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ARTICLE

# Olive Oil Phenols Modulate the Expression of Metalloproteinase 9 in THP-1 Cells by Acting on Nuclear Factor-κB Signaling

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In vivo studies suggest that the phenolic component contributes to the anti-inflammatory and anti-atherosclerotic actions of olive oil; however, the effects in circulating cells are not fully characterized. Monocytes play a key role in inflammation-based diseases by expressing several molecules, including metalloproteinases (MMPs). In the present study, we investigated the effects of olive oil phenolic extract and individual compounds on MMP-9 in THP-1 cells, a human monocyte-like cell line. Olive oil extract prevented the stimulation of MMP-9 expression and secretion in tumor necrosis factor α-treated THP-1 cells. Oleuropein aglycone, a typical olive oil phenol, was active at concentrations found in the extract, although other compounds probably contribute to the biological activity. We also found that the effect of the extract and individual compounds on MMP-9 is due to impaired nuclear factor-κB signaling. Our findings provide further evidence on the mechanisms by which olive oil reduces the inflammatory burden associated with disorders, such as atherosclerosis.

**KEYWORDS: Olive oil phenols; oluropein aglycone; inflammation; monocytes; metalloprotease-9; NF-kB**

## INTRODUCTION

Cardiovascular diseases are the leading cause of death worldwide, with increasing prevalence in developing countries and Eastern Europe (*1*). Evidence gathered over the last decades indicates that atherosclerosis, the main cause of coronary artery diseases, is an inflammatory disease in which immune mechanisms interact with metabolic risk factors to initiate, propagate, and activate lesions in the arterial wall (*2*).

Notably, diet is still the preferred option in primary prevention for reducing the risk of cardiovascular disease (*3*); therefore, elucidation of the biological actions of nutrients and food components is a crucial issue. Numerous studies have demonstrated that the beneficial effects of olive oil consumption on human health can be ascribed to minor phenol components such as flavonoids, in particular luteolin (LU) and apigenin (AP), and the secoiridoids oleuropein aglycone (OleA) and ligstroside aglycone and their hydrolysis products hydroxytyrosol and tyrosol, respectively. Olive oil is a unique dietary source of phenolic compounds since, in contrast to other natural extracts, most of them are present as aglycones (*4*), which have been shown to be more active than the corresponding glucosidic derivatives. Furthermore, in vivo studies showed a higher degree of excretion when phenols were ingested in the olive oil matrix in comparison to other vehicles, thus reflecting a more efficient absorption (*5*,*6*).

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Regarding the biological actions, olive oil phenols, either in the form of extract or as pure compounds, have been shown to counteract the effects of pro-inflammatory stimuli on target molecules (i.e., adhesion molecules) in different cell types, including cells of the arterial wall (*7*−*10*). These findings therefore suggest that olive oil phenols may be anti-inflammatory and responsible for the antiatherosclerotic actions of olive oil observed in vivo. Despite this evidence, less is known on the effect of olive oil phenols on circulating cells. We have recently reported the ability of olive oil phenolic extracts to inhibit platelet aggregation (*11*), in line with the antithrombotic potential of olive oil administration to rabbits (*12*).

Among circulating cells, monocytes play a key role in the initiation of atherosclerosis, and the expression of cytokine-induced matrix metalloproteinases (MMPs) by activated monocytes may contribute to atherosclerotic alteration of the arterial intima by facilitating the migration of recruited monocytes in the subendothelial layer (*2*). In particular, monocytes express MMP-9, a member of the MMPs family acting on the extracellular matrix but also on nontraditional substrates, including precursors of inflammatory cytokines, thus contributing to the amplification of the inflammatory response (*13*).

With the long-range goal of elucidating the mechanisms through which olive oil-derived phenols exert beneficial effects in the prevention of cardiovascular diseases, we investigated the actions of one olive oil phenolic extract and individual phenolic compounds on the modulation of MMP-9 in THP-1 cells, a

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widely used human monocyte-like cell line. We also attempted to elucidate the molecular mechanisms underlying the biological effect.

## MATERIALS AND METHODS

**Reagents.** All cell culture reagents were purchased from Invitrogen (Milan, Italy). Phloretin, homovanillyl alcohol, tyrosol, quercetin, epigallocatechin-3-gallate (EGCg), and dexamethasone were purchased from Sigma-Aldrich (Milan, Italy). AP and LU were purchased from Extrasynthèse (Lyon, France). OleA was obtained from oleuropein glucoside (Extrasynthèse) by enzymatic digestion (*14*), and the purity (99%) was confirmed by both thin-layer chromatography and electrospray ionization−mass spectrometric (ESI-MS) analysis. Hydroxytyrosol was from Cayman Chemical Co. (Tallinn, Estonia). DEAE-dextran was purchased from Amersham Biosciences (Milan, Italy). Parthenolide was a kind gift from Dr. D'Ambrosio, University of Trento. All compounds used for the analytical determinations and for the biological assays were of high-performance liquid chromatography (HPLC) purity grade.

