The Protective Effect of the Olive Oil Polyphenol (3,4-Dihydroxyphenyl)-ethanol Counteracts Reactive Oxygen Metabolite–Induced Cytotoxicity in Caco-2 Cells[1,2]

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ABSTRACT We investigated the injurious effects of reactive oxygen metabolites on the intestinal epithelium and the possible protective role played by two olive oil phenolic compounds, (3,4-dihydroxyphenyl)ethanol and (*p*-hydroxyphenyl)ethanol, using the Caco-2 human cell line. We induced oxidative stress in the apical compartment, either by the addition of 10 mmol/L H_2O_2 or by the action of 10 U/L xanthine oxidase in the presence of xanthine (250 μmol/L); after the incubation, we evaluated the cellular and molecular alterations. Both treatments produced significant decreases in Caco-2 viability as assessed by the neutral red assay. Furthermore, we observed a significant increase in malondialdehyde intracellular concentration and paracellular inulin transport, indicating the occurrence of lipid peroxidation and monolayer permeability changes, respectively. The H_2O_2-induced alterations were completely prevented by preincubating Caco-2 cells with (3,4-dihydroxyphenyl)ethanol (250 μmol/L); when the oxidative stress was induced by xanthine oxidase, complete protection was obtained at a concentration of polyphenol as small as 100 μmol/L. In contrast, (*p*-hydroxyphenyl)ethanol was ineffective up to a concentration of 500 μmol/L. Our data demonstrate that (3,4-dihydroxyphenyl)ethanol can act as a biological antioxidant in a cell culture experimental model and that the ortho-dihydroxy moiety of the molecule is essential for antioxidant activity. This study suggests that dietary intake of olive oil polyphenols may lower the risk of reactive oxygen metabolite–mediated diseases such as some gastrointestinal diseases and atherosclerosis. J. Nutr. 127: 286–292, 1997.

KEY WORDS: • *Caco-2* • *polyphenol* • *olive oil* • *antioxidant* • *Mediterranean diet*

Reactive oxygen metabolites (ROM)[4] induce a number of molecular alterations in cellular components, leading to changes in cell morphology and viability; these changes include DNA lesions, protein cross-links and side-chain oxidation (Halliwell 1994, Sies 1991). Moreover, phospholipids constitute a major target for the cytotoxic effect of ROM (Halliwell and Chirico 1993). Lipid peroxidation, indeed, is one of the main factors responsible for the structural and functional alterations of the cell membrane following oxidative stress. Reactive oxygen metabolites are generated during both normal and xenobiotic metabolism, and they can be overproduced in several pathological conditions. Cells are naturally provided with an extensive array of protective enzymatic and non-enzymatic antioxidants that counteract these potentially injurious oxidizing agents (Halliwell 1994, Sies 1991).

Even this multifunctional protective system cannot completely counteract the deleterious effects of ROM, however, and consequently oxidatively damaged molecules accumulate in cells. The clinical implications of these alterations can be severe; in fact, the accumulation of ROM in several cellular components is thought to be a major cause of molecular injury leading to cell aging and to age-related degenerative diseases such as cancer, immune system decline, brain dysfunction, cataracts and coronary heart disease (Ames et al. 1993, Halliwell et al. 1992). In this respect, the so-called "oxidation hypothesis" of atherosclerosis implies that the oxidative modifications of LDL represent a key step in the pathogenesis of this disease (Witztum 1994). Oxidized LDL are potentially more atherogenic than native LDL (Witztum 1993), and inhibition of lipoprotein oxidation slows the progression of atherosclerotic lesions (Mancini and Rubba 1995).

There has also been an increasing interest in the possible role of ROM as mediators of cellular damage in several gastrointestinal diseases, and ROM have been implicated in ischemia-induced permeability changes of the intestine, in Crohn's disease and in ulcerative colitis (Grisham 1994).

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[4] Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DPE, (3,4-dihydroxyphenyl)ethanol; EMEM, Eagle's minimum essential medium; FCS, fetal calf serum; MDA, malondialdehyde; MUFA, monounsaturated fatty acid; PE, (*p*-hydroxyphenyl)ethanol; PUFA, polyunsaturated fatty acids; ROM, reactive oxygen metabolites; SFA, saturated fatty acids.

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A possible way to prevent ROM-mediated cellular injury is to augment endogenous oxidative defenses through the dietary intake of antioxidants such as vitamins A, C and E (Block et al. 1992, Di Mascio et al. 1991, Krinsky 1991). Recently, attention has also focused on a variety of non-vitamin antioxidants, such as phenolic compounds, that might also contribute to cellular defense mechanisms (Decker 1995). These phenolic compounds are found in many plant species and are present at very high concentrations in many components of the Mediterranean diet, including olive oil, fruit and vegetables (Ho et al. 1992). The amount of phenols consumed per day probably exceeds 1 g, which supports the nutritional relevance of these compounds.

