

Involvement of Hydrogen Peroxide Formation on Apoptosis Induction by Olive Oil Phenolic Compounds

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Abstract: In the present investigation the ability of different phenolic compounds, either present or not in olive oil, to induce both apoptosis on tumour cells and H₂O₂ accumulation in cell culture medium was assessed. Among the phenols studied we found that tyrosol (*p*-HPEA), homovanillic alcohol and protocatechuic, *o*-coumaric, vanillic, homovanillic, ferulic and syringic acids did not induce either apoptosis on HL60 cells or H₂O₂ accumulation, while hydroxytyrosol (3,4-DHPEA), 3,4-dihydroxyphenylacetic acid (3,4-DHPA), 3,4-dihydroxy-hydrocinnamic acid (3,4-DHHC) and gallic acid induced both apoptosis and accumulation of H₂O₂ in the culture medium which were significantly reduced by catalase. In contrast, the dialdehydic form of elenoic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) and to tyrosol (*p*-DHPEA-EDA) induced high level of apoptosis not reduced by catalase. Finally, oleuropein exerted a weak pro-apoptotic effect not mediated by H₂O₂ release. From these results it is evident that: (i) the cathecol moiety of phenols is necessary but not sufficient to induce apoptosis and H₂O₂ accumulation; (ii) the 3,4-DHPEA metabolism may partially reduce its pro-apoptotic potential; (iii) the pro-apoptotic activity of 3,4-DHPEA-EDA and *p*-DHPEA-EDA is not mediated by H₂O₂ releasing activity.

Keywords: olive oil; phenols; apoptosis; hydrogen peroxide

INTRODUCTION

Olive oil contains different phenolic compounds which have been shown to possess preventive activities toward chronic-degenerative diseases such as cardiovascular diseases and cancer (KRIS-ETHERTON *et al.* 2002). Hydroxytyrosol (3,4-DHPEA) and tyrosol (*p*-HPEA) are the most representative olive oil phenols present both as free compounds and linked to the dialdehydic form of elenoic acid (3,4-DHPEA-EDA and *p*-HPEA-EDA) (SERVILI & MONTEDORO 2002). Previous studies carried out in our laboratory have showed that these compounds exert a pro-apoptotic activity toward cancer cell lines (FABIANI *et al.* 2002, 2006). In particular, we have found that 3,4-DHPEA at high concentrations (100 μM) induces apoptosis on HL60 cells through an oxidative stress caused by the extracellular production of hydrogen peroxide (H₂O₂) (FABIANI *et*

al. 2008). The apoptotic effect of 3,4-DHPEA was effectively inhibited by N-acetyl-cysteine, dietary antioxidants (ascorbate and α-tocopherol) and the enzyme catalase (FABIANI *et al.* 2008).

In this study, preliminary results on the pro-apoptotic activities of different phenolic compounds structurally similar to 3,4-DHPEA are reported, together with the extracellular H₂O₂ accumulation and the effect of catalase on the apoptosis induction in HL60 cells.

MATERIALS AND METHODS

Materials. 3,4-DHPEA was obtained from Cayman Chemicals Ltd; protocatechuic, vanillic, ferulic, 3,4-dihydroxy-hydrocinnamic, *o*-coumaric and syringic acids were obtained from Fluka Co. (Buchs, Switzerland); gallic acid was from Carlo

Erba (Milan) while oleuropein was purchased by Extrasynthese (Genay, France). 3,4-DHPEA-EDA and *p*-HPEA-EDA were purified by semi-preparative HPLC from a methanolic extract obtained from a virgin olive oil containing 650 mg/kg of total phenols as previously reported (FABIANI *et al.* 2006). Tyrosol (*p*-HPEA) and all other reagents were purchased from Sigma-Aldrich (Irvine, UK) unless differently specified

Cell treatment and apoptosis analysis. Human promyelocytic leukemia cells (HL60) were cultured in complete RPMI 1640 medium supplemented with 10% FCS, 2.0mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. The cells were seeded at a density of 0.25×10^6 /ml and incubated for 24 h at 37°C and 5% CO₂ with the different phenolic compounds (100µM) in the absence and in the presence of catalase (CAT: 100 U/ml). The percentage of apoptotic cells was determined by a fluorescent microscopy assay as previously described (FABIANI *et al.* 2006).

Measurement of H₂O₂ concentration in the culture medium. The concentration of H₂O₂ in the culture medium was measured by the ferrous ion oxidation-xylenol orange method as follows: medium (20 µl) was mixed with a reaction solution (200 µl) containing 250µM ammonium iron (II) sulphate, 25mM H₂SO₄, 100mM sorbitol and 125µM xylenol orange and incubated at room temperature for 30 min. The absorbance was then read at 595 nm and the concentration of H₂O₂ was derived from a standard curve.

