Food & Function

PAPER

View Article Online
View Journal | View Issue

Cite this: *Food Funct.*, 2014, **5**, 1270

Extra virgin olive oil polyphenolic extracts downregulate inflammatory responses in LPS-activated murine peritoneal macrophages suppressing NFκB and MAPK signalling pathways

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Received 9th January 2014
Accepted 8th March 2014

DOI: 10.1039/c4fo00014e

www.rsc.org/foodfunction

Extra virgin olive oil (EVOO) is obtained from the fruit of the olive tree *Olea europaea* L. Phenolic compounds present in EVOO have recognized anti-oxidant and anti-inflammatory properties. However, the activity of the total phenolic fraction extracted from EVOO and the action mechanisms involved are not well defined. The present study was designed to evaluate the potential anti-inflammatory mechanisms of the polyphenolic extract (PE) from EVOO on LPS-stimulated peritoneal murine macrophages. Nitric oxide (NO) production was analyzed by the Griess method and intracellular reactive oxygen species (ROS) by fluorescence analysis. Moreover, changes in the protein expression of the pro-inflammatory enzymes, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and microsomal prostaglandin E synthase-1 (mPGES-1), as well as the role of nuclear transcription factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK) signalling pathways, were analyzed by Western blot. PE from EVOO reduced LPS-induced oxidative stress and inflammatory responses through decreasing NO and ROS generation. In addition, PE induced a significant down-regulation of iNOS, COX-2 and mPGES-1 protein expressions, reduced MAPK phosphorylation and prevented the nuclear NFκB translocation. This study establishes that PE from EVOO possesses anti-inflammatory activities on LPS-stimulated murine macrophages.

Introduction

Over the years, the so-called Mediterranean diet has become widely associated with improved health and well-being as well as protection against cardiovascular diseases and colon, breast and skin cancers.[1] Epidemiological and clinical studies have demonstrated that low chronic disease risk observed in Mediterranean areas seems to be ascribed to high intake of fruits and oils from the olive tree *Olea europaea* L.[2] Among them, extra virgin olive oil (EVOO) is obtained from the fruits solely by mechanical or other physical means under conditions that do not lead to oil alteration. Within this context, the beneficial effect of EVOO consumption has been ascribed to non-polar lipids[3] or its high monounsaturated fatty acid (MUFA) content present in the major fraction of EVOO (98–99%).[4] However, EVOO also contains multiple minor components with important biological properties. Nowadays, it is clear that many of the beneficial effects of ingesting EVOO are due to its minor polyphenol compounds such as flavonoids, lignans (acetoxypinoresinol), secoiridoids (oleuropein-aglycone and ligstroside aglycone) and their hydrolysis products hydroxytyrosol (HT) and tyrosol (Ty), respectively, among others. These compounds have shown a broad spectrum of bioactive properties, including anti-oxidant, free radical scavenging, anti-inflammatory and chemopreventive effects.

The anti-inflammatory effects of polyphenolic compounds have been largely described and attributed primarily to their capacity to scavenge and prevent both reactive oxygen species (ROS) and nitrogen species formation.[1,5] However, at cellular level, the activity of the total phenolic fraction extracted from EVOO and the plausible action mechanisms have not been completely described.

Macrophages are major inflammatory and immune effector cells, having a key role in the pathogenesis and development of inflammatory chronic diseases.[6–8] The exposition to bacterial lypopolyssacharide (LPS), which acts as an endotoxin, drives the macrophages to an activated state where an excess of inflammatory mediators such as nitric oxide (NO) and prostaglandin (PGE_2), as well as several pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, are produced. In addition, enzymes for example, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) are major

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effectors of the inflammation, which are well-regulated by mitogen-activated protein kinase (MAPK) family activation and nuclear transcription factor kappa B (NFκB) activation.[9–11]

Taking this background into account, the present study was designed to investigate the antioxidant and anti-inflammatory effects of the total phenolic fraction extracted from EVOO (PE) on LPS-stimulated murine macrophages. Also, we attempted to characterize the cellular mechanisms underlying PE possible anti-inflammatory activities, evaluating NO production, intracellular ROS and protein expression of COX-2, iNOS and mPGES-1. Moreover, we studied the role of MAPK and NFκB signalling pathways involved in these beneficial effects.