These nonessential dietary components presumably play a major role in controlling oxidative reactions in vivo, thus exhibiting anticarcinogenic and antiatherogenic activity (Decker 1995, Stavric 1994). Previous studies of possible mechanisms of phenol action indicate that these compounds are able to scavenge free radicals and to break peroxidative chain reactions. Phenolic acid can prevent lipid peroxidation by metal chelation. Khan et al. (1992) reported that oral feeding of the polyphenolic fraction of green tea to female SKH-1 hairless mice induced an increase of antioxidant enzymes, including glutathione peroxidase and catalase.

In addition to their antioxidant properties, polyphenols exert several indirect, mediated effects, including prevention of arachidonic acid release from membrane phospholipids through the inhibition of phospholipase A_2, thus reducing the production of chemotactic and inflammatory compounds (Middleton and Kandaswami 1992). Polyphenols are also potent inhibitors of lipoxygenase and cyclo-oxygenase (Laughton et al. 1991). Furthermore, the antiatherogenic effect of polyphenols has also been ascribed to the observed capacity of these molecules to reduce platelet aggregation (Ferro-Luzzi and Ghiselli 1993, Petroni et al. 1995). Finally, the anticarcinogenic activity of phenols may be due to not only their antioxidant properties but also to their ability to reduce the bioavailability of food carcinogens and to inhibit their metabolic activation (Stavric 1994).

Despite the large body of evidence concerning the beneficial effect of dietary phenols, only a limited number of reports have indicated that these compounds directly suppress oxidative damage in biological assay systems. Nakayama's group (1992a) found that a plant polyphenol, nordihydroguaiaretic acid, protects Chinese hamster V79 cells from the oxidative injury caused by H_2O_2, and they subsequently identified other polyphenols capable of similar biological activity, such as gallic acid and caffeic acid derivatives (Nakayama et al. 1992b, Nakayama 1994).

To our knowledge, however, no data are available in the literature concerning the antioxidant activity of polyphenols present in olive oil in a cell culture model system. These compounds, although minor constituents of olive oil, participate in the mechanism involved in sensory organoleptic properties (i.e., flavor and aroma) and contribute to the prevention of oil autooxidation. The types of phenols and their concentrations differ greatly among olive oils, depending on fruit varieties and their degree of maturation as well as other agronomic and technological factors, such as the extraction procedures (Montedoro et al. 1992a). Referring to the total phenol concentrations, olive oils can therefore be divided into three groups, containing low (0.05–0.2 g/kg), medium (0.2–0.5 g/kg) and high (0.5–1.0 g/kg) total phenol concentrations (Montedoro et al. 1992b).

The chemical characterization of olive oil phenolic compounds has been performed using several methods, including HPLC separation (Montedoro et al. 1992b and 1992c). Among the different kinds of polyphenols, we have focused our attention on (3,4-dihydroxyphenyl)ethanol (DPE), also called hydroxytyrosol. This phenol, either in the free or esterified forms, is chiefly responsible for the intrinsic defense of olive oil against the autooxidation of unsaturated fatty acids (Montedoro et al. 1992c, Papadopoulos and Boskou 1991). In addition to DPE, we also investigated the antioxidant activity of (*p*-hydroxyphenyl)ethanol (PE), also called tyrosol, which lacks a phenolic hydroxyl group. The chemical structures of PE and DPE are shown in **Figure 1.**

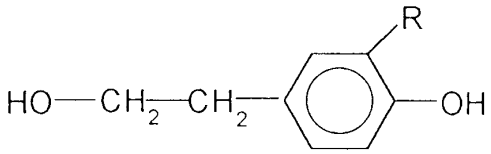

FIGURE 1 Structure of (*p*-hydroxyphenyl)ethanol (PE; R = H) and (3,4-dihydroxyphenyl)ethanol (DPE; R = OH)

The aim of our study was to investigate the role of ROM injury on the structural and functional integrity of the intestinal epithelium and the possible protective effect of PE and DPE. We used Caco-2 epithelial intestinal cells as the model system. When this cell line, originally derived from a human colon carcinoma, is grown in culture, it undergoes enterocytic differentiation to form a polarized monolayer closely resembling, both morphologically and functionally, the human small intestine epithelium (Hidalgo et al. 1989). Therefore, differentiated Caco-2 cells are a suitable model for evaluating the physiological response of enterocytes to oxidative injury (Baker et al. 1993 and 1995).

