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Hydroxytyrosol, a Natural Molecule Occurring in Olive Oil, Induces Cytochrome *c*-Dependent Apoptosis

Fulvio Della Ragione,*[,1] Valeria Cucciolla,* Adriana Borriello,* Valentina Della Pietra,* Gabriele Pontoni,* Luigi Racioppi,† Caterina Manna,* Patrizia Galletti,* and Vincenzo Zappia*
**Institute of Biochemistry of Macromolecules, Medical School, Second University of Naples, Naples, Italy; and*
†Department of Cellular and Molecular Biology and Pathology, Medical School,
University of Naples "Federico II," Naples, Italy

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2-(3,4-Dihydroxyphenyl)ethanol (DPE), a naturally occurring phenolic antioxidant molecule found in olive oil, has been reported to exert several biological and pharmacological activities. We studied the effect of this compound on the proliferation and survival of HL60 cell line. Concentrations from 50 to 100 μM DPE, comparable to its olive oil content, caused a complete arrest of HL60 cell proliferation and the induction of apoptosis. This was demonstrated by flow cytometric analyses, poly(ADP-ribose) polymerase cleavage, and caspase 3 activation. The apoptotic effect requires the presence of two *ortho*-hydroxyl groups on the phenyl ring, since tyrosol, 2-(4-hydroxyphenyl)ethanol, did not induce either cell growth arrest or apoptosis. DPE-dependent apoptosis is associated with an early release of cytochrome c from mitochondria which precedes caspase 8 activation, thus ruling out the engagement of cell death receptors in the apoptotic process. 2-(3,4-Dihydroxyphenyl)ethanol induced cell death in quiescent and differentiated HL60 cells, as well as in resting and activated peripheral blood lymphocytes, while did not cause cell death in two colorectal cell lines (HT-29 and CaCo2). These results suggest that DPE down-regulates the immunological response, thus explaining the well-known antinflammatory and chemopreventive effects of olive oil at the intestinal level.

Key Words: **2-(4-hydroxyphenyl)ethanol; hydroxytyrosol; olive oil; Mediterranean diet; reactive oxygen species; ROS; apoptosis; programmed cell death; chemoprevention; colon cancer.**

Current approaches to cancer treatment are mostly based on cytotoxic and cytostatic mechanisms to eliminate malignant cells. These pharmacological strategies, although efficacious towards the malignant cells, show a number of toxic side effects which frequently hamper or drastically reduce their use. A newer dimension in cancer management is the increasing awareness that chemoprevention, namely the administration of chemical (both natural and synthetic) agents to prevent the early events of carcinogenesis, could be the most direct way to counteract malignancy development and progression (1).

In the search for new cancer chemopreventive compounds, hundreds of naturally occurring molecules have been evaluated over the past few years. Among the agents able to lower the rate of malignant transformation, antioxidants appear to be very promising. Indeed, diets rich in antioxidant molecules are clearly associated with a diminished risk of cancer at various anatomical sites (2, 3). However, some intervention studies have questioned the effectiveness of specific antioxidants in tumor prevention (3 and references therein). This suggests that the use of these compounds as chemopreventive (or pharmacological) agents must await more detailed knowledge of their mechanism of action and their interactions with genetic phenotypes and environment (3).

Among the most accepted correlations between dietary habit and cancer risk is the observation that Mediterranean diet is associated with a statistically significant reduction of breast and colon neoplasias (4, 5). Particularly, vegetable and fruit consumption appears to account for a quoticeable part of this protective effect. Moreover, there is clear evidence that olive oil intake contributes to the reduced incidence of neoplasias (4–6). Since Mediterranean olive oil is rich in polyphenol antioxidant molecules, it is highly probable that such components might be responsible, at least in part, for the nutrient chemopreventive activity.

[1] To whom correspondence should be addressed at Institute of Biochemistry of Macromolecules, Second University of Naples, via Costantinopoli 16, 80138 Naples, Italy. Fax: +39-081-441688. E-mail: dellarag@cds.unina.it.

