Research Article

Olive oil compounds inhibit vascular endothelial growth factor receptor-2 phosphorylation

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Abstract

Vascular endothelial growth factor (VEGF) triggers crucial signaling processes that regulate tumor angiogenesis and, therefore, represents an attractive target for the development of novel anticancer therapeutics. Several epidemiological studies have confirmed that abundant consumption of foods from plant origin is associated with reduced risk of developing cancers. In the Mediterranean basin, the consumption of extra virgin olive oil is an important constituent of the diet. Compared to other vegetable oils, the presence of several phenolic antioxidants in olive oil is believed to prevent the occurrence of a variety of pathological processes, such as cancer. While the strong antioxidant potential of these molecules is well characterized, their antiangiogenic activities remain unknown. The aim of this study is to investigate whether tyrosol (Tyr), hydroxytyrosol (HT), taxifolin (Tax), oleuropein (OL) and oleic acid (OA), five compounds contained in extra virgin olive oil, can affect in vitro angiogenesis. We found that HT, Tax and OA were the most potent angiogenesis inhibitors through their inhibitory effect on specific autophosphorylation sites of VEGFR-2 (Tyr951, Tyr1059, Tyr1175 and Tyr1214) leading to the inhibition of endothelial cell (EC) signaling. Inhibition of VEGFR-2 by these olive oil compounds significantly reduced VEGF-induced EC proliferation and migration as well as their morphogenic differentiation into capillary-like tubular structures in Matrigel. Our study demonstrates that HT, Tax and OA are novel and potent inhibitors of the VEGFR-2 signaling pathway. These findings emphasize the chemopreventive properties of olive oil and highlight the importance of nutrition in cancer prevention.

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Abbreviations: EC, endothelial cell; ERK, extracellular signal–regulated kinase; FBS, fetal bovine serum; HT, hydroxytyrosol; HMVEC, human dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cell; MAPK, mitogen-activated protein kinase; OA, oleic acid; OL, oleuropein; Tax, taxifolin; Tyr, tyrosol; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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Introduction

Tumor angiogenesis is a critical step by which tumor cells stimulate the formation of new blood capillaries from existing vessels that sustain the development of cancer by providing oxygen and nutrients to tumor cells [1]. This neovascularization occurs through a series of steps, including stimulation of endothelial cells (ECs) by autocrine and/or paracrine growth factors, proteolytic degradation of the basement membrane and surrounding extracellular matrix, EC migration and proliferation, and morphological differentiation/reorganization of ECs into a three-dimensional tubular structure [2]. Vascular endothelial growth factor (VEGF), a major factor secreted by tumor cells, plays an important role in the expansion of the microvascular network [3,4]. It is a specific EC mitogen that binds with high affinity to the EC receptors VEGF receptor-1 (VEGFR-1, Flt-1) and VEGF receptor-2 (VEGFR-2, Flk-1, KDR), the latter being responsible for most of the mitogenic and chemotactic effects of VEGF [5]. Therefore, the binding of VEGF to VEGFR-2 activates the intrinsic VEGF-2 tyrosine kinase activity required for EC migration, proliferation and survival of vascular ECs [6]. To date, five major autophosphorylation sites within VEGFR-2 have been documented. Tyr951 lies in the kinase-insert domain [7], Tyr1054 and Tyr1059 are in the kinase domain and are critical for the catalytic activity of the receptor [8,9], while Tyr1175 and Tyr1214 are in the C-terminal tail [10]. Since VEGF regulates angiogenesis, targeting new vessel growth via the inhibition of VEGF-VEGFR-2 kinase axis therefore represents a promising strategy for cancer chemoprevention and therapy [11,12].

