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

R. FABIANI*, P. ROSIGNOLI, R. FLICCELLI, F. PIERAVANTI, A. DE BARTOLOMEO and G. MOROZZI

Dipartimento di Specialità Medico-Chirurgiche e Sanità Pubblica, Sezione di Epidemiologia Molecolare e Igiene Ambientale, University of Perugia, 06123 Perugia, Italy

*E-mail: fabirob@unipg.it

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 assed. 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 (100µM) 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 x 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 catalised 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-di-hydroxyphenylacetic acid (3,4-DHPA), 3,4-di-hydroxy-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](image-url)
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 \( \text{H}_2\text{O}_2 \) 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 catecol moiety of phenols is necessary but not sufficient to induce apoptosis and \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) 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.

References