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Among the olive oil antioxidants, 2-(3,4-dihydroxyphenyl)ethanol (DPE) seems to have a pivotal role. Several reports demonstrated that DPE has remarkable protective effects against oxidative stress-related damages. *In vitro* experiments showed that DPE might efficaciously counteract chemical oxidation of low density lipoproteins (7) and exerts a scavaging action with respect to superoxide anion generation (8). Furthermore, DPE is able to interfere with the leukotriene generation from arachidonic acid by inhibiting 5-lipoxygenase and 12-lipoxygenase activities from rat platelets (9) and polymorphonuclear leukocytes of rat and human origin (10). Conversely, the olive oil phenol does not modify the activity of mammalian cyclooxygenase (10). Additional experiments demonstrated that DPE inhibits platelet aggregation (11), and that a brief preincubation with the molecule protects intact cells against the toxic effects of reactive oxygen species (ROS) (12–14). Importantly, these effects are probably operative *in vivo*, since recent studies from our group demonstrated that DPE is rapidly taken up by intestinal cell lines via a passive diffusion (15).

Although olive oil antioxidants, and particularly DPE, exert a number of biological protective effects, so far no investigations have been carried out to highlight the activity of the molecule on the proliferation of human cells. These studies might be extremely important to shed light on the molecular bases of the diminished risk to develop cancer associated with olive oil increased intake (5, 6).

This paper reports the first study devoted to evaluate the effect of DPE on cultured human cells. We found that 2-(3,4-dihydroxyphenyl)ethanol actively induces apoptosis in white blood cells (both transformed and normal cells) as a consequence of a rapid cytochrome c release from intermembrane mitochondrial space. The importance of this result will be discussed in the light of olive oil chemopreventive value and of the potential therapeutical use of DPE.

MATERIALS AND METHODS

Materials. 2-(3,4-dihydroxyphenyl)ethanol was a kind gift of G.F. Montedoro, University of Perugia, Italy. 2-(4-hydroxyphenyl)ethanol (tyrosol), 2-(3,4-dihydroxyphenyl)acetic acid, 2,5-(dihydroxyphenyl)-acetic acid (homogentisic acid), phenylmethylsulfonyl fluoride, leupeptin, chymostatin, soybean trypsin inhibitor were from Sigma Chemical Company (St. Louis, MO). These compounds were dissolved in DMSO at a concentration of 100 mM. All these solutions were stored at −80°C. Vitamin D3 (Sigma) was resuspended in absolute ethanol and stored at −20°C. Anti-PARP monoclonal antibodies were from BioMol Research Laboratories, Inc. (PA), while anti-CPP32/Caspase 3 monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Antibodies directed against caspase 8 were from Upstate Biotechnology or Santa Cruz Biotechnologies (Santa Cruz, CA). Polyclonal antibodies to Fas and FasL were provided by Santa Cruz. Anti-cytochrome c (clone 7H8.2C12) monoclonal antibodies were from Pharmingen (San Diego, CA).

Cells and cell treatments. Human myeloid leukemia HL60 cells were cultured as in ref. 16. The cells were induced to differentiate (towards a myelo-monocitic phenotype) by incubation with 1 μM vitamin D3 for 5 days (16). HT-29 and Caco-2 cells were grown as described in 17. Resting peripheral lymphocytes were prepared and activated as in 18.

Differentiation assays and flow cytometry analyses. The differentiation of HL60 cells after vitamin D3 treatment was evaluated by analyzing the nitroblue tetrazolium reduction and the nonspecific esterase activity (16). Nonspecific acid esterase activity was assayed microscopically by employing a standard kit (Sigma) and following manufacturer's instruction. Flow cytometry analyses were carried out as described in 16.

Immunochemical methodologies. Total cell extracts for direct immunoblotting analysis were prepared as in 16 and 17. Cells were counted in a Burker's chamber and pellets of 300,000 cells were directly resuspended in sample loading buffer (16), boiled for 5 min and centrifuged at 15,000*g*. Samples were then analyzed for immunoblotting as in reference 17.