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (EMEM) and fetal calf serum (FCS) were purchased from Hyclone (Logan, UT); nonessential amino acids, L-glutamine, hydrogen peroxide, xanthine, xanthine oxidase, neutral red and 1,1,3,3-tetraethoxypropane were purchased from Sigma Chemical (St. Louis, MO); Triton X-100 was purchased from BioRad Laboratories (Hercules, CA); penicillin-streptomycin, trypsin from porcine pancreas, EDTA, dimethyl sulfoxide (DMSO) and PBS tablets were purchased from ICN-Flow (Costa Mesa, CA); Transwell polycarbonate microporous cell culture inserts, 6.5 mm in membrane diameter (surface area 0.33 cm^2) and with 0.4-μm pore size were from Costar (Cambridge, MA); [^{14}C]inulin (specific radioactivity 348 MBq/mmol) was obtained from Amersham International plc, Little Chalfont, U.K.

Cell lysis buffer. The cell lysis buffer consisted of 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L dithiothreitol and Triton X-100 (1%, v/v).

Cell culture. The Caco-2 cell strain was obtained from E. Rodriguez-Boulan (Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY) and used between passages 75 to 90. The cells were routinely maintained in DMEM, containing 200 mL/L FCS, 10 mL/L of 100× nonessential amino acids, 2 mmol/L L-glutamine, 5×10^4 IU/L penicillin, 50 mg/L streptomycin at 37°C in a 5% CO_2 atmosphere at 90–100% relative humidity. Cells were grown in 10-cm petri dishes, and the medium was changed every 48 h. For experiments, the cells were seeded at a density of 90,000 cells/cm^2 in a Transwell insert, and the medium (0.1 mL in the insert and 0.8 mL in the well) was changed every 48 h. Fourteen to sixteen days after confluence, the integrity of the monolayer of differentiated cells was monitored by measuring the transepithelial electrical resistance value, according to the method of Hidalgo et al. (1989), using the Millicell-ERS system (Millipore, Bedford, MA).

Induction of oxidative stress. For the oxidative stress induction experiments, an iron-free medium (EMEM) was used. The oxidative

stress was induced in the apical compartment of the Transwell insert by two methods: *1*) addition of H_2O_2; *2*) an enzymatic system, composed of different amounts of xanthine oxidase and its substrate xanthine (250 μmol/L). After 20 h of incubation, several oxidative stress markers were measured.

Neutral red assay. We assessed the cytotoxicity of ROM on Caco-2 by the viability test of neutral red uptake, performed according to the procedure of Fautz et al. (1991). After oxidative stress induction, the medium in the insert was removed and replaced with 0.1 mL of fresh medium containing 1.14 mmol/L neutral red. At the end of 3 h of incubation, the medium was removed and the cells washed twice with PBS; finally, the incorporated neutral red was released from the cells by incubation for 15 min at room temperature in the presence of 1 mL of the cell lysis buffer containing acetic acid (1%, v/v) and ethanol (50%, v/v). To measure the dye taken up, the cell lysis products were centrifuged and supernatants spectrophotometrically measured at 540 nm.

Determination of malondialdehyde. Malondialdehyde (MDA) determination was performed by HPLC, according to the method of Esterbauer et al. (1984). After oxidative stress induction, the cells were lysed with 0.2 mL of cell lysis buffer, and MDA was extracted. To this end, one volume of cell lysate was mixed with one volume of acetonitrile, vortexed and centrifuged, and the clear supernatant was used for the HPLC analysis.

Malondialdehyde was separated using normal phase HPLC. A Supelcosil LC-NH_2 (5 μm) column (25 cm $\times$ 4.6 mm i.d.) was eluted isocratically at 1.0 mL/min with an acetonitrile–30 mmol/L Tris-HCl, pH 7.4 (1:9, v/v) mixture and detected at 267 nm. Quantification of MDA was obtained from a calibration curve constructed by injecting increasing amounts of standard MDA. The standard MDA was prepared by acid hydrolysis of 1,1,3,3-tetraethoxypropane using the method of Esterbauer et al. (1984) and was used within 48 h. The concentration of the standard solution was calculated using the value of 34,000 $(mol/L)^{-1} \cdot cm^{-1}$ as the extinction coefficient of MDA at 267 nm.

[^{14}C]Inulin paracellular transport. The paracellular inulin transport was measured according to the method of Hidalgo et al. (1989). After oxidative stress induction, the medium in the insert was removed and replaced with 0.2 mL of fresh medium containing 1 kBq of [^{14}C]inulin. At the end of 2 h of incubation, the radioactivity of the medium contained in the well was measured. The apical to basolateral paracellular transport of the labeled species was expressed as the percentage of total radioactivity used.

