Olive oil compounds inhibit the paracrine regulation of TNF-α-induced endothelial cell migration through reduced glioblastoma cell cyclooxygenase-2 expression☆

Sylvie Lamy[*,1], Aroua Ben Saad[1], Alain Zgheib, Borhane Annabi

Laboratoire d'Oncologie Moléculaire, Centre de Recherche BioMed, Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Montréal, QC, Canada H3C 3P8

Abstract

The established causal relationship between the chronic inflammatory microenvironment, tumor development and cancer recurrence has provided leads for developing novel preventive strategies. Accumulating experimental, clinical and epidemiological data has provided support for the chemopreventive properties of olive oil compounds traditionally found within the Mediterranean diet. In this study, we investigated whether tyrosol (Tyr), hydroxytyrosol, oleuropein and oleic acid (OA), four compounds contained in extra virgin olive oil, can prevent tumor necrosis factor (TNF)-α-induced expression of cyclooxygenase (COX)-2 (an inflammation biomarker) in a human glioblastoma cell (U-87 MG) model. We found that Tyr and OA significantly inhibited TNF-α-induced COX-2 gene and protein expression, as well as PGE_2 secretion. Both compounds also inhibited TNF-α-induced JNK and ERK phosphorylation, whereas only Tyr inhibited TNF-α-induced NF-κB phosphorylation. Paracrine-regulated migration of human brain microvascular endothelial cells (HBMECs) was assessed using growth factor-enriched conditioned media (CM) isolated from U-87 MG cells. We found that while PGE_2 triggered HBMEC migration, the CM isolated from U-87 MG cells, where either COX-2 or NF-κB had been silenced or had been treated with Tyr or OA, exhibited decreased chemotactic properties. These observations demonstrate that olive oil compounds inhibit the effect of the chronic inflammatory microenvironment on glioblastoma progression through TNF-α actions and may be useful in cancer chemoprevention.

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1. Introduction

Glioblastoma multiforme (GBM) is the most common and malignant type of astrocytoma (WHO Grade IV glioma) of the central nervous system (CNS) [1]. Despite extensive research in the treatment

* Corresponding author at: Laboratoire d'Oncologie Moléculaire, Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Québec, Canada, H3C 3P8. Tel.: +1-514-987-3000x0317; fax: +1-514-987-0246.

E-mail addresses: lamy.sylvie@uqam.ca (S. Lamy), ben_saad.aroua@courrier.uqam.ca (A. Ben Saad), zgheib.alain@uqam.ca (A. Zgheib), annabi.borhane@uqam.ca (B. Annabi).

[1] These authors contributed equally to this work.

of GBMs, the combination treatment of surgery, radiotherapy and chemotherapy does not yet allow patients to live more than approximately 15 months [2]. Indeed, GBMs are among the most difficult cancers to treat due to their genetic heterogeneity, high invasive growth and vascularization [3]. The invasive nature of GBMs not only accounts for local tumor recurrence but is also responsible for breakdown of the blood–brain barrier (BBB) and cerebral edema formation causing serious symptoms in these patients [4].

Inflammation processes promote tumor development and contribute to glioma recurrence [5]. Proinflammatory mediators therefore play an essential role in the regulation of CNS disorders as well as in modulating BBB functions [6]. There is a correlation between patients with GBM and the presence of specific biomarkers in the serum that could regulate angiogenesis and inflammation processes [7]. Tumor necrosis factor alpha (TNF-α) is one of the proinflammatory cytokines which have received particular attention over the past few years, in part due to its ability to contribute to glioblastoma development [8].

Cyclooxygenase (COX)-2, one of the enzymes responsible for causing inflammation, has been detected in a variety of human malignant tumors [9] and has been shown to induce brain edema [10]. There are two isoforms, COX-1 and COX-2, which convert arachidonic acid (AA) into several eicosanoids such as prostaglandin, thromboxanes and prostacyclin [11]. Whereas COX-1 is constitutively expressed in most tissues, COX-2 is an inducible enzyme, stimulated by growth

factors, oncogenes, tumor promoters or inflammatory cytokines such as interleukin (IL)-1β and TNF-α [12]. COX-2 plays a key role in the release and activity of proangiogenic proteins, such as prostaglandin E$_2$ (PGE$_2$), that directly stimulate endothelial cell migration and angiogenesis [13] and has recently been associated with vascular endothelial growth factor (VEGF)-independent angiogenesis [14]. Furthermore, COX-2 inhibition led to VEGF pathway blockade and suppressed tumor vascularization and prevented metastasis. These results demonstrate the importance of the COX-2/PGE$_2$ pathway in mediating autocrine/paracrine mechanisms, which support tumor growth and which may represent a potential target for the prevention/treatment of cancer.

To overcome some of the therapeutic challenges in the treatment of GBM patients, novel approaches are required to prolong survival. Over the past few years, there has been a growing interest in nutraceutical interventions, which have been investigated for application toward different types of tumors, including brain tumors [15,16]. This approach uses the antiinflammatory and chemopreventive properties of naturally occurring agents, especially those which originate from the vegetables, spices and fruits in our diet [17,18]. Along these lines, accumulating experimental, clinical and epidemiological data indicates the advantages to the traditional Mediterranean diet which is characterized by high consumption of foods from plant origin as well as relatively low consumption of red meat [19]. The benefits of such a diet have been shown against cardiovascular diseases, chronic degenerative diseases and some types of cancers [20,21].

This diet is rich in olive oil, especially extra virgin olive oil (EVOO), which exhibits antioxidant and antiinflammatory actions contributing to the prevention of colorectal, prostate, lung, endometrial and breast cancers [20–26]. The chemopreventive ability of EVOO is not only due to fatty acids but also to its content of phenolic compounds such as polyphenols and flavonoids [27]. In support of this, it has been reported that oleuropein (OL), the most abundant phenolic compound in olives [28], inhibits LN-18 glioblastoma cell migration [29]. Moreover, oleocanthal, an antiinflammatory compound which has a chemical structure similar to ibuprofen [30], activates adenosine monophosphate-activated protein kinase to down-regulate COX-2 expression in HT-29 colon cancer cells [31]. However, to our knowledge, aside from a study by our own group [32], no one has yet measured the activity of olive oil compounds against proinflammatory cytokines in glioblastoma cells. Considering the need for chemoprevention intervention against glioblastoma progression, fundamental studies are required to gain insight into the impact of olive oil compounds on cancer-associated processes. Here, we investigated the effects of three phenolic compounds (Hydroxytyrosol, HT; OL; Tyrosol, Tyr) and of a monounsaturated fatty acid (oleic acid, OA) on TNF-α-induced COX-2 expression in glioblastoma cells. Moreover, the effect of COX-2 inhibition within the tumor microenvironment on endothelial cell migration was also examined.