Cytochrome c analysis in HL60 cytosol. Preparation of cytosolic extracts of HL60 cells was performed as reported in 19 with minor modifications. The cells were collected by centrifugation at 200*g* for 5 min at 4°C. The pellets were then washed twice with ice-cold phosphate-buffered saline (120 mM NaCl/2.7 mM KCl/10 mM sodium phosphate, pH 7.4), and resuspended in 300 μl of extraction buffer (EB), containing 220 mM mannitol, 68 mM sucrose, 50 mM Hepes-NaOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, and protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 0.83 μg/ml chymostatin, 10 μg/ml soybean trypsin inhibitor). After 15 min incubation on ice, cells were lysed by pushing them through a 22-gauge needle and the lysate spun at 14,000*g* for 15 min. Finally, supernatants were removed and analyzed by gel electrophoresis or stored at −80°C if not used immediately. Cytosolic protein extracts (2–5 μg) were loaded onto each lane of a 15% SDS–polyacrylamide gel and then transferred to Hybond-ECL nitrocellulose membrane (Amersham). The membranes were then incubated with the anti-cytochrome c monoclonal antibody 7H8.2C12 and the immunoreactive bands were revealed using enhanced chemoluminescence (17).

Apoptosis assessment by annexin-V staining. In the case of peripheral lymphocytes, apoptosis was evaluated by analyzing the labelling of cell membrane by annexin-V technique. Briefly, after DPE treatment the cells were washed in PBS and resuspended in 100 μl of staining solution (containing annexin-V fluorescein and propidium iodide in Hepes buffer). After incubation at room temperature for 15 min, cells were analyzed by flow cytometry. Annexin-V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the DNA of cells with a compromised cell membrane. This allows for the discrimination of living cells (unstained with either fluorochrome) from apoptotic cells (stained only with Annexin-V) and necrotic cells (stained with both annexin-V and propidium iodide).

Nuclear magnetic resonance analysis. Cell culture media (300 μl) were taken before and after a 24 h incubation with cells treated as described elsewhere (20 and references therein), and analyzed at pH 2.5 in a Bruker AC 200 E NMR Spectrometer (4.7 Tesla, proton resonance at 200 MHz). Perdeuterated sodium trimethylsylilpropionate was used as a qualitative and quantitative standard. Under these conditions, the proton spectra of the two samples showed the typical singlet of homogentisic acid at 6.76 ppm (aromatic singlet, unambiguously identified by copeaking with added standard homogentisic acid). The concentration of homogentisic acid was estimated, exploiting the area of the peak at 6.76 ppm, as about 100 ± 20 μM; no detectable difference in homogentisic acid concentration was evidenced after the 24 h incubation.