Polyphenols. Olive oil phenolic compounds were kindly supplied by Gianfrancesco Montedoro (Perugia University, Perugia, Italy). (3,4-Dihydroxyphenyl)ethanol was synthesized and purified by the method of Baraldi et al. (1983); the compound was 97–98% pure as verified by gas chromatography and NMR (Baraldi et al. 1983). (*p*-Hydroxyphenyl)ethanol was from Janssen Chemical (Beerse, Belgium).

To assay the capacity of these compounds to protect Caco-2 cells from ROM-mediated oxidative injury, the cells were preincubated for 4 h in the presence of the chosen polyphenol, which had been added to the apical side of the monolayer. At the end of the preincubation time, the medium was changed before the addition of the oxidative stress–inducing agents. Finally, the above mentioned markers were evaluated.

Statistical analysis. All variables were tested in three independent cultures for each experiment, and each experiment was repeated three times (n = 9); results are reported as means ± SD. One-way ANOVA was used to evaluate the data. In Tables 1 and 2 and Figures 3 and 5, significantly different means were identified using the Newman-Keuls test for multiple comparisons (Steel and Torrie 1980); Duncan's test was used to analyze the effect of increasing concentrations of H_2O_2 (Fig. 2) and xanthine oxidase (Fig. 4) on Caco-2 cell viability. Differences were considered significant at $P < 0.05$.

RESULTS

To investigate ROM-induced cytotoxic effects on differentiated Caco-2 cells, we added increasing amounts of H_2O_2 to the medium, bathing the apical side of the cells and after the incubation we evaluated the cellular and molecular alterations.

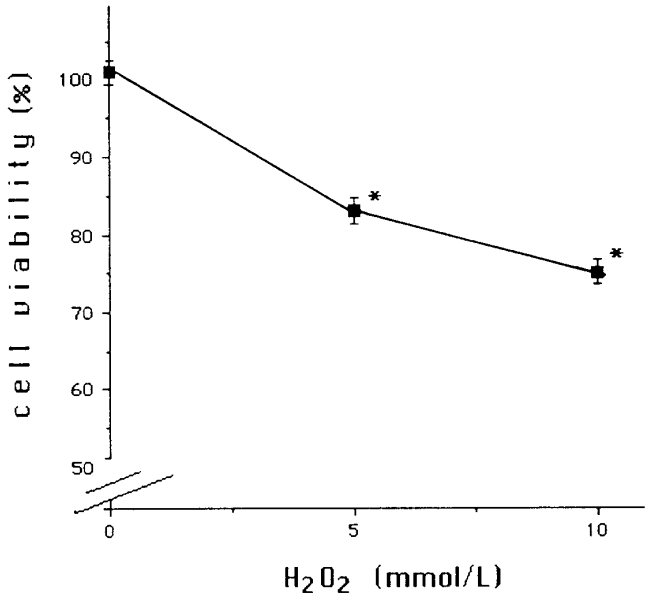

FIGURE 2 Hydrogen peroxide–induced cytotoxicity in Caco-2 cells. The cells were incubated for 20 h at 37°C in the presence of increasing amounts of H_2O_2, and cell viability was measured by neutral red uptake assay as described in Materials and Methods. All variables were tested in three independent cultures for each experiment, and each experiment was repeated three times (n = 9). Values are means ± SD. The data were analyzed by Duncan's test, and the values with asterisks are significantly different from the control ($P < 0.01$).

We checked the overall cellular injury by means of the neutral red assay, one of the most reliable methods of assessing cell viability. Viable cells take up the dye by active transport, incorporating it into lysosomes, whereas nonviable cells do not; therefore differences in the amount of neutral red incorporated by the cells indicate either a variation in the number of the cells or simply a change in their physiological state. Incubation of the cells in the presence of millimolar concentrations of H_2O_2 resulted in a significant decrease in Caco-2 viability (**Fig. 2**). After 20 h of treatment with 10 mmol/L H_2O_2, we observed about 25% loss of cell viability.

This marker was then utilized to verify the protective effect of the olive oil polyphenols PE and DPE against the H_2O_2-induced oxidative injury to the intestinal Caco-2 cells. To evaluate the real effects of the polyphenol taken up by the cells or simply incorporated into the cell membrane, we preincubated Caco-2 cells for 4 h with different concentrations of either PE or DPE and changed the medium before oxidative stress was induced, thereby avoiding a direct reaction in the medium between the polyphenol and the oxidant source.