In the last couple of years, several studies linked abundant consumption of foods from plant origin with decreased risk of developing various cancers [13]. It is now well established that the Mediterranean diet represents a model of healthy eating and is associated with a favorable health status, a better quality of life, and a decreased incidence of mortality from cardiovascular diseases, chronic degenerative diseases and cancers [14–16]. These beneficial effects have partially been explained by the consumption of extra virgin olive oil, the first-pressed olive oil [17,18], which has been reported to be more beneficial than any other dietary lipids against cancer such as colorectal, prostate, lung, endometrial and breast cancers [15,19–21]. The healthful properties of extra virgin olive oil can in particular be attributed not only to the high relationship between unsaturated and saturated fatty acids but also to the antioxidant and anti-inflammatory properties of its phenolic compounds [22]. The total phenolic content is higher in extra virgin olive oil than in refined olive oil, since 80% or more of the phenolic compounds are lost in the refinement process [23]. The main groups among these phenolic compounds are phenolic acids, phenolic alcohols, flavonoids, secoiridoids and lignans, which confer some of the anticancer effects observed in both epidemiological and experimental studies [24–26]. Indeed, it has been reported that hydroxytyrosol, a phenolic alcohol, inhibits proliferation of both human promyelocytic leukemia cells (HL60) and colon adenocarcinoma cells (HT29) [27]. Moreover, hydroxytyrosol and oleuropein, a secoiridoid, were found to reduce cell viability, inhibit cell proliferation, and induce cell apoptosis in human breast cancer cells (MCF-7) [28]. Recently, Scoditti et al. [29] demonstrated that these compounds reduce inflammatory angiogenesis through the metalloproteinase (MMP)-9 and the proinflammatory enzyme cyclooxygenase (COX)-2 in human vascular ECs.

The aim of the present study was to investigate and compare the effect of four phenolic compounds (hydroxytyrosol, HT; oleuropein, OL; Taxifolin, Tax; Tyrosol, Tyr) and a monounsaturated fatty acid, oleic acid (OA) on EC functions essential for angiogenesis. Since the mechanisms underlying the inhibition of neovascularization by olive oil compounds remain to be established, this prompted us to investigate whether these specific compounds affect VEGF-induced angiogenesis. We observed that olive oil compounds inhibited EC proliferation and migration induced by VEGF as well as their morphogenic differentiation into capillary-like structures through the inhibition of the VEGFR-2/mitogen-activated protein kinase (MAPK) signaling pathways.

Materials and methods

Materials

Cell culture media were obtained from Life Technologies (Burlington, ON) and serum was purchased from HyClone Laboratories (Logan, UT). Matrigel basement membrane matrix growth factor reduced factor was obtained from Becton Dickinson Labware (Bedford, MA). Hydroxytyrosol, oleic acid, oleuropein, (+)-taxifolin and tyrosol, were purchased from Extrasynthese (Lyon, France). Human recombinant VEGF was obtained from R&D Systems (Minneapolis, MN). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The anti-ERK-1/2 (extracellular signal-regulated kinase 1 and 2) (K-23) polyclonal antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibodies for VEGF receptor-2, the stress-activated protein kinase/Jun-amino-terminal kinase (JNK) and anti-phospho-VEGF receptor-2 (Tyr951), anti-phospho-VEGF receptor-2 (Tyr1059), anti-phospho-VEGF receptor-2 (Tyr1175), anti-phospho-VEGF receptor-2 (Tyr1214), anti-phospho-JNK polyclonal antibody and anti-phospho-p44/42 MAPK monoclonal antibodies were from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences (Boston, MA). Micro bicinchoninic acid protein assay reagents were from Thermo Scientific (Rockford, IL). All other reagents were from Sigma-Aldrich (Oakville, ON).

Cell culture

Human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HMVECs-d-AD) were purchased from Clonetics (San Diego, CA). Both types of ECs were maintained in EC basal medium-2 (EBM-2; Lonza, Walkersville, MD) with EGM-2 growth factor mixture (Lonza) supplemented with 2% fetal bovine serum (FBS) for HUVECs and 5% FBS for HMVECs. Cells used in this study were restricted to use between passages 3 and 6. They were cultured at 37 °C under a humidified 95–5% (v/v) mixture of air and CO₂. Cells were treated with vehicle (0.1% ethanol) or with olive oil compounds and stimulated with VEGF.
Endothelial cell tube formation assays

The formation of capillary-like structures was assessed on growth factor reduced (GFR) Matrigel (7.85 mg/mL). Matrigel was thawed at 4°C, and 50 μL were quickly added to each well of a 96-well plate and allowed to solidify for 10 min at 37°C. The cells were then incubated for 6 h at 37°C with HUVECs (20,000 cells/well), which had previously been treated for 18 h with 50 μM olive oil compounds in EBM-2 containing 1% FBS. VEGF was added to the cells at 1 μg/mL. Tube formation was monitored by an inverted phase contrast microscopy (Nikon Eclipse TE2000-U microscope) and pictures (× 60 magnification) were taken using a Retiga 1300 camera. The extent to which capillary-like structures formed in the gel was quantified by analysis of digitized images to determine the thread length of the capillary-like network, using ImageJ software (NIH).