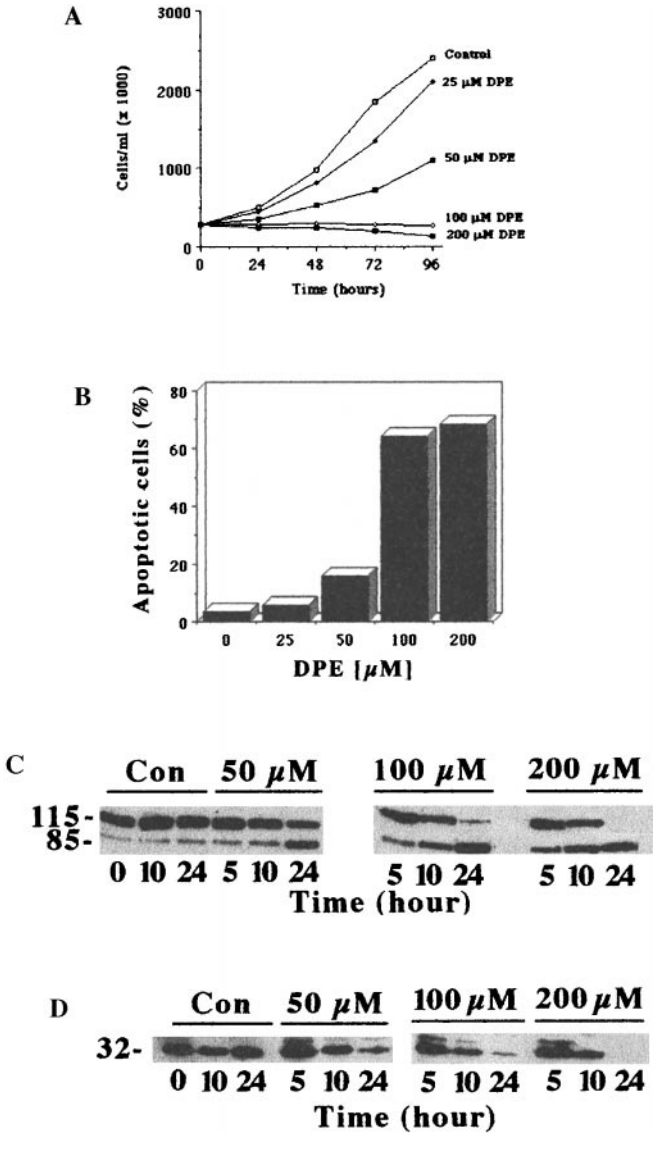

FIG. 1. Growth inhibitory and apoptogenic effect of DPE on HL-60 cells. (A) HL60 cells were plated at 300,000 cells/ml and incubated with or without different DPE concentrations. Cells were then counted daily. (B) Percentage of apoptosis in HL60 cells treated with DPE. (C) Poly(ADP ribose) polymerase (PARP) cleavage demonstrated by the accumulation of the 85 kDa fragment with respect of the intact protein (signal at 115 kDa). (D) Activation of procaspase 3 determined by the disappearance of the 32 kDa band.

RESULTS

Effect of DPE on Proliferation and Survival of HL60 Cells

The effect of different DPE concentrations on HL60 cell growth was reported in Fig. 1A. This cell line, established from a acute promyelocytic leukemia, was selected as model system since it is largely employed in studies devoted to the evaluation of antiproliferative, differentiative, and apoptotic effect of potentially active molecules. As shown in Fig. 1A, 100 μM DPE completely inhibited cell proliferation, while the total cell number was reduced at higher level (i.e., 200 μM). The estimated DPE concentration inducing a 50% cell growth inhibition is about 35–40 μM (data not shown).

In order to clarify the effect of DPE on cell proliferation, hydroxytyrosol-treated HL60 cells were analyzed by flow cytometry (data not reported). DPE caused a noticeable increase of cells with a subdiploid DNA (i.e., apoptotic cells) which corresponded to the sub-G1 population on the cell division cycle analysis. Figure 1B reports the percentage of cells undergoing to programmed cell death after DPE treatment. It must be underlined that 100 μM DPE induced apoptosis in more than 65% of HL60 cells within 24 h.

Subsequently, in order to confirm biochemically the findings reported in Fig. 1B, we carried out a detailed analysis on extracts from HL60 cells incubated with different DPE amounts. It is definitely established that the key biochemical event of apoptosis is the activation of the caspases cascade which results in the degradation of a number of target proteins, including: poly-(ADP ribose)polymerase (PARP), DNA-dependent protein kinase, lamins, and fodrin. Thus, the evaluation of PARP cleavage by Western blotting represents an *in vivo* direct proof of caspase activation. An additional important experimental evidence of the programmed cell death is the proteolytic activation of caspases, which can be demonstrated by the disappearance of proenzyme signals on immunoblotting analyses. Both PARP cleavage and caspase 3 activation are early events of apoptosis and generally precede DNA cleavage.

As shown in Fig. 1C, DPE induced PARP cleavage, evidenced by the accumulation of a 85 kDa fragment compared to the 115 kDa intact protein, in a dose- and time-dependent fashion. The caspase 3 activation is demonstrated by the disappearance of procaspase 3 band (Fig. 1D) and the occurrence of the active cleavage products (data not shown). Also in this case dosage- and time-dependent hydroxytyrosol effects were evidential.

In conclusion, DPE, when added to the culture medium of HL60 cells, arrests the cellular growth and actively induces programmed cell death.

Apoptotic Activity of DPE on Normal and Transformed Human Cells

Prompted by the finding of the apoptogenic capability of DPE, we wondered whether this effect was cell phenotype specific or if the molecule was able to induce apoptosis in different human cell types. Moreover, we were interested in establishing if the molecule exerts its effects on either proliferating or resting cells. This latter information might be particular intriguing, since it is generally easier to induce programmed cell death in rapidly dividing rather than in quiescent cells.