When cells were pretreated with DPE before being challenged with 10 mmol/L H_2O_2, in conditions similar to those used in the experiments mentioned above, no decrease in cell viability was observed, indicating that DPE suppresses the H_2O_2-induced cytotoxicity (**Fig. 3**). This polyphenol was active in a micromolar concentration range, providing complete protection against oxidative stress at 250 μmol/L. In contrast, no biological antioxidant activity was exerted by PE in concentrations up to 500 μmol/L. Neither PE nor DPE alone was toxic at the concentrations used (data not shown).

The intracellular MDA concentration, directly resulting from membrane lipid peroxidation, was also measured as a second marker of the H_2O_2-induced cellular injury of Caco-2

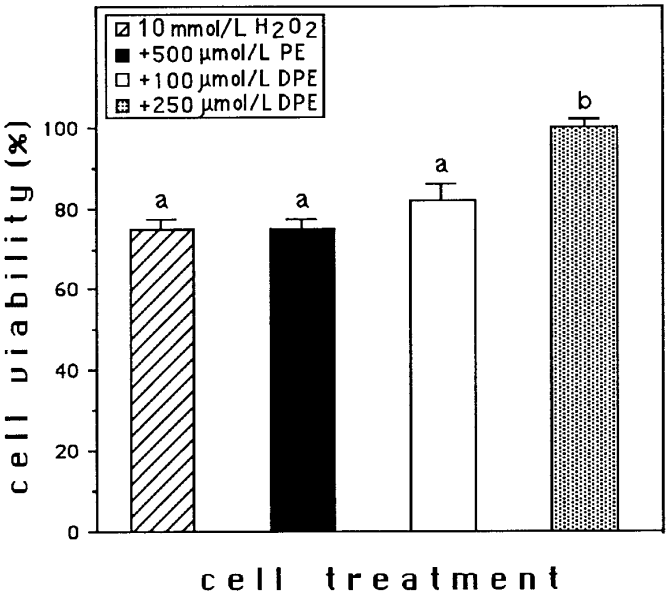

FIGURE 3 Effect of (*p*-hydroxyphenyl)ethanol (PE) and (3,4-dihydroxyphenyl)ethanol (DPE) on hydrogen peroxide–induced cytotoxicity of Caco-2 cells. The cells were preincubated for 4 h at 37°C with increasing amounts of either PE or DPE and then treated for 20 h with 10 mmol/L H_2O_2; parallel sets of samples received only H_2O_2 treatment or no treatment at all (100% viability). At the end of incubation, cell viability was measured by the neutral red uptake assay as described in Materials and Methods. All variables were tested in three independent cultures for each experiment, and each experiment was repeated three times (n = 9). Values are means ± SD. The data were analyzed by Newman-Keuls test. Values that do not share a letter are significantly different ($P < 0.01$). Cells incubated only in the presence of PE or DPE showed neutral red uptake values similar to those of untreated cells (data not shown).

cells. The polyunsaturated fatty acids (PUFA) of the cellular membranes are particularly prone to peroxidation by free radicals, leading to the formation of hydroperoxides, which are subsequently degraded to MDA (Halliwell and Chirico 1993).

The intrinsic rate of lipid peroxidation resulted in a cellular MDA concentration of 0.99 ± 0.13 nmol/10^6 control cells (**Table 1**). When Caco-2 cells were treated with 10 mmol/L H_2O_2, a significant increase in MDA concentration was observed, indicating a severe peroxidative degradation of the enterocytic cellular membrane. Preincubation of cells with 250 µmol/L DPE completely prevented the phospholipid molecular alteration. However, PE, even at 500 µmol/L, did not show biological antioxidant activity (Table 1). The MDA concentrations in Caco-2 cells incubated in the presence of PE or DPE alone were similar to those in control cells.

TABLE 1

Effect of (p-hydroxyphenyl)ethanol (PE) and (3,4-dihydroxyphenyl)ethanol (DPE) on malondialdehyde levels of hydrogen peroxide–treated Caco-2 cells[1]

Sample and treatment	Malondialdehyde level
	nmol/10^6 cells
Control	0.99 ± 0.13[a]
+250 *µmol/L* DPE	0.98 ± 0.15[a]
+500 *µmol/L* PE	1.02 ± 0.17[a]
+10 *mmol/L* H_2O_2	2.10 ± 0.42[b]
+250 *µmol/L* DPE + 10 *mmol/L* H_2O_2	0.87 ± 0.40[a]
+500 *µmol/L* PE + 10 *mmol/L* H_2O_2	2.28 ± 0.27[b]

[1] Values are means ± SD, n = 9. Data were analyzed by Newman-Keuls test. Values that do not share a superscript are significantly different ($P < 0.01$).

