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Research Article

Production of hydrogen peroxide is responsible for the induction of apoptosis by hydroxytyrosol on HL60 cells

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Hydroxytyrosol [3,4-dihydroxyphenylethanol (3,4-DHPEA)], a phenolic compound found exclusively in olive oil, exerts growth-suppressive and pro-apoptotic effects on different cancer cells. Although some molecular mechanisms involved in the pro-apoptotic activity of 3,4-DHPEA have been proposed, the initial stress signals responsible of this phenomenon are not known. Our aim was to assess the involvement of reactive oxygen species as mediators of apoptosis induced by 3,4-DHPEA on HL60 cells. Apoptosis was determined by analyzing the nuclear fragmentation by both fluorescence microscopy and flow cytometry. The externalization of phosphatidylserine was evidenced using an Annexin V-FITC kit. The concentration of H_2O_2 in the culture medium was measured by the ferrous ion oxidation-xylenol orange method. The pro-apoptotic effect of 3,4-DHPEA (100 μM) was prevented by *N*-acetyl-cysteine, ascorbate, and α-tocopherol. Catalase suppressed the 3,4-DHPEA-induced apoptosis, while the Fe(II)-chelating reagent *o*-phenantroline showed no effect, suggesting the involvement of H_2O_2 but not of $OH^{\bullet}$. Indeed, 3,4-DHPEA caused accumulation of H_2O_2 in the culture medium. Tyrosol (*p*-hydroxyphenylethanol) and caffeic acid, compounds structurally similar to 3,4-DHPEA but not able to generate H_2O_2, did not induce an appreciable apoptotic effect. This is the first study demonstrating that apoptosis induction by 3,4-DHPEA is mediated by the extracellular production of H_2O_2.

Keywords: Apoptosis / Cancer chemoprevention / Hydrogen peroxide / Hydroxytyrosol / Olive oil

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1 Introduction

Recently, olive oil components received particular attention because several epidemiological and animal studies have provided convincing evidences that olive oil may prevent carcinogenesis [1]. In particular, *in vitro* studies have demonstrated that phenolic compounds present in virgin olive oil possess chemopreventive properties acting on both initiation and promotion/progression steps of the carcinogenesis process. Among them, *o*-diphenol hydroxytyrosol [3,4-dihydroxyphenylethanol (3,4-DHPEA)] is the most representative and it is present in olive oil as a free compound and both linked to the dialdehydic form of elenoic acid (3,4-DHPEA-EDA) and as the isomer of oleuropein aglycon (3,4-DHPEA-EA) [2]. 3,4-DHPEA has been intensely investigated over the last few years because of its potent antioxidant activity. Indeed, it possesses a clear scavenging activity toward different free radicals [3] and it is able to prevent several forms of damage caused by reactive oxygen species (ROS) such as hydrogen peroxide-induced DNA strand breaks in Jurkat [4] and prostate [5] cancer cells, and DNA-base modification induced by peroxynitrite [6]. However, the chemopreventive capacity of 3,4-DHPEA could also be related to its ability to inhibit proliferation and to induce apoptosis and differentiation in different cancer cell lines as demonstrated by previous studies conducted in our laboratory [7] and by other investigators [8].

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Abbreviations: **CA**, caffeic acid; **CAT**, catalase; **3,4-DHPEA**, 3,4-dihydroxyphenylethanol (hydroxytyrosol); **FCS**, fetal calf serum; **HO 342**, Hoechst 33342; **p-HPEA**, *p*-hydroxyphenylethanol (tyrosol); **NAC**, *N*-acetyl-cysteine; **PI**, propidium iodide; **PNT**, *o*-phenanthroline; **PP2A**, protein phosphatase 2A; **ROS**, reactive oxygen species

We previously found that 3,4-DHPEA at a concentration of 100 μM induces a total arrest of cell growth on human promyelocytic leukemia cells (HL60) by inhibiting the $G_1 \rightarrow S$ transition, blocking the cell cycle in the G_0/G_1 phase, and causing massive apoptotic cell death [9]. It was also found that 3,4-DHPEA alters the expression of important cell cycle and apoptosis regulatory proteins reducing the expression of cyclin-dependent kinase 6 (CDK6) and increasing that of CDK inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ [10]. On the other hand, 3,4-DHPEA induced apoptosis on colon carcinoma cells (HT-29) at high concentrations (200–400 μM) and caused a cell cycle arrest in the S/G_2 phases [11]. In this case it was shown that the main mechanism by which 3,4DHPEA induces apoptosis was mediated by the activation of the Ser/Thr protein phosphatase 2A (PP2A). In addition, several other genes (Bcl-2, Bax, Egr1, c-Jun) involved in the control of cell cycle arrest, differentiation, and apoptosis, were shown to be modulated by 3,4-DHPEA, both on their expression level and on phosphorylation state of their translation products [12].