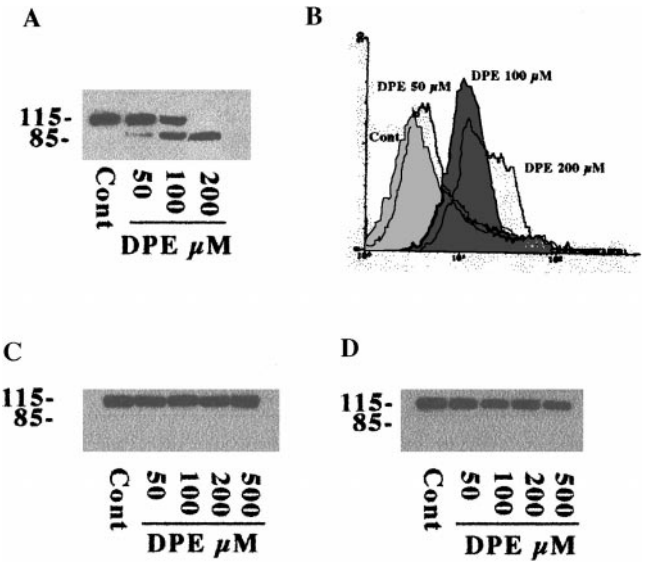

FIG. 2. Effect of DPE on different cell types. Various cell types were cultured in the presence of different amounts of DPE. After 24 h the cells were collected and analyzed for PARP cleavage (except A) (A) Differentiated HL60 cells. HL60 cells were incubated for 5 days with 1 μM vitamin D3. After this period more than 90% of the cells were differentiated towards a myelo-monocytic lineage as demonstrated by different markers (see Material and Methods). Then the cells were treated for 24 h with DPE. (B) Resting peripheral lymphocytes. Peripheral lymphocytes were prepared as under Materials and Methods and incubated for 24 h with different amounts of DPE. The apoptogenic effect was evidenced by annexin-V labelling. (C and D) Growing CaCo2 and HT-29 cells were incubated for 24 h with different DPE amounts.

In order to unravel these questions, we investigated the effect of DPE on different cell models, including: exponentially proliferating and quiescent HL60 cells; differentiated (by previous incubation with vitamin D3) HL60 cells; resting and proliferating peripheral blood lymphocytes (PBLs), and two colon cell lines, namely $CaCo_2$ and HT-29 cells.

As shown in Fig. 2, differentiated HL-60 (A) and resting PBLs (B) were remarkably responsive to programmed death induced by DPE addition. This was demonstrated by different approaches, including the analysis of membrane phospholipid asymmetry (detected as phosphatidylserine translocation by means of annexin V staining, Fig. 2B) and PARP cleavage (Fig. 2A). Identical results, namely induction of apoptosis, were obtained in quiescent HL60 cells and proliferating PBLs (data not reported). In addition, DPE caused a strong inhibition of PBLs cell growth (results not shown). Conversely, neither $CaCo_2$ nor HT-29 growth was affected by DPE treatment (data not shown). Importantly, these two cell lines were completely resistant to the apoptogenic capability of DPE up to 500 μM concentration (Figs. 2C and 2D).

Therefore, the programmed cell death due to the olive oil phenol is specific for cell phenotype and, remarkably important, both normal and malignant white blood cells are very good targets of DPE action.

Structural Requirements and Molecular Basis of DPE-Dependent Apoptosis

Having established the apoptotic effect of DPE, we were interested in clarifying the major structural features of the molecule required to induce programmed cell death. Particularly, we investigated the importance of the two *ortho*-hydroxyl groups on the phenyl moiety. Thus, we compared the apoptogenic effect of hydroxytyrosol with those of tyrosol (which presents only one hydroxyl group on the phenyl ring), dihydroxyphenylacetate (presenting an acetate chain in spite of the ethanol moiety) and homogentisic acid (which has two *para*-hydroxyl groups on the phenyl moiety and the acetate chain). The structures of the molecules are reported in Fig. 3A. The compounds were tested by analyzing their capability of inhibiting HL60 cell proliferation and of inducing apoptosis. PARP cleavage was chosen as biochemical hallmark of programmed cell death.