Cell proliferation and migration assay by xCELLigence biosensor system

Experiments were carried out using the Real-Time Cell Analyzer (RTCA) Dual-Plate (DP) Instrument, the xCELLigence system (Roche Diagnostics, QC). This system was used according to the instructions of the supplier. Firstly, the optimal seeding concentration of cells and VEGF concentration for proliferation and migration assays, were determined. For the cell proliferation assay, HUVECs were seeded at 5000 cells/well in 200 μL complete medium into an E-plate 16, specifically designed to measure cellular impedance (Roche Diagnostics), and incubated at 37°C under a humidified atmosphere containing 5% CO2 for 24 h. Then, cells were incubated with serum-free medium containing VEGF (25 ng/mL) or olive oil compounds for 35 h. Cell proliferation was monitored every 5 min for 18 h. The impedance measurement of cell migration. Prior to cell seeding, the arrays on the bottom side of the membrane, which provide a real-time cell proliferation, viability and cytotoxicity analyzer, was conducted. The assay involved exposing cells to 0–50 μM of olive once with ice-cold PBS containing 1 mM each of NaF and Na2VO4, and were incubated in the same medium for 30 min at 4°C. The cells were solubilized on ice in lysis buffer [150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM ethyleneglycol-0,0′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), 0.5% (vol/vol) Nonidet P-40 and 1% (vol/vol) Triton X-100]. The resulting lysates (25–50 μg protein) were solubilized in Laemmli sample buffer [125 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.00125% bromophenol blue], boiled for 4 min, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes which were then blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (TBS-T; 147 mM NaCl, 20 mM Tris–HCl, pH 7.5, and 0.1% Tween 20). Membranes were further washed in TBS–T and incubated with the primary antibody in TBS–T containing 3% bovine serum albumin (BSA) and 0.01% sodium azide, followed by a 1 h incubation with HRP-conjugated antimouse or anti-rabbit antibodies in TBS–T containing 5% nonfat dry milk. Immunoreactive material was visualized with an ECL detection system. The immunoreactive bands were quantified with ImageJ software (NIH).

Statistical analysis

Statistical analyses were performed using 1-way ANOVA with a post hoc Dunnett’s test. Differences with P < 0.05 were considered significant.

Results

Olive oil compounds inhibit VEGF-induced tube formation of endothelial cells

In order to determine whether olive oil compounds affect angiogenesis, we compared the effect of five olive oil compounds (HT, OA, OL, Tax, and Tyr) (Fig. 1A) on the morphological differentiation capacity of ECs into capillary-like structures. Tubulogenesis was investigated using GFR Matrigel, in which the levels of cytokines and growth factors have been markedly reduced compared to the standard Matrigel. This basement membrane matrix is suitable to study the induction of tubule elongation of ECs in response to specific angiogenic factor. For our assay, we added exogenous VEGF (1 μg/mL) to induce robust tube-like structures (Fig. 1B). Treatment of HUVECs with 50 μM of the compounds for 24 h, prior to seeding on top of Matrigel, significantly abrogated tube formation in OL, HT, Tax and OA conditions. The length of endothelial tubular structures were reduced by 9.3%, 17%, 58.4% and 74%, respectively. However, Tyr did not affect the formation of capillary-like structures.