Results

Effects of PE on cell viability in LPS-stimulated murine peritoneal macrophages

After 18 h, our data demonstrated that cell viability was not significantly reduced at concentrations up to 100 μg mL^{-1} for EVOO's PE on murine macrophages (data not shown). Based on these results, subsequent assays were carried out at concentrations of 25 and 50 μg mL^{-1}. The concentration of HT (50 μM) was selected based on the HT concentration present in 50 μg mL^{-1} of PE (41.71 μM).

PE inhibits LPS-induced intracellular ROS production in murine peritoneal macrophages

Oxygen free radicals are suggested to be signalling messengers in the LPS-mediated inflammatory response. Thus, we tested the effects of EVOO's PE and HT on LPS-induced intracellular ROS production using the fluorescent probe DCFH-DA, which can be oxidized to the highly fluorescent compound DCF. As shown in Fig. 1, cells incubated using different treatments for 18 h exhibited significant decreases in intracellular ROS production; EVOO's PE (25 and 50 μg mL^{-1}) ($P < 0.01$ *vs.* DMSO control) and HT (50 μM) ($P < 0.05$ *vs.* DMSO control). H_2O_2 (100 μM) was used as the pro-oxidant positive control.

NO_2^- production and iNOS expression are inhibited by PE in LPS-stimulated murine peritoneal macrophages

In macrophages and invading immune cells, the high amount of NO produced by iNOS in response to LPS and/or inflammatory cytokines plays a crucial role in inflammation and cytotoxicity. Thus we examined whether EVOO's PE inhibited NO release from activated macrophages. As shown in Fig. 2A, nitrite production as an indicator of NO production was substantially higher in cells treated with LPS than in those treated with the vehicle control. However both EVOO's PE and HT treatments significantly exhibited lower NO_2^- levels, 12.54% for 25 μg mL^{-1}, 9.09% for 50 μg mL^{-1} EVOO's PE ($P < 0.001$ *vs.* DMSO control) and 20.06% for HT treated cells ($P < 0.001$ *vs.* DMSO control). This diminution of NO_2^- production suggested a possible down-regulation of iNOS enzyme activity. Thus, this hypothesis was assessed by measuring the iNOS protein levels. Immunoblotting analysis demonstrated that the decrease in NO_2^- levels was paralleled by a significant decrease in iNOS protein levels after incubation for 18 h using

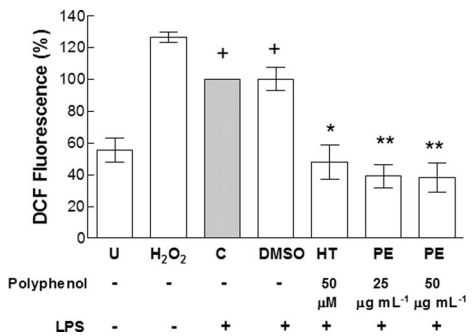

Fig. 1 Intracellular ROS generation reduced by PE and HT. Intraperitoneal murine isolated macrophages pretreated with LPS were incubated with PE (25 and 50 μg mL^{-1}) or HT (50 μM) for 18 h. Then, cells were harvested and incubated with 10 μM of DCFH-DA for 30 min at 37 °C in the dark. Results were expressed as mean fluorescence intensity obtained ± S.E.M. ($n = 3$). +$P < 0.05$ *vs.* untreated cells and *$P < 0.05$, **$P < 0.01$ *vs.* LPS–DMSO treated control cells. H_2O_2 was used as the pro-oxidant positive control.

