Eur J Nutr (2012) 51:455–464
DOI 10.1007/s00394-011-0230-3

ORIGINAL CONTRIBUTION

Anti-proliferative and pro-apoptotic activities of hydroxytyrosol on different tumour cells: the role of extracellular production of hydrogen peroxide

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Received: 25 March 2011 / Accepted: 15 July 2011 / Published online: 30 July 2011

Abstract
Purpose Several recently published data suggest that the anti-proliferative and pro-apoptotic properties of hydroxytyrosol [3,4-dihydroxyphenyl ethanol (3,4-DHPEA)] on HL60 cells may be mediated by the accumulation of hydrogen peroxide (H_2O_2) in the culture medium. The aim of this study was to clarify the role played by H_2O_2 in the chemopreventive activities of 3,4-DHPEA on breast (MDA and MCF-7), prostate (LNCap and PC3) and colon (SW480 and HCT116) cancer cell lines and to investigate the effects of cell culture medium components and the possible mechanisms at the basis of the H_2O_2-producing properties of 3,4-DHPEA.
Methods The proliferation was measured by the MTT assay and the apoptosis by both fluorescence microscopy and flow cytometry. The concentration of H_2O_2 in the culture medium was measured by the ferrous ion oxidation–xylenol orange method.
Results It was found that the H_2O_2-inducing ability of 3,4-DHPEA is completely prevented by pyruvate and that the exposure of cells to conditions not supporting the H_2O_2 accumulation (addition of either catalase or pyruvate to the culture medium) inhibited the anti-proliferative effect of 3,4-DHPEA. Accordingly, the sensitivity of the different cell lines to the anti-proliferative effect of 3,4-DHPEA was inversely correlated with their ability to remove H_2O_2 from the culture medium. With regard to the mechanism by which 3,4-DHPEA causes the H_2O_2 accumulation, it was found that superoxide dismutase increased the H_2O_2 production while tyrosinase, slightly acidic pH (6,8) and absence of oxygen (O_2) completely prevented this activity. In addition, different transition metal-chelating compounds did not modify the H_2O_2-producing activity of 3,4-DHPEA.
Conclusions The pro-oxidant activity of 3,4-DHPEA deeply influences its 'in vitro' chemopreventive activities. The main initiation step in the H_2O_2-producing activity is the auto-oxidation of 3,4-DHPEA by O_2 with the formation of the semiquinone, superoxide ions (O_2^-) and $2H^+$.

Keywords Hydroxytyrosol · Apoptosis · Hydrogen peroxide · Olive oil · Tumour cells

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Introduction

It is generally accepted that olive oil phenolic compounds may exert their cancer preventive activity by acting as both anti-initiation and anti-promotion/progression compounds [21]. One of the possible anti-promotion/progression mechanisms is represented by the ability of olive oil phenols to interfere with proliferation and apoptosis of cancer cells. The most studied olive oil phenolic compound is the *orto*-diphenol hydroxytyrosol [3,4-dihydroxyphenyl ethanol (3,4-DHPEA)], which is abundantly and exclusively present in olive oil both as a free compound and linked to the dialdehydic form of elenoic acid (3,4-DHPEA-EDA), and as the isomer of oleuropein aglycon (3,4-DHPEA-EA) [40].

3,4-DHPEA has been found both to inhibit proliferation and to induce apoptosis in different tumour cells [2, 7, 9, 11, 13, 14, 18–20, 29, 41]. However, the results obtained on tumour cells derived from different organs disagree one to each other. Promyelocytic leukaemia cells (HL60) that are completely inhibited in the growth and undergo a massive apoptosis by treatment with 100 μM of 3,4-DHPEA appeared to be particularly sensitive to 3,4-DHPEA action [11, 13]. In HL-60 cells, 3,4-DHPEA alters the cell cycle progression by inhibiting G_1–S transition and modifies the expression of important cell cycle and apoptosis regulatory proteins by reducing the expression of cyclin-dependent kinase 6 (CDK6) and increasing that of CDK inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ [14]. In contrast, a different behaviour has been reported for breast cancer MCF-7 and SKBR3 cells that are quite resistant to the treatment with 100 μM of 3,4-DHPEA [29] and require higher concentrations of the phenol (MCF-7, 324 μM) to show an effect on apoptosis and proliferation [20]. On the other hand, other authors reported that MCF-7 cells are resistant to the pro-apoptotic effect of 3,4-DHPEA at a concentration as high as 400 μM, a behaviour suggested to be due to the absence of a functional caspase-3 activity in these cells [18]. In sharp contrast with the above-reported data, a recent study showed that MCF-7 cells are very sensitive to the anti-proliferative activity of 3,4-DHPEA, which reduced 50% of growth at a concentration of 12.5 μM [19]. In addition, it has been recently shown that 3,4-DHPEA is able to inhibit the estradiol-induced proliferation of MCF-7 breast cancer cells by interfering with ERK 1/2 activation [41]. Contrasting results have also been obtained on colon cancer HT-29 cells that are shown to be resistant to the anti-proliferative effect of 3,4-DHPEA up to 400 μM in one study [35] but to be sensible to the pro-apoptotic effect at a concentration of 200-400 μM in other studies [2, 18]. Furthermore, treatment of CaCo2 colon tumour cell line with 3,4-DHPEA (50-100 μM) caused a reduction in cell growth concomitant to both accumulation of the cells in the G2 phase of the cell cycle and inhibition of ERK1/2 phosphorylation [7]. Finally, it has been reported that human melanoma M14 cells respond to the exposure of 3,4-DHPEA only at concentrations above 600–800 μM [9]. In this context, it should be noted that the doses used in vitro to highlight the pro-apoptotic and anti-proliferative effects of hydroxytyrosol are well above those achieved in plasma of individuals who normally consume olive oil [30, 43].