Olive oil compounds inhibit VEGF-induced proliferation of endothelial cells

In response to angiogenic stimuli, ECs proliferate, migrate, and form vascular tube networks. To determine the effect of olive oil compounds on proliferation of HUVECs, the xCELLigence system, a real-time cell proliferation, viability and cytotoxicity analyzer, was conducted. The assay involved exposing cells to 0–50 μM of olive

Western blot analysis

Following treatment with olive oil compounds for 24 h in 1% FBS, HUVECs or HMVECs were stimulated with VEGF (50 ng/mL). To study the effect of these compounds on the VEGFR-2 phosphorylation or VEGF signaling pathway, VEGF was added to the cells for 2 min or 10 min, respectively. Cells were then washed
oil compounds in the presence of VEGF. The cell–electrode impedance response generated from the interaction of cells with electronic biosensors was used to derive the cell index, representing growth over time (Fig. 2C–E). At 50 μM for 24 h, Tax, OA and HT inhibited EC proliferation by 16% for Tax, 23% for HT and 62% for OA (Fig. 2A) without significant toxicity (Fig. 2B). These inhibitions occurred in a concentration- and time-dependent manner (Fig. 2C–E). In order to determine a half-maximal inhibition (IC₅₀) of these compounds, cells were also treated with higher concentrations (100–200 μM) (data not shown). The inhibitory potency of HT, Tax and OA toward VEGF-induced EC proliferation was significantly different, with IC₅₀ values being observed at concentrations of 60 μM for HT (Fig. 2D), 41 μM for Tax (Fig. 2C) and 22 μM for OA (Fig. 2E).

Olive oil compounds inhibit VEGF-induced migration of endothelial cells

To assess the effect of olive oil compounds on the chemotactic motility of HUVECs, we measured their migration by the xCELLigence system. After 18 h of olive oil compounds treatment at 50 μM, VEGF-induced HUVECs migration was significantly inhibited by 28% for OL, 46% for Tax, 58% for OA and 59% for HT (Fig. 3A). We further studied these inhibition effects for the three best potent compounds, and the results showed that Tax, OA and HT caused a concentration- and time-dependent inhibition of cell migration in the presence of VEGF with IC₅₀ values of 50 μM, 34 μM and 28 μM, respectively (Fig. 3B–D).

Hydroxytyrosol, taxifolin and oleic acid inhibit VEGF-induced tyrosine phosphorylation of VEGFR-2

To achieve a better understanding of how olive oil compounds affected the molecular mechanisms involved in the inhibition of growth, migration and vascular tube formation, we next explored their inhibition effects on signaling through the VEGF/VEGFR-2 axis, which is already known to mediate angiogenesis in ECs. VEGF induces the dimerization of VEGFR-2 that leads to receptor autophosphorylation on several tyrosine residues and activation [30]. Failure in the phosphorylation of one of these tyrosine residues could alter the kinase activity of VEGFR-2 or downstream signaling pathways. Quiescent HUVECs were incubated for 24 h in EBM-2 medium containing 1% FBS in the presence or absence of olive oil compounds and then, stimulated with VEGF for 2 min. Protein expression and extent of phosphorylation of tyrosine residues Tyr951, Tyr1059, Tyr1175 and Tyr1214 were assessed by immunoblotting. As shown in Fig. 4A, olive oil compounds affected the relative levels of phosphorylation of specific tyrosine amino acid residues, as determined by the ratio of unphosphorylated to phosphorylated forms of VEGFR-2. Blotting of membranes with an antibody against VEGFR-2 showed that olive oil compounds did not affect the amount of VEGFR-2 in cell lysates. These results showed that HT, Tax and OA were potent inhibitors of VEGF-induced tyrosine phosphorylation of VEGFR-2, whereas OL was less potent. Thus, we further analyzed the inhibitory effect of the most potent inhibitors and we observed that Tax, OA and HT caused a concentration-dependent inhibition of VEGF-induced tyrosine residues phosphorylation (Fig. 4B–D). Effectively, the phosphorylation of Tyr951, Tyr1059, Tyr1175 and Tyr1214 were inhibited with an IC₅₀ at concentrations of 23–33 μM for Tax, 10–50 μM for OA, and 7–30 μM for HT (Table 1). These values indicate that certain olive oil compounds could efficiently interfere with the activity of VEGFR-2 in HUVECs.
Hydroxytyrosol, taxifolin and oleic acid inhibit VEGF-mediated signaling pathways in endothelial cells

