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

R. FABIANI, P. ROSIGNOLI, R. FUCCELLI, 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 H_2O_2 accumulation in cell culture medium was assesed. 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 H_2O_2 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 H_2O_2 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 H_2O_2 release. From these results it is evident that: (*i*) the cathecol moiety of phenols is necessary but not sufficient to induce apoptosis and H_2O_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 H_2O_2 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 (H_2O_2) (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 H_2O_2 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% CO_2 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 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 as follows: medium (20 μl) was mixed with a reaction solution (200 μl) containing 250μM ammonium iron (II) sulphate, 25mM H_2SO_4, 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 H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 in the culture medium. Such activities were efficiently reduced by catalase so suggesting that the pro-apoptotic effect is mediated by H_2O_2. 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 H_2O_2 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

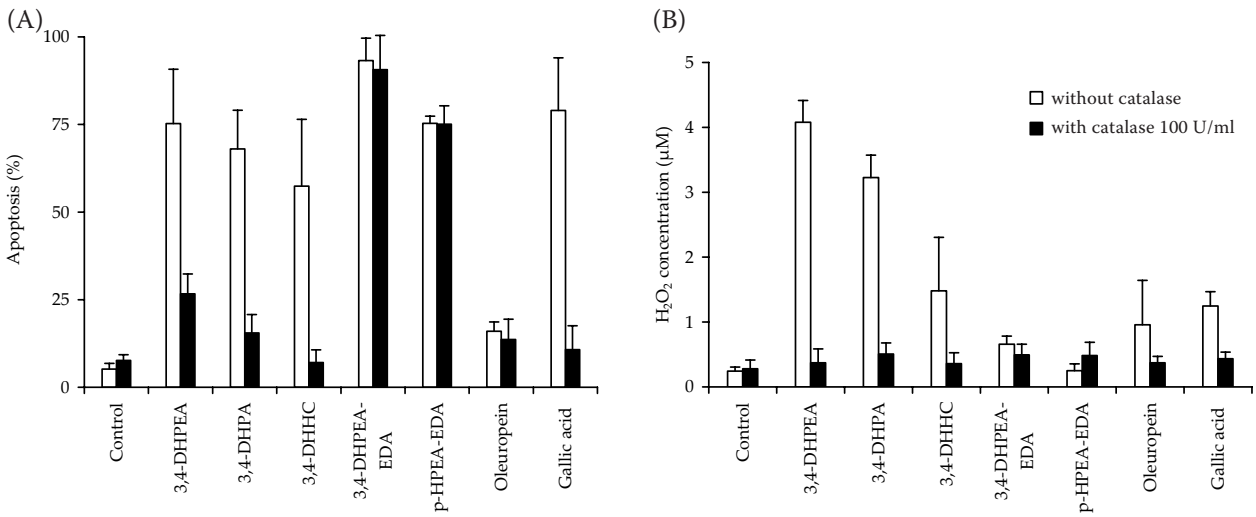

Figure 1. Effect of different phenolic compounds on apoptosis of HL60 cells (A) and H_2O_2 accumulation in the cell culture medium (B)

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 mediate by H_2O_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 cathecol moiety of phenols is necessary but not sufficient to induce apoptosis and H_2O_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 H_2O_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

Babich H., Visioli F. (2003): *In vitro* cytotoxicity to human cells in culture of some phenolics from olive oil. Farmaco, **58**: 403–407.

D'Angelo S., Ingrosso D., Migliardi V., Sorrentino A., Donnarumma G., Baroni A., Masella L., Tufano M.A., Zappia M., Galletti P. (2005): Hydroxytyrosol, a natural antioxidant from olive oil, prevents protein damage induced by long-wave ultraviolet radiation in melanoma cells. Free Radicals Biology & Medicine, **38**: 908–919.

Fabiani R., De Bartolomeo A., Rosignoli P., Servili M., Montedoro G.F, Morozzi G. (2002): Cancer chemioprevention by hydroxytyrosol isolated from virgin olive oil through G_1 cell cycle arrest and apoptosis. European Journal of Cancer Prevention, **11**: 351–358.

Fabiani R., De Bartolomeo A., Rosignoli P., Servili M., Selvaggini R., Montedoro G.F, Di Saverio C., Morozzi G. (2006): Virgin olive oil phenols inhibit proliferation of human promyelocytic leukemia cells (HL60) by inducing apoptosis and differentiation. Journal of Nutrition, **136**: 614–619.

Fabiani R. Fuccelli R., Pieravanti F., De Bartolomeo A., Morozzi G. (2008): Production of hydrogen peroxide is responsible for the induction of apoptosis by hydroxytyrosol on HL60 cells. Molecular Nutrition & Food Research. (In press)

Kris-Etherton P.M., Hecker K.D., Bonanome A., Coval S.M., Binkoski A.E., Hilpert K.F., Griel A.E., Etherton T.D. (2002): Bioactive compounds in foods: their role in the prevention of cardiovascular diseases and cancer. The American Journal of Medicine, **113**: 71S–88S.

Menendez J.A., Vazquez-Martin A., Colomer R., Brunet J., Carrasco-Pancorbo A., Garcia-Villalba R., Fernandez-Gutierrez A., Segura-Carretero A. (2007): Olive oil's bitter principle reverses acquired autoresistance to trastuzumab (Herceptin) in HER2-overexpressing breast cancer cells. BMC Cancer, **7**: 80.

Servili M., Montedoro G.F. (2002): Contribution of phenolic compounds to virgin olive oil quality. European Journal of Lipid Science and Technology, **104**: 602–613.