**Extraction and Analysis of Phenolic Extract from Olive Oil.** Three different lots of a commercially available virgin olive oil of the same brand were purchased from various Italian drugstores. Oils were produced in Italy by using 100% Italian olives.

Olive oils were extracted according to the method of Montedoro et al. (*15*) with minor modifications. Briefly, 100 g of olive oil was delipidized with hexane (100 mL) and extracted twice with methanol−water (80:20 v/v; 100 mL) for 20 min on a mechanical shaker. The methanolic phase was removed under vacuum at 35 °C, and the remaining aqueous phase was extracted with an equal volume of hexane to remove lipid traces. The extract was stored at −20 °C until analysis. Spectrophotometric analyses of total phenols reactive to Folin−Ciocalteu (*16*), expressed as oleuropein equivalents, were carried out as described (*17*) on aliquots of extracts resuspended in ethanol. HPLC-UV analyses were performed as recently described (*11*). OleA, tyrosol, hydroxytyrosol, AP, quercetin, and LU were identified by comparison of the retention times and mass spectra with those obtained from authentic standards. Quantitative determination of the single phenols was carried out by ESI-MS/MS using a linear ion-trap mass spectrometer (LTQ; ThermoElectron Corp., United States) equipped with an ESI source operating in the negative mode. The operating parameters were previously described (*11*). Calibration curves were prepared with phloretin (100 ng/sample) as an internal standard and increasing amounts of the authentic phenols (0−200 ng for AP and quercetin and 0−500 ng for LU); for the determination of OleA, samples for the calibration curve contained 0−100 $\mu$g of OleA and 100 $\mu$g of phloretin. Tyrosol and hydroxytyrosol were quantified by gas chromatography−mass spectrometry (GC-MS) using deuterium-labeled hydroxytyrosol as an internal standard (*18*).

**Cell Cultures.** Human THP-1 monocytic leukemia cells (ATCC, Teddington, United Kingdom) were grown in RPMI 1640 supplemented with 100 units penicillin G/mL, 100 mg streptomycin/mL, 1 mM sodium pyruvate, 0.05 mM $\beta$-mercaptoethanol, 10 mM Hepes, and 10% heat-inactivated fetal calf serum (FCS).

Cells were exposed to 10 ng/mL tumor necrosis factor α (TNF-α) (Sigma-Aldrich) in medium without FCS for 6 h in the absence or in the presence of increasing concentrations of olive oil phenolic extract or individual phenolic compounds. EDTA (2 mM) and EGCg (20 $\mu$M) were used as reference inhibitors of MMP-9 activity and/or expression (*19*, *20*), whereas 10 $\mu$M parthenolide was used as an inhibitor of nuclear factor-$\kappa$B (NF-$\kappa$B)-driven transcription. Control samples received the vehicle only (0.1% ethanol or dimethylsulfoxide). Olive oil phenolic extract and individual phenolic compounds were used at nontoxic concentrations, that is, concentrations causing alteration of mitochondrial functionality <20% as assessed by MTT test (*21*).

**Sodium Dodecyl Sulfate (SDS)−Polyacrylamide Gel Electrophoresis Zymography.** The gelatinolytic activity of MMP-9 secreted from control and treated THP-1 cells was evaluated as described previously (*20*). Aliquots of conditioned media underwent electrophoresis on 7.5% polyacrylamide gels containing 10% SDS and gelatin (1 mg/mL). The gels were then washed in 2.5% Triton X-100 (Sigma-Aldrich) at room temperature and then incubated overnight at 37 °C (50 mM Tris pH 7.5 containing 150 mM NaCl, 10 mM $CaCl_2$, and 1 $\mu$M $ZnCl_2$; activation buffer). At the end of the incubation, the gels were stained with Coomassie brilliant blue R-250 (Sigma-Aldrich). To test the effect of olive oil phenolic extract on the activity of secreted MMP-9, conditioned medium was electrophoresed as described above, and the extract was added in the activation buffer. For the quantification of zymograms, densitometric scanning was performed using QuantityOne software (Bio-Rad), and each lysis area was normalized against intracellular protein content determined by bicinchoninic acid assay (*22*).

**Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR).** THP-1 cells were treated as described above. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, S.p.a., Milan, Italy) according to the manufacturer's instructions. Total RNA was quantified using the Ribo Green RNA Quantitation Assay from Molecular Probes (Invitrogen). Aliquots corresponding to 80−200 ng of total RNA were reverse transcribed with random hexamers by using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Milan, Italy) following the manufacturer's protocol. Aliquots of the cDNAs (corresponding to 10 ng of the original RNA) were subjected to real-time PCR with a SYBR Green kit (Bio-Rad Laboratories) following the manufacturer's instructions. 18S rRNA was used as the housekeeping gene for sample normalization and was amplified in separate wells within the same plate. Primers for real-time PCRs were designed with Primer Express software (Applied Biosystems, Monza-Milan, Italy) and optimized to work in a two-step protocol (40 cycles of amplifications each consisting of a denaturation step at 95 °C for 15 s and an annealing/extension step at 60 °C for 60 s). The oligonucleotides were synthesized by Primm (Milan, Italy), and the sequences were the following: MMP-9, forward primer, 5′AAACCGAGTTGGAACCACGA3′, reverse primer, 5′TCAGGGAGACGCCCATTTC3′; 18S rRNA, forward primer, 5′CGGCTACCACATCCAAGGAA3′, reverse primer, 5′CCTGTATTGTTATTTTTCGTCACTACCT3′. The specificity of the amplified products was monitored performing melting curves at the end of each amplification reaction. All amplicons generated a single peak, thus reflecting the specificity of the primers. Experiments were repeated at least three times. mRNA levels of the tissue inhibitor of metalloprotease-1 (TIMP-1) were measured as previously described (*23*). Dexamethasone was used as a reference inhibitor of MMP-9 expression (~80% inhibition at 100 nM).

**Assay of Nucler Factor-$\kappa$B Promoter Activity.** The NF-$\kappa$B promoter activity was evaluated by means of transient transfection assays in THP-1 cells transfected by the DEAE-dextrane method (*24*). Briefly, cells were exposed to a mixture of DNA-dextran (750 $\mu$g/mL final concentration) for 30 min at 37 °C, using 700 ng NF-$\kappa$B-luc reporter plasmid/$1.5 \times 10^6$ cells. Cells were then incubated for 48 h in complete medium (FCS-supplemented RPMI). pNF-$\kappa$B-luc containing the luciferase gene under the control of three $\kappa$B responsive elements of the promoter of the E-selectin gene was a kind gift of N. Marx (Department of Internal Medicine II-Cardiology, Ulm, Germany); PRC/CMV-p65, an expression vector of p65 subunit, was kindly provided by L. Guerrini (Department of Genetics and Biology of Microorganisms, Milan, Italy).

Parthenolide was used as an inhibitor of NF-$\kappa$B-driven transcription (~50% inhibition at 10 $\mu$M). Luciferase assays were performed as described previously (*20*) and are expressed as means ± standard deviations (SDs) of triplicate samples.

**Statistical Analysis.** All experiments were reproduced at least three times, and where indicated, representative experiments are shown. Statistical analyses were performed with GraphPad Prism 4 software, using *t* test or one-way analysis of variance followed by Bonferroni's posthoc test. The significance was set at $p < 0.05$.

## RESULTS

**Characterization of Olive Oil Phenolic Extract.** The study was performed using the phenolic extract obtained from one commercially available virgin olive oil. The amounts of total phenols and of individual compounds, which are found in the phenolic fraction of olive oil, were measured as described in the Materials and Methods. **Table 1** shows data obtained from three lots of the same item. Total phenols accounted for about 650 mg/kg of oil. As expected, OleA was the most abundant phenolic compound (150 mg/kg of oil) representing the 20−30% of total phenols,

**Table 1.** Characterization of Olive Oil Phenolic Extract[a]

| mg/kg | | | | | |
|---|---|---|---|---|---|
| total phenols | OleA | HT | TY | LU | AP |
| 678 ± 187 | 180 ± 37 | 15.1 ± 1.1 | 7.9 ± 0.9 | 4.7 ± 2.6 | 2.5 ± 0.9 |

[a] The phenolic fraction of a commercial olive oil was extracted as described in the Materials and Methods. The amount of total phenols was measured by means of a spectrophotometric assay based on the reactivity to Folin−Ciocalteu reagent, using a solution of OleA at a known concentration as the reference. The concentrations of OleA, LU, and AP were determined by ESI-MS/MS, while the concentrations of hydroxytyrosol (HT) and tyrosol (TY) were assessed by GC-MS. For each individual compound, calibration curves were used as described in the Materials and Methods. Bars represent the mean ± SD of the values obtained from three different lots of the same item.

while AP and LU were present at much lower concentrations (≤2 mg/kg of oil). Quercetin was undetectable. On the basis of these results, this virgin olive oil was selected as representative of various olive oils widely distributed on the Italian market and characterized by high phenol content, with similar qualitative−quantitative profiles (*4*), as reported in previous studies (*11*).