Although the broad effects exerted by 3,4-DHPEA on different intracellular signaling pathways, the initial stimulus(i) responsible for the anti-proliferative and pro-apoptotic effect of 3,4-DHPEA is/are at present not known. One hypothesis aimed at explaining the effects of 3,4-DHPEA considered that ROS are implicated in the regulation of proliferation, differentiation and apoptosis [13]. The production of ROS is an unavoidable consequence of aerobic metabolism and all cells are continuously exposed to these damaging agents. In addition, ROS may serve as physiological second messengers in signal transduction pathways. In fact, an increased production of ROS has been observed upon stimulation of cells by different ligands such as tumor necrosis factor-α, interleukin-1 and growth factors [14]. Therefore 3,4-DHPEA could alter the oxidative equilibrium acting either as antioxidant or as pro-oxidant. We can rule out that 3,4-DHPEA exerts the anti-proliferative and pro-apoptotic effect by its antioxidant properties because our recent results have clearly shown that 3,4-DHPEA exert a potent antioxidant activity at a low concentration range (1–10 μM) [15] that is not sufficient to induce appreciable effects on proliferation and apoptosis [9]. Instead, the growth arrest is a crucial phenomenon of the cellular response to oxidative stress, and ROS can induce cell death either by apoptosis or by necrosis as a consequence of both the level and the duration of the exposure [13]. Therefore, our working hypothesis was that 3,4-DHPEA may cause apoptosis through a pro-oxidant mechanism. Indeed, other works have shown that molecules such as β-carotene [16], ascorbate [17] and quercetin [18], which act as antioxidant at low concentrations, exert a pro-oxidant activity and induce apoptosis at high concentrations.

The results of the present investigation demonstrate for the first time a clear pro-oxidant activity of 3,4-DHPEA that is responsible for its anti-proliferative and pro-apoptotic effects on HL60 cells. This pro-oxidant effect is principally mediated by the generation of hydrogen peroxide (H_2O_2) in the culture medium and it is efficiently prevented by the H_2O_2-scavenging enzyme catalase and by dietary antioxidants.

2 Materials and methods

2.1 Materials and cell culture

RPMI 1640 medium and heat-inactivated fetal calf serum (FCS) were obtained from Gibco (Gibco BRL, Life Technologies, Paisley, Scotland). 3,4-DHPEA was obtained from Cayman Chemicals Ltd. (United States), supplied as an ethanol solution at a concentration of 320 mM and stored at −20°C in the dark. The compound was diluted in RPMI 1640 medium just before use to the desired concentration. All the solutions were sterilized by filtration on 0.22-μm filters (Celbio S.r.l., Milan, Italy). All other reagents were purchased from Sigma unless differently specified (Sigma-Aldrich, Irvine, UK).

Human promyelocytic leukemia cells (HL60), obtained from the American Type Culture Collection (Rockville, MD, USA), were cultured in complete RPMI 1640 medium supplemented with 10% FCS, 2.0 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin. The cells were maintained at 37°C and 5% CO_2 in a humidified atmosphere and seeded every 4–5 days at a density of 0.2×10^6/mL.

2.2 Cell treatments

The following compounds were tested to identify their effects on the anti-proliferative and pro-apoptotic properties of 3,4-DHPEA: *N*-acetyl-cysteine (NAC; 10 mM), ascorbate (100 μM), α-tocopherol (10–100 μM), β-carotene (10–100 μM), catalase (CAT, 100 U/mL), *o*-phenanthroline (PNT, 2.5–50 μM). NAC was prepared as stock solution at 0.4 M in RPMI medium (the pH was adjusted to 7.4 with 0.2 M NaOH). α-Tocopherol and β-carotene were dissolved in tetrahydrofuran (THF) preparing stock solutions at 8 and 30 mM, respectively. Before each experiment, aliquots were added to the culture medium to obtain the indicated concentration, while the control cultures received the same amount of THF, which was never greater than 0.05%. In all experiments, before the treatment with 3,4-DHPEA, the HL60 cells were pre-incubated with the above reported compounds for 1 h at 37°C. After the different times aliquots of the cell suspension were taken to perform the various assays.

2.3 Cell proliferation and apoptosis assays

The proliferation of HL60 cells was followed by counting the viable cells using the trypan blue exclusion method.

HL60 cells were seeded at a density of 0.25×10^6/mL in culture medium in 25-cm^2 flasks (Falcon; Becton Dickinson, Oxnard, CA, USA) and incubated for the indicated time at 37°C and 5% CO_2 with 3,4-DHPEA (100 μM) in the absence and in the presence of the different effectors. At each established experimental time, 20-μL aliquots were withdrawn from the cell suspensions, diluted 1:10 with a trypan blue solution (2.22 mg/mL PBS), and, after standing at room temperature for 5 min, the viable cells were counted using a Burker chamber. The blue-stained cells were considered non-viable.

The percentage of apoptotic cells was determined by a fluorescent microscopy assay [19]. The cell suspensions (100 μL) were centrifuged ($400 \times g$, 7 min) and the pellets were resuspended in complete RPMI medium containing the DNA binding dyes Hoechst 33342 (HO 342, 20 μg/mL in PBS) and propidium iodide (PI, 10 μg/mL in PBS). After 10-min incubation at room temperature, the cells were examined using a fluorescent microscope (Zeiss, R.G., equipped with a 50-W mercury lamp) with ultraviolet excitation at 340 – 380 nm. HO 342 is a plasma membrane-permeable compound, which freely enters cells with intact membranes, as well as those with damaged membranes, and stains the DNA blue, whereas PI is a highly polar dye that is impermeable to cells with intact plasma membrane and stains the DNA red. Consequently, viable cells are identified by their intact nuclei with blue fluorescence (HO 342), and necrotic cells by their intact nuclei with yellow fluorescence (HO 342 plus PI). Apoptotic cells are detected by their fragmented nuclei exhibiting either a blue (HO 342) or yellow (HO 342 plus PI) fluorescence depending on the stage of the process. Under each of the experimental condition, three slides were prepared and 100 cells were counted for each slide.

In some experiments apoptosis was also evaluated by PI staining and flow cytometry. In this case, the cell pellet was resuspended in 0.5 mL hypotonic fluorochrome solution (50 μg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100) in 12×75 mm polypropylene tubes (Becton Dickinson, Lincoln Park, NJ, USA). The tubes were kept at 4°C for 30 min before flow cytometric analysis. PI fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) at a wavelength of 488 nm.