To further investigate the mechanisms involved in the inhibitory actions of Tax, OA and HT on the VEGFR-2 signaling pathway, we next examined whether these olive oil compounds affected the phosphorylation of several downstream substrates involved in angiogenesis, such as ERK-1/2 [6] and SAPK/JNK [31]. Quiescent HUVECs were incubated for 24 h in EBM-2 medium containing 1% FBS in the presence or absence of olive oil compounds and then, stimulated with VEGF for 10 min. Treatment with these compounds resulted in a concentration-dependent inhibition of the VEGF-induced tyrosine phosphorylation of ERK-1/2 and SAPK/JNK (Fig. 5A–C). For all these treatments, the IC50 values obtained for pERK-1/2 were very close (ranging from 21 to 27 μM) comparatively for those obtained for pSAPK/JNK (varying from 10 to 28 μM) (Table 1). For both signaling pathways, pERK-1/2 and pSAPK/JNK, HT was the most potent inhibitor followed by OA then Tax (Table 1 and Fig. 5A–C). In order to strengthen these results, we next investigated whether HT, Tax and OA were also capable of inhibiting VEGF-induced tyrosine phosphorylation of VEGFR-2 and downstream signaling in microvascular ECs, HMVECs. We found that HT inhibited the phosphorylation of all tyrosine residues (Tyr951, Tyr1059, Tyr1175 and Tyr1214) as well as ERK-1/2 and SAPK/JNK (Fig. 5D). For Tax, the inhibition was observed at Tyr1059 while OA inhibited both Tyr951 and Tyr1059 phosphorylations. For these two compounds, SAPK/JNK activation was inhibited but not for ERK. Overall, these results show that HT is the most effective olive oil compound inhibiting VEGF-induced in vitro angiogenesis.

Discussion

VEGF has emerged as an attractive target in antiangiogenesis treatment strategies [11,32]. However, the chronic therapeutic use
of anti-VEGF agents is limited due to significant side effects in some patients [33]. Therefore, in the last few years, major efforts have focused on identifying naturally occurring VEGF inhibitors as chemopreventive agents, which will not significantly alter quality of life, are inexpensive, safe, well tolerated and effective [12]. In this respect, we have demonstrated that EGCG from green tea [34], delphinidin from berries [35] and apigenin from parsley [36] are potent VEGF signaling inhibitors. Several studies have shown that the presence of phenolic antioxidants in olive oils could also prevent the occurrence of a variety of pathological processes, including the development of cancer [17,22,23]. While the strong antioxidant potential of these molecules is well characterized, their antiangiogenic activity remains unknown. Recently, it has been reported that HT affected tumor growth and tumor-associated angiogenesis in a HT-29 (colon cancer cells) xenograft model through the downregulation of hypoxia inducible factor-1α, VEGF, and microsomal prostaglandin-E synthase-1 [37]. Moreover, Yuan et al. [38,39] demonstrated that an ethanolic extract of dried shark muscle mixed with olive oil interfered with VEGF binding to VEGFRs. These results prompted us to further investigate olive oil compounds as a diet-derived source of anti-VEGF agents. The central findings of the current study are that we demonstrate that specific olive oil compounds, such as Tax, OA and HT block VEGF-induced angiogenesis, by inhibiting ECs growth and migration as well as tubulogenesis.