2. Materials and methods

2.1. Materials

Olive oil compounds HT, OL, Tyr (purity≥98%) and OA (purity≥99%) were purchased from Extrasynthese (Lyon, France). Human recombinant TNF-α and PGE$_2$ were obtained from EMD Millipore Corporation (Billerica, MA, USA). AA was from Cayman Chemical Company (Ann Arbor, MI, USA). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON, USA). The anti-ERK (extracellular signal-regulated kinase 1 and 2) (K-23) polyclonal antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Advanced Immunochemical Inc. (Long Beach, CA, USA). Antibodies for NF-κB p65, SAPK/JNK (c-Jun amino-terminal kinase), phospho-NF-κB p65, phospho-SAPK/JNK polyclonal antibodies and phospho-ERK monoclonal antibodies were from Cell Signaling Technology (Beverly, MA, USA). The anti-COX-2 monoclonal antibody was from BD Transduction Laboratories™ (Franklin Lakes, NJ, USA). Antimouse and antirabbit horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and enhanced chemiluminescence (ECL) reagents were from Denville Scientific Inc. (Metuchen, NJ, USA). Microbicinchoninic acid protein assay reagents were from Thermo Scientific (Rockford, IL, USA). All other reagents were from Sigma–Aldrich (Oakville, ON, USA).

2.2. Cell culture

A human glioblastoma cell line (U-87 MG) was purchased from the American Tissue Culture Collection (HTB-14™) and maintained in modified Eagle's Minimum Essential Medium (Wisent, 320-036-CL) containing 10% calf serum (HyClone Laboratories, SH30541.03), 1mM sodium pyruvate (Sigma–Aldrich, P2256), 2mM L-glutamine, 100units/ml penicillin and 100μg/ml streptomycin (Wisent, 450-202-EL). Human brain microvascular endothelial cells (HBMECs) were from ScienCell™ Research Laboratories (Carlsbad, CA, USA) and maintained in RPMI Medium (Wisent, 350-007-CL) containing 10% fetal bovine serum (Life Technologies, 12483–020), Nu-serum™ (VWR, CACB355500), endothelial cell growth supplement (EMD Millipore Corporation, 02–102) and 1mM sodium pyruvate (Sigma–Aldrich, P2256). HBMEC used in this study were restricted to use between passages 4 and 8. Cells were cultured at 37 °C under a humidified 95%–5% (v/v) mixture of air and CO$_2$. Cells were treated with vehicle (0.1% ethanol) or with olive oil compounds and stimulated with TNF-α. All cellular assays were performed at 85% confluence.

2.3. Western blot analysis

To study the effects of olive oil compounds on COX-2 protein expression, U-87 MG cells were serum-starved in the presence of one of these molecules for 24 h, and then the medium was replaced by fresh medium containing 25ng/ml TNF-α for 24 h. To study the phosphorylation status of NF-κB p65, ERK and JNK, the cell medium was replaced by fresh, serum-free medium for 30 min prior to cell stimulation with 25ng/ml TNF-α for 5 min. Cells were then washed once with ice-cold phosphate-buffered saline (PBS) containing 1 mM each of NaF and Na$_3$VO$_4$ and followed by incubation in the same buffer solution for 30 min at 4 °C. The cells were solubilized on ice in lysis buffer [150mM NaCl, 10mM Tris–HCl, pH7.4, 1mM EDTA, 1mM ethyleneglycol-O, O'-bis(2-aminoethyl)-N, N, N', N'-tetraacetic acid (EGTA), 0.5% (vol/vol) Nonidet P-40 and 1% (vol/vol) Triton X-100]. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes which were then blocked 1 h at 4 °C with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (TBS-T; 147mM NaCl, 20mM Tris–HCl, pH 7.5 and 0.1% Tween 20). Membranes were further washed in TBS-T and incubated overnight with an appropriate primary antibody in TBS-T containing 3% bovine serum albumin and 0.01% sodium azide (NaN$_3$), followed by a 1-h incubation with an HRP-conjugated antimouse or antirabbit secondary antibody in TBS-T containing 5% nonfat dry milk. Immunoreactive material was visualized with an ECL detection system. The immunoreactive bands were quantified using ImageJ software (NIH).

2.4. Total RNA isolation, cDNA synthesis and real-time quantitative PCR

Total RNA was extracted from U-87 MG monolayers using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). cDNA was stored at -80 °C prior to PCR. Gene expression was quantified by real-time quantitative PCR using iQ SYBR Green Supermix (BIO-RAD, Hercules, CA, USA). DNA amplification was carried out using an Icycler iQ5 (BIO-RAD, Hercules, CA, USA), and product detection was performed by measuring the binding of the fluorescent dye SYBR Green I to double-stranded DNA. The following primer sets were provided by QIAGEN (Valencia, CA, USA): COX-2 (QT00040586), β-Actin (QT01680476), GAPDH (QT00079247) and peptidylpropyl isomerase A (PPIA; QT01866137). The relative quantities of target gene mRNA against an internal control, β-Actin/GAPDH/PPIA RNA, were measured by following a ΔCt method employing an amplification plot (fluorescence signal vs. cycle number). The difference (ΔCt) between the mean values in the triplicate samples of target gene and those of β-Actin/GAPDH/PPIA RNA were calculated using the iQ5 Optical System Software version 2.0 (BIO-RAD, Hercules, CA, USA), and the relative quantified value was expressed as $2^{-\Delta Ct}$. Semiquantitative PCR was also performed to validate single amplification products which were resolved on 1.8% agarose gels containing 1mg/ml ethidium bromide (not shown).

2.5. Cytotoxicity assays

The sensitivity of U-87 MG cells to olive oil compounds was determined in vitro by using the WST-1 assay (Roche Diagnostics, Montreal, QC, Canada). Briefly, after cotreatment of cells with TNF-α and olive oil compounds for 24 h, U-87 MG cells were exposed to 10 μL of the WST-1 tetrazolium salt reagent. The soluble formazan dye produced by metabolically active cells was monitored for 60 min at 37 °C. The absorbance at 450 nm was measured using a SpectraMax Plus reader (Molecular Devices, Sunnyvale, CA, USA).

2.6. Determination of PGE$_2$ levels from cell supernatants

U-87 MG cells were exposed to either vehicle or olive oil compounds (100 μM) for 24 h before the addition of 25ng/ml TNF-α for 24 h. Cell culture media were subjected to low-speed centrifugation to remove cell debris, and the amount of PGE$_2$ protein secreted by U-87 MG cells was determined using the PGE$_2$ EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA) in accordance with the supplier's instructions.

2.7. Transfection method and RNA interference

U-87 MG cells were transiently transfected with 20nM siRNA (Qiagen Sciences; Germantown, MD, USA) against COX-2 (SI03038672), NF-κB p65 (SI01399622) or scrambled sequences (AllStar Negative Control siRNA, 1027281) using the Lipofecta-mine 2000 transfection reagent (Invitrogen, CA, USA). Cells were treated for 24 h in the presence or absence of 25ng/ml TNF-α. Small interfering RNA and mismatch siRNA were synthesized by QIAGEN and annealed to form duplexes.