Differently from the well-known antioxidant and DNA-damaging preventive properties exerted by 3,4-DHPEA in a low concentration range (1–10 μM) [15], recently it has been demonstrated that the anti-proliferative and pro-apoptotic properties of this phenol on HL60 cells at 100 μM are mediated by a pro-oxidant activity consisting in the generation of hydrogen peroxide (H_2O_2) in the cell culture medium [16]. The H_2O_2-releasing activity is not a peculiar characteristic of 3,4-DHPEA, but it has also been described for other plant-derived phenolic compounds both when tested as complex mixtures, such as those derived from grape seeds [3], apples [25], tea and wine [4], and when used as purified compounds such as gallic acid and quercetin [26], ascorbate [44], and epigallocatechin gallate [28]. The influence of the cell culture conditions and the possible chemical reactions involved in gallic acid- and epigallocatechin gallate-induced H_2O_2 generation have been recently investigated [44], while nothing is known about 3,4-DHPEA. It is possible that the effects exerted by 3,4-DHPEA on different cell lines may be mediated by its ability to release H_2O_2 in the culture medium, and therefore the different observed responses may depend, in addition to the capability of the cells to eliminate H_2O_2 by specific enzymes such as catalase and glutathione peroxidase, on the effectiveness by which 3,4-DHPEA releases H_2O_2 in the presence of the different cell culture media used for the treatment of cells.

In the present study, the role played by H_2O_2 in the chemopreventive activities of 3,4-DHPEA on different tumour cells, together with the involvement of the cell culture medium components and the possible mechanisms behind its H_2O_2-producing activity, has been investigated.

Materials and methods

Materials and cell culture

RPMI 1640 (Catalogue No. 21875), MEM (Catalogue No. 21090), McCOY'S (Catalogue No. 36600), RPMI + HEPES (Catalogue No. 42402), D-MEM (Catalogue No. 31885) and heat-inactivated foetal calf serum (FCS) were obtained from Gibco (Gibco BRL, Life Technologies, Paisley, Scotland). 3,4-DHPEA was obtained from Cayman Chemicals Ltd. (USA), supplied as ethanol solution at a concentration of 320 mM and stored at −20 °C in the dark. The compound was diluted in RPMI 1640 medium to the desired concentration just before use. All the solutions were sterilised 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 CO. Ltd., Irvine, UK).

Human promyelocytic leukaemia cells (HL60) and breast (MDA and MCF-7), prostate (LNCap and PC3) and colon (SW480 and HCT116) cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in complete medium

(RPMI 1640 for HL60, D-MEM for the adherent cell lines MDA, MCF-7, LNCap, PC3, SW480 and HCT116) supplemented with 10% FCS, 2.0 mM L-glutamine, 100U/mL penicillin and 100U/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 proper density as suggested by the supplier.

Cell treatments

HL60 cells were seeded at a density of 0.25×10^6/mL in the indicated culture medium on 24-well plates (Celbio S.r.l., Milan, Italy) and incubated with 3,4-DHPEA (100 μM) or H_2O_2 (0–10 μM) at 37 °C and 5% CO_2. At each established experimental time, 100- to 200-μL aliquots were withdrawn from the cell suspensions and either used to measure apoptosis or centrifuged to recover the supernatant for the subsequent quantification of H_2O_2.

Adherent cell lines (MDA, MCF-7, LNCap, PC3, SW480 and HCT116) were seeded in the indicated culture medium on 96-well plates (5000 *per* well). After 24 h of incubation at 37 °C and 5% CO_2, the medium was replaced with fresh medium containing 3,4-DHPEA at the established concentration and then (72 h) proliferation was determined by the MTT assay.

Cell proliferation

The proliferation of the adherent cells was measured by the MTT (3-[4,5-dimethyl(thiazol-2-yl)-3,5-diphenyltetrazolium bromide) assay carried out essentially as previously described [24]. This assay measures the production of highly coloured formazan as a result of the reduction of MTT by metabolically active cells due to the dehydrogenase enzymes. After 72 h of exposure to 3,4-DHPEA, the medium was removed and the cells were incubated at 37 °C with MTT solution (0.5 mg/mL of MTT in Hank's balanced salt solution). After 2 h, the MTT solution was substituted by a lysing solution (100 μL, isopropanol containing 0.1 M HCl), and the plates were incubated for 10 min at 37 °C to dissolve the blue formazan crystals. The absorbance of the released formazan was then determined at 565 nm.