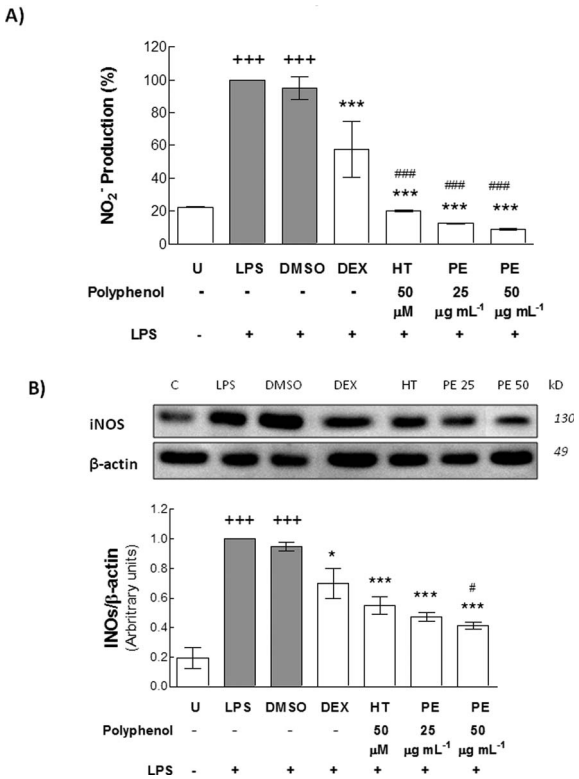

Fig. 2 Effect of PE and HT on release of nitrite generation. (A) Nitrite generation and (B) densitometric analysis of iNOS protein expression. The plots represent the band intensity and were measured using Image J software. β-Actin served as an equal loading control for normalization. Each value represents the mean ± S.E.M. for three independent experiments. +++$P < 0.001$ *vs.* untreated cells, *$P < 0.05$, ***$P < 0.001$ *vs.* LPS–DMSO treated control cells and #$P < 0.05$, ###$P < 0.001$ *vs.* LPS–DEX. Dexamethasone (1 μM) was used as the positive control.

both treatments, EVOO's PE (25 and 50 μg mL^{-1}) ($P < 0.001$ *vs.* DMSO control) and HT (50 μM) ($P < 0.001$ *vs.* DMSO control) (Fig. 2B). Dexamethasone (1 μM) was used as the anti-inflammatory positive control. PE and HT treatments were significantly more efficient in inhibiting NO_2^- production and PE than dexamethasone treatment ($P < 0.001$ PE or HT *vs.* DEX) (Fig. 2A). Moreover, statistical significant differences between data from cells treated with PE or DEX were found in iNOS protein levels ($P < 0.05$ PE *vs.* DEX) (Fig. 2B).

PE produced down-regulation in COX-2 and mPGES-1 overexpression induced by LPS

We subsequently investigated the possible effects of EVOO's PE and HT on COX-2 inflammation-related enzyme. COX-2 protein expression was markedly induced by LPS treatment (Fig. 3A). However, a significant down-regulation on this proinflammatory protein expression was observed in cells treated with 50 μg mL^{-1} PE ($P < 0.05$ *vs.* DMSO control). Likewise, no statistical significant down-regulation from cells treated with 25 μg mL^{-1} PE or 50 μM HT on COX-2 expression was found. However 50 μM HT seems to be more active than 25 μg mL^{-1} PE.

Similarly, LPS stimulation resulted in a marked expression of the mPGES-1 protein ($P < 0.001$ *vs.* untreated cells) (Fig. 3B). Though, 50 μg mL^{-1} PE, but not 50 μM HT treatments prior to LPS stimulation, resulted in significant inhibition of LPS-induced mPGES-1 protein expression ($P < 0.05$ *vs.* DMSO control).

PE reduces p38 and JNK phosphorylation in LPS-stimulated murine peritoneal macrophages

To further explore the molecular mechanism underlying the anti-inflammatory effect of PE, we also determined its role in MAPK activation by Western blot analysis using phosphospecific MAPK antibodies. Cells were incubated in the absence or presence of different concentrations of PE before LPS stimulation. LPS induced the appearance of phosphorylated JNK and p38 ($P < 0.05$ and $P < 0.01$ *vs.* untreated cells), whereas PE treatment demonstrated to inhibit significantly JNK and p38 activation (25 μg mL^{-1}, $P < 0.05$ and $P < 0.01$; 50 μg mL^{-1}, $P < 0.05$ and $P < 0.01$ *vs.* DMSO control, respectively) (Fig. 4). However, after 18 h, HT treatment demonstrated the inability to inhibit the activation of JNK or p38 (Fig. 4).