2.8. Cell migration assay by xCELLigence biosensor system

Experiments were carried out using the Real-Time Cell Analyser (RTCA) Dual-Plate (DP) Instrument, the xCELLigence system (Roche Diagnostics, QC, Canada). This system was used according to the instructions of the supplier. Firstly, U-87 MG cells were serum-starved for 24 h. Then, cells (25,000 cells/well) were seeded in serum-free medium onto a CIM-Plates 16 (Roche diagnostics). These plates are similar to conventional Transwells (8μm pore size) with gold electrode arrays on the bottom side of the membrane, which provide a real-time measurement of cell migration. Prior to cell seeding, the underside of the wells from the upper chamber was coated with 25 μL of 0.15% gelatin in PBS and incubated for 1 h at 37 °C. The lower chamber was filled with either growth factor-enriched conditioned media (CM) derived from U-87 MG cells treated or not with olive oil compounds (100 μM), TNF-α (25 ng/ml), or siCOX-2 (20 nM), or with serum-free medium containing (or lacking) PGE$_2$ (50 ng/ml) or olive oil compounds. Prior to cell seeding, the underside of the wells from the upper chamber was coated with 25 μL of 0.15% gelatin in PBS and incubated for 1 h at 37 °C. The upper chamber of each well was filled with 100 μL of U-87 MG cells (2.5×10^5 cells/ml). After 30 min of adhesion, cell migration was monitored every 5 min for 8 h. The impedance value was measured by the RTCA DP Instrument and was expressed as an arbitrary unit called the *Cell Index* which reflecting the amount of migration-active cells. Each experiment was performed in duplicate wells.

2.9. Statistical analyses

Statistical analyses were assessed with Student's *t* test when one group was compared with the control group. To compare two or more groups with the control group, one-way analysis of variance with Dunnett's post hoc test was used. Differences with $P<0.05$ were considered significant. All statistical analyses and graphs were performed using the GraphPad Prism software Version 5.0b (San Diego, CA, USA).

3. Results

3.1. Olive oil compounds inhibit TNF-α-induced COX-2 gene and protein expression in human glioblastoma cells

IL-β-induced COX-2 expression in U-87 glioblastoma cells was previously reported by us to be inhibited by the flavonoid luteolin, which is found in olive oil [32]. The effects of other major olive oil compounds (Fig. 1) on another cytokine which induces COX-2, that is, TNF-α, were examined. U-87 MG cells were incubated for 24 h in serum-free medium in the presence or absence of 100μM olive oil compounds. The medium was then replaced with fresh serum-free medium, and the cells were stimulated with 25 ng/ml of recombinant TNF-α for 24 h. Under these conditions, TNF-α caused a marked increase in COX-2 expression (Fig. 2A), whereas it was significantly inhibited by 61.7% for OA and 36.5% for Tyr but not by HT or OL (Fig. 2B). As shown in Fig. 2C–D, transcriptional inhibition of COX-2 by the same compounds was observed (65.0% inhibition for OA and 36.2% inhibition for Tyr) and was not related to cell death (Fig. 2D). The half maximal inhibition concentrations (IC$_{50}$) of OA and Tyr on TNF-α-induced COX-2 expression were 15.7 μM and 69.6 μM, respectively (Fig. 2E–F).
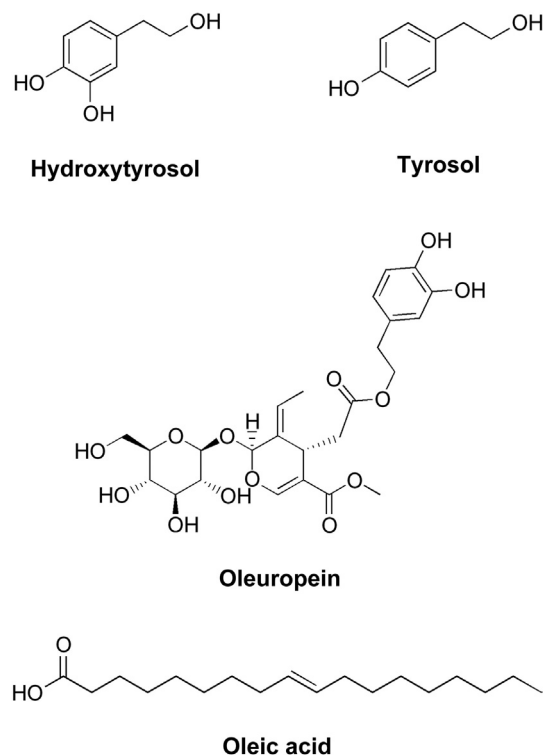

Fig. 1. Chemical structures of olive oil compounds.