Apoptosis assays

The percentage of apoptosis was determined by both fluorescence microscopy and flow cytometry [23]. In the first case, the cell 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 of incubation at room temperature, the cells were examined by 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 the cells with intact membranes, as well as cells 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, the viable cells have been identified by their intact nuclei with blue fluorescence (HO 342), and the necrotic cells by their intact nuclei with yellow fluorescence (HO 342 plus PI). Apoptotic cells were detected by their fragmented nuclei that exhibited 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.

For the flow cytometry detection of apoptosis (Sub G_1), the cells were stained with propidium iodide (PI) as follows. The cell pellet was resuspended in 0.5 mL of hypotonic fluorochrome solution containing 50 μg/mL of PI in 0.1% sodium citrate plus 0.1% Triton X-100 in 12 × 75 mm polypropylene tubes (Becton and Dickinson, Lincoln Park, NJ, USA). The tubes were kept at 4 °C for 30 min, and then the PI fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA, USA) at a wavelength of 488 nm.

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 [34] as follows: medium (20 μL) was mixed with a reaction solution (200 μL) containing 250 μM ammonium iron(II) sulphate, 25 mM H_2SO_4, 100 mM sorbitol and 125 μM xylenol orange and incubated at room temperature for 30 min. The absorbance was then 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.

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 both Student's *t* test and one-way ANOVA. When a significant ($P < 0.05$) treatment effect was detected, the mean values were compared using Tukey's post hoc comparisons.

Results

Production of H_2O_2 by 3,4-DHPEA and HL60 cell death

The addition of 3,4-DHPEA (100 μM) to the complete RPMI culture medium (RPMI + FCS) caused a time-dependent increase in H_2O_2 concentration (Fig. 1a). The presence of HL60 cells (0,2 × 10^6/mL) drastically inhibited the accumulation of H_2O_2 that raised to about 5 μM during the first hour and then remained constant throughout the entire incubation time (Fig. 1a). The rate of H_2O_2 production after 3 h of incubation was directly correlated (linear) with the concentration of 3,4-DHPEA, and it was inhibited (35%) by the addition of 10% FCS (Fig. 1b). Treatment of HL60 cells with 100 μM of 3,4-DHPEA for 24 h caused a marked apoptosis, which was reduced when catalase (CAT: 100 U/mL) was added to the cell culture medium (Fig. 1c). CAT addition also significantly reduced the H_2O_2 accumulation in the medium (Fig. 1c). Treatment of HL60 with exogenously added H_2O_2 showed that these cells were highly susceptible to H_2O_2 treatment because they underwent death through apoptosis within a low H_2O_2 concentration range (2.5–7.5 μM) similar to that generated by 3,4-DHPEA (Fig. 1d). These results clearly indicate that 3,4-DHPEA induces a cell- and serum-independent extracellular production of H_2O_2, which is responsible for its pro-apoptotic effect of on HL60 cells.

Effect of different cell culture media on 3,4-DHPEA-mediated H_2O_2 production and apoptosis

The time-dependent H_2O_2-producing activity of 3,4-DHPEA (100 μM) incubated in different culture media without the addition of the tumour cell line showed a similar rate of H_2O_2 production in RPMI, MEM and McCOY'S (Fig. 2a). RPMI formulated with the addition of HEPES (a widely used pH buffering compound) significantly reduced the H_2O_2 concentration by about 50% when compared to the above-reported media, while D-MEM completely prevented H_2O_2 formation (Fig. 2a). Similar qualitative results were obtained when the H_2O_2-producing activity of 3,4-DHPEA (100 μM) was evaluated in the presence of HL60 cells after 24 h of incubation in a different media (Fig. 2b). Comparable concentrations of H_2O_2 were detected in RPMI, MEM and McCOY'S, whereas the concentration was significantly reduced by 50 and 100% in RPMI + HEPES and D-MEM, respectively (Fig. 2b). Consequently, the pro-apoptotic activity of 3,4-DHPEA was not different when the cells were treated in RPMI, MEM and McCOY'S media, while it was significantly reduced in both RPMI + HEPES and D-MEM (Fig. 2c).