In addition, the externalization of phosphatidylserine, which is an early event during the apoptotic process, was evidenced using an Annexin V-FITC kit (Beckman Coulter, Instrumentation Laboratory, Milan, Italy) according to the manufacturer's indications. Briefly, the HL60 cells were harvested by centrifugation and washed with ice-cold PBS; the pellet was resuspended in binding buffer to 2×10^6 cells/mL. Annexin-V-FITC (1 μL to 100 μL) and PI (5 μL to 100 μL) were added, the samples were incubated on ice in the dark for 15 min and then analyzed by flow cytometry (Beckman Coulter).

2.4 Measurement of H_2O_2 concentration in the culture medium

The concentration of H_2O_2 in the culture medium was measured by the ferrous ion oxidation-xylenol orange method [20] as follows: 20 μL medium was mixed with 200 μL reaction solution containing 250 μM ammonium iron(II) sulfate, 25 mM H_2SO_4, 100 mM sorbitol and 125 μM xylenol orange, and incubated at room temperature for 30 min. The absorbance was read at 595 nm and the concentration of H_2O_2 was derived from a standard curve obtained by adding different concentrations of H_2O_2 in the RPMI medium just before the assay.

2.5 Data analysis

All tests were run in triplicate for each experimental condition and each experiment was repeated at least three times; the results are reported as the mean ± SD. Significant differences among treatments were assessed using a one-way ANOVA. When a significant ($p < 0.05$) treatment effect was detected, the mean values were compared using Tukey's post-hoc comparisons. The effect of CAT on apoptosis and H_2O_2 release were compared with Student's *t*-test.

3 Results

3.1 NAC reverses the anti-proliferative and pro-apoptotic activities of 3,4-DHPEA

N-acetylcysteine (NAC) is a scavenger of free radicals interacting with ROS such as $OH^{\bullet}$ and H_2O_2. It is a source of sulfhydryl groups in the cells and is the precursor of the major intracellular antioxidant thiol glutathione (GSH) [21]. Therefore, NAC has been widely used to demonstrate the involvement of ROS in different cell systems and experimental conditions. The effect of NAC on the anti-proliferative activity of 3,4-DHPEA (100 μM) was examined by pre-incubating the HL60 cells for 1 h with the compound before the addition of 3,4-DHPEA and then counting the viable cells during the following 96 h. As reported in our previous study [9], treatment with 3,4-DHPEA (100 μM) completely inhibited the HL60 growth over time (Fig. 1A). The inclusion of NAC in the culture medium partially restored proliferation to about 50% of the control value after 96 h of incubation (Fig. 1A). Nevertheless, NAC effectively inhibited apoptosis induced by 3,4-DHPEA in HL60 cells after 24 h of treatment as evidenced by changes in the nuclear morphology and apoptotic cell count determined by fluorescent microscopy (Fig. 1B). The inhibitory activity of NAC toward 3,4-DHPEA-induced apoptosis was also confirmed by both the analysis of the DNA fragmentation, demonstrated by the sub-G_1 peak (Fig. 2A), and the externalization of phosphatidylserine as evidenced by the double-staining method using FITC-conjugated annexin-V and PI (Fig. 2B).

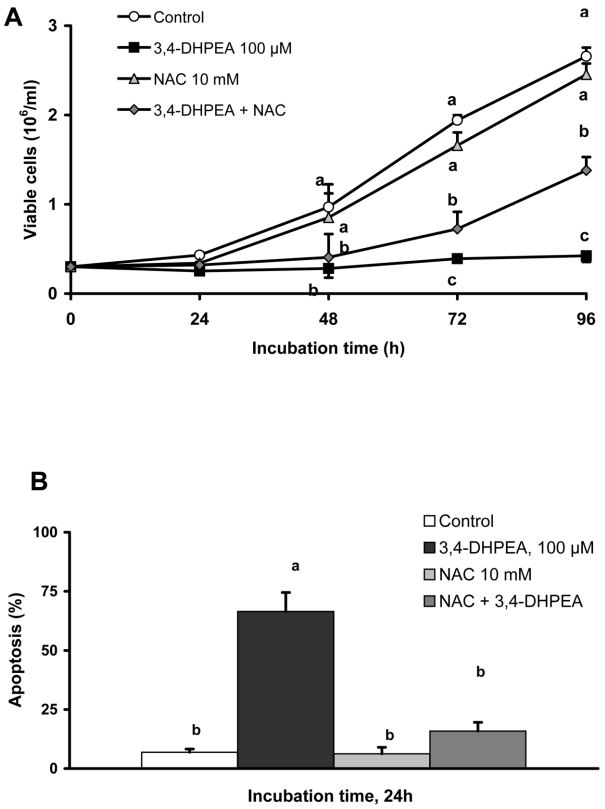

Figure 1. Effect of NAC on the anti-proliferative and pro-apoptotic effects of 3,4-DHPEA on HL60 cells. (A) The cells were seeded at 0.25×10^6/mL in complete RPMI medium in the absence (control) and in the presence of 3,4-DHPEA (100 μM), NAC (10 mM) and 3,4-DHPEA plus NAC; the viable cells were counted over time by the trypan blue method. (B) The cells (0.25×10^6/mL) were incubated for 24 h in complete RPMI medium in the absence (control) and in the presence of 3,4-DHPEA (100 μM), NAC (10 mM) and 3,4-DHPEA plus NAC and the percentage of apoptotic cells was determined by fluorescence microscopy after double-staining with HO 342 and PI. The data represent the mean ± SD of three independent experiments. Points and bars with a different letter are significantly different, $p < 0.05$.