**Virgin Olive Oil Phenolic Extract and Phenolic Compounds Dampen the TNF-α-Induced Expression of MMP-9 by THP-1 Cells.** The intake of olive oil in the Mediterranean countries is estimated to be 30−50 g/day (*4*), corresponding to about 20−30 mg of total phenols/day referring to the olive oil considered in this study (650 mg of total phenols/kg olive oil). Taking into account the absorption (around 55−66% of the ingested olive oil phenols) (*4*) and the distribution volume (considering the plasma compartment), it may be assumed that the circulating levels of olive oil phenols are in the range of μg/mL. Therefore, we decided to perform our investigations using concentrations from 0.5 to 7.5 μg/mL of total phenols.

First of all, we evaluated the ability of olive oil phenolic extract to affect the secretion of MMP-9 in THP-1 cells. To this end, cells were stimulated with 10 ng/mL TNF-α in the presence of increasing concentrations of olive oil phenolic extract. As shown in **Figure 1A**, the addition of olive oil phenolic extract significantly reverses the stimulatory effect of TNF-α on the secretion of MMP-9 in a concentration-dependent manner. In particular, the addition of 7.5 μg/mL of total phenols completely abolished the effect of TNF-α.

When individual phenolic compounds were considered, we found that OleA (**Figure 1B**), AP, and LU (**Figure 1C**) were active at micromolar concentrations in counteracting the effect of TNF-α. However, AP and LU were active at lower concentrations since, at 10 μM, both flavonoids reduced MMP-9 secretion by about 80%, whereas OleA by only 25% (**Figure 1B** vs **C**). We also evaluated the effect of other phenolic compounds, particularly tyrosol; hydroxytyrosol, a compound that occurs in oil extracts as a product of OleA hydrolysis; and homovanillyl alcohol, a derivative that can be formed in vivo from metabolism of hydroxytyrosol (*25*). None of these compounds tested at the concentration up to 50 μM significantly affected MMP-9 secretion (data not shown).

We also tested the ability of olive oil phenolic extract to directly affect MMP-9 enzymatic activity in a cell-free system. An inhibitory effect of very low relevance (12.2 ± 3.2%, mean ± SD) was observed at concentrations active on MMP-9 secretion (7.5 μg/mL).

The next step was to establish the mechanism(s) by which olive oil phenolic extract affects MMP-9 expression. First, we tested whether the effect exerted by olive oil phenolic extract and by phenolic compounds on MMP-9 secretion was associated to a reduced mRNA levels. We measured MMP-9 mRNA levels in THP-1 cells treated with olive oil phenolic extract at the concentration of 7.5 μg/mL that was able to completely abolish