The potential mechanisms behind these inhibitory effects in HUVECs could be attributed to the inhibition of VEGF-induced tyrosine phosphorylation of VEGFR-2, leading to the inhibition of cellular signaling triggered by this receptor. Here, we showed that Tax, OA and HT effectively inhibit VEGF-dependent tyrosine phosphorylation of the VEGFR-2 in a concentration-dependent manner and that this inhibitory effect is associated with an impairment of downstream signaling events triggered by VEGFR-2, such as phosphorylation of p42/p44 MAPK (ERK-1/2), and p46/p54 SAPK/JNK. Moreover, these olive oil compounds exert their effect through the phosphorylation of precise tyrosine residues on the cytoplasmic portion of VEGFR-2. Our data showed that Tax, OA and HT inhibited the major autophosphorylation sites on VEGFR-2 in HUVECs, such as Tyr951, Tyr1059, Tyr1175 and Tyr1214. Interestingly, failure in the phosphorylation of one of these tyrosine residues and/or that of ERK-1/2 or SAPK/JNK pathways has been proven to alter EC migration and proliferation [6,31,40–43]. As such, Tyr1059 of VEGFR-2 is required for VEGF-induced MAPK activation and EC proliferation, whereas Tyr951 is essential for EC migration [40]. The phosphorylation of Tyr1175 residue conveys the VEGF signal to the ERK pathway, which is critically required for the subsequent stimulation of EC proliferation [6]. However, the autophosphorylated tyrosines within VEGFR-2 responsible to SAPK/JNK pathway activation, which is critical for VEGF-induced angiogenesis [31,43], still remain unknown. It was reported that the phosphorylation of Tyr1214 is responsible to the activation of SAPK2/p38 by VEGF but not of ERK [41]. Our data revealed a possible involvement of Tyr1214 in SAPK/JNK activation. Indeed, the HUVECs treatment using HT showed an IC_{50} of 8.4 μM for pTyr1214, similar to that obtained for pSAPK/JNK with an IC_{50} of 9.9 μM. Furthermore, since HT...
inhibited both Tyr1059 phosphorylation and SAPK/JNK activity with a similar IC₅₀, it seems that the phosphorylation of Tyr1059 residue would also mediate SAPK/JNK activation in HUVECs. We extend these observations by showing that the inhibition of Tyr1059 phosphorylation, but not of Tyr1214, by Tax and OA is correlated to the inhibition of pSAPK/JNK in HMVECs. Although the exact mechanisms underlying the inhibitory effect of olive oil compounds on this pathway remain to be determined, it is tempting to speculate that Tyr1059 of VEGFR-2 could mediate SAPK/JNK pathway activation. Overall, it is, therefore, possible that blocking ERK and SAPK/JNK pathways and/or VEGFR-2 phosphorylation with Tax, OA and HT may lead to the inhibition of VEGF-induced proliferation and migration of HUVECs in a concentration- and time-dependent manner, and consequently, reduced EC ability to differentiate into capillary-like structures. These inhibitory effects seem to be specific to Tax, OA and HT, because the other olive oil compounds tested (Tyr and OL) were less potent. However, it is not excluded that these two compounds may affect other aspects of angiogenesis such as matrix metalloproteinase activities [44] or basement membrane synthesis [45].

For assessing the relationship between the in vivo antiangiogenic effects of isolated compounds and their concentration in olive oil, their concentrations in human plasma must be determined [46]. The majority of the studies on bioavailability of olive oil compounds have focused on phenolic compounds, because the fatty acids such as OA, are rarely available as free fatty acids in vivo and thus, represent the basic structural components of triglycerides, phospholipids and cholesterol esters [47]. These studies have shown that the phenolic compounds are concentration-dependently absorbed and highly bioavailable, further supporting the putative health promoting effects of these compounds [48,49]. In humans, it was estimated that at least 55–66% of ingested olive oil phenolic compounds is absorbed [49], metabolized and distributed throughout the body, even across the blood–brain barrier [50]. However, the amount of these compounds detected in human plasma is highly variable and depends on the type of olive cultivars, the extraction and analytical procedure of olive oil, the chemical structure of the phenolic compounds and the nutrition customs [49,51]. It was reported that the concentration of phenols in extra virgin olive oil could vary from 50 to 800 mg/kg [52]. Nevertheless, the total plasma concentration of HT after olive oil consumption in humans has been reported to be in the range of 0.1–25 μM [22,52–54]. In our study, we observed that low concentrations of olive oil compounds were sufficient to significantly inhibit VEGFR-2 activity. Indeed, a half maximal inhibition of VEGF-dependent tyrosine residues phosphorylation in VEGFR-2 by the strongest inhibitor