3.2. OA and Tyr alter TNF-α-induced downstream signaling events in human glioblastoma cells

To investigate the mechanisms involved in the inhibitory actions of OA and Tyr on TNF-α-induced COX-2 expression, we further examined the effects of these compounds on TNF-α-induced NF-κB and MAPK signaling pathways. Although ERK and JNK signaling pathways have been reported to be activated by this proinflammatory cytokine [33], little is known about their activation status in glioblastoma cells, unlike TNF-α-induced NF-κB [34,35]. U-87 MG cells were pretreated for 24 h with various concentrations of olive oil compounds in serum-free medium. The medium was then replaced with fresh serum-free medium lacking compounds, and the cells were incubated for 30 min. After that, the cells were stimulated with 25 ng/ml of recombinant TNF-α for 5 min. Total protein expression and phosphorylation status of downstream signaling intermediates possibly targeted by OA or Tyr were assessed by immunoblotting using specific antibodies. Exposure of U-87 MG cells to TNF-α markedly induced phosphorylation of NF-κB, ERK and JNK, as determined by the ratio of unphosphorylated to phosphorylated proteins (Fig. 3). Both OA and Tyr compounds suppressed TNF-α-induced phosphorylation of ERK (Fig. 3B) and JNK (Fig. 3C), whereas pNF-κB was unaffected by OA (Fig. 3A). Treatment with these olive oil compounds resulted in a concentration-dependent inhibition of the TNF-α-induced down-stream signaling pathways. For OA and Tyr treatments, the IC$_{50}$ values obtained were 51.0 μM and 20.5 μM for pERK inhibition (Fig. 3B) and 11.5 μM and 81.3 μM for pJNK inhibition (Fig. 3C), respectively. The phosphorylation of NF-κB was inhibited by Tyr treatment with an IC$_{50}$ value of 15.2 μM (Fig. 3A; right panel). Overall, these results suggest that OA is efficient at inhibiting TNF-α-induced COX-2 expression via a JNK-dependent pathway, whereas Tyr better targets the ERK and NF-κB signaling in U-87 MG cells.
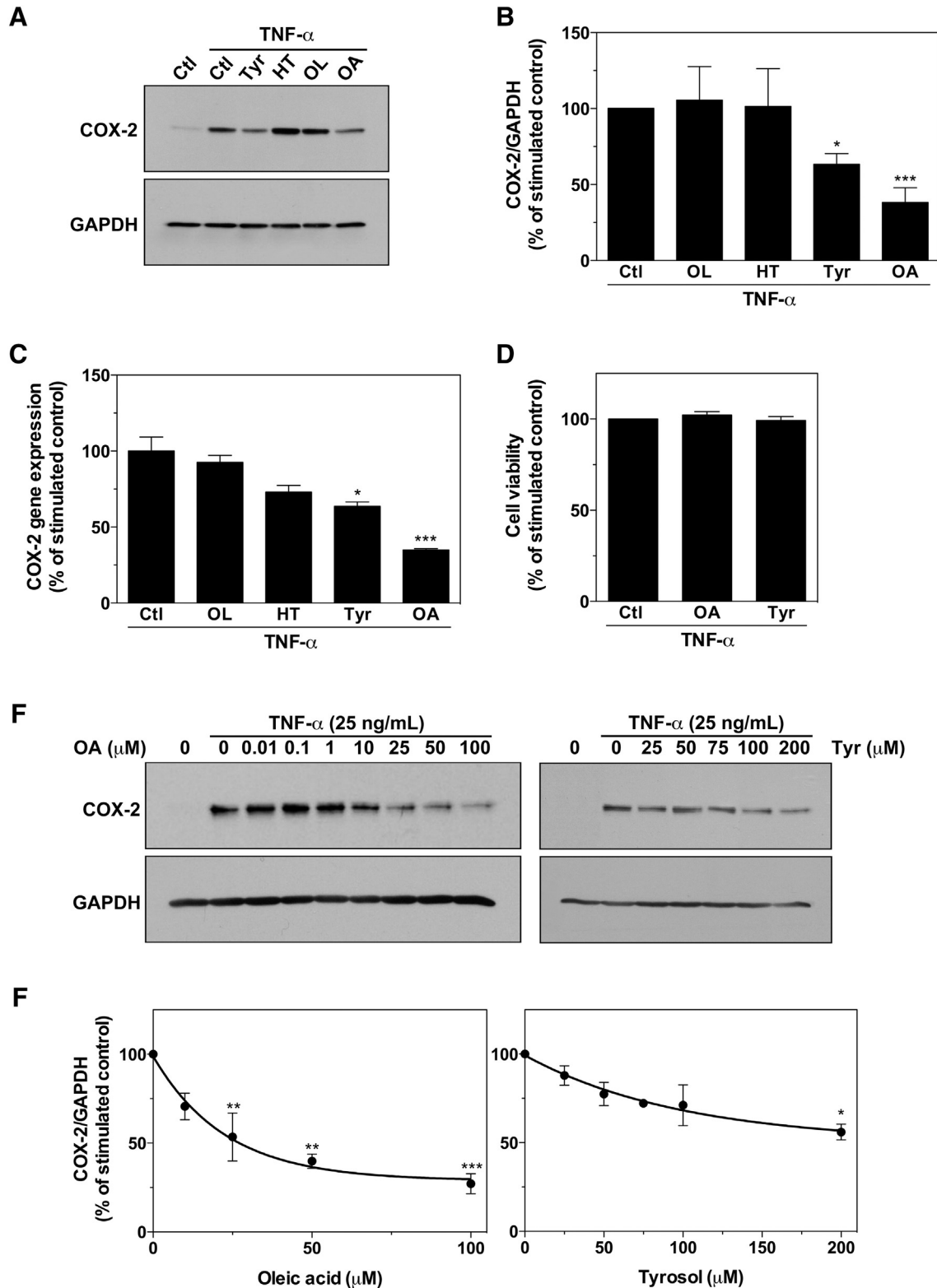
Fig. 2. Olive oil compounds inhibit TNF-α-induced COX-2 gene and protein expression in human glioblastoma cells. U-87 MG cells were serum-starved in the presence or absence of 100 μM of the indicated olive oil compounds. The medium was then replaced with fresh, serum-free medium, and the cells were stimulated with 25 ng/ml of recombinant TNF-α for 24 h. (A) Cells were lysed, and the levels of COX-2 protein expression were monitored by immunoblotting. Immunodetections obtained from representative experiments are shown. (B) The band intensities were analyzed by scanning densitometry using ImageJ software, and the quantification of three independent experiments is shown. Values are means±S.E.M. ($^{*}P<0.05$ and $^{***}P<0.001$ vs. TNF-α alone). For each sample, the COX-2 level was normalized for GAPDH. (C) Total RNA was isolated from conditions described above; cDNA synthesis and qPCR were performed to assess COX-2 gene expression. Values are means±S.E.M. of four independent experiments ($^{*}P<0.05$ and $^{***}P<0.001$ vs. TNF-α alone). (D) Cell viability was assessed by WST-1 assay, as described in the Methods section. Values are means of two independent experiments performed in sextuplicate. (E) U-87 MG cells were serum-starved in the presence or absence of various concentrations of the indicated olive oil compounds for 24 h, and then the medium was replaced by fresh medium containing 25ng/ml TNF-α for 24 h. Immunodetections obtained from representative experiments are shown. (F) The band intensities obtained for each olive oil treatment were analyzed and corrected for GAPDH. The quantification of four independent experiments is shown. The relative levels of COX-2 protein expression were also normalized to those seen in TNF-α control (value=100). Values are means±S.E.M. ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. TNF-α alone).

3.3. OA and Tyr inhibit TNF-α-induced PGE₂ secretion by human glioblastoma cells

We next investigated whether TNF-α increased PGE₂ release by U-87 MG cells. As shown in Fig. 4, TNF-α triggered higher release of PGE₂ in the medium (28-fold increase) compared to untreated U-87 MG cells, and this result was comparable to that obtained with AA (31-fold increase), known to produce PGE₂ by COX-2 [36]. OA and Tyr at 100 μM significantly reduced TNF-α-induced PGE₂ levels in cell culture media by 45.4% and 71.5%, respectively.