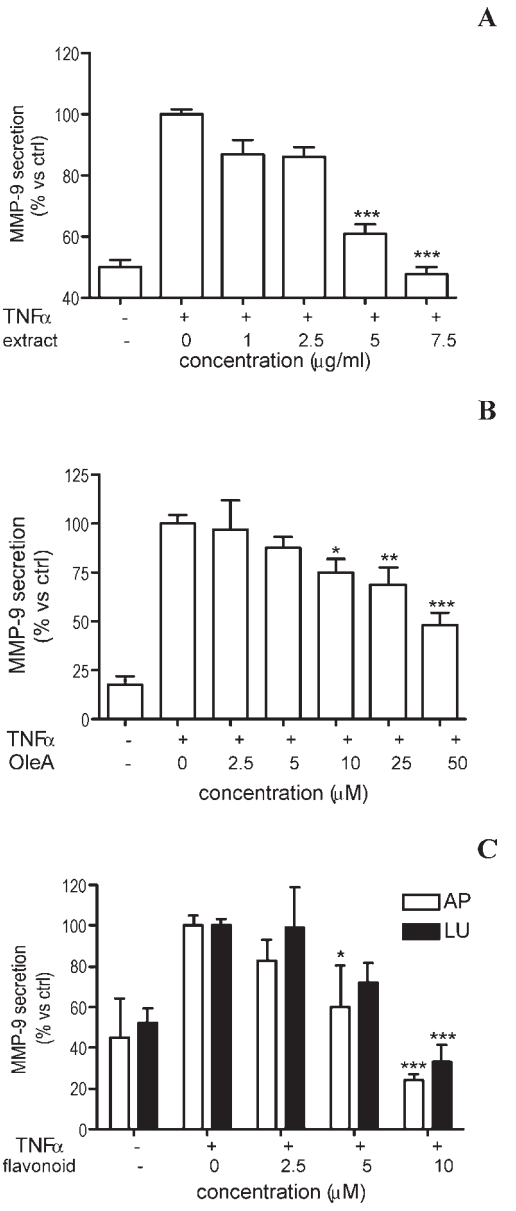


**Figure 1.** Effect of olive oil phenolic extract and pure polyphenols on MMP-9 secretion. THP-1 cells were incubated for 6 h in the presence of 10 ng/mL TNF-α and increasing concentrations of olive oil phenolic extract (**A**), OleA (**B**), AP, and LU, (**C**). The amount of MMP-9 secreted into the media was evaluated by zymography as described in the Materials and Methods. Results are expressed as arbitrary optical density units normalized against the protein content and calculated as percentages versus the normalized values measured in stimulated cells (+TNF-α). EGCg inhibited MMP-9 secretion as expected (data not shown). Each bar represents the mean ± SD of three independent experiments performed in duplicate (**$p < 0.01$).

the effect of TNF-α on MMP-9 secretion (**Figure 1A**). As shown in **Figure 2A**, we found that olive oil phenolic extract prevented the stimulatory effect of TNF-α by 40%. Similarly, OleA (50 μM) and LU (10 μM) significantly reduced the mRNA levels of MMP-9 by 50%, whereas AP (5 μM) completely abolished the stimulatory effect induced by TNF-α (**Figure 2B**). These results indicated that the reduced levels of MMP-9 secreted from treated THP-1 cells were due, at least in part, to the decrease in protein expression, thus implying that olive oil phenolic extract and phenolic compounds may affect gene expression. At the same conditions, olive oil phenolic extract (7.5 μg/mL) did not reduce the mRNA levels of the TIMP-1 (data not shown).

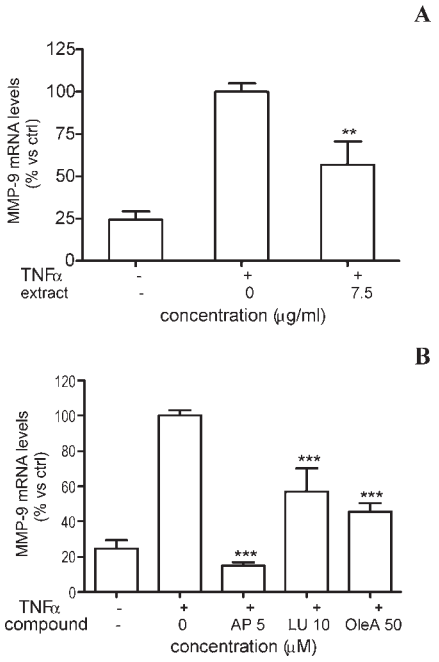


**Figure 2.** Effect of olive oil phenolic extract and pure polyphenols on MMP-9 mRNA levels. THP-1 cells were incubated for 6 h in the presence of 10 ng/mL TNF-α alone or in combination with 7.5 μg/mL olive oil phenolic extract (**A**), 5 μM AP, 10 μM LU, or 50 μM OleA (**B**). RNA was extracted as described in the Materials and Methods. Data were first normalized against 18S rRNA and then expressed setting at 100 the value measured in stimulated cells (+TNF-α). Dexamethasone reduced MMP-9 mRNA levels as expected (data not shown). Each bar represents the mean ± SD of three independent experiments performed in duplicate (**$p < 0.01$).

To rule out the possibility that the effect of olive oil phenolic extract on MMP-9 expression reflected a general inhibitory action on gene transcription, we measured the mRNA levels of other genes that are constitutively expressed by monocyte-macrophages, that is, the apolipoprotein E and the glycolytic enzyme glyceraldeheide 3-phosphate dehydrogenase in THP-1 cells treated with 7.5 μg/mL olive oil phenolic extract. We found that the expression of these genes was not affected either by TNF-α or by olive oil phenolic extract (Figure 1 of the Supporting Information). In contrast, the induction of TNF-α mRNA levels by TNF-α itself was inhibited by 7.5 μg/mL olive oil phenolic extract by about 60% (data not shown). This observation is in line with the effect on MMP-9 expression, suggesting that olive oil phenolic extract may attenuate the inflammatory potential of monocytes.

**Effect of Olive Oil Phenolic Extract on MMP-9 Involves the NF-κB System.** To get further insights on the mechanisms by which olive oil phenolic extract acts on MMP-9 expression, we focused our attention on the NF-κB system. In fact, it is known that TNF-α acts at different levels, ultimately promoting the binding of the NF-κB complex to target sequences and inducing the transcription of several genes (*26*), including MMP-9 (*27*, *28*). To evaluate whether the olive oil phenolic extract affects the NF-κB driven transcription, we transiently transfected THP-1 cells with a reporter plasmid bearing the luciferase gene under the control of a promoter containing NF-κB binding sites and stimulated with TNF-α.