These results give the first convincing evidence that ROS are involved in the 3,4-DHPEA-induced growth arrest and apoptosis in HL60 cells.

3.2 Natural antioxidants protect HL60 cells from 3,4-DHPEA-induced apoptosis

The effect of different natural antioxidants that are regularly assimilated with the diet were tested for their effect on apoptosis induced by 3,4-DHPEA. At doses equimolar to that of 3,4-DHPEA (100 μM), only ascorbate was efficiently able to reduce the 3,4-DHPEA-induced apoptosis (Table 1). α-Tocopherol at 100 μM did not significantly change both apoptotic and necrotic cells, while at 10 μM it caused a protective effect similar to that observed with ascorbate (Table 1). A completely different behavior was observed with β-carotene, which was highly toxic at 100 μM both in the absence and in the presence of 3,4-DHPEA (Table 1). On the other hand, at 10 μM β-carotene was weakly pro-apoptotic when tested alone, while in combination with 3,4-DHPEA it slightly reduced apoptosis, although the effect was not statistically significant. These results further support the hypothesis that apoptosis induced by 3,4-DHPEA is mediated by an overproduction of ROS.

3.3 H_2O_2 is primarily involved in 3,4-DHPEA-induced apoptosis

ROS include H_2O_2 and hydroxyl radicals ($OH^{\bullet}$) derived from H_2O_2 through a Fe(II)-dependent Fenton reaction. To assess whether these species were involved in the 3,4-DHPEA-induced apoptotic effect, the HL60 cells were exposed to 3,4-DHPEA in the presence of CAT (100 U/mL) and the Fe(II)-chelating reagent PNT (50 μM). The results depicted in Fig. 3A show that apoptosis was effectively prevented by CAT, whereas PNT did not reduce the effect of 3,4-DHPEA, but induced apoptosis by itself. Therefore, lower concentrations of PNT were tested; the lowest, 2.5 μM, was not apoptotic (Fig. 3C). However, at this dose PNT also did not inhibit of the 3,4-DHPEA-induced apoptosis (Fig. 3C). These results suggest that the main compound responsible for the 3,4-DHPEA-induced apoptosis is H_2O_2. In addition, since CAT is not cell permeable, the results suggest that the production of H_2O_2 must take place in the extracellular space. To test this hypothesis we next measured the concentration of H_2O_2 in the HL60 culture medium after 24 h of incubation with 3,4-DHPEA. As shown in Fig. 3B, 3,4-DHPEA caused an evident accumulation of H_2O_2 in the HL60 culture medium that was totally inhibited by the addition of CAT. In contrast, PNT did not significantly modify the accumulation of H_2O_2 in the HL60 culture when tested either at 50 μM (Fig. 3B) or at lower concentrations (Fig. 3D). These results strongly support the view that the pro-apoptotic effect of 3,4-DHPEA is mediated by an overproduction of H_2O_2.

3.4 The 3,4-DHPEA-mediated generation of H_2O_2 is actively metabolized by HL60 cells

To elucidate the source of H_2O_2 and whether the HL60 cells and/or FCS were involved in the 3,4-DHPEA-mediated H_2O_2 generation, further experiments were performed measuring the H_2O_2 accumulation after different incubation times of 3,4-DHPEA in HL60 cell culture and in cell-free medium with or without FCS. The results reported in Fig. 4A show that the maximum accumulation of H_2O_2 was observed in the medium without FCS, whereas both FCS and HL60 cells reduced the concentration of H_2O_2. In particular, HL60 cells drastically reduced the accumulation of H_2O_2 in the medium after 24 h of incubation. Indeed, when

Table 1. Effect of different antioxidants on the percentage of necrotic and apoptotic HL60 cells after treatment without (control) and with 3,4-DHPEA 100 μM for 24h.

q		None	Ascorbate	α-Tocopherol		β-Carotene	
			100 μM	100 μM	10 μM	100 μM	10 μM
Control	Necrosis	$0.5 \pm 0.2^{B,b}$	$1.4 \pm 0.4^{B,b}$	$1.5 \pm 1.3^{B,b}$	n.d.	$77.0 \pm 6.0^{A,a}$	$0.7 \pm 1.0^{C,b}$
	Apoptosis	$2.5 \pm 1.0^{B,c}$	$2.7 \pm 0.9^{B,c}$	$5.5 \pm 0.6^{B,c}$	$4.3 \pm 1.8^{B,c}$	$20.7 \pm 5.0^{B,a}$	$15.0 \pm 2.8^{B,b}$
3,4-DHPEA	Necrosis	$0.5 \pm 0.5^{B,b}$	$1.0 \pm 0.5^{B,b}$	$1.0 \pm 0.8^{B,b}$	n.d.	$91.0 \pm 2.0^{A,a}$	$2.5 \pm 0.7^{C,b}$
	Apoptosis	$43.2 \pm 5.0^{A,b}$	$17.2 \pm 6.0^{A,c}$	$54.5 \pm 6.0^{A,a}$	$18.2 \pm 6.5^{A,c}$	$8.0 \pm 1.4^{B,c}$	$35.0 \pm 7.0^{A,b}$

n.d., not determined. Values are means ± SD, $n = 5$. Means without a common letter differ, $p < 0.05$. Capital letters refer to the comparisons made within the columns, while the small letters refer to the comparisons performed within the rows.