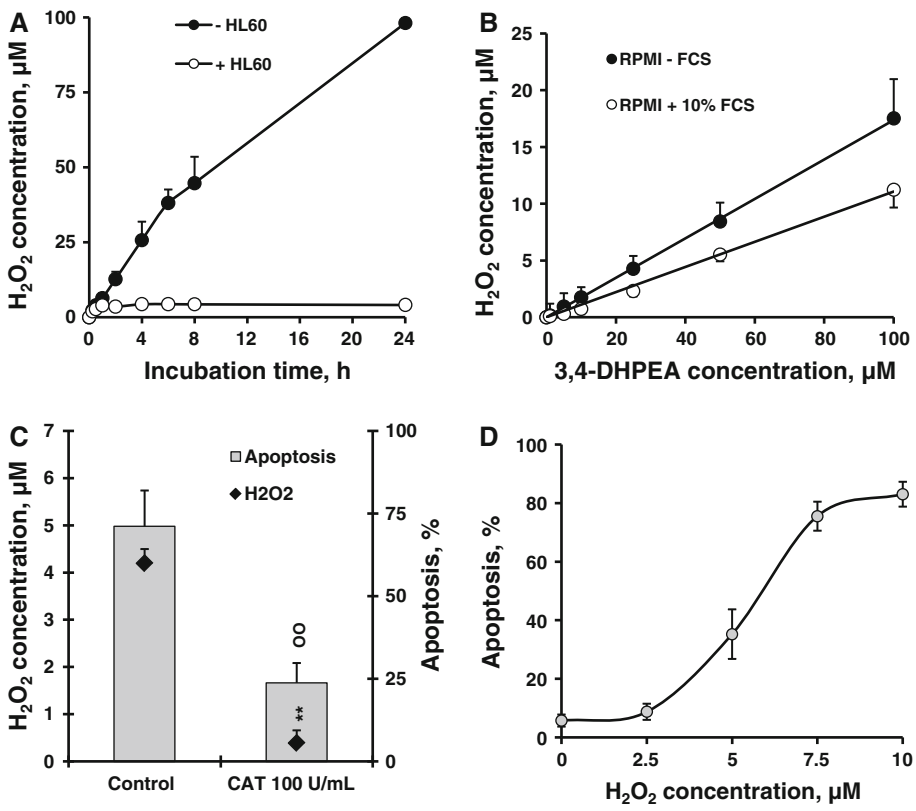


Fig. 1 **a** Time-dependent H_2O_2 accumulation in RPMI medium induced by 3,4-DHPEA (100 μM) either in the presence or in the absence of HL60 cells (0.2 × 10^6/mL). **b** Dose-dependent accumulation of H_2O_2 after 3 h of incubation induced by 3,4-DHPEA in the cell-free RPMI culture medium, either containing or not foetal calf serum (FCS). **c** Effect of catalase (CAT) on both H_2O_2 accumulation and apoptosis on HL60 cells induced by 3,4-DHPEA (100 μM) in RPMI medium; ** and °° $P < 0.01$ compared to the control. **d** Effect of exogenously added H_2O_2 on HL60 apoptosis after 24 h of incubation in RPMI medium. The data are presented as the means ± S.D., $n = 5$

