

Minor Components of Olive Oil Modulate Proatherogenic Adhesion Molecules Involved in Endothelial Activation

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The Mediterranean diet reduces the risk of coronary artery disease as a consequence of its high content of antioxidants, namely, hydroxytyrosol (HT) and oleuropein aglycone (OleA), typical of virgin olive oil. Because intercellular and vascular cell adhesion molecules (ICAM-1 and VCAM-1) and E-selectin are crucial for endothelial activation, the role of the phenolic extract from extra virgin olive oil (OPE), OleA, HT, and homovanillyl alcohol (HVA) on cell surface and mRNA expression in human umbilical vascular endothelial cells (HUVEC) was evaluated. OPE strongly reduced cell surface expression of ICAM-1 and VCAM-1 at concentrations physiologically relevant ($IC_{50} < 1 \mu M$), linked to a reduction in mRNA levels. OleA and HT were the main components responsible for these effects. HVA inhibited cell surface expression of all the adhesion molecules, whereas the effect on mRNA expression was weaker. These results supply new insights on the protective role of olive oil against vascular risk through the down-regulation of adhesion molecules involved in early atherogenesis.

KEYWORDS: Olive oil; proatherogenic adhesion molecules; endothelial activation; ICAM-1; VCAM-1; hydroxytyrosol; oleuropein aglycone; E-selectin

INTRODUCTION

An increasing number of epidemiological and experimental studies report that the Mediterranean diet is associated with a reduced risk of coronary artery disease (1). In particular, it has been extensively reported that these beneficial effects are ascribable, at least in part, to some dietary polyphenols which occur in the Mediterranean diet, although the precise mechanisms by which they exert these properties have not been completely defined (2, 3). Olive oil, the principal fat component of this diet, contains several phenolic "minor components", which are also responsible for its typical taste and contribute to the stability of the nutrient. Among these compounds, hydroxytyrosol (3,4 dihydroxyphenylethanol; HT) and oleuropein aglycone (OleA), which share a catecholic (ortho-diphenolic) structure (Figure 1), exhibit a series of *in vitro* biological activities (4, 5) such as protection of low-density lipoprotein (LDL) against peroxy radical- or copper-induced oxidation (6–10), inhibition of platelet aggregation (11), and potentiation of the nitric oxide-mediated macrophagic immune response (12).

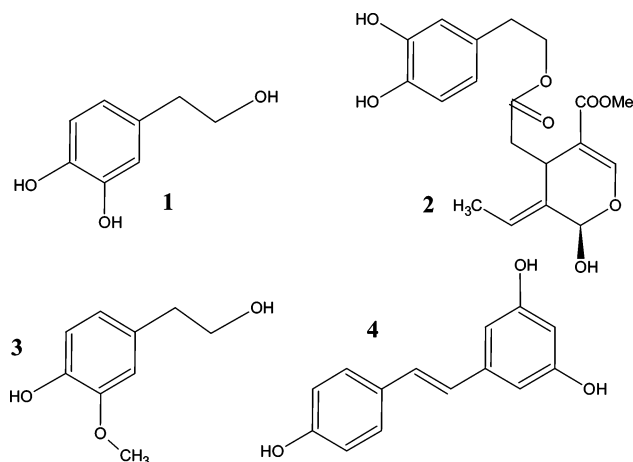


Figure 1. Compounds of olive oil used for the experiments: HT, **1**; OleA, **2**; HVA, **3** (the main human metabolite of HT). *trans*-Resveratrol (**4**) was used as reference compound.

In vivo, they can play an important role in inflammatory pathologies such as atherosclerosis (13). The well-accepted oxidative hypothesis of atherosclerosis emphasized that oxidative modification of LDL is one of the key events in atherogenesis (6–8, 14). Accumulation of oxLDL in the intima, due to changes in permeability of the endothelium, contributes sig-