TABLE 2

Effect of (p-hydroxyphenyl)ethanol (PE) and (3,4-dihydroxyphenyl)ethanol (DPE) on paracellular transport of [14C]inulin in hydrogen peroxide–treated Caco-2 cells[1]

Sample and treatment	[14C]Inulin paracellular transport
	%
Control	0.6 ± 0.05[a]
+250 *µmol/L* DPE	0.7 ± 0.05[a]
+500 *µmol/L* PE	0.6 ± 0.04[a]
+10 *mmol/L* H_2O_2	3.5 ± 0.12[b]
+250 *µmol/L* DPE + 10 *mmol/L* H_2O_2	0.6 ± 0.04[a]
+500 *µmol/L* PE + 10 *mmol/L* H_2O_2	3.4 ± 0.15[b]

[1] Values are means ± SD, n = 9. Data were analyzed by Newman-Keuls test. Values that do not share a superscript are significantly different ($P < 0.01$).

To evaluate the effect of the oxidative stress on the integrity of the intestinal monolayer, paracellular transport of [^{14}C]-inulin was measured. Under physiological conditions, the epithelium is completely impermeable to this fructopolysaccharide, and only trace amounts cross the epithelium via an extracellular route (Hidalgo et al. 1989). When [^{14}C]inulin was added to the apical medium of Caco-2 after the cells were incubated for 20 h in the presence of 10 mmol/L H_2O_2, transepithelial [^{14}C]inulin flux increased fivefold compared with control cells, suggesting that the oxidative treatment severely affected the barrier function of the intestinal epithelium (**Table 2**). The increase in the paracellular transport of inulin was completely prevented by preincubating Caco-2 cells with 250 µmol/L DPE; 500 µmol/L PE was not protective against the H_2O_2-induced permeability changes (Table 2). Values for the paracellular transport of [^{14}C]inulin into Caco-2 cells incubated in the presence of PE or DPE were similar to those for control cells.

We also investigated the cytotoxic effects induced in Caco-2 cells by another ROM-generating system, consisting of xanthine oxidase and its substrate xanthine. This system enzymatically generates the superoxide radical and H_2O_2 during the conversion of xanthine to uric acid. **Figure 4** shows the effect of xanthine oxidase activity on Caco-2 cell viability, as monitored by the neutral red assay. When the cells were preincubated in the presence of 250 µmol/L xanthine and increasing concentrations of xanthine oxidase, a marked decrease of neutral red uptake was observed compared with the control, reaching a significant decrease of 20% upon incubation of the cells with 10 U/L of the enzyme. The addition of xanthine or xanthine oxidase alone did not affect cell viability (data not shown). Subsequently, the effect of PE and DPE in protecting Caco-2 cells from oxidative injury was also studied under these experimental conditions. Under these conditions, again only DPE acted as a biological antioxidant. The pretreatment of cells with as little as 100 µmol/L DPE completely prevented

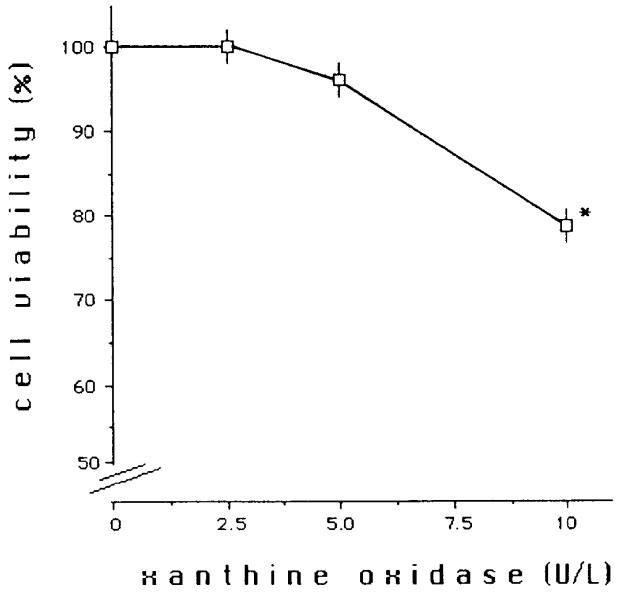

FIGURE 4 Xanthine oxidase–induced cytotoxicity in Caco-2 cells. The cells were incubated for 20 h at 37°C in the presence of 250 μmol/L xanthine and increasing amounts of xanthine oxidase. At the end of incubation, cell viability was measured by the neutral red uptake assay as described in Materials and Methods. All variables were tested in three independent cultures for each experiment, and each experiment was repeated three times ($n = 9$). Values are means ± SD; the data were analyzed by Duncan's test, and the value with an asterisk is significantly different from the control ($P < 0.01$).

xanthine oxidase–induced loss of viability, whereas 500 μmol/L PE had no effect (**Figure 5**).