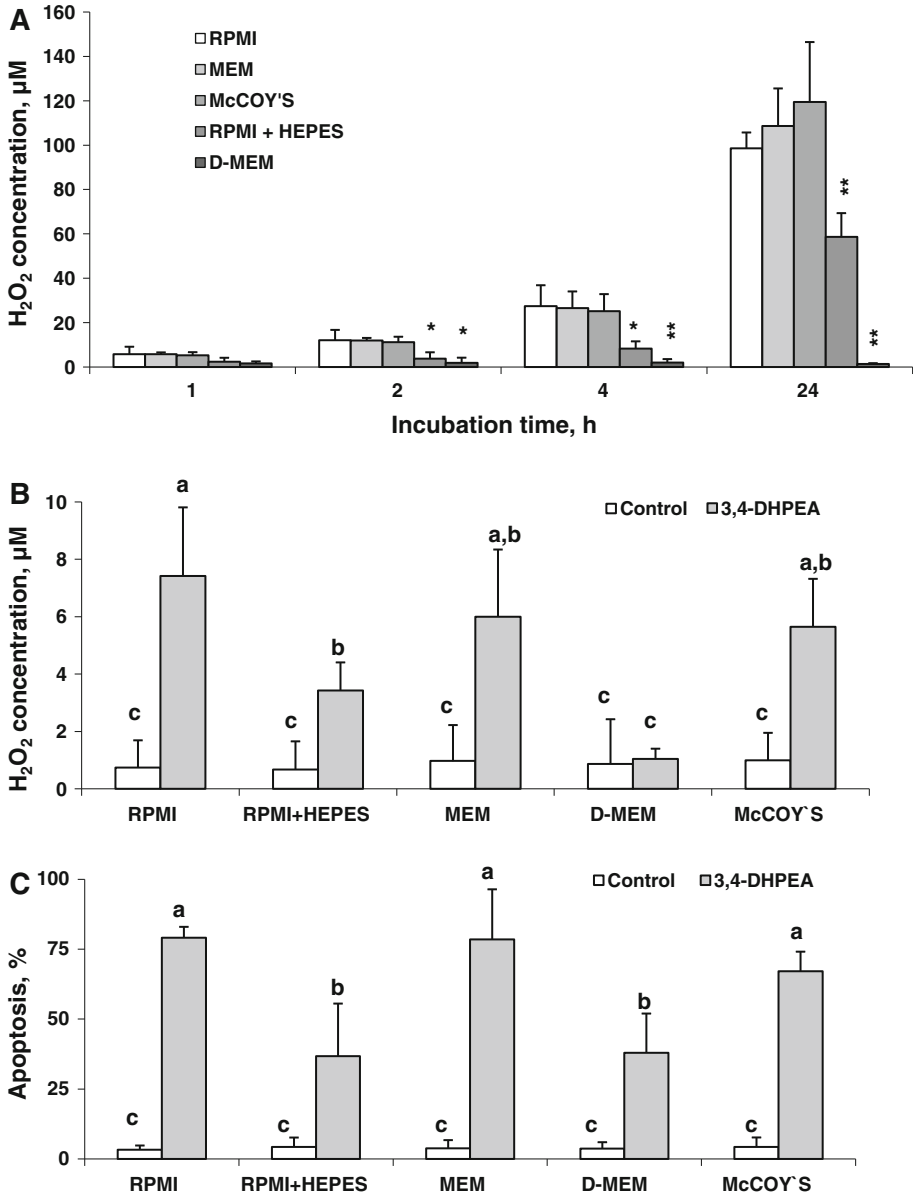


Fig. 2 **a** Time-dependent accumulation of H_2O_2 induced by incubation of 3,4-DHPEA (100 µM) for 24 h in different cell-free culture media; * $P < 0.05$, ** $P < 0.01$ compared to the RPMI medium. Effect of 3,4-DHPEA (100 µM) on **b** H_2O_2 accumulation and **c** apoptosis of HL60 cells (0.2×10^6/mL) after 24 h of incubation in different culture media. The data are presented as the means ± S.D., $n = 5$. Means without a common letter differ, $P < 0.05$

In order to identify the D-MEM medium components responsible for the inhibition of H_2O_2 accumulation, its chemical composition was compared with that of the other culture media used. Because the analysis revealed that D-MEM is the only medium containing pyruvate (110 mg/L, 1 mM), it was investigated whether this compound could be responsible for the reduction in H_2O_2 concentration. Indeed, RPMI medium enriched with 1 mM pyruvate significantly prevented H_2O_2 accumulation (Fig. 3a) and reduced the pro-apoptotic activity of 3,4-DHPEA (Fig. 3b).

It must be pointed out that when the HL60 cells were incubated with 3,4-DHPEA in conditions not supporting H_2O_2 accumulation, *i.e.* RPMI + CAT, RPMI + pyruvate and D-MEM, they were still able to undergo apoptosis (23.8 ± 6.0% in RPMI + CAT; 27.6 ± 7.3% in RPMI + pyruvate; 38.0 ± 14.0% in D-MEM). These results suggest that other mechanisms, in addition to the H_2O_2-releasing activity, could be involved in the pro-apoptotic activity of 3,4-DHPEA.

Effect of the culture medium on the anti-proliferative effect of 3,4-DHPEA

The anti-proliferative activity of 3,4-DHPEA towards cell lines derived from breast (MDA and MCF-7), prostate (PC3 and LNCap) and colon (SW480 and HCT116) was determined in different culture media either supporting (RPMI) or not (RPMI + CAT and D-MEM) the H_2O_2 accumulation (Fig. 4). It is clear that in all cases, 3,4-DHPEA induced an evident dose-dependent anti-proliferative effect only in RPMI medium, while in both RPMI + CAT and D-MEM, the effect was drastically

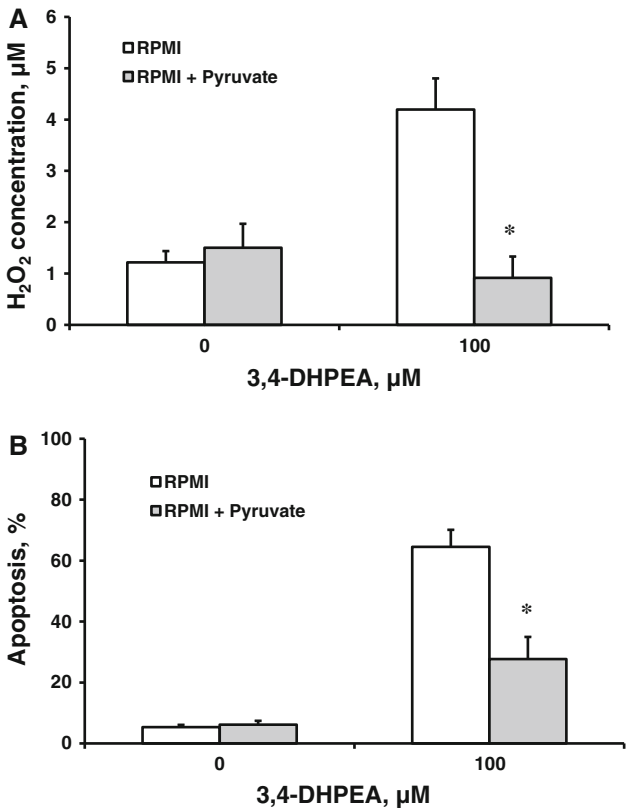


Fig. 3 Effect of pyruvate (1 mM) on 3,4-DHPEA-induced H_2O_2 accumulation (**a**) and apoptosis (**b**) of HL60 cells (0.2×10^6/mL) after 24 h of incubation; * $P < 0.05$ compared to the RPMI (RPMI medium without pyruvate). The data are presented as the means ± S.D., $n = 5$

reduced. The cell lines showed a different response to the 3,4-DHPEA treatment in RPMI medium. The $IC_{50\%}$ values (the concentration of 3,4-DHPEA that caused the 50% inhibition of growth compared with the control after 72 h of treatment) for the different cell lines are shown in Table 1. In addition, the accumulation of H_2O_2 in the RPMI medium was also measured in the presence of different cell lines after 24 h of incubation with 100 μM of 3,4-DHPEA (Table 1). The $IC_{50\%}$ values (x) were negatively correlated with the concentration of H_2O_2 (y) obtained in the presence of different cell lines ($x = -0.1432y + 29.408$; $R^2 = 0.7431$). These results suggest that the ability of the different cell lines to remove H_2O_2 from the medium influences their sensitivity to the anti-proliferative effect of 3,4-DHPEA.