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nificantly to monocyte recruitment and foam cell formation and initiates the chronic inflammatory processes that might produce advanced atherosclerotic lesions. Activation of the vascular endothelium, increased adhesion of leukocytes, monocytes, and T-lymphocytes to the injured endothelial layer, and their subsequent extravasation into the vessels wall are the initial events in inflammatory response and atherogenesis (15–17). The adhesion process is facilitated by the action of endothelial-leukocyte adhesion molecules, which include intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. ICAM-1 is widely expressed at low levels in unstimulated cells and can be up-regulated by proinflammatory cytokines in leukocytes, platelets, and endothelium; soluble forms of ICAM-1 have been shown to result from shedding (18–21). VCAM-1 is transcriptionally induced in endothelial cells and participates in the recruitment of blood cells by activated endothelium, inducing their firm adhesion. E-selectin participates in different, although overlapping, ways in the early steps of leukocyte recruitment at the endothelial surface under shear forces, such as leukocyte rolling and tethering. The expression of these molecules can be transcriptionally regulated by inflammatory cytokines such as interleukin-1 α , tumor necrosis factor- α (TNF- α), and lipopolysaccharide (LPS), which stimulate ICAM-1, VCAM-1 and E-selectin mRNA synthesis and cell surface expression, promoting atherogenic processes, as supported by several studies (22, 23). In humans, the expression of these molecules has been consistently observed in atherosclerotic plaques (24–26). In addition, high levels of soluble adhesion molecules have been postulated to be useful risk predictors of cardiovascular events in healthy populations (27) even though their pathological role remains to be further investigated.

The aim of the present research was to elucidate the role of extra virgin olive oil on endothelial activation. For this purpose, the effect of phenolic extract from extra virgin olive oil (OPE) on VCAM-1, ICAM-1, and E-selectin cell surface and mRNA expression in human umbilical vascular endothelial cells (HUVEC) was investigated. We have also verified which are the phenols [OleA, HT, and homovanillyl alcohol (HVA), the major human metabolite of HT in humans] responsible for the observed effects.

MATERIALS AND METHODS

Reagents. M199 medium, L-glutamine, penicillin, and streptomycin were purchased from GIBCO (Grand Island, NY). Fetal calf serum (FCS) was obtained from Mascia Brunelli S.p.A. (Milano, Italy).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), TNF- α , HT, HVA, *trans*-resveratrol, and Dulbecco's modified Eagle's medium were purchased from Sigma Aldrich (Milano, Italy).

OleA was obtained from oleuropein glucoside by enzymatic digestion (28). OPE was obtained from extra virgin olive oil commercially available according to the method of Montedoro et al. (29).

The primary antibodies anti-ICAM-1, anti-VCAM-1, anti-E-selectin, and anti-plant allergen (mouse monoclonal IgG1) were purchased from Research Diagnostic Inc. (Flanders, NJ). The secondary antibody goat anti-mouse-IgG-horseradish peroxidase was obtained from Promega (Milano, Italy).

Endothelial Cell Culture. HUVEC were harvested from freshly obtained human umbilical cords by digestion with collagenase as previously described (30, 31). HUVEC were grown in M199 medium supplemented with 2 mmol/L L-glutamine, 50 units/mL penicillin, 50 μ g/mL streptomycin, 20% inactivated FCS, 0.1 mg/mL heparin from porcine intestinal mucosa, and 0.1 mg/mL crude extract endothelial cell growth factor.

HUVEC at the first passage were treated with OPE, HT, OleA, and HVA. *trans*-Resveratrol was used as reference compound. After 18 h,