3.4. Gene silencing of COX-2 or of NF-κB p65 abrogates the TNF-α-mediated paracrine regulation of HBMECs migration

The actions of PGE₂ on tumor-associated angiogenesis are thought to involve endothelial cell migration, proliferation and tube formation [13,37,38]. Since PGE₂ is produced in large quantities by COX-2-overexpressing tumors [39], we analyzed the role of COX-2-mediated paracrine regulation of microvascular endothelial cell migration. Gene silencing was performed in U-87 MG cells, and the specificity of COX-2 knockdown was confirmed (Fig. 5A–B, left panel). Moreover, COX-2
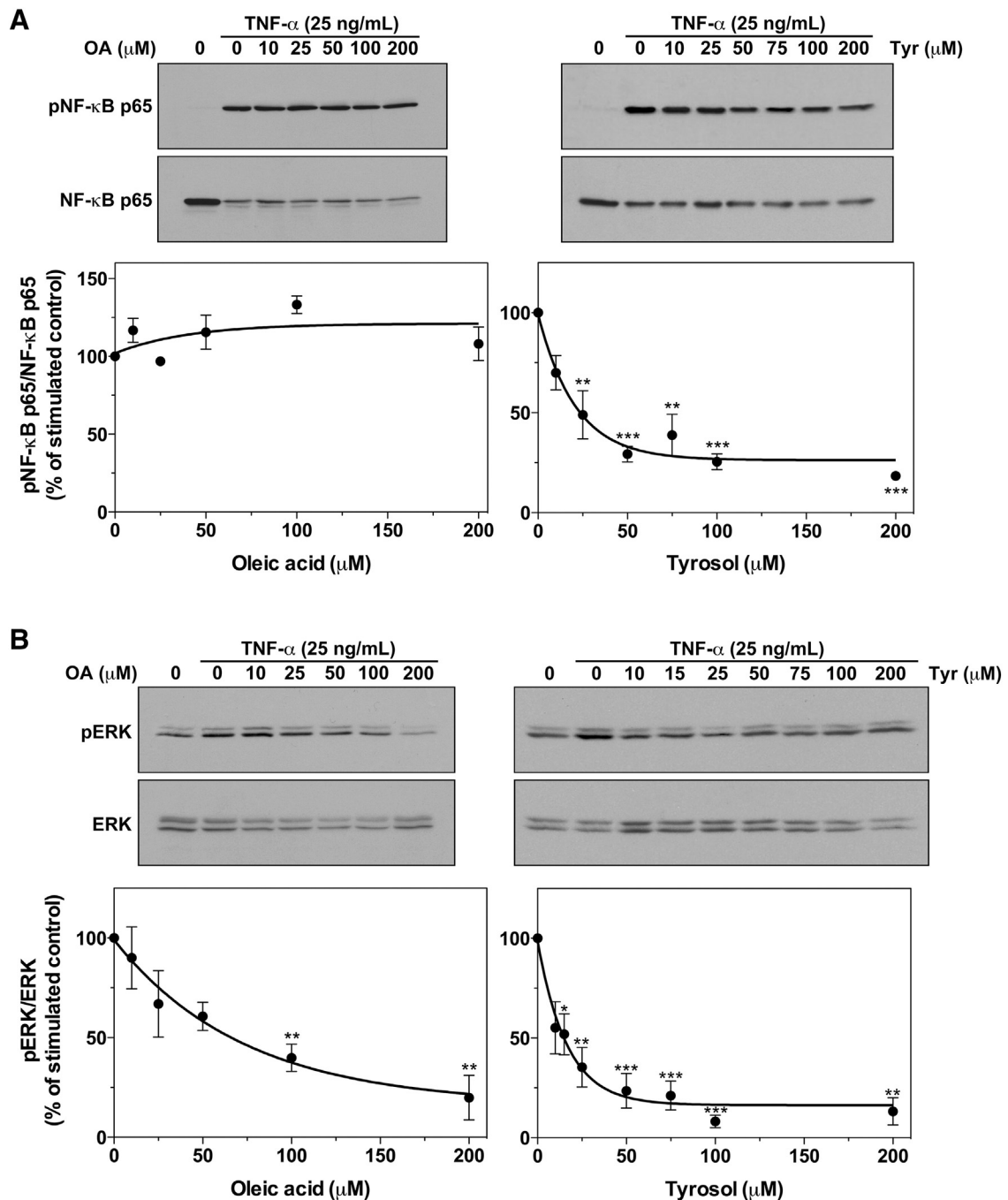

Fig. 3. OA and Tyr alter TNF-α-induced downstream signaling events in human glioblastoma cells. U-87 MG cells were serum-starved in the presence of various concentrations of the indicated olive oil compounds for 24 h. The medium was then replaced by fresh serum-free medium for 30 min prior to cell stimulation with 25ng/ml TNF-α for 5 min. After these treatments, the phosphorylated forms of (A) NF-κB, (B) ERK or (C) JNK, along with their total protein levels, were monitored by immunoblotting. Immunodetections obtained from representative experiments are shown (top panels). The band 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 (bottom panel). The relative levels of phosphorylated protein were also normalized to those seen in TNF-α control (value=100). Values are means±S.E.M. of three independent experiments (*P<0.05, **P<0.01 and ***P<0.001 vs. TNF-α alone).
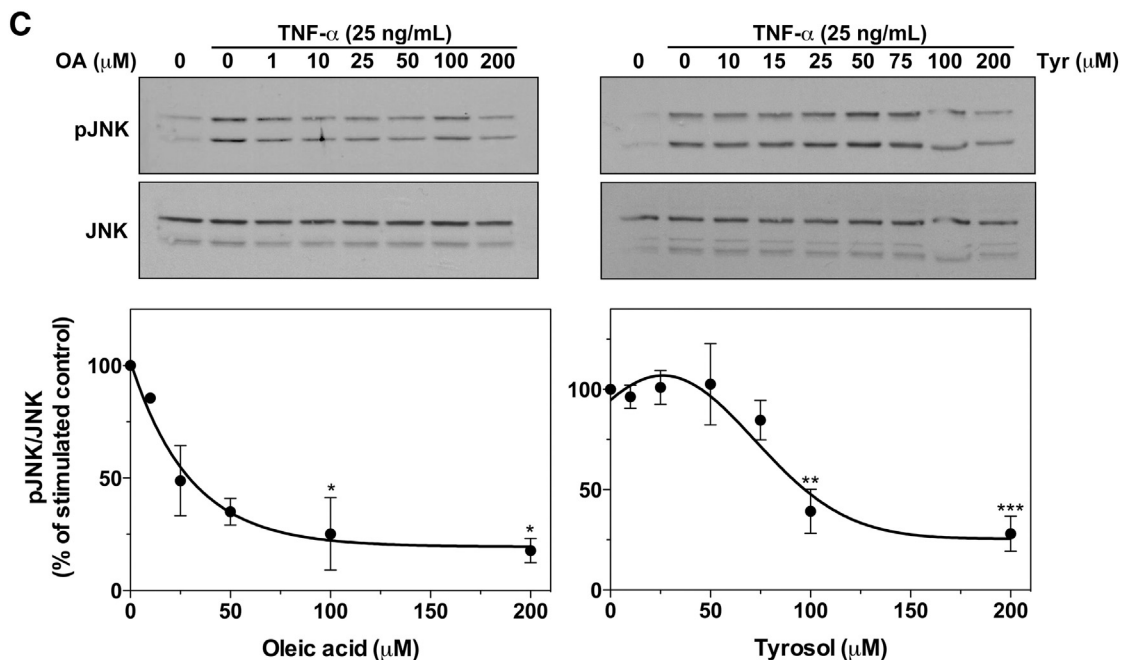
Fig. 3. (continued)

silencing also prevented TNF-α from affecting the COX-2/PGE$_2$ signaling axis involved in HBMEC migration. In evidence of this, the CM harvested from U-87 MG cells where COX-2 expression was silenced was able to prevent TNF-α-mediated HBMEC migration (Fig. 5C, left panel) suggesting that the secretion of PGE$_2$ was abrogated. Interestingly, the TNF-α-induced migration of HBMECs was also inhibited beyond the control level upon COX-2 silencing. Since VEGF is up-regulated by TNF-α [8] and that COX-2 is directly involved with VEGF production [40], COX-2 silencing, as used in our assay, may inhibit HBMEC migration through both the suppression of TNF-α-induced COX-2 expression and VEGF secretion in U-87 MG cells.