Dihydroxyphenylacetate was able to restrain cell growth (Fig. 3B) and to cause apoptosis at an extent similar to that of DPE (Fig. 3C). Conversely, both tyrosol and homogentisic acid did not interfere with HL60 proliferation and did not activate the genetic death program (Figs. 3B and 3C).

Since homogentisate is a physiological intermediate of phenylalanine and tyrosine catabolism, we wondered whether the lack of action might be due to its rapid removal. Therefore, we analyzed by nuclear magnetic resonance methodology the homogentisic acid level in the culture media. These experiments demonstrated that the medium concentration of the molecule did not vary during 24 h incubation with HL60 cells (see Materials and Methods for technical details), thus suggesting that the absence of apoptogenic effect was not related to the compound catabolism. In conclusion, these findings demonstrate that two *ortho*-hydroxyl groups on the phenyl ring are absolutely necessary for the activation of cell death.

Apoptosis is generally believed to be activated, at molecular level, by a heterogeneous initiation phase when cells receive death-inducing stimuli via: (i) engagement of specific membrane receptors (especially Fas receptor); (ii) shortage of obligatory growth factors, and (iii) physical or chemical damages. Different initiation events result in the activation of at least two non-exclusive distinct "executioner" pathways. One is related to the activation of caspase 8, which is downstream to the cell death receptor(s) engagement. The other relies on the increase of mitochondrial membrane permeability, which causes cytochrome c release from the intermembrane space. Both these executioner pathways cause the proteolytic activation of caspase 3

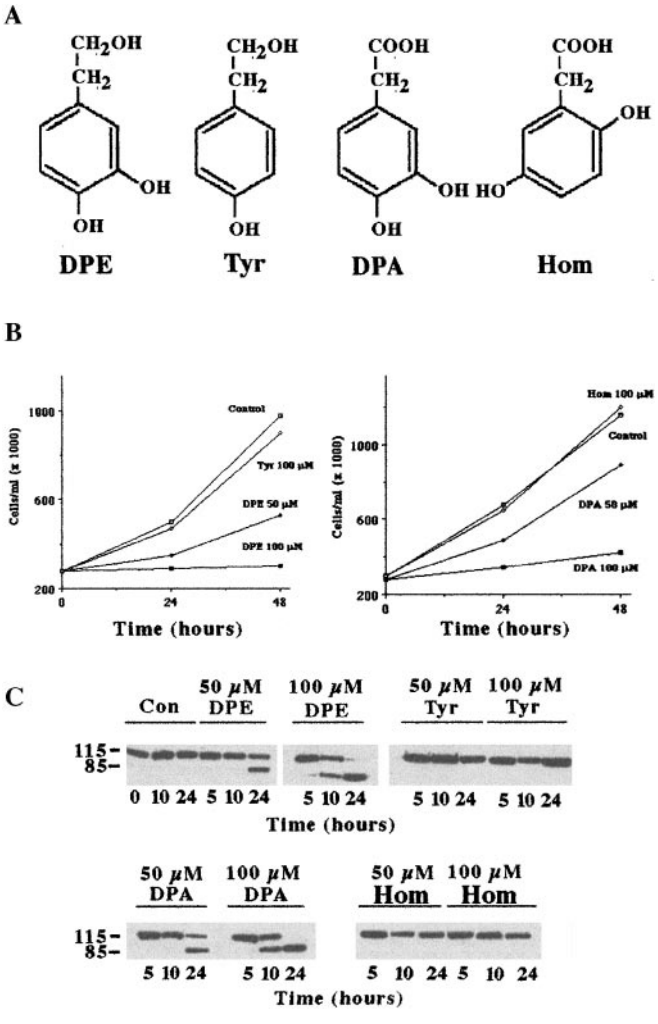

FIG. 3. Apoptogenic effect of DPE and its analogs on HL60 cells. HL-60 cells were grown in the presence of different amounts of DPE and its analogs for various time periods. Cells were then counted daily. Cellular extracts were analyzed by immunoblotting employing anti-PARP antibodies. (A) Chemical structure of hydroxytyrosol (DPE), tyrosol (Tyr), hydroxyphenylacetate (DPA), and homogentisic acid (Hom). (B) Growth inhibitory effect of DPE and its analogs. The experiments were carried out as described in Fig. 1. (C) Cleavage of PARP in HL60 cells incubated with the molecules reported in A for the reported time periods.