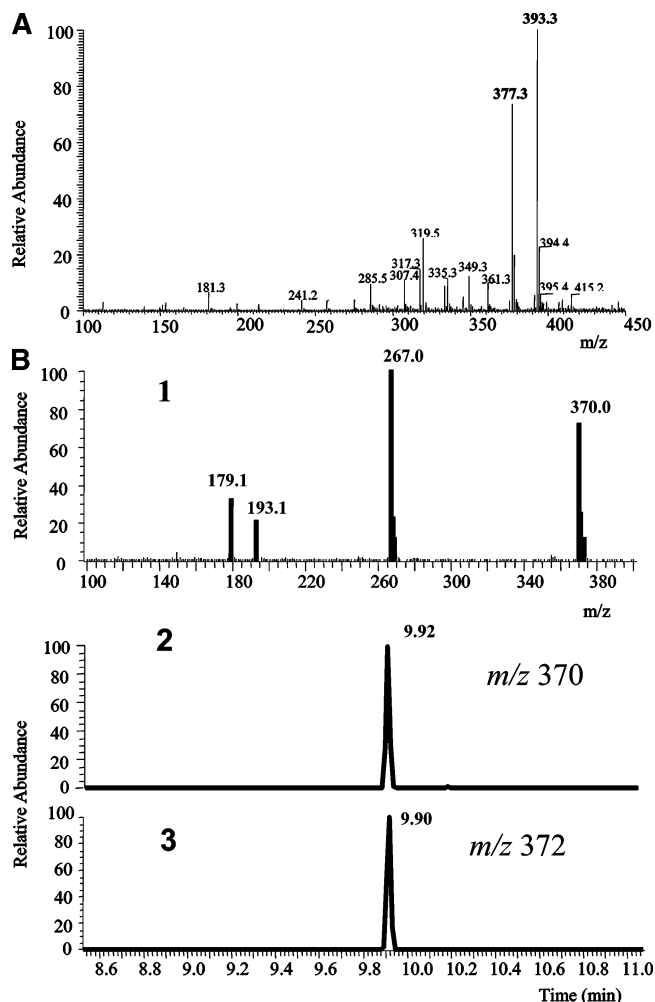


Figure 2. (A) ESI-MS spectrum obtained by loop injection of the OPE used for the experiments: ion at m/z 241, elenolic acid; m/z 319, deacetoxy oleuropein aglycone; m/z 361, ligstroside aglycone; m/z 377, oleuropein aglycone; m/z 393, 10-hydroxyoleuropein. (B1) GC-MS-IT mass spectrum of native HT. (B2) Ion chromatogram of the native (m/z 370). (B3) Ion chromatogram of the deuterated HT (m/z 372).

the medium was renewed with the compounds in the presence of TNF- α (20 ng/mL) for 6 h. All of the compounds were dissolved in ethanol (0.1%). Cellular toxicity caused by exposure for 24 h to increasing concentrations (0.25–25 μ M) of HT, HVA, OleA, and OPE was assessed by MTT colorimetric assay according to the method of Denizot et al. (32). No sign of cytotoxicity was observed for the compounds at the concentrations tested.

Preparation and Characterization of the OPE. OPE was extracted according to the method of Montedoro et al. (29) with minor modifications. Briefly, samples (3 mL) were extracted twice with hexane (1 volume) and methanol/water (80:20; v/v; 1 mL) for 2 min on a mechanical shaker. The collected methanolic phases were then taken to dryness under nitrogen. Extracts were stored at -20 °C until analysis.

For quantitative analysis of OleA, OPE samples were spiked with phloretin (100 μ g/sample, MW = 272) to be used as an internal standard. Five aliquots (20 μ L) of each extract were analyzed for ESI-MS/MS (LTQ, ThermoElectron Co.) monitoring ions at m/z 307 and 167 derived from the collision of the $[M - H]^-$ ions of OleA (m/z 377) and phloretin (m/z 273), respectively (33). HT was quantified by GC-MS (GCQ, ThermoQuest) using deuterium-labeled HT as already described (34). The concentration of free HT was 0.170 mg/mL, whereas the OleA level was 0.380 mg/mL. The molarity of OPE was expressed as HT equivalent. **Figure 2** reports a representative ESI spectrum of OPE (panel A) and an example of the HT ion chromatogram used for quantitative determination (panel B).

Detection of Cell Surface Molecules. The assay on cell surface molecules was performed by ELISA, using primary mouse anti-human monoclonal antibodies against ICAM-1, VCAM-1, and E-selectin (35). HUVEC were cultured and incubated as described above in 96 flat-bottom well plates. The cells were fixed with 0.025% glutaraldehyde (Sigma Aldrich, Milano, Italy), and the plates were washed with phosphate-buffered saline (PBS) and exposed for 2 h to primary antibodies (100 μ L/well) diluted in PBS–Tween 20 (0.05% v/v) + 10% goat serum (Sigma Aldrich) to obtain the following final concentrations: anti-ICAM-1 and anti-VCAM-1, 0.2 ng/mL; anti-E-selectin, 0.5 ng/mL. An anti-plant allergen mouse monoclonal IgG1 (final concentration = 0.4 ng/mL) was used as a negative control. The plates were washed with PBS–Tween 20 and incubated for 1 h with the secondary antibody GAM–IgG–HRP (100 μ L/well) diluted 1:2200 in PBS–Tween 20–goat serum. The plates were washed with PBS–Tween 20 and incubated with a 3,3',5,5'-tetramethylbenzidine–urea–hydrogen peroxide solution (100 μ L/well) for 10 min (Sigma Aldrich). The reaction was stopped by adding 4 N H₂SO₄. The OD was read at 450 nm in a plate reader within 30 min.