It was reported that the activation of NF-κB in tumor cells contributed to the expression of several proangiogenic genes essential for endothelial cell migration [41]. Therefore we analyzed the role of NF-κB-mediated paracrine regulation of HBMECs. The specificity of NF-κB p65 knockdown in U-87 MG cells was first confirmed (Fig. 5A–B, right panel). The CM harvested from U-87 MG cells where NF-κB p65 expression was silenced prevented TNF-α-mediated HBMEC migration (Fig. 5C, right panel). The fact that siNF-κB p65 down-regulated COX-2 protein expression in U-87 MG cells (Fig. 5A, right panel) confirms the involvement of the NF-κB pathway in the up-regulation of the COX-2/PGE$_2$ system by TNF-α, which is important in the paracrine regulation of HBMEC migration.

3.5. OA and Tyr inhibit HBMEC migration

Since the vascular microenvironment is important in promoting glioblastoma growth, we analyzed the paracrine effect of CM derived from U-87 MG cells on HBMEC migration when treated with the two most potent olive oil compounds inhibitors, OA and Tyr, in the presence of TNF-α. As shown in Fig. 6A–B, the HBMEC migration induced by the CM from U-87 MG cells treated with TNF-α was inhibited in a concentration- and time-dependent manner by OA and Tyr with IC$_{50}$ values being observed at concentrations of 60.4 μM for OA (Fig. 6A) and 70.3 μM for Tyr (Fig. 6B).

After having demonstrated that OA and Tyr inhibited both TNF-α-induced COX-2 expression and PGE$_2$ secretion from U-87 MG cells, we next addressed whether OA or Tyr inhibit HBMEC migration by directly targeting the chemotactic activity of PGE$_2$. We measured cell migration after incubation of HBMECs with 50 ng/ml of recombinant PGE$_2$ in the presence or absence of 100 μM of olive oil compounds. We found that PGE$_2$ stimulated HBMEC migration as compared to control cells and that increase was completely abolished by Tyr, whereas OA had no inhibitory effect (Fig. 6C). These results indicate that these olive oil compounds blocked endothelial cell migration through different cellular mechanisms.

4. Discussion

The incidence of brain cancer has markedly increased during the last few decades. GBMs present several challenges related to cancer cell proliferation and resistance to antiangiogenic, antimetastatic and antiinflammatory therapies [3]. A high level of COX-2 expression has been detected in gliomas, and its expression has been correlated with the histopathological grade [9]. Recent studies have shown that many COX-2 inhibitors, such as nonsteroidal antiinflammatory drugs, could act as efficient agents for cancer prevention as well as for intervention alone or in combination with current chemotherapy and radiation modalities [42,43].

TNF-α has been reported to be up-regulated following radiation therapy in GBM patients [44] and that dietary antioxidants can reduce the incidence of brain tumors by down-regulating TNF-α or by scavenging free radicals [45]. Here, we demonstrate for the first time that two specific olive oil compounds, Tyr and OA, inhibited TNF-α-induced COX-2 expression in U-87 MG cells and PGE$_2$ released through different signaling pathways. Although both compounds inhibited TNF-α-induced phosphorylation of MAPK, Tyr preferentially inhibited the phosphorylation of ERK as opposed to OA which reduced JNK phosphorylation. Moreover, the TNF-α-induced phosphorylation of NF-κB is inhibited by Tyr, but not by OA. Consequently, the observed inhibition of PGE$_2$ secretion was more affected by Tyr.
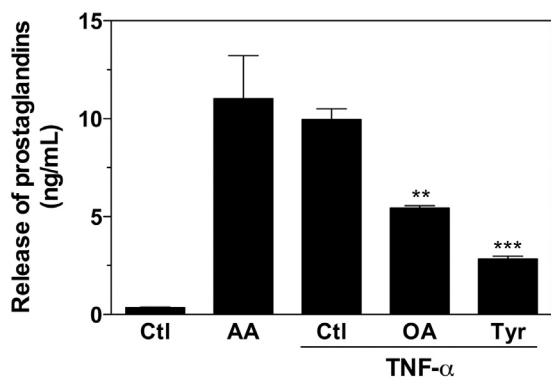
Fig. 4. OA and Tyr inhibit TNF-α-induced PGE$_2$ secretion by human glioblastoma cells. U-87 MG cells were serum-starved in the presence or absence of 100 μM of the indicated olive oil compounds before the addition of 25ng/ml TNF-α for 24 h. Cell culture media were collected, and the levels of PGE$_2$ were measured by enzyme immunoassay. Values are means±S.E.M. of two independent experiments performed in triplicate ([**]$P<0.01$ and [***]$P<0.001$ vs. TNF-α alone).

It was reported that the transforming growth factor-β-induced activation of ERK triggered the release of PGE$_2$ in osteoblastic cells which, in turn, mediated cell proliferation [46]. Although speculative, such a mechanism could be similarly involved with TNF-α in U-87 MG cells. The phosphorylation of ERK could either be directly activated by TNF-α or indirectly through an autocrine growth factor-mediated process involving TNF-α. Consequently, activated ERK could up-regulate COX-2 expression [47] as well as PGE$_2$ synthesis and secretion [46]. The findings that TNF-α-induced COX-2 expression was more affected by OA than TNF-α-induced ERK activation and PGE$_2$ secretion in U-87 MG cells, and that the angiogenic action of PGE$_2$ on HBMEC migration remained unaltered, support the separate involvements of these two crucial mechanisms in the regulation of TNF-α-mediated inflammation.