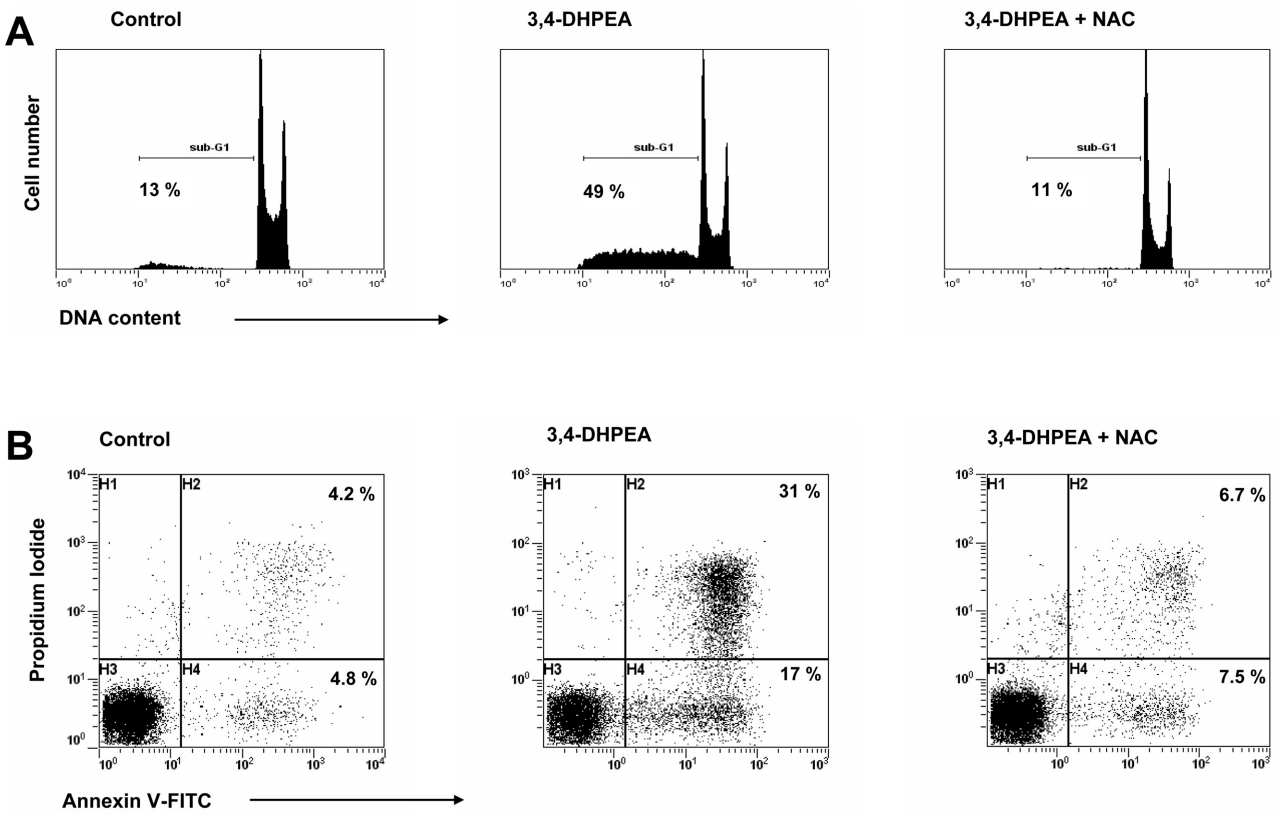

Figure 2. Effect of NAC on the pro-apoptotic effect of 3,4-DHPEA on HL60 cells. The cells (0.25×10^6/mL) were incubated in complete RPMI medium for 24 h in the absence (control) and in the presence of 3,4-DHPEA (100 μM) and 3,4-DHPEA plus NAC (10 mM) and the percentage of apoptotic cells was determined by flow cytometry after PI staining (A) and annexin V-FITC double staining (B). The results of a single experiment representative of three performed (which produced similar results) are shown.

HL60 cells were incubated with exogenously added H_2O_2 (50 μM), they were particularly efficient in eliminating the compound from the medium (Fig. 4B). In addition, the kinetic of H_2O_2 elimination was highly dependent from the cell density; at 0.1×10^6 cells/mL the half life of H_2O_2 was about 15 min, while this value was reduced to about 3 min when the cell density was increased to 0.8×10^6 cells/mL. If the accumulation of H_2O_2 in the culture medium is the primary event responsible for the induction of apoptosis, then we would expect that treatment of cell cultures with different cell densities would respond differently to the pro-apoptotic effect of 3,4-DHPEA. To test this hypothesis, different densities of cell suspensions were treated with 3,4-DHPEA (100 μM) for 24 h and both apoptosis and the concentration of H_2O_2 accumulated in the medium were measured. The results clearly indicate that both the percentage of apoptotic cells and the concentration of H_2O_2 in the medium were inversely correlated to the cell number (Fig. 5A). Con-