PE inhibits NF-κB-mediated transcriptional activation and IκBα degradation in murine peritoneal macrophages

Since NFκB activity is controlled by the steady state level of IκBα, we further investigated IκB degradation in murine peritoneal

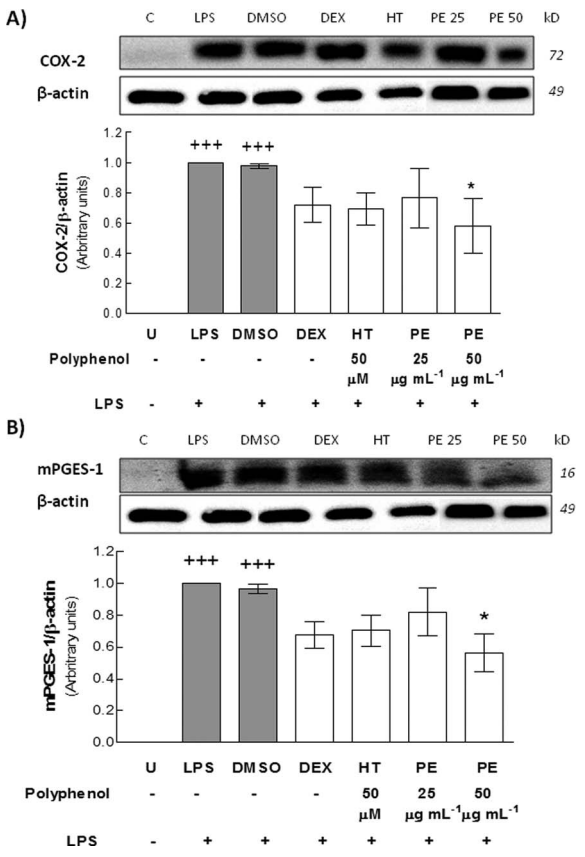

Fig. 3 PE inhibits COX-2 (A) and mPGES-1 (B) protein expression in murine intraperitoneal isolated macrophages. Cells were treated with PE (25 and 50 μg mL^{-1}) or HT (50 μM) for 18 h in the presence of LPS. As controls, cells were also treated with DMSO (solvent control) or left untreated in the absence of LPS. The plots represent the band intensity. β-Actin served as an equal loading control for normalization. Data are represented as mean ± S.E.M. +++$P < 0.001$ *vs.* untreated cells and *$P < 0.05$ *vs.* LPS–DMSO treated control cells. Dexamethasone (1 μM) was used as the positive control.

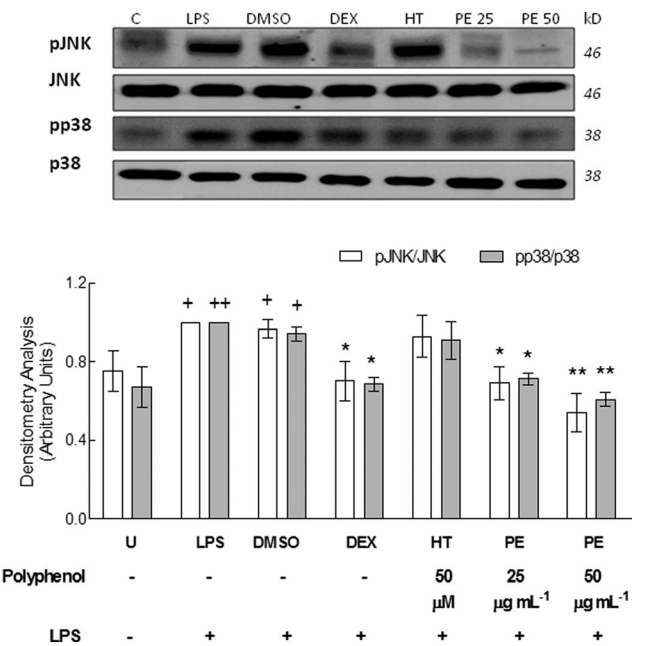

Fig. 4 Effects of PE and HT on pJNK and pp38 signalling pathways in murine intraperitoneal isolated macrophages. The results are representative of three independent experiments. Densitometry was performed following normalization to the control (JNK and p38 housekeeping genes, respectively). Data are expressed as the means ± S.E.M. +$P < 0.05$ and ++$P < 0.01$ *vs.* untreated cells and *$P < 0.05$ and **$P < 0.01$ significantly different from LPS–DMSO treated control cells. Dexamethasone (1 μM) was used as the positive control.