mRNA Analysis by Real-Time Quantitative RT-PCR. HUVEC were treated with the compounds as described above. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Milano, Italy) and quantified by using RiboGreen RNA Quantitation Kit (Molecular Probe Europe BV, Leiden, The Netherlands). Total RNA was retrotranscribed by using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Milano, Italy). Real-time PCR reactions were carried out in an iCycler iQ detection system (Bio-Rad, Hercules, CA) with a SYBR Green Kit (Qiagen) to amplify genes coding for ICAM-1, VCAM-1, and E-selectin in the following conditions: activation step at 95 °C followed mRNA analysis by real-time quantitative RT-PCR by 40 cycles with 95 °C denaturation step for 15 s and annealing/extension at 60 °C for 60 s. All of the samples were tested in triplicate, and the relative expression of ICAM-1, VCAM-1, E-selectin mRNA was calculating by normalizing the threshold cycle (*C_t*) of each gene with the *C_t* of 18S mRNA to correct for variation in RNA loading.

RESULTS

Characterization of the OPE. The characterization of the olive oil extract used for the experiments was performed by electrospray ionization mass spectrometry (ESI-MS) without liquid chromatography (LC) separation (33). The obtained mass spectra is reported in **Figure 2A**. The main ions were derived from oleuropein (*m/z* 377) and 10-hydroxyoleuropein (*m/z* 393). Other phenolic components known to occur in oil extracts were identified (see **Figure 2** caption) and in particular hydroxytyrosol (*m/z* 153), elenolic acid (*m/z* 241), deacetoxy oleuropein aglycon (*m/z* 319), and ligstroside (*m/z* 361), containing a phenolic residue instead of the catecholic residue of oleuropein. Among those compounds only hydroxytyrosol, oleuropein, and deacetoxy- and 10-hydroxyoleuropein contain a catecholic moiety and may therefore contribute to the antioxidant properties of the virgin olive oil. No oleuropein glycoside was detected in the extract.

The quantitative determination of hydroxytyrosol in olive oil extract was performed by means of GC-MS-IT technique using deuterated hydroxytyrosol as internal standard, according to our previous paper (34). The mass spectra of native and deuterated HT are shown in **Figure 2B**. Because the main ion detected (*m/z* 267) is common to native and deuterated HT, the ions used for the quantification of HT were at *m/z* 370 and 372.

Effects of the Compounds on VCAM-1, ICAM-1, and E-Selectin Cell Surface Expression. In the first set of experiments, we investigated the possible capacity of the compounds to modulate the proatherogenic cell surface expression of VCAM-1, ICAM-1, and E-selectin; for this purpose, HUVEC were treated with OPE (0.25–25 μ M), HT, HVA, OleA, and *trans*-resveratrol (5–25 μ M) as described under Materials and

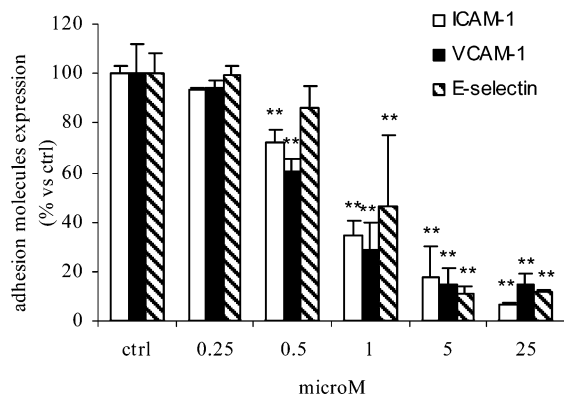


Figure 3. Concentration-dependent curves of OPE (0.25–25 μ M) on ICAM-1, VCAM-1, and E-selectin cell surface expression. IC₅₀ values were analyzed using Graph Pad Prism 4. Results represent the mean \pm SD of two experiments performed in triplicate. *, *p* < 0.05; **, *p* < 0.01, versus control. *trans*-Resveratrol (25 μ M) was used as reference compound.