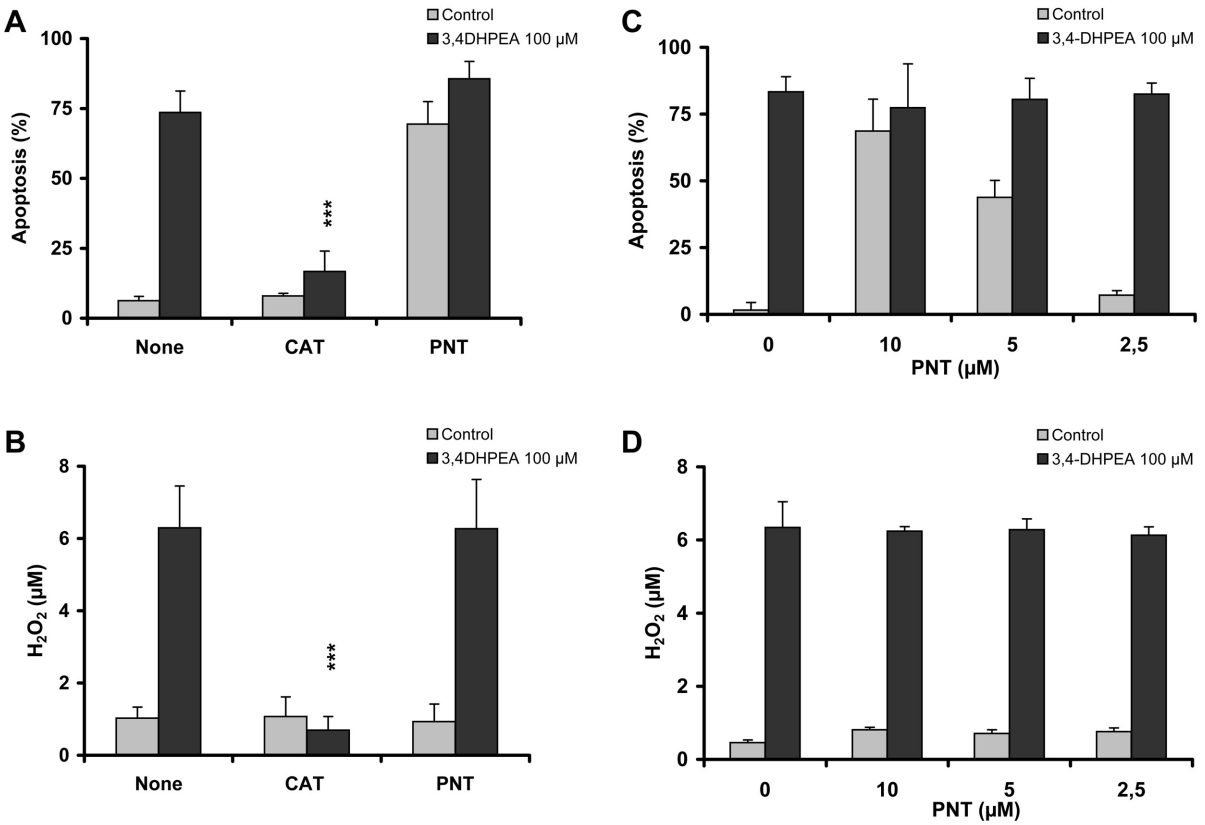

Figure 3. Effect of CAT and PNT on 3,4-DHPEA-induced apoptosis and H_2O_2 production. The cells (0.25×10^6/mL) were treated or not with 3,4-DHPEA (100 μM) in complete RPMI medium for 24 h in the absence and in the presence of CAT (100 U/mL, A and B) and PNT (50 μM, A and B; 2.5–10 μM C and D) and the percentage of apoptotic cells was evaluated by fluorescence microscopy after double-staining with HO 342 and PI (A and C), and the concentration of H_2O_2 in the culture medium was determined by the ferrous ion oxidation-xylenol orange method (B and D). The data represent the mean ± SD of three independent experiments. ***$p <$ 0.001 as compared with the effect of 3,4-DHPEA in the absence of CAT.

versely, the percentage of apoptotic cells and the concentration of H_2O_2 in the medium were directly and significantly correlated (Fig. 5B).

3.5 Lack of H_2O_2-generating activity and apoptosis induction by tyrosol and caffeic acid

To determine the structural components of the 3,4-DHPEA molecule involved in H_2O_2 generation and apoptosis induction, two compounds structurally similar to 3,4-DHPEA, tyrosol [*p*-hydroxyphenylethanol (*p*-HPEA)] and caffeic acid (CA) (Fig. 6A), were tested. As shown in Fig. 6B, *p*-HPEA and CA did not significantly increase the percentage of apoptotic cells (Fig. 6B) or the production of H_2O_2 in the culture medium (Fig. 6C). These results suggest that the *ortho*-hydroxyl group of the phenol ring present in the 3,4-DHPEA molecular structure is fundamental but not sufficient for the above-reported effect. Therefore, the ethanolic part of the 3,4-DHPEA may also be important for the generation of H_2O_2 and induction of apoptosis.

4 Discussion

In the present study we confirmed previous results showing that 100 μM 3,4-DHPEA completely arrests the proliferation and induces apoptosis of HL60 cells. In addition, it was exhaustively demonstrated, for the first time, that these effects were due to an oxidative stress primarily mediated by the ability of 3,4-DHPEA to generate extracellular H_2O_2 in the culture medium. These conclusions are supported by the following evidence: (i) the anti-proliferative and pro-apoptotic activities of 3,4-DHPEA were efficiently inhibited by several antioxidants, NAC, ascorbate and a-tocopherol; (ii) the extracellular detoxification of H_2O_2 by CAT into water and oxygen prevented the 3,4-DHPEA-induced apoptosis; (iii) the enhanced removal of H_2O_2 obtained by increasing the cell density during the 3,4-DHPEA exposure resulted in a direct relationship between the dose of H_2O_2 found in the culture medium and the percentage of apoptotic cells; and (iv) molecules structurally similar to 3,4-DHPEA (p-HPEA and CA), but not able to generate high level of H_2O_2, did not induce apoptosis.