As shown in **Figure 3A**, olive oil phenolic extract was able to reduce the transcriptional activity in a concentration-dependent manner. Notably, at concentrations corresponding to 5 μg/mL, the stimulatory effect of TNF-α was completely abrogated, thus resembling the effect observed on MMP-9 secretion. Therefore,

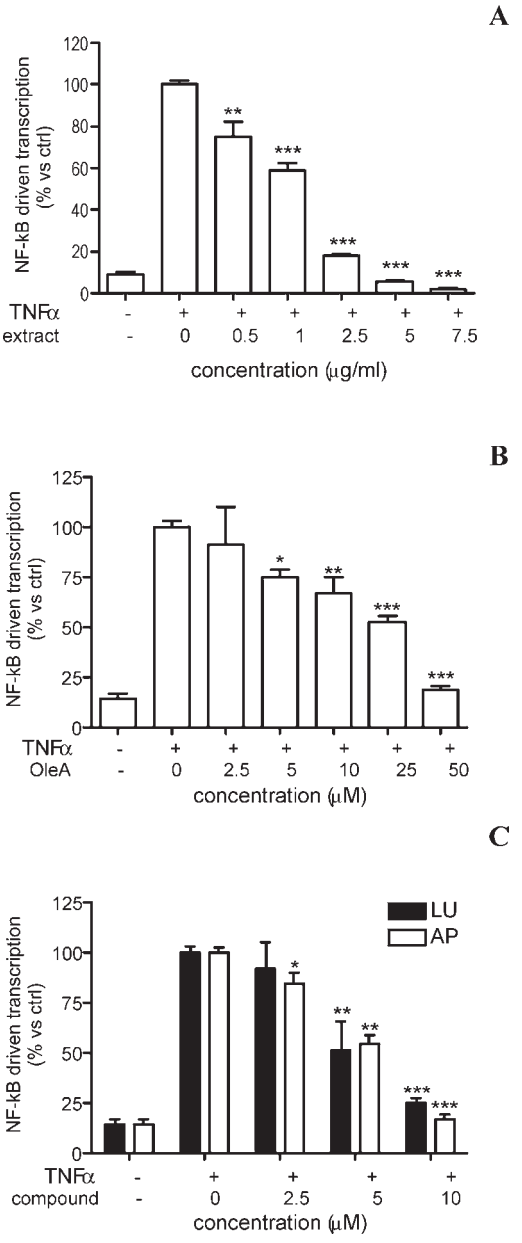


**Figure 3.** Effect of olive oil and pure polyphenols on NF-κB-driven transcription. THP-1 cells were transiently transfected with the plasmid carrying the luciferase gene under the control of a promoter containing three κB responsive elements (pNF-κB-luc, 700 ng reporter plasmid/1.5 × $10^6$ cells) as described in the Materials and Methods. Transfected cells were then treated for 6 h with 10 ng/mL TNF-α in the presence of increasing concentrations of olive oil phenolic extract (**A**), OleA (**B**), LU, or AP (**C**). The promoter activity was expressed as a percentage of the samples incubated in the presence of 10 ng/mL TNF-α alone. Parthenolide reduced pNF-κB-luc promoter activity as expected (data not shown). The graphs show the results of three different experiments performed in triplicate (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

this finding strongly suggests that the ability of olive oil phenolic extract to prevent the TNF-α-induced MMP-9 secretion and expression can be ascribed to the impairment of NF-κB system. When we evaluated the effect of pure phenolic compounds, we found that all tested substances were able to reduce the stimulatory effect of TNF-α on NF-κB promoter in a concentration-dependent manner. In particular, 50 μM OleA inhibited the promoter activity by 80% but showing a statistically significant effect at concentrations as low as 5 μM (**Figure 3B**). AP and LU