Methods. The experiments were performed for all of the compounds in the presence of 20 ng/mL TNF- α as pro-inflammatory stimulus. The results clearly show that OPE was able to down-regulate the adhesion molecule cell surface expression in a concentration-dependent manner (**Figure 3**); the IC₅₀ values calculated for ICAM-1, VCAM-1, and E-selectin were 0.72 ± 0.11 , 0.54 ± 0.02 , and 0.87 ± 0.01 μ M (\pm SD), respectively. Among the compounds that occur in olive oil, OleA showed a significant down-regulation of E-selectin and ICAM-1 cell surface expression at 5 μ M (92 and 65% inhibition, respectively), whereas the inhibition on VCAM-1 was significant only at 25 μ M (60% inhibition) (**Figure 4**). Indeed, the inhibition exerted by OleA, in particular on ICAM-1 and E-selectin, was similar to that found for OPE, indicating OleA as the major responsible for this activity (**Figure 3** versus **Figure 4**). HT significantly inhibited ICAM-1 (**Figure 4A**) already at 5 μ M (70% inhibition), whereas weaker effects on VCAM-1 (**Figure 4B**) and E-selectin (**Figure 4C**) were found (40 and 30% inhibition at 25 μ M, respectively).

To investigate if the effect on the adhesion molecules exerted by OleA and HT could be due to the presence of the catecholic group, we tested HVA, the major metabolite of HT in humans. ICAM-1 and E-selectin cell surface expressions were significantly down-regulated (92 and 75% inhibition at 25 μ M, respectively), whereas a weaker inhibition was found on VCAM-1 (33% inhibition at 25 μ M).

trans-Resveratrol, an antioxidant stilbene that occurs in red wine, was used as reference compound. In our experimental conditions, *trans*-resveratrol strongly inhibited VCAM-1, ICAM-1, and E-selectin cell surface expression (78, 77, and 92% inhibition at 5 μ M, respectively), according to Carluccio et al. (36). The effect was comparable to that observed for the tested compounds.

Effects of the Compounds on VCAM-1, ICAM-1, and E-Selectin mRNA Levels. To verify whether the down-regulation of cell surface expression was consequent to a decreased rate of the gene transcription, the effects of OPE, OleA, HT, and its metabolite HVA on the corresponding mRNA levels were investigated. For this purpose, HUVEC were incubated with the tested compounds in the presence of TNF- α (20 ng/mL). After 24 h, the total RNA was extracted, quantified, reverse transcribed, and analyzed as described under Materials and Methods.

OPE at 5 μ M strongly reduced VCAM-1 and ICAM-1 mRNA levels after endothelial activation with TNF- α (85 and 60%

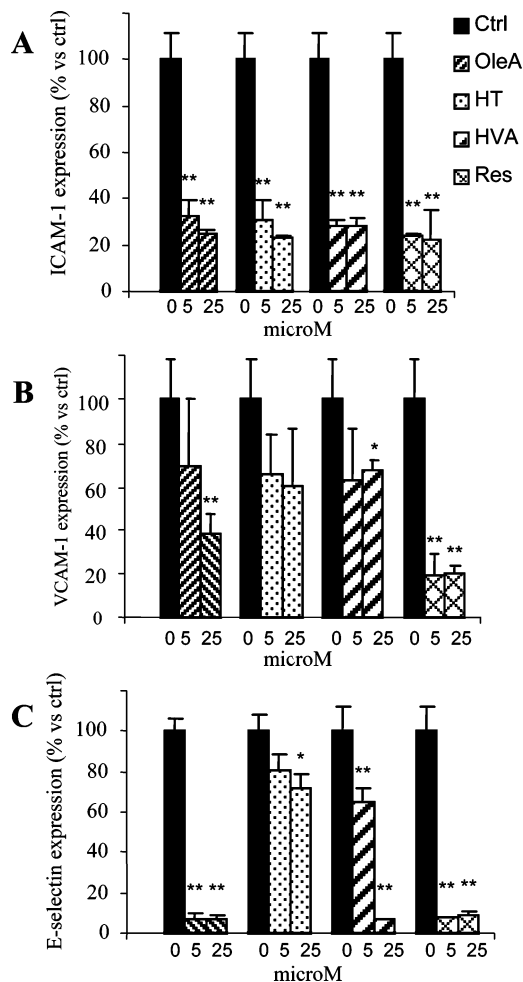


Figure 4. Effect of HT, OleA, and HVA on (A) ICAM-1 cell surface expression, (B) VCAM-1 cell surface expression, and (C) E-selectin cell surface expression. Results represent the mean \pm SD of two experiments performed in triplicate. *, $p < 0.05$; **, $p < 0.01$, versus control. *trans*-Resveratrol (5–25 μ M) was used as reference compound.