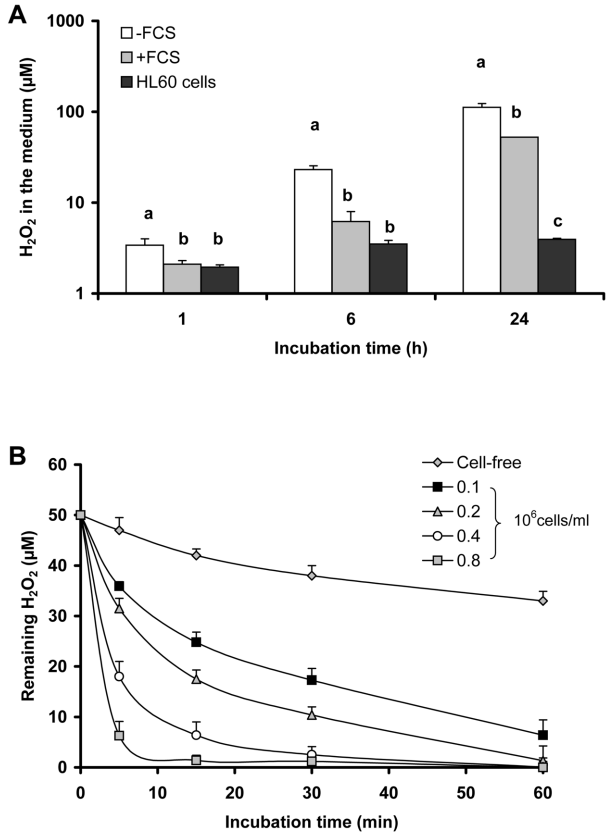

Figure 4. H_2O_2-removing activity of HL60 cells. (A) 3,4-DHPEA (100 μM) was added to the RPMI medium either devoid of FCS (-FCS) or containing FCS both alone (+FCS) and in the presence of 0.2×10^6/mL HL60 cells (HL60 cells). After 1, 6 and 24 h of incubation the concentration of H_2O_2 in the culture medium was determined by the ferrous ion oxidation-xylenol orange method. The data represent the mean ± SD of three independent experiments. Bars with a different letter are significantly different, $p < 0.05$. (B) H_2O_2 (50 μM) was added to the complete RPMI medium either containing different cell densities ($0.1 \times 10^6 - 0.8 \times 10^6$/mL) or without cells (Cell-free) and incubated at 37°C. After different times the concentration of H_2O_2 in the culture medium was determined by the ferrous ion oxidation-xylenol orange method. The data represent the mean ± SD of three independent experiments.

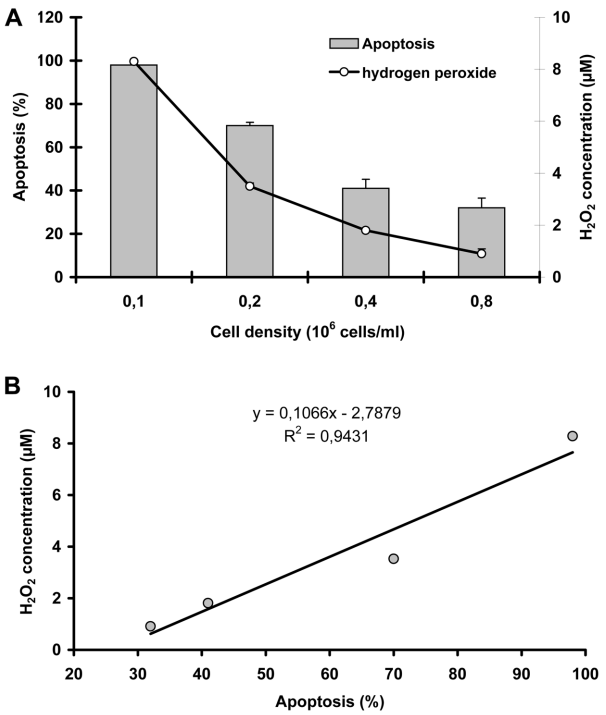

Figure 5. Effect of cell density on 3,4-DHPEA-induced apoptosis and H_2O_2 production. (A) The HL60 cells at different densities ($0.1 \times 10^6 - 0.8 \times 10^6$/mL) were exposed to 3,4-DHPEA (100 μM) in complete RPMI medium for 24 h and the percentage of apoptotic cells was evaluated by fluorescence microscopy after double-staining with HO 342 and PI; the concentration of H_2O_2 in the culture medium was determined by the ferrous ion oxidation-xylenol orange method. (B) Correlation analysis of data reported in (A) between the concentration of H_2O_2 accumulated in the cell culture medium and the percentage of apoptosis. The data represent the mean ± SD of three independent experiments.

The pro-oxidant activity of 3,4-DHPEA is not the first example showing that a compound notoriously known as an antioxidant may have, under certain conditions, a pro-oxidant effect. Previous reports have demonstrated that other plant polyphenolic compounds can induce an oxidative stress [22, 23]. In particular, a pro-oxidant activity correlated to the toxicity on HL60 cells has been demonstrated for flavonoids, gallic acid, CA and their derivatives [24]. It should be noted that the concentration of CA necessary to reduce the cell viability by 50% was 800 μM [24], a value well above 100 μM used in the present experiments that did not give any appreciable effect. Also resveratrol, a polyphenol present in red wine, was recently found to exert pro-oxidant effects inducing both senescence on colon HCT 116WT tumor cells [25] and DNA damage on human peripheral blood lymphocytes [26]. However, in all these cases the release of extracellular H_2O_2 was not shown, whereas a pro-oxidant effect mediated by the increase of H_2O_2 concentration in the culture medium has been previously demonstrated for the tea polyphenol (−)-epigallocatechin-3-gallate [27–29].