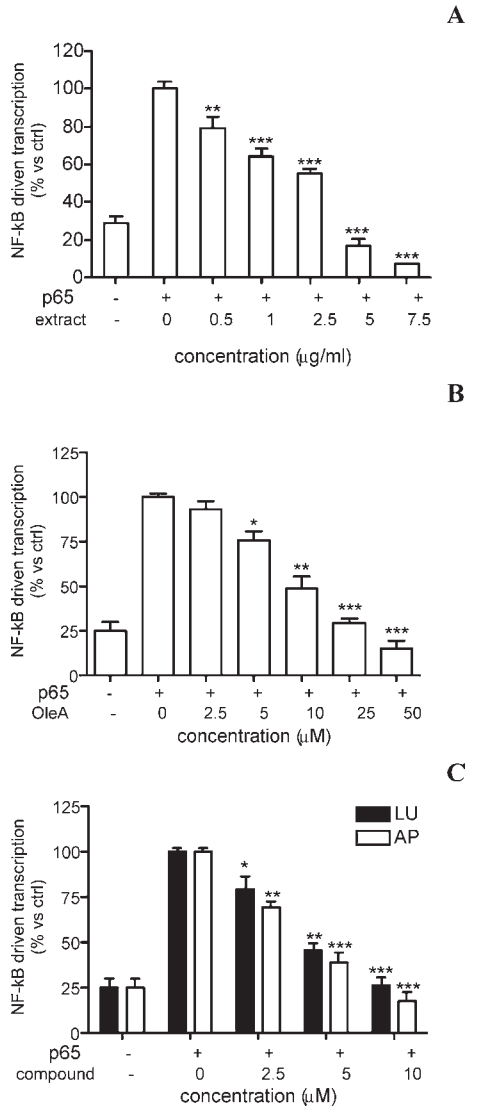


**Figure 4.** Effect of olive oil and pure polyphenols on p65-mediated transcription of NF-κB promoter. THP-1 cells were transiently transfected with the plasmid carrying the luciferase gene under the control of a promoter containing three κB responsive elements (pNF-κB-luc, 700 ng reporter plasmid/$1.5 \times 10^6$ cells) and the plasmid expressing p65 (350 ng/$1.5 \times 10^6$ cells) as described in the Materials and Methods. Transfected cells were then treated for 6 h with increasing concentrations of olive oil phenolic extract (**A**), OleA (**B**), LU, or AP (**C**). The promoter activity was expressed as a percentage of the samples incubated in the presence of the vehicle alone. The graphs show the results of three individual experiments performed in triplicate (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

displayed similar inhibitory effects and were active at lower concentrations in comparison to OleA since, at 10 μM, 80% inhibition was observed (**Figure 3C**).

The final step in NF-κB signaling is the activation of the basal transcription machinery following the association of NF-κB complex to responsive sequences of target gene promoters. To investigate whether olive oil phenol extract and pure compounds may affect gene transcription by acting at this level, p65, the transcriptionally active subunit of NF-κB, was overexpressed in THP-1 cells cotransfected with the NF-κB promoter. As shown in **Figure 4A**, olive oil phenol extract reduced the transcriptional activity of this promoter, showing a statistically significant inhibition already at 0.5 μg/mL and a complete prevention of the stimulation at 5 μg/mL. When the effect of pure phenolic compounds was tested, we found that all of the substances were able to reduce the stimulatory effect of p65 on NF-κB promoter in a concentration-dependent manner. In particular, 50 μM OleA inhibited the promoter activity by 80%, showing a statistically significant effect at concentrations as low as 5 μM (**Figure 4B**). AP and LU displayed similar inhibitory effects and were active at lower concentrations in comparison to OleA since, at 10 μM, 80% inhibition was observed (**Figure 4C**). Because olive oil extract and pure compounds inhibit, at higher concentrations, expression to levels below those of control without stimulation, it is likely that an effect of the compounds on non-p65 mediated transcription of the NF-kB promoter.

## DISCUSSION

Although the beneficial effects of olive oil consumption on human health are widely recognized, the functions and the mechanisms through which specific components of olive oil exert their effects still need to be fully deciphered. Olive oil phenols represent a group of bioactive compounds with a variety of activities on biological systems besides their antioxidant properties. The specific aim of our study was to investigate the ability of an olive oil phenolic extract and individual phenolic compounds to modulate MMP-9 in THP-1 cells. We also attempted to elucidate the molecular mechanisms underlying the biological effect.

The observations reported in the present paper indicate that phenolic olive oil extract modulates MMP-9, and possibly other NF-κB-dependent genes, for example, TNF-α, by acting at the level of gene transcription at concentrations that are commonly thought to be reached in individuals consuming olive oil-rich diets (*4*). This observation suggests that olive oil phenolic extract attenuates the inflammatory potential of monocytes. The relevance of this observation under pathological conditions is currently under investigation.

We also tested the ability of olive oil phenolic extract to directly affect MMP-9 enzymatic activity in a cell-free system. A negligible effect was observed at concentrations active on MMP-9 secretion and expression, thus suggesting that the active components of the extract do not exert their effect by inhibiting the enzymatic activity of MMP-9. Therefore, we expect that the effect on MMP-9 in vivo will be mainly mediated through modulation of gene expression.