Mechanisms behind the 3,4-DHPEA-induced H_2O_2 formation

To determine whether the H_2O_2-releasing activity was due to the interaction of 3,4-DHPEA with some components of the culture media, the phenolic compound was incubated in a simple 100 mM sodium phosphate buffer (PBS, adjusted at different pH) and subsequently the H_2O_2 concentration was determined over time. 3,4-DHPEA (100 μM) induced the formation of H_2O_2 in PBS at pH 7.8 but not at pH 6.8 (Table 2). Further, the presence of superoxide dismutase (SOD 100 U/mL), enzyme that catalyses the dismutation of superoxide to H_2O_2 and O_2, effectively enhanced the 3,4-DHPEA-induced H_2O_2 accumulation, while the addition of tyrosinase (TYR 1 μg/mL), which through its diphenolase activity catalyses the oxidation of 3,4-DHPEA to the corresponding *o*-quinone [12], completely prevented H_2O_2 formation (Table 2). Similarly, when the reaction was carried out under N_2 in the absence of O_2 (PBS pH 7.8 was subjected to a N_2 flow for 1 h both before and after the addition of 3,4-DHPEA), the production of H_2O_2 was totally abolished (Table 2). This last result suggests that H_2O_2 is the reductive product of O_2. Finally, the inclusion of different transition metal-chelating compounds (*o*-phenanthroline, deferoxamine, bathocuproine and neocuproine) tested at a concentration of 100 μM in PBS at pH 7.8 did not modify the H_2O_2-producing activity of 3,4-DHPEA (data not shown).

Discussion

This study clearly shows that the pro-apoptotic activity of 3,4-DHPEA on HL60 cells is due, at least in part, to the extracellular production of H_2O_2. However, other mechanisms seems to be involved since a significant percentage of apoptosis was registered also when the cells where incubated in conditions not supporting H_2O_2 accumulation (RPMI + CAT, RPMI + pyruvate and D-MEM). This conclusion is further supported by the observation that the amount of apoptosis induced by 3,4-DHPEA (71%) was higher that that caused by the direct addition of 5 μM of H_2O_2 (35%). This phenomenon could be explained considering that, as suggested later in this discussion, during the auto-oxidation of 3,4-DHPEA, in addition to H_2O_2, the corresponding *o*-quinone is also produced. This compound, similarly to what has been demonstrated for tocopherol quinones, acting as a strong arylating electrophile may form Michael adducts to thiol groups in cellular proteins and promote the endoplasmic reticulum stress response and the consequent activation of pro-apoptotic signals [5, 18].

In addition, we show that the accumulation of H_2O_2 influenced the anti-proliferative effect of 3,4-DHPEA on the other tumour cell lines independently from their organ origin, *i.e.* breast (MDA and MCF-7), prostate (PC3 and LNCap) and colon (SW480 and HCT116). Indeed, when the cells were exposed to conditions supporting the H_2O_2 accumulation (RPMI medium), 3,4-DHPEA effectively suppressed cell growth while both the addition of catalase