A mechanism has been proposed to explain the pro-oxidant activity of plant-derived phenols involving both O_2 and transition metals [23]. In this system the initial oxidation of catechol moiety by Cu(II) or Fe(II) generate semiquinone that can react with O_2 to form $O_2^{-\bullet}$ the disproportionation of which can form H_2O_2 [23]. Although in the present study the mechanisms of 3,4-DHPEA-mediated H_2O_2 production has not been investigated in detail, we can conclude that in our experimental conditions the catechol moiety is not sufficient for this effect since CA was not effective. In addition, the results with the Fe(II)-chelating reagent PNT sug-

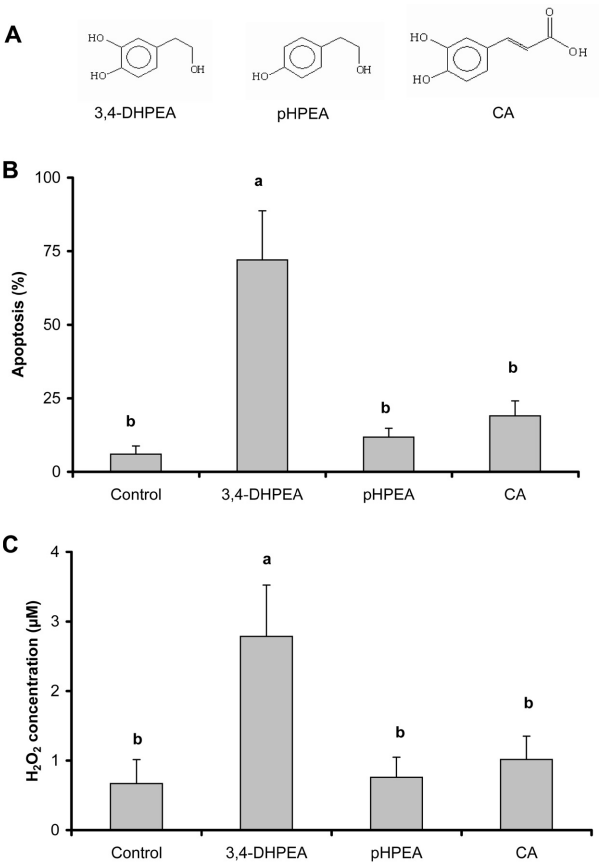

Figure 6. Structure-related effect of different phenols on HL60 apoptosis and H_2O_2 accumulation in the culture medium. (A) Structures of phenols investigated. HL60 cells (0.2×10^6/mL) were treated with 100 μM phenols in complete RPMI medium for 24 h. After incubation the percentage of apoptotic cells was evaluated by fluorescence microscopy after double-staining with HO 342 and PI (B), and the concentration of H_2O_2 in the culture medium was determined by the ferrous ion oxidation-xylenol orange method (C). The data represent the mean ± SD of three independent experiments. Bars with a different letter are significantly different, $p < 0.05$.

gest that Fe(II) is not involved in the ability of 3,4-DHPEA to release H_2O_2 in the culture medium.

Whatever the mechanism involved, we suggest that the pro-apoptotic effect exerted by 3,4-DHPEA on other cells (HT-29) is also mediated by the production of H_2O_2. The concentrations of 3,4-DHPEA necessary to induce apoptosis in HT-29 cells were two to four times higher than those effective on HL60 cells [11]. Since we have demonstrated that the pro-apoptotic effect of 3,4-DHPEA is strongly correlated to cell density, a direct comparison is not feasible. Nevertheless, it is possible that either the HT-29 cells are more efficient at removing H_2O_2 from the extracellular medium or 3,4-DHPEA produces less H_2O_2 in the HT-29 medium. Indeed, the DMEM medium used to grow the HT-29 cells contains abundant amounts of pyruvate (110 mg/L), which is known to react non-enzymatically with H_2O_2 causing the detoxification of this compound and the consequent production of acetate, carbon dioxide and water [30]. Furthermore, it was shown that in HT-29 cells 3,4-DHPEA interferes with several signaling pathways that control proliferation and apoptosis, and these effects were mediated by the activation of serine/threonine PP2A [11]. The involvement of the H_2O_2 generation in the 3,4-DHPEA-induced activation of PP2A is supported by the finding that H_2O_2 is able to activate this enzyme in endothelial cells [31]. The hypothesis that a similar activation of PP2A can be responsible for the effects induced by 3,4-DHPEA on HL60 cells remains to be investigated, although our preliminary experiments using okadaic acid as specific inhibitor of PP2A do not seem to support this view (results not shown).

The production of H_2O_2 as common molecular mediator may also explain other effects exerted by 3,4-DHPEA on the expression of different genes and on the phosphorylation state of proteins involved in some signal transduction pathways. In HL60 cells, 100–200 μM 3,4-DHPEA stimulates the up-regulation of *c-jun* and *egr1* both at the transcription and translation level, while *p*-HPEA has no effect [12]. 3,4-DHPEA also stimulates the phosphorylation of the c-Jun protein and the up-regulation of *egr1* is mediated by the activation of Erk1/2 pathway [12]. All these effects are also mediated by H_2O_2 in different cell systems [32–34]. In a previous study we found that 3,4-DHPEA increased the level of cyclin D in HL60 cells [10], an effect common to H_2O_2 in fibroblasts [35].

In conclusion, the results of the present study may be relevant for the design of human intervention studies aimed at demonstrating the ability of olive oil phenols to prevent oxidative-DNA damage. Indeed, some recent studies investigating the DNA-protective potential of olive oil phenols have produced discordant results showing a positive effect in some cases [36, 37] and no effect in others [38, 39]. On the other hand, since 3,4-DHPEA is efficiently absorbed in the small intestine and subjected to an extensive first-pass intestinal/hepatic metabolism with the production of both methylated and conjugates forms [40, 41], caution should be taken in extrapolating our *in vitro* results to the *in vivo* effects of this compound. Experiments are in progress in our laboratory to investigate the apoptosis-inducing ability of 3,4-DHPEA metabolites.

The authors have declared no conflict of interest.

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