It has been demonstrated that several phenolic constituents exhibit anti-inflammatory properties in a variety of cell systems, contributing to the overall effect of olive oil. Thus, in our study, we also assayed the individual components that we identified in the extract (**Figure 1**). We demonstrated that OleA was active in all assays at micromolar concentrations. These levels can be found in olive oil phenolic extracts, as reported by us and other investigators; therefore, we suggest that OleA is one of the compounds that mainly contribute to the effects that we observed on the modulation of MMP-9. Notably, OleA was shown to inhibit the expression of vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, other NF-κB-regulated genes, by interfering with nuclear translocation and DNA binding of p65, in human endothelial cells at concentrations similar to those used in our study (*7*, *8*).

In contrast, tyrosol, hydroxytyrosol, and homovanillyl alcohol were not active on MMP-9 secretion, even at concentrations as high as 50 μM, thus ruling out their contribution to the biological activity of olive oil phenolic extract.

Flavonoids such as AP and LU are present in olive oil at low concentrations, and we estimated that 7.5 μg/mL of the olive oil phenolic extract used in the present study corresponds to about a

0.1−0.2 $\mu$M concentration of both compounds. Our findings regarding the ability of both flavonoids to down-regulate the NF-$\kappa$B-driven transcription are consistent with other studies reporting the ability of AP and LU to significantly inhibit the NF-$\kappa$B signaling, acting at different levels, that is, I$\kappa$B kinase activity and degradation, the NF-$\kappa$B translocation, and DNA binding (*29*−*32*). Nevertheless, it should be pointed out that the concentrations necessary to obtain a significant effect on the NF-$\kappa$B system ($\geq$2.5 $\mu$M) are much higher than those found in the olive oil phenolic extract used in our experiments, thus excluding a role of these compounds in the effects exerted by the phenolic extract on MMP-9. However, because flavonoids, especially in the glycosidic form, are abundant in fruits and vegetables and are absorbed, it is reasonable to assume that they can reach in vivo concentrations exhibiting biological activities as those described in the present paper. In this regard, it has recently been shown that the chronic intake of a Mediterranean diet enriched in virgin olive oil, fruits, and vegetables decreases the activation of NF-$\kappa$B in peripheral monocytes and the plasma levels of VCAM-1 in humans (*33*).

Olive oil phenolic extract also contains other compounds such as ligstroside aglycone; however, the evaluation of their biological activities has been hampered so far because they are not commercially available. Because the chemical structure of ligstroside aglycone is highly similar to that of OleA, its contribution in the modulation of MMP-9 by olive oil phenolic extract cannot be excluded. Furthermore, oleocanthal, the dialdehydic form of (−)-deacetoxy-ligstroside aglycone, was shown to exhibit, at micromolar concentrations, anti-inflammatory properties through direct inhibition of cyclooxygenases (*34*), therefore, by means of mechanisms others than NF-$\kappa$B signaling.

In human body, olive oil phenolics circulate after conjugation with glucuronic acid and sulfate. Pharmacological activity of sulfate and glucuronide derivatives is lacking; however, generally speaking, glucuronation and sulfation are metabolic routes to facilitate excretion. Therefore, it is unfeasible to predict the actual effect in humans from the present results obtained using aglycones form.

Extracellular matrix degradation occurs in several pathological conditions such as atherosclerosis and tumor invasion. Therefore, the inhibition of proteolytic activity by pharmacological or dietary means may be an effective strategy to improve the pathological status. In this respect, dietary polyphenols represent a promising class of compounds since numerous in vitro studies demonstrated their ability to modulate the expression and activity of MMPs (*35*). It is worthwhile to mention that Hashim et al. recently demonstrated that olive oil phenolic extract inhibits the in vitro invasiveness of human tumor cells at concentrations similar to those used in our study and postulated that the overall effect is due to several mechanisms, among which inhibition of extracellular matrix degradation (*36*). In the present study, we clearly demonstrated that olive oil phenolic extract inhibits MMP-9 expression, thus supporting the hypothesis that inhibition of proteolytic activity by olive oil phenols could be, at least in part, responsible for the reduction of invasiveness of tumor cells. In this context, our study elucidates some of the molecular mechanisms through which olive oil, a phenolic-rich source, can be beneficial to human health, as widely demonstrated by in vivo studies.

## ABBREVIATIONS USED

AP, apigenin; EGCg, epigallocatechin-3-gallate; ICAM-1, intercellular adhesion molecule-1; LU, luteolin; MMP, metalloprotease; OleA, oleuropein aglycone; TNF-$\alpha$, tumor necrosis factor $\alpha$; VCAM-1, vascular cellular adhesion molecule-1; NF-kB, nuclear factor-kB; TIMP-1, tissue inhibitor of metalloprotease-1.

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**Supporting Information Available:** Figure of the effect of olive oil extract on other genes constitutively expressed in THP-1 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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