtually operative in RBCs is protein methyl esterification, catalyzed by L-isoaspartyl/D-aspartyl protein methyltransferase. This enzyme selectively recognizes and methyl esterifies proteins at the level of D-aspartyl and L-isoaspartyl residues, a crucial step in the process leading to the "repair" of the damaged proteins.[36] Therefore, the decreased efficiency of the protein "repair" machinery in conditions of reduced AdoMet synthesis could contribute to the accumulation of damaged proteins following oxidative stress and to the decreased survival in the circulation of oxidized RBCs. Preliminary results obtained in our laboratory[37] suggest that oxidative stress does affect the methyl esterification reaction in human RBCs. On the other hand, the protection of methionine transport by DPE may be a

critical factor in the ability of cells to recover from sublethal oxidative insult. It should be underlined, in this respect, that AdoMet is a key intermediate of the transsulphuration pathway by which methionine is converted into cysteine, a precursor of glutathione. This tripeptide, which is a key component of the antioxidant defense system, is vital in the maintenance of RBC shape and viability.

As far as the molecular mechanisms of DPE antioxidant effect are concerned, it is well known that this phenol is endowed with peroxyl radical scavenging activity[7] and likely it prevents H_2O_2-induced cytotoxicity in the RBCs by acting as a chain-breaking inhibitor of lipid peroxidation. Moreover, DPE might contribute to the regeneration of vitamin E by reacting with its α-tocopheroxyl radical, as has been demonstrated[9] when human lipoproteins are peroxidated in vitro. In addition, the ability of DPE to counteract oxidative damages could also be related to the ability of dihydroxyphenols to chelate iron ions. Indeed, this metal is known to initiate and propagate lipid peroxidation via a series of reactions involving ROS formation. On the other hand, the RBC model system, in which protein synthesis is not operative, allows us to rule out the possibility that the antioxidant activity of DPE might be due to the induction of newly synthesized antioxidant proteins. Khan et al.[38] reported that oral feeding of the polyphenolic fraction of green tea to female SKH-1 hairless mice induces an increase of antioxidant enzymes, including glutathione peroxidase and catalase.

Together our data give experimental support to the hypothesis that polyphenols greatly contribute to the nutritional value of virgin olive oil. In this respect, indirect evidence of an animal model from Scaccini et al.[39] already indicated that the dietary intake of olive oil polyphenols could contribute to modulate in vivo the antioxidant balance. These authors demonstrated that rats fed with olive oil showed a higher serum antioxidant capacity and an increased resistance to lipoperoxidation compared with rats receiving a synthetic diet, based on the same fatty acid composition and vitamin E content. More recently, similar data on in vitro peroxidation of low density lipoproteins purified from New Zealand white rabbits fed with semipurified diet were reported.[40] Moreover, even though the DPE concentrations achievable in vivo by the dietary intake of olive oil have not been evaluated, it should be stressed that, in oils with high total phenol content, DPE is present in concentrations of up to 3 mM, far exceeding those used in our experimental conditions.

Conclusion

Extra virgin olive oil, characterized by a high DPE content, may be helpful in designing dietary strategies to maximize the antioxidant potential in humans. Moreover, its intake could contribute to the dietary prevention of those pathologies, whose etiology is related to ROS-mediated cellular injury. In this respect, the reported data allow us to propose DPE as a possible therapeutic tool in the treatment of hematologic disorders such as β-thalassemia, sickle cell anemia, and glucose-6-phosphate dehydrogenase deficiency. It is noteworthy that DPE does not appear to be toxic to RBCs in concentrations up to 1 mM, a concentration far exceeding that required for the antioxidant activity.

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