DISCUSSION

The hypothesis that olive oil intake may contribute to the lower risk of several diseases, especially coronary heart disease, observed in the Mediterranean populations first came from Ancel Keys' observation, who defined the Mediterranean diet as a way "to eat well and stay well" (Keys and Keys 1975). Since then, a large body of evidence has been accumulated showing the beneficial health effects of dietary monounsaturated fatty acids (MUFA) compared with both saturated (SFA) and PUFA. Indeed, the analysis of the serum lipid profiles of patients consuming diets enriched in either MUFA or PUFA shows a lower level of total cholesterol, compared with that observed for people consuming SFA-rich diets. In addition, MUFA do not lower plasma HDL cholesterol as observed for PUFA (Mattson and Grundy 1985, Riccardi and Rivellese 1993). According to the "oxidation hypothesis" of atherosclerosis, diets enriched in olive oil are believed to slow the progression of atherosclerosis because oleic acid–rich LDL are highly resistant to oxidative modifications (Parthasarathy et al. 1990). Moreover, several investigators have stressed the possible role of the antioxidant vitamins of olive oil in the prevention of LDL oxidation (Mancini and Rubba 1995).

A fascinating hypothesis raised in the past few years is that the health-promoting action of olive oil could also be due to the presence of nonessential components, such as polyphenols (many with antioxidant potential), that could contribute to the modulation of the oxidative balance in vivo (Ferro-Luzzi and Ghiselli 1993, Visioli et al. 1995). The nutritional relevance of olive oil polyphenols is confirmed by data obtained by Scaccini et al. (1992) using animal models. These authors demonstrated that rats fed olive oil show a higher serum antioxidant capacity and an increased resistance to lipoperoxidation than rats receiving a purified diet, with the same fatty acid composition and vitamin E concentration.

The data reported in this paper represent the first direct evidence that DPE at a micromolar concentration plays a protective role against ROM-induced oxidative injury, using a cell culture model as the experimental system.

In the first part of our study, we treated differentiated Caco-2 cells with different sources of ROM and evaluated several oxidative stress markers. The incubation of cells in the presence of millimolar concentrations of H_2O_2 resulted in a significant reduction of cell viability as measured by the neutral red assay; moreover, the increased MDA intracellular concentration indicated that an induction of phospholipid peroxidation is also operative in our experimental model. In turn, ROM-induced cellular damage resulted in an increased paracellular transport of impermeable molecules, such as inulin, suggesting that H_2O_2 treatment also affects the structural integrity of the intestinal monolayer. These data are in agreement with previous reports of increased paracellular permeability of the cultured renal epithelial cell line MDCK following exposure to various oxidant sources (Welsh et al. 1985). These authors reported that increased paracellular permeability is associated with cytoskeleton alterations leading to damage to the junctional complex and cell shape.

A severe decrease in cell viability was also observed when Caco-2 cells were incubated in the presence of xanthine oxidase and its substrate, xanthine. This enzymatic system was