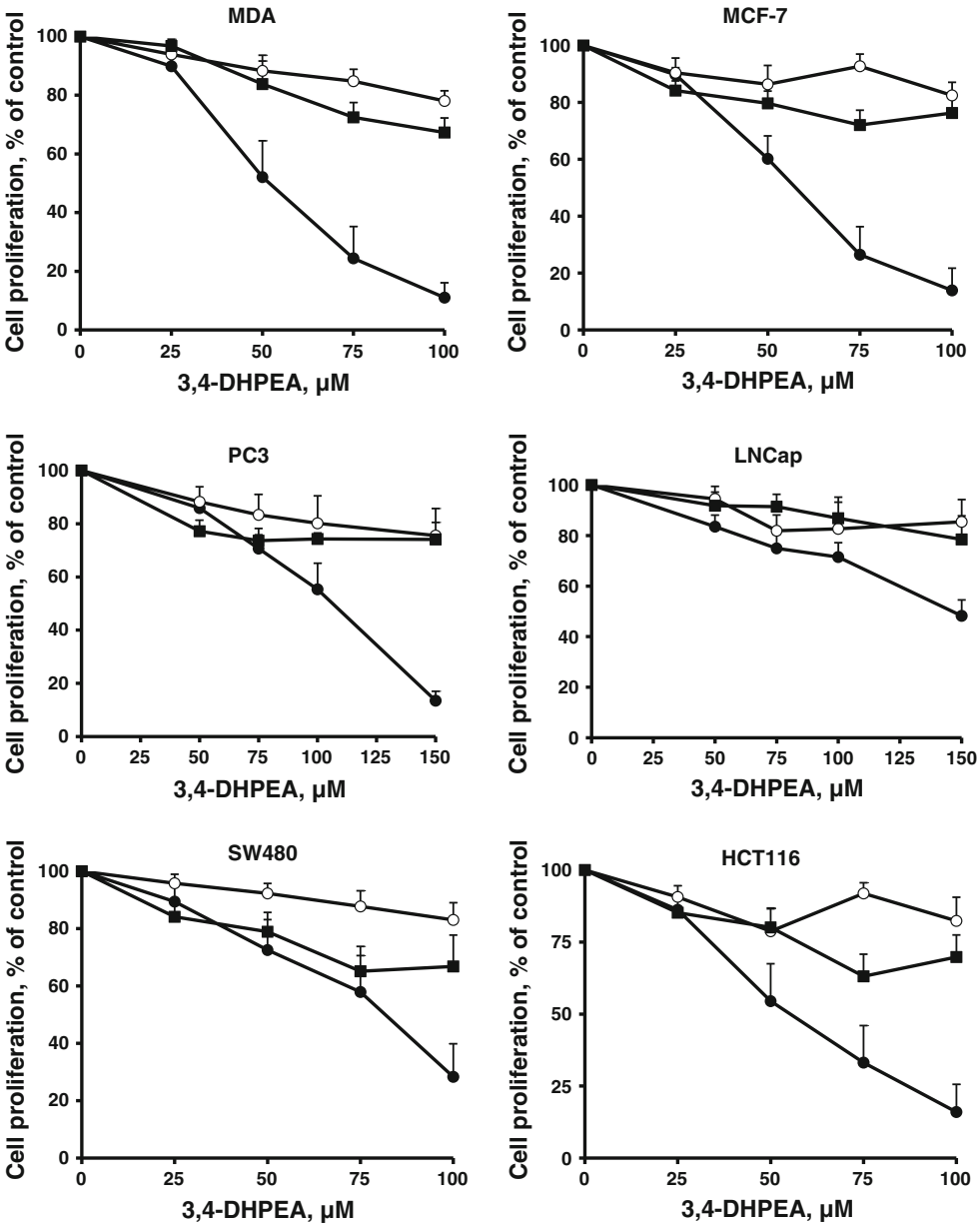


Fig. 4 Dose-dependent effect of 3,4-DHPEA on proliferation of different cell lines incubated for 72 h in RPMI (*filled circle*), RPMI + CAT (*open circle*) and D-MEM (*filled square*). The data are presented as the means ± S.D., $n = 5$

Table 1 $IC_{50\%}$ (72 h of treatment) and concentrations of H_2O_2 accumulated in the culture medium after treatment of the different cell lines with 3,4-DHPEA (100 μM) for 24 h

Cell line	$IC_{50\%}$ (μM)	H_2O_2 (μM)
MDA	52 ± 4^{d}	23.8 ± 2.8^{a}
HCT116	55 ± 7^{d}	23.3 ± 5.1^{a}
MCF-7	58 ± 8^{d}	$15.7 \pm 3.7^{b,c}$
SW480	82 ± 10^{c}	$21.5 \pm 2.7^{a,b}$
PC3	103 ± 7^{b}	12.0 ± 2.9^{c}
LNCap	146 ± 12^{a}	8.8 ± 3.4^{c}

The data are presented as the means ± S.D., $n = 5$. Means in a column without a common letter differ; Tukey's post hoc comparisons, $P < 0.05$

and the use of D-MEM medium widely prevented this effect. Furthermore, the amount of H_2O_2 accumulated in the culture medium in the presence of different tumour cells was directly correlated with the anti-proliferative activity of 3,4-DHPEA, suggesting that the different sensitivity of the cells to the chemopreventive effect of 3,4-DHPEA can be correlated to their capability to remove the H_2O_2 from the culture medium.

The ability of 3,4-DHPEA to release H_2O_2 is not due to an interaction with some components of the culture medium because this phenomenon was also evident in a simple PBS buffer. Instead, the H_2O_2-releasing activity depends upon cell culture conditions such us the medium buffer capacity, the pH and the presence of pyruvate. The effect of

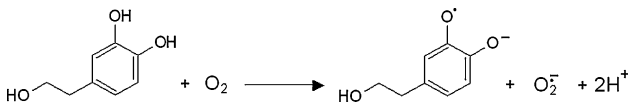
Table 2 Time-dependent accumulation of H_2O_2 (μM) induced by 3,4-DHPEA incubated in PBS buffer under different experimental conditions

Experimental conditions	Incubation time (h)			
	1	2	4	24
pH 6.8	2.4 ± 0.6^{d}	2.5 ± 0.3^{d}	2.7 ± 0.8^{d}	1.5 ± 0.5^{d}
pH 7.8	3.3 ± 0.4^{d}	7.2 ± 1.1^{d}	$11.4 \pm 1.4^{c,d}$	$23.1 \pm 3.7^{b,c}$
pH 7.8 + SOD	25.9 ± 7.9^{b}	39.6 ± 7.7^{a}	46.1 ± 5.1^{a}	47.5 ± 9.9^{a}
pH 7.8 + TYR	0.5 ± 0.3^{d}	0.9 ± 0.7^{d}	1.6 ± 0.7^{d}	5.2 ± 0.6^{d}
pH 7.8 + N_2	N.D.	N.D.	N.D.	2.2 ± 1.2^{d}