RESULTS AND DISCUSSION

Among the different phenols tested, at 100µM concentration, tyrosol (*p*-HPEA), homovanillic alcohol and protocatechuic, *o*-coumaric, vanillic, homovanillic, ferulic and syringic acids did not induce either apoptosis on HL60 cells or H₂O₂ accumulation in the culture medium (results not shown). Since homovanillic acid and homovanillic alcohol are important methoxy metabolites of 3,4-DHPEA, derived from the reaction catalysed by the catechol-*O*-methyltransferase (D'ANGELO *et al.* 2005), the present data suggest that the 3,4-DHPEA biotransformation may in part decrease its activity. The results obtained with the other phenolic compounds regarding both the pro-apoptotic activity and the ability to release H₂O₂ in the culture medium are reported in Figure 1A and 1B, respectively. It is clear that hydroxytyrosol (3,4-DHPEA), 3,4-dihydroxyphenylacetic acid (3,4-DHPA), 3,4-dihydroxy-hydrocinnamic acid (3,4-DHHC) and gallic acid induced both apoptosis and accumulation of H₂O₂ in the culture medium. Such activities were efficiently reduced by catalase so suggesting that the pro-apoptotic effect is mediated by H₂O₂. In contrast, 3,4-DHPEA-EDA and *p*-HPEA-EDA induced high level of apoptosis which was not reduced by catalase (Figure 1A). This result is in accordance with the low accumulation of H₂O₂ in the culture medium induced by 3,4-DHPEA-EDA and *p*-HPEA-EDA (Figure 1B). It should be noted that, in olive oil, these two compounds are about

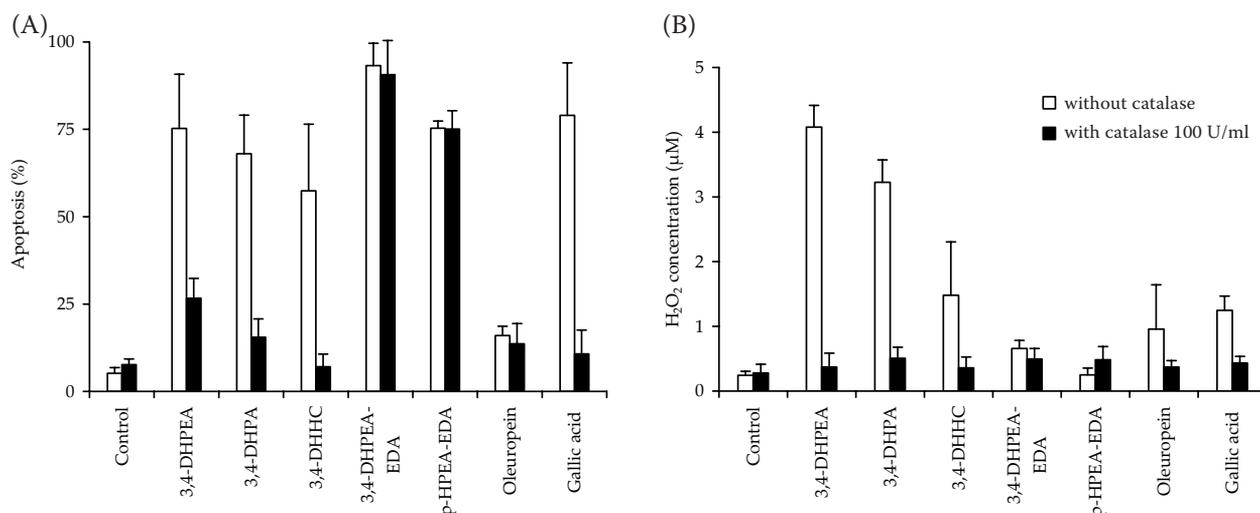


Figure 1. Effect of different phenolic compounds on apoptosis of HL60 cells (A) and H₂O₂ accumulation in the cell culture medium (B)

100 and 10 times more abundant than 3,4-DHPEA and *p*-HPEA, respectively (FABIANI *et al.* 2006), therefore they could significantly influence the cancer preventive activity of olive oil. Finally, oleuropein exerted a weak pro-apoptotic effect which was not reduced by catalase and therefore it is not mediated by H₂O₂ release. The low cytotoxicity of oleuropein has been previously observed in human salivary gland (BABICH & VISIOLI 2003)) and breast cancer cells (MENENDEZ *et al.* 2007). All together these results suggest that; (i) the catechol moiety of phenols is necessary but not sufficient to induce apoptosis and H₂O₂ accumulation; (ii) the 3,4-DHPEA metabolism may partially reduce its pro-apoptotic potential; (iii) the pro-apoptotic activity of 3,4-DHPEA-EDA and *p*-DHPEA-EDA is not mediated by the H₂O₂ releasing activity. Further studies are currently in progress to elucidate the mechanisms involved in the pro-apoptotic activity of 3,4-DHPEA-EDA and *p*-DHPEA-EDA.

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