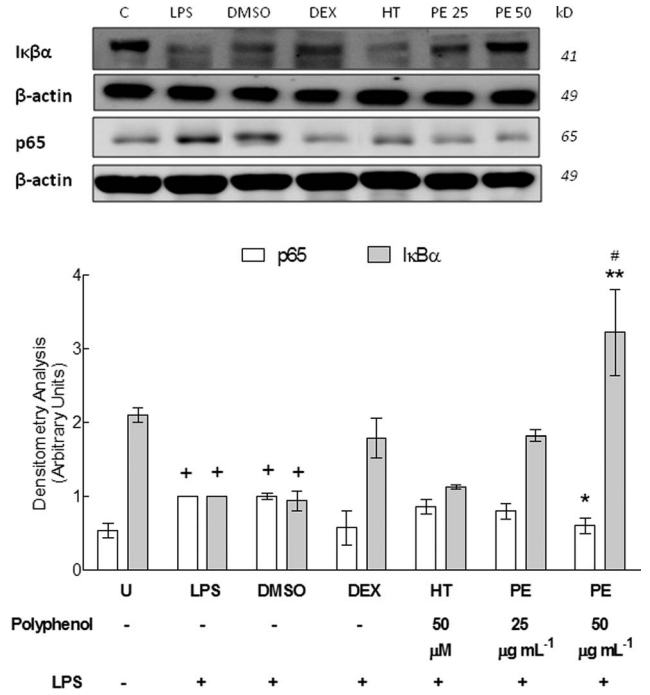

Fig. 5 PE treatment inhibits NFκB-mediated transcriptional activation and IκBα degradation in murine intraperitoneal isolated macrophages. Results are representative of three independent experiments. Densitometry was performed following normalization to the control (β-actin house-keeping gene). Data are expressed as the means ± S.E.M. $+P < 0.05$ *vs.* untreated cells, $*P < 0.05$ and $**P < 0.01$ *vs.* LPS–DMSO treated control cells and $\#P < 0.05$ *vs.* LPS–DEX. Dexamethasone (1 μM) was used as the positive control.

macrophages. LPS stimulation produced IκBα degradation which was consistent with an up-regulation of NFκB-binding activity (Fig. 5) ($P < 0.05$ *vs.* untreated cells). In contrast, PE treatment at 50 μg mL^{-1} prevented IκBα degradation and subsequently ubiquitination ($P < 0.01$ *vs.* DMSO control). Moreover, PE treatment caused a significant parallel inhibition of NFκB-mediated transcriptional activation, preventing the nuclear translocation level of p65 protein in murine macrophages, which was previously increased after LPS stimulation ($P < 0.05$ *vs.* DMSO control). HT treatments did not produce any change in IκBα or p65 protein expression. Dexamethasone (1 μM) was used as the positive anti-inflammatory control in all protein assays. 50 μg mL^{-1} PE treatment was significantly more efficient in inhibiting IκBα degradation than dexamethasone treatment ($P < 0.05$ PE *vs.* DEX) (Fig. 5).

Discussion

Our findings have shown, for the first time, that PE from EVOO prevented the progression of cellular damage induced by LPS reducing the ROS levels and acting as an effective anti-oxidant.

Balance disruption of the intracellular reduction–oxidation state has been observed in activated macrophages, which leads to oxidative stress characterized by a major shift in the cellular redox balance and usually accompanied by ROS-mediated damage. Besides, ROS are capable of eliciting a variety of pathological changes, including the peroxidation of lipids, proteins, and DNA. Therefore, modulators of ROS production and ROS-induced signalling pathways, especially in macrophages, could represent potential targets for anti-inflammatory intervention