The data are presented as the means ± S.D., $n = 5$. Means without a common letter differ; Tukey's post hoc comparisons, $P < 0.05$

pyruvate was particularly important because commercial formulations of D-MEM and MEM exist either containing or not this compound; moreover, in designing the experimental conditions of different studies, the pyruvate status of the cell culture medium is not always reported. This evidence is true in studies in which the chemopreventive activity of 3,4-DHPEA towards breast and colon tumour cells has been investigated [18–20, 29, 35]. For this reason, it can be hypothesised that the necessity to use a wide range of 3,4-DHPEA concentration to observe a chemopreventive effect in different tumour cells may be due to the interference of pyruvate. In agreement with the results of the present investigation, recent studies have reported that pyruvate can scavenge H_2O_2 produced in the medium by epigallocatechin gallate and ascorbate [28] and that it can protect HF-1 fibroblasts from the cytotoxic effect exerted by both authentic oxidants compounds such us *tert*-butyl hydroperoxide and hydrogen peroxide and pro-oxidant compounds such us caffeic acid, epigallocatechin gallate and theaflavin-3′3′-digallate [1].

With regard to the mechanism by which 3,4-DHPEA causes the H_2O_2 accumulation, it can be suggested that the main initiation step would be the auto-oxidation of 3,4-DHPEA by O_2 as follows:



The semiquinone formed can be further oxidised by O_2 to give the corresponding *o*-quinone and O_2^-. According to our results, this reaction can be both inhibited by the action of tyrosinase, which removes the two hydrogen atoms from the catechol structure of 3,4-DHPEA to directly form the *o*-quinone, and enhanced by decreasing the $[H^+]$ (increasing the pH), which moves the equilibrium to the right. Although the oxidation of catechol can also be due to the transition metals such us Cu and Fe [38], in our system this reaction does not seem to be important since the transition metal-chelating reagents did not inhibit the H_2O_2-producing activity of 3,4-DHPEA. The superoxides (O_2^-) produced in the above-reported reaction can be converted to H_2O_2 through either spontaneous or SOD-catalysed dismutation. Although SOD can influence H_2O_2 formation, either increasing or decreasing it depending on the presence in the system of an oxidant or a reductant of O_2^-, respectively [27], in our conditions this enzyme accelerates H_2O_2 accumulation. Contrasting results regarding the effect of SOD on H_2O_2 formation induced by different compounds have been previously published. Indeed, it has been reported that SOD decreases the H_2O_2 accumulation caused by epigallocatechin gallate [32, 33], myricetin [31] and theaflavin-3′3′-digallate [39], while it enhances the accumulation of H_2O_2 induced by *para*-aminophenol [17] and canned coffee [22]. In this context, it should be underlined that the reactions behind the H_2O_2-releasing activity of 3,4-DHPEA may be particularly complex and deeply influenced by the experimental conditions. In addition, it should be considered that 3,4-DHPEA may interact with the reactive oxygen species (O_2^- and H_2O_2) in a way that is not completely clear since some studies have shown a potent scavenger activity of 3,4-DHPEA against superoxide anions [37, 42] while others have found a scavenger activity against H_2O_2 but not against O_2^- [36].

Overall, these findings are of particular relevance because they point out that 3,4-DHPEA may have a pro-oxidant activity, highly dependent on the experimental conditions, which can deeply influence all its 'in vitro' biological activities. When interpreting the 'in vivo' activities of our results, it should be considered that 3,4-DHPEA is well absorbed in the gastrointestinal tract but its bioavailability is poor [10]; therefore, we may suppose that such high concentration (100 μM) will not be reached in the circulation even under an olive oil–rich diet. Indeed, previous studies have shown that after ingestion of 50 g of olive oil per day, the plasma concentrations of phenols can be at most 0.06 μM [43]. These results are also supported by the data reported by Miro-Casas et al. [30] who found 25 μg/L (less than 0.2 μM) of 3,4-DHPEA in plasma after ingestion of 25 mL of olive oil. However, another

intervention study showed that a single ingestion of 40 mL of olive oil containing modest amount of phenols (366 mg/kg) resulted in a plasma concentration of hydroxytyrosol close to 20 μM [8]. In any case, high concentrations of 3,4-DHPEA could be reached in the colon lumen considering that oleuropein, a secoiridoid derivatives of 3,4-DHPEA, is stable in human gastric juice and duodenal fluid; it is not absorbed and metabolised in the small intestine, but it is biotransformed by the colonic microflora with the production of 3,4-DHPEA [6]. Further studies are necessary to clarify these important points.

Acknowledgments This work was supported by grants of the MIUR, FISR 'Bando 2002, Qualità Alimentare e Benessere'.

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