(or 7), the central event in the apoptotic process, followed by the final cascade characterized by the cleavage of multiple downstream caspase substrates. Thus, in order to understand the molecular mechanisms of DPE-induced apoptosis, it appeared essential to unravel the executioner event(s) which cause(s) caspase 3 activation. However, since the two pathways are strongly linked (for example activated caspase 3 rapidly activates caspase 8, ref. 21), only very detailed time-course experiments might shed light on the primary events of the apoptotic cascade.

Initially, we tested the effect of DPE on cytochrome c release by evaluating the cytosolic cytochrome c content of HL60 cells incubated with various amounts of DPE for different time-periods. As shown in Fig. 4 (A to C) a remarkable release of the protein from the mitochondrial intermembrane space towards the cytosolic compartment was clearly demonstrated. This phenomenon precedes caspase 3 (Fig. 1D) activation, PARP cleavage (Fig. 1C), and most importantly, caspase 8 activation (Fig. 4D), thus suggesting that it might represent the executioner event of DPE-induced cell death. In order to further confirm this conclusion, we analyzed Fas and Fas ligand protein levels in HL60 extracts (not shown). We were unable to demonstrate any variation between control and DPE-treated cells.

Altogether, these data point to the increase of mitochondrial membrane permeability as a critical step in the DPE-dependent apoptosis.

DISCUSSION

Epidemiological studies indicate that olive oil exerts a remarkable preventive effect against the development of breast and colon cancers (2, 3). Although this action might be ascribed to different components of the nutrient, several findings suggest that the phenolic fraction, and particularly 2-(3,4-dihydroxyphenyl)-ethanol, is responsible (at least in part) for the beneficial effects. This view is supported by a number of reports which demonstrate that DPE has several intriguing biological activities (7–15).

Moreover, a wealth of studies has demonstrated that a number of naturally occurring phenols (especially polyphenols like flavonoids, isoflavonoids, resveratrol, etc.) show chemopreventive activity, particularly towards colon carcinomas (22, 23). Thus, studies devoted to clarify their mechanism(s) of action appear of remarkable importance.

In this investigation, we analyzed the effect of DPE on the proliferation of a human promyelocytic cell line, namely HL60 cells. Initial results demonstrated that the compound inhibits HL60 growth and, very intriguing, induces apoptosis in a concentration range (50–100 μM) which is most probably reached *in vivo* (at specific anatomical sites), being olive oil DPE content up to 3 mM (12).

A more detailed mechanistic analysis demonstrated that the phenol induces the release of cytochrome c from the intermembrane mitochondrial space (Figs. 4A–4C). This event precedes caspase 8 activation (Fig. 4D), thus demonstrating that DPE apoptotic initiation phase does not involve the engagement of cell death receptors, including Fas (Fig. 4 and data not shown).

The DPE apoptotic activity also prompted us to unravel the structural requirements necessary for the biological effects. The results obtained point to the occurrence of two *ortho*-hydroxyl groups on the phenyl moiety as an essential feature. This finding suggests that DPE might exert its biological apoptotic effect