inhibition, respectively), whereas it was quite inactive on E-selectin (cf. panels A and B of Figure 5 with panel C). OleA significantly reduced VCAM-1 and E-selectin mRNA levels, whereas the effect on ICAM-1 was less pronounced. Conversely, HT (5–25 μ M) down-regulated VCAM-1 and ICAM-1 mRNA expression in a statistically significant manner, whereas no effect was elicited on E-selectin (Figure 5C). HVA exerted 50% inhibition only on E-selectin at 5 μ M. As expected, *trans*-resveratrol at 25 μ M reduced all of the adhesion molecules' mRNA levels (Figure 5). The 18S mRNA, used as internal standard for normalization, was not affected by the tested compound.

DISCUSSION

In recent years, the formulation of a strong correlation between the pathogenesis of atherosclerosis and reactive oxygen species (ROS) stimulated experimental and epidemiological studies on the possible role of antioxidants in the prevention of cardiovascular diseases, as observed in the Mediterranean area. Consumption of virgin olive oil has been associated with a lower cardiovascular diseases risk; in particular, among the several minor constituents of this nutrient, the major protective effect has been attributed to the occurrence of phenolic compounds, mainly HT and OleA (5). In this paper we describe the

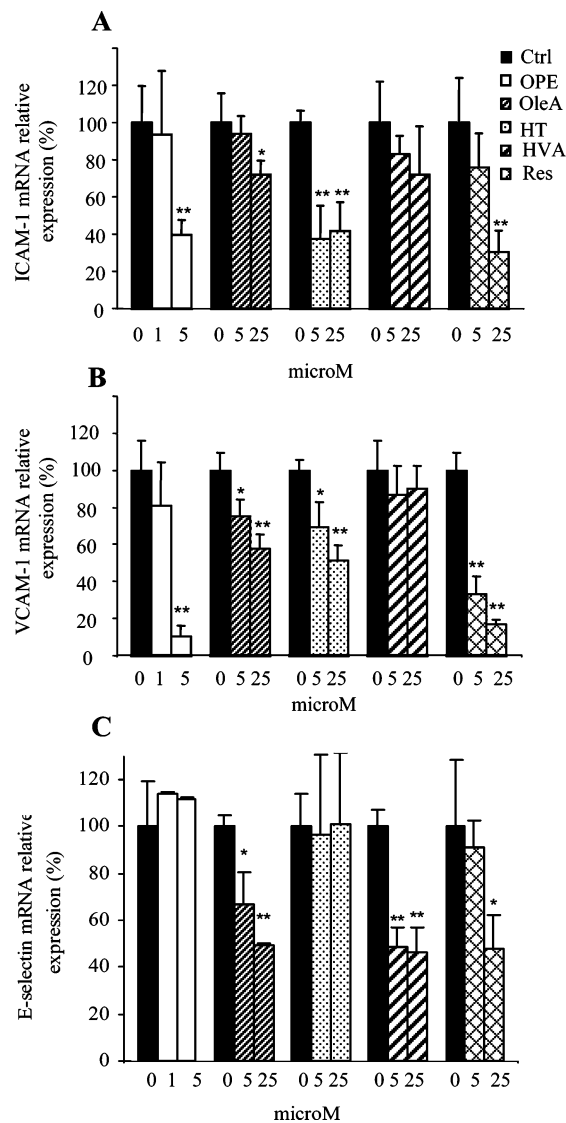


Figure 5. Effect of OPE, HT, OleA, and HVA on (A) ICAM-1 mRNA expression, (B) VCAM-1 mRNA expression, and (C) E-selectin mRNA expression. Results represent the mean \pm SD of two experiments performed in triplicate. *, $p < 0.05$; **, $p < 0.01$, versus control. *trans*-Resveratrol (5–25 μ M) was used as reference compound.

modulation of a phenolic extract from extra virgin olive oil, HT, OleA, and HVA on VCAM-1, ICAM-1 and E-selectin cell surface and mRNA expression.