The molecular regulation of COX-2 gene expression is tightly regulated at both the transcriptional and posttranscriptional levels in physiological conditions or dysregulated in pathophysiological conditions such as cancer [31]. To our knowledge, the effect of olive oil compounds on the transcription of COX-2 mRNA or its stability still remains poorly understood. However, it has been reported that JNK pathway is involved in the up-regulation of COX-2 expression by LPS (bacterial lipopolysaccharide) possibly through enhanced COX-2 mRNA decay in activated murine J774 macrophages [48]. Since both Tyr and OA suppressed TNF-α-induced phosphorylation of JNK in our study, it is tempting to speculate that such a mechanism could also be involved in the inhibitory effect of these compounds. Among the nuclear factors shown to stabilize and increase the expression of COX-2 mRNA, HuR has been reported to regulate COX-2 stability [49]. The binding of HuR within the 3'-untranslated region of COX-2 seems to be critical for its posttranscriptional mRNA stabilization. It has been reported that triptolide, a natural compound isolated from *Tripterygium wilfordii* Hook.f., reduced TNF-α-induced COX-2 expression in human non-small cell lung cancer, and the mechanism of action was related to an HuR-mediated decrease of mRNA stability [50]. In support, we previously reported that the green tea polyphenol EGCG decreased the mRNA stabilizing factor HuR expression in HL-60 myeloid leukemia cells [51]. However, Tyr and OA did not inhibit HuR mRNA expression in the presence of TNF-α in U-87 MG cells suggesting that these compounds did not exert their inhibitory effect on COX-2 mRNA expression via HuR (data not shown). However, olive oil compounds could suppress other stabilizing factors such as tristetraproline [52] or CUG-BP2 [53] or COX-2 gene transcription.

GBMs are among the most invasive and vascularized tumors. Interaction of GBM cells with their tumor microenvironment is necessary for their growth, which is limited by the emergence of new blood vessels via angiogenesis [54]. Angiogenesis and inflammation are closely linked [55], and it was reported that TNF-α induces expression of VEGF in gliomas, leading to increased brain tumor angiogenesis [8]. In the present study, in order to recapitulate some features of the GBM microenvironment *in vitro*, growth factor-enriched media from U-87 MG cells were generated, and their paracrine effects on the migration of HBMECs were analyzed. We first demonstrated that HBMEC migration was attenuated with CM isolated from U-87 MG cells with COX-2 or NF-κB p65 silenced genes, supporting a role for COX-2 in paracrine-regulated angiogenesis. Second, the CM from U-87 MG cells stimulated with TNF-α and treated with OA or Tyr altered endothelial cell migration. Interestingly, it was reported that PGE$_2$ could synergize with TNF-α to promote the transcriptional activity of NF-κB p65 in tumor cells [56] which, in turn, regulates the expression of genes involved in endothelial cell migration [41]. Thus, the observed inhibition of PGE$_2$-induced endothelial cell migration by Tyr correlates well with the inhibitory effects of this compound on the activation of key pathways mediating PGE$_2$ release from U-87 MG cells, such as NF-κB p65 and ERK, and, to a much lesser extent, COX-2.

It has recently been reported that two olive oil polyphenols, HT and OL, inhibited inflammatory angiogenesis through the suppression of ROS-mediated NF-κB p65-dependent COX-2 and MMP-9 expression in human vascular endothelial cells [57]. In fact, these compounds inhibited the phorbol myristate acetate (PMA)-induced COX-2 protein expression and prostanoid production as well as MMP-9 protein release and gelatinolytic activity. It has also been reported that HT inhibited PMA-induced MMP-9 and COX-2 activity and expression in activated human monocytes via NF-κB and protein kinase C (PKC)α and PKCβ1 suppression [41]. Since it has been reported that TNF-α did not induce significant secretion of MMP-9 in both the cell lines used in our study, U-87 MG cells and HBMECs [30,56], this then indicates that the modulation of MMP-9 is not implicated.

We previously reported that OA but not Tyr inhibited VEGFR-2 phosphorylation and its downstream pathway, leading to the inhibition of VEGF-induced human umbilical vein endothelial cell proliferation and migration [58]. Since OA exerted no inhibitory effect on PGE$_2$-induced HBMEC migration, it is possible that the inhibition observed by the CM from U-87 MG cells stimulated with TNF-α and treated with OA is attributable not only to COX-2 expression inhibition but also to alternate angiogenic factors secreted in the medium such as VEGF.

Given the pleiotropic modes of action of OA and Tyr, a combination of these compounds could be beneficial for preventing tumor progression. EVOO provides a considerable amount of OA, but it is rarely available as a free fatty acid *in vivo* and is found as a basic structural component in triglycerides, phospholipids and cholesterol esters [59]. Thus, the majority of the studies on olive oil compound bioavailability have only focused on the potential healthful effects of phenolic compounds. These studies have shown a concentration-dependent absorption of olive oil phenols in humans [60]. The apparent absorption of these compounds was at least 55–66% of the ingested olive oil, which was metabolized and distributed throughout the body, even across the BBB, and was reexcreted as HT and Tyr in urine [61,62]. Depending on the type of olive cultivars and on nutritional custom, the concentration of phenols in EVOO could vary from 50 to 800 mg/kg [63]. Moreover, the plasma concentration of phenolic compounds after olive oil consumption in humans has been reported to be in the range of 0.1–59 μM [64,65]. It is thus tempting to speculate that the concentrations required for inhibitory effects, as observed in the current study, are behaviorally achievable in humans.