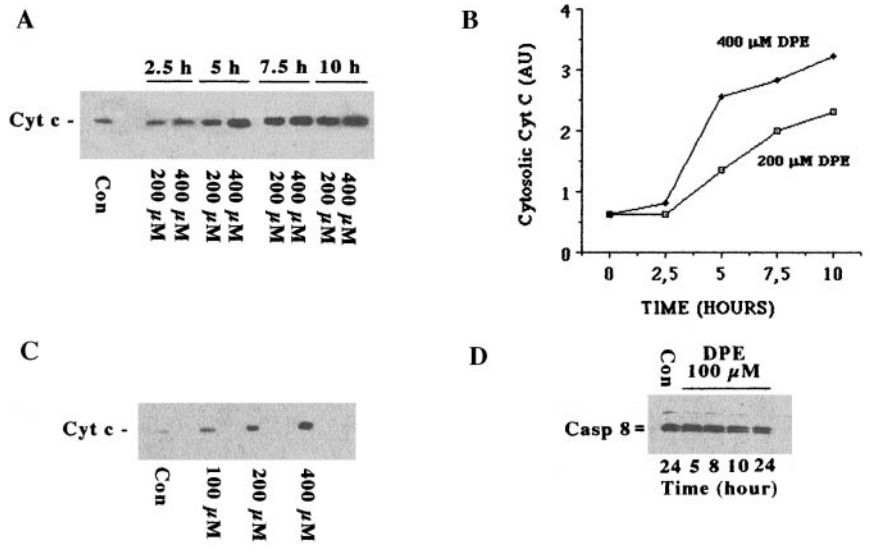

FIG. 4. Effect of DPE on Fas ligand and cytosolic cytochrome c levels in HL60 cells. HL60 cells were incubated for various time period with different amounts of DPE. Then, the cellular extract (3×10^5 cells) or the cytosolic fraction was prepared and analyzed by immunoblotting for the specific protein. (A) Cytosol fraction of HL60 cells treated with DPE for different time periods was analyzed with monoclonal antibodies against cytochrome c. (B) The results of B were quantified by laser scanning. Cytosolic cytochrome c content was reported as arbitrary unit (AU). (C) Cytosol of HL60 cells treated with different amounts of DPE for 5 h was analyzed for cytochrome c content. (D) Cytosols of HL-60 cells treated with 100 μM DPE were analyzed for caspase 8 activation. The arrows show the two different forms of caspase 8 (α and β). The activation was determined by evaluating the disappearance of the signal.

acting as scavenger of free radical and/or as a metal ions (possibly iron) chelating molecule (24 and references therein). It is to underline that the ability of phenolic compounds, like DPE, to interrupt the propagation of a reaction chain is due to the stability of phenoxy radical (24 and references therein). On the other hand, it is totally conceivable that DPE (and mostly of the so-called "antioxidant compounds"), under specific environmental conditions, can behave like a "prooxidant" and initiate a new chain of radical reactions (24). It must be pointed out, though, that the higher activity of orthodiphenols as opposed to paradiphenols (such as homogentisic acid) in triggering apoptosis was not expected on the basis of simple interference with radicalic processes. Indeed, ortho- and paradiphenols are reported to be very similar in this respect (24). Based on this finding, a mechanism involving the specific interaction of orthodiphenols (and not of paradiphenols) with cellular target molecule(s) might be proposed.

Independently of the precise mechanism of DPE action, it is intriguing that 2-(3,4-dihydroxyphenyl)-ethanol induces programmed cell death in white blood cells, while two cell lines of colon origin (HT-29 and CaCo2) were completely resistant to the apoptogenic activity. Studies are in progress in order to unravel the molecular bases of the intestinal cell resistance and to investigate the DPE effects on additional cell phenotypes.

The ability of DPE to induce apoptosis in proliferating and resting normal peripheral lymphocytes represents a very promising finding. This effect might be important in explaining the molecular bases of the observed olive oil beneficial activities on human health, and particularly in the prevention of colon cancer. Indeed, it is well-established that inflammatory processes are involved in all steps of cancer transformation. The capability of DPE to reduce the lymphocytic response, by inhibiting proliferation and inducing apoptosis, might be particularly important at intestinal level where the content of the molecule is higher.

Moreover, our data might open new routes in the development of therapeutical strategies. Indeed, the down-regulation of lymphocyte proliferation (without any effect on intestinal cells) might be extremely useful in treating chronic inflammatory bowel pathologies, like Chron's disease. The possibility of inducing apoptosis in quiescent lymphocytes has also a noticeable importance in the treatment of indolent hematological tumors (like chronic leukemias, low grade lymphomas, and others) which are characterized by a slow growth rate, an increase of malignant cell survival, and a strong resistance to traditional chemotherapeutic intervention. Studies are needed to address these promising therapeutical developments.

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