OPE was able to strongly reduce the cell surface expression of the adhesion molecules at concentrations that can be considered to be physiologically relevant ($IC_{50} < 1 \mu$ M) (37). The decrease was linked to a reduction in mRNA levels, the correlation being more evident at 5 μ M. Among the minor components from olive oil, OleA and HT were shown to inhibit VCAM-1 and ICAM-1 expression at both levels; by comparing the effects of OleA and HT with those observed for OPE, it is noteworthy that both of the components could contribute to the down-regulation elicited by the extract. In contrast, the effect of OPE on E-selectin expression was not mirrored by a decrease of the corresponding mRNA levels; the effect of OleA, at the gene level, was higher in comparison to the extract, whereas HT influenced very little the E-selectin expression at both levels. The evidence that OleA represents ~70% of the total content of OPE (as described under Materials and Methods) suggests that OPE shows no effect on E-selectin mRNA expression,

probably because, in the extract, OleA does not reach the effective concentration.

Interestingly, HVA inhibited cell surface expression of all the tested adhesion molecules, whereas the effect on mRNA expression was evident only for E-selectin (Figures 4 and 5). By comparing the effect on the cell surface expression to that observed on the mRNA expression, the tested compounds more efficiently inhibit the first step; this evidence induces us to suggest further effects, in addition to the down-regulation of mRNA levels. It has been shown that an oxidative stress and the formation of ROS can induce a strong remodeling of the actin cytoskeleton in endothelial cells, which causes the expression of the adhesion molecules on the cell surface. This modification of the cytoskeleton structure by ROS is particularly relevant in response to inflammation (38). Thus, the compounds under study, which possess strong antioxidant properties (39, 40), could inhibit more efficiently the cell surface expression process through their "scavenging" properties.

Among the tested compounds, only HT and OleA share a catecholic structure, whereas all of the compounds tested possess an antioxidant activity, as previously described (9, 39–41). This evidence, in addition to the observation that *trans*-resveratrol and HVA, which are antioxidants without the catecholic moiety, significantly down-regulate the cell surface adhesion molecules expression, supports the hypothesis that the effect at this stage could be mainly ascribable to their antioxidant activity and not directly related to a "scavenger" effect.

Transfection studies using different VCAM-1 gene promoter constructs showed that antioxidants from olive oil and *trans*-resveratrol repressed VCAM-1 gene transcription (36). Because the inhibition of promoter activity was decreased by deletion of binding sites for AP-1 and GATA and totally abolished by deletion of the two kB binding sites, an interference by these molecules with redox-sensitive nuclear transcription factors NF- κ B and AP-1 could be the mechanism by which OPE and the related compounds down-regulate mRNA levels (36, 42, 43).

Although the inhibition of the adhesion molecules by HT and OleA has been previously reported (36), this is the first work that investigates the effect of a phenolic extract from extra virgin olive oil on VCAM-1, ICAM-1, and E-selectin cell surface and mRNA expression, demonstrating that, among the components that occur in olive oil, OleA and HT, although with different efficacies, are the main ones responsible for the effect observed. Interestingly, the effect on the cell surface expression was relevant also for HVA, the main metabolite of HT in humans, indicating that the effect of HT could be maintained, although with minor efficacy, also after *in vivo* metabolization.

It should be highlighted that all of the experiments were performed using HUVEC at the first passage, which our previous experiments have indicated to be the best model for these studies (data not shown).

In another set of experiments, the compounds were also tested without the proinflammatory stimulus: in these experimental conditions, all of the compounds were inactive or showed only a negligible effect (data not shown), confirming the key role of these molecules in modulating the expression of the proatherogenic adhesion molecules just on the activated endothelium. Taken together with several *in vitro* and *in vivo* investigations showing the beneficial effect of olive oil phenolics, the results of the present study supply new insights on how these constituents, daily consumed with the diet, could be able to protect against cardiovascular risk also through the down-regulation of adhesion molecules involved in early atherogenesis.

ABBREVIATIONS USED

OPE, phenolic extract from extra virgin olive oil; HT, hydroxytyrosol; OleA, oleuropein aglycone; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; TNF- α , tumor necrosis factor-alpha; LPS, lipopolysaccharide; HVA, homovanillyl alcohol; HUVEC, human umbilical vascular endothelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FCS, fetal calf serum; ROS, reactive oxygen species; PBS, phosphate-buffered saline; ESI-MS, electrospray ionization mass spectrometry; GC-MS-IT, gas chromatography–mass spectrometry–ion trap.

ACKNOWLEDGMENT

We thank Aurelio Toia and Flavio Giavarini for skillful technical assistance in MS analyses. We gratefully acknowledge Elda Desiderio Pinto for invaluable support.

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Received for review November 22, 2005. Revised manuscript received March 7, 2006. Accepted March 13, 2006.

JF0529161