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Fig. 4 – Olive oil compounds inhibit VEGF-induced tyrosine phosphorylation of VEGFR-2 in HUVECs. (A) Quiescent HUVECs were incubated in 1% FBS containing (or lacking) 50 μM of indicated olive oil compounds for 24 h or with various concentrations (5, 10, 15, 25, 35 or 50 μM) of (B) Tax, (C) OA or (D) HT. Then, cells were stimulated with 50 ng/ml of VEGF for 2 min. HUVECs were lysed and the levels of various tyrosine-phosphorylated residues in VEGFR-2, along with their total protein level were monitored by immunoblotting using specific antibodies. Immunodetection obtained from representative samples are shown, and data are representative of three independent experiments. The bands intensities were analyzed by densitometry using ImageJ software and expressed in arbitrary units as a ratio of levels of phosphorylated protein to those of the total protein to correct for variation in the amount of protein. The relative levels of phosphorylated protein were also normalized to those seen in VEGF control (value = 1).
Table 1 – Comparative overview of IC50 values of olive oil compounds on VEGFR-2/MAPK phosphorylation.

<table>
<thead>
<tr>
<th>Olive oil compounds</th>
<th>IC50 (μM)</th>
<th>VEGFR-2 tyrosine residues</th>
<th>pERK-1/2</th>
<th>pSAPK/JNK</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Tyr951</td>
<td>Tyr1059</td>
<td>Tyr1175</td>
</tr>
<tr>
<td>Tax</td>
<td>23.3</td>
<td>26.4</td>
<td>33.0</td>
<td>25.6</td>
</tr>
<tr>
<td>OA</td>
<td>10.8</td>
<td>13.8</td>
<td>50.0</td>
<td>35.0</td>
</tr>
<tr>
<td>HT</td>
<td>19.6</td>
<td>7.4</td>
<td>30.2</td>
<td>8.4</td>
</tr>
</tbody>
</table>

NOTE: IC50 is defined as the concentration (μM) at which olive oil compounds inhibit 50% of VEGF-induced phosphorylation of tyrosine residues within VEGFR-2 or downstream substrates. The IC50 values were calculated using dose-response curves for each condition. Data are representative of three independent experiments.

Fig. 5 – Taxifolin, oleic acid and hydroxytyrosol inhibit VEGF downstream signaling events in ECs. Quiescent HUVECs (A–C) or HMVECs (D) were incubated in 1% FBS containing (or lacking) various concentrations (5, 10, 15, 25, 35 or 50 μM) of indicated olive oil compounds for 24 h before adding VEGF (50 ng/mL). After cell treatments, the extent of the phosphorylated forms of ERK, SAPK/JNK, along with their total protein level, were monitored by immunoblotting using specific antibodies. Immunodetection obtained from representative samples are shown, and data are representative of three independent experiments. The bands intensities were analyzed by densitometry using ImageJ software and expressed in arbitrary units as a ratio of levels of phosphorylated protein to those of the total protein to correct for variation in the amount of protein. The relative levels of phosphorylated protein were also normalized to those measured in VEGF control (value = 1).
HT could be observed at concentrations around 7–30 μM. It is thus tempting to speculate that these concentrations are behaviorally achievable in humans and that the inclusion of olive oil in the diet may exert some chemopreventive effects through the inhibition of tumor-associated angiogenesis.

Taken together, this study provides molecular evidence that Tax, OA and HT potently suppress angiogenesis by targeting VEGFR-2 activation in a concentration-dependent manner. These results provide new mechanisms of action for these olive oil compounds, and contribute to explain, in part, the beneficial effect of the Mediterranean diet in the prevention of cancer.

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Conflict of interest statement

The authors have no conflict of interest.

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REFERENCES

[29] E. Scoditti, N. Galabriso, M. Massaro, M. Pellegrino, C. Storelli, G. Martines, R. De Caterina, M.A. Carlucio, Mediterranean diet polyphenols reduce inflammatory angiogenesis through MMP-9 and COX-2 inhibition in human vascular endothelial cells: a...
S. Lamy, M. Blanchette, J. Michaud-Levesque, R. LaT. Kamba, D.M. McDonald, Mechanisms of adverse effects of anti-
L. Lamalice, F. Houle, J. Huot, Phosphorylation of Tyr1214 within VEGFR-2 triggers the recruitment of Nck and activation of Fyn leading to SAPK/JNK activation and endothelial cell migration in response to VEGF, J. Biol. Chem. 281 (2006) 34009–34020.