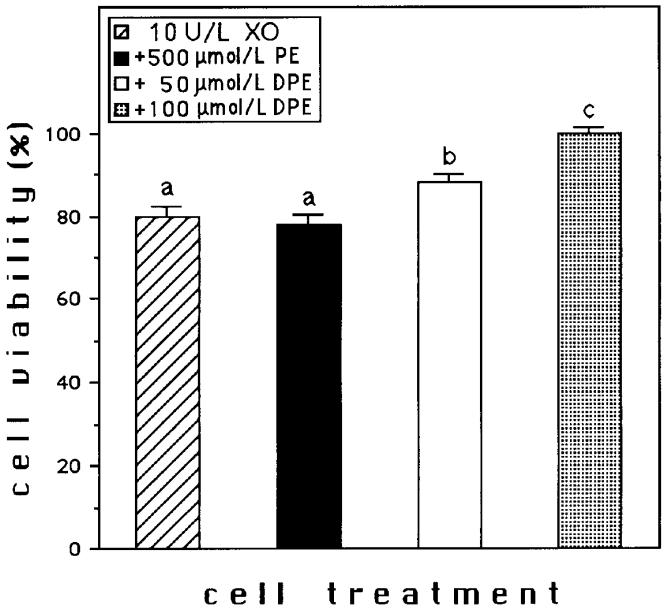

FIGURE 5 Effect of (*p*-hydroxyphenyl)ethanol (PE) and (3,4-dihydroxyphenyl)ethanol (DPE) on xanthine oxidase–induced cytotoxicity on Caco-2 cells. The cells were preincubated for 4 h at 37°C with increasing amounts of either PE or DPE and then treated for 20 h with 250 μmol/L xanthine and 10 U/L of xanthine oxidase (XO); parallel sets of samples received only XO treatment or no treatment at all (100% viability). At the end of incubation, cell viability was measured by the neutral red uptake assay as described in Materials and Methods. All variables were tested in three independent cultures for each experiment, and each experiment was repeated three times ($n = 9$). Values are means ± SD. The data were analyzed by Newman-Keuls test. Values that do not share a letter are significantly different ($P < 0.01$). Cells incubated only in the presence of PE or DPE showed neutral red uptake values similar to those of untreated cells (data not shown).

chosen because of the key role proposed for this enzyme in the small intestine physiopathology. In fact, ROM have been implicated as mediators of the tissue injury observed in several diseases of the gastrointestinal tract, and their formation from xanthine oxidase is considered a primary mechanism of cellular damage (Granger et al. 1986, Parks 1989).

We thus evaluated the possible protective roles of the olive oil polyphenols PE and DPE against ROM-mediated Caco-2 cytotoxicity with respect to their effects on the oxidative stress markers discussed above. Pretreatment of cells with 250 μmol/L DPE completely prevented H_2O_2-induced cytotoxicity as monitored by neutral red assay, MDA formation and [^{14}C]-inulin paracellular transport; when oxidative stress was induced by xanthine oxidase activity, complete protection was observed at concentrations as low as 100 μmol/L. Conversely, in the system investigated, PE exerted no protective effect up to a concentration of 500 μmol/L, indicating that the ortho-dihydroxy structure is essential for the biological antioxidant activity. These data confirm previous observations on the relationship between the chemical structure and the antioxidant activity of polyphenols (Nakayama 1994).

These findings suggest that olive oil, because of its DPE content, could exert a protective effect against those intestinal pathologies whose etiology has been related to ROM-mediated injuries, especially those characterized by changes in epithelium permeability such as inflammatory diseases. Our data also provide experimental support for the hypothesis of the key role played by the phenolic antioxidant fraction of olive oil in the dietary prevention of atherosclerosis, thus contributing to the positive effect of olive oil in lowering the risk of coronary heart disease in Mediterranean populations.

In Southern Italy, the dietary intake of olive oil can reach 50 g/d (Ferro-Luzzi and Sette 1989), corresponding to an average of about 25 mg of total phenols. Moreover, olive oil with medium or high concentrations of total phenols contains DPE at concentrations ranging from 0.6 to 3.0 mmol/L, indicating that this compound could exert its beneficial action in vivo. Unfortunately, however, no data are present in the literature on DPE absorption and bioavailability in humans.

Even though the exact molecular mechanisms by which DPE exerts its antioxidant effects are still undefined, the antioxidant activity of phenols depends on the hydrogen-donating capacity of the hydroxyl group. The oxidation of a hydroquinone such as DPE to an orthoquinonic structure normally occurs via a radical intermediate, the resonance-stabilized phenoxyl radical. Moreover, the stabilization of this radical is further enhanced by its dismutation equilibrium with quinonic and hydroquinonic forms. In contrast, the PE-derived phenoxyl radical cannot dismutate, which probably explains the lack of a protective effect of this molecule against ROM.

Important chemical features of DPE are its lipophilic and hydrophilic behaviors. Therefore, this phenol could be lodged in the membrane, acting as a chain-breaking inhibitor of lipid peroxidation. Moreover, DPE might help regenerate α-tocopherol by reacting with its tocopheroxyl radical, as has been demonstrated for caffeic acid (Laranjinha et al. 1995). Therefore, DPE probably functions, at least in part, as a free radical scavenger. However, the possibility that its antioxidant effect is mediated by the induction of the endogenous defense mechanisms cannot presently be ruled out.

Further research is needed to understand the molecular mechanisms of DPE action, especially regarding DPE cellular targets and its metabolic fate. Studies are in progress in our laboratory to clarify whether a DPE-derived metabolite(s) or DPE itself is responsible for the observed antioxidant effect.

Finally, epidemiological as well as biochemical studies have confirmed the beneficial health effects of antioxidant vitamins. This article calls attention to the non-vitamin dietary antioxidants, such as polyphenols, whose role in human nutrition could be crucial. As discussed, endogenous defense mechanisms are inadequate for the complete prevention of oxidative damage, and different sources of dietary antioxidants may be especially important. Identification of new food components with antioxidant effects in biological systems would therefore be helpful in designing dietary strategies to maximize the in vivo antioxidant potential in humans.

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