While most studies focus on one compartment independently of the other, our study provides evidence for a molecular and cellular
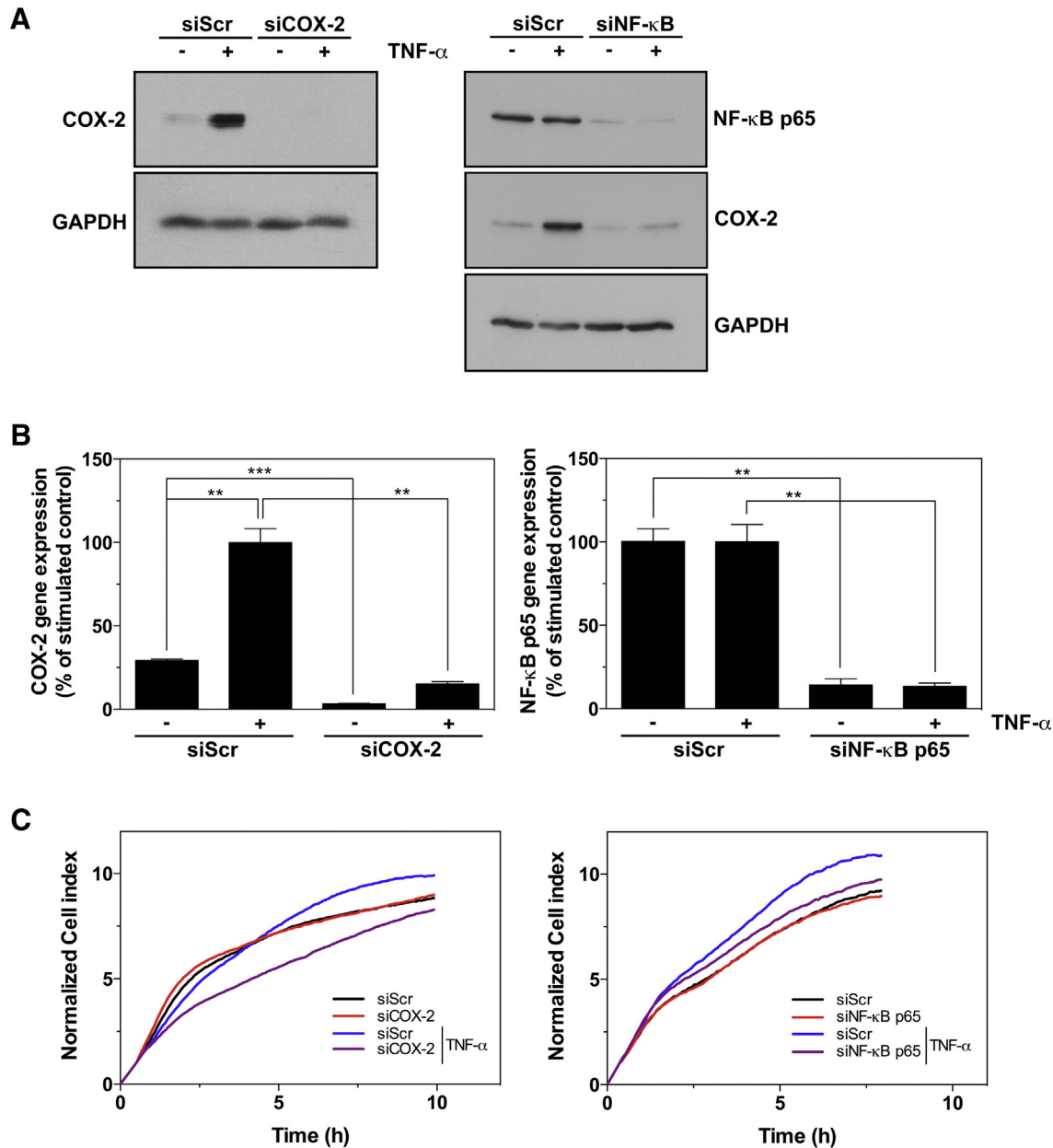
Fig. 5. Gene silencing of COX-2 or NF-κB p65 abrogates TNF-α-mediated paracrine regulation of HBMEC migration. (A–B) U-87 MG cells were transiently transfected with siRNA against COX-2 (siCOX-2) or NF-κB p65 (si NF-κB p65) or with a scrambled sequence (siScr) as described in the Methods section. (A) Representative Western blots are shown for the expression of COX-2 and NF-κB p65, with GAPDH used as a loading control. (B) Total RNA was extracted, and qRT-PCR was used to assess COX-2 (left panel) and NF-κB p65 (right panel) gene expression in the siScr, siCOX-2 and siNF-κB p65-transfected cells upon treatment with 25ng/ml TNF-α for 24 h. Values are means±S.E.M. of two independent experiments ([**]P<0.01 and [***]P<0.001 vs. TNF-α alone). (C) HBMECs were starved for 24 h before adhesion onto a CIM-Plates coated with 0.15% gelatin. Then cells were incubated with CM derived from U-87 MG cells treated (or not) with TNF-α (25 ng/ml) or siCOX-2 (20 nM) (left panel) or siNF-κB p65 (20 nM) (right panel) as described in the Methods section. The rate of cell migration was monitored in real-time using the xCELLigence system. Representative examples from two experiments measuring impedance responses are shown for each condition. The normalized Cell Index at the base time is set to 1 in all wells.

interplay between the brain tumor and the brain endothelial compartments. Overall, our data therefore suggest that the chronic inflammatory microenvironment, which drives glioblastoma growth and which contributes to their neovascularization and invasive characteristics, may be efficiently prevented through the consumption of EVOO. Given that cancer development and progression is a multistep process, supplementation with olive oil may represent an efficient dietary intervention in the prevention and/or management of glioblastoma.

Conflict of interest

The authors have no conflicts of interest.
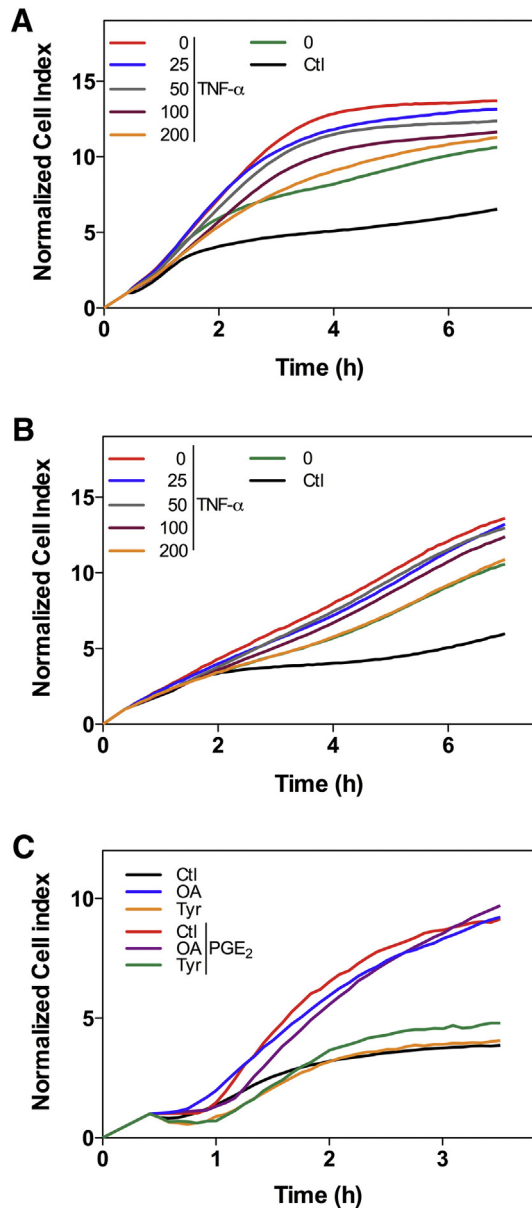
Acknowledgements

Fig. 6. OA and Tyr inhibit HBMEC migration. HBMEC migration was induced with either CM from U-87 MG cells treated or not with TNF-α (25 ng/ml), various concentrations of (A) OA or (B) Tyr, or (C) with serum-free medium containing (or lacking) PGE$_2$ (50 ng/ml) or olive oil compounds (100 μM) as described in the Methods section. The rate of cell migration was monitored in real-time using the xCELLigence system. Representative examples from two experiments measuring impedance responses are shown for each condition. The normalized Cell Index at the base time is set to 1 in all wells. For (A) and (B), the negative controls were represented by green line (CM from U-87 MG cells) and black line (serum-free media for U-87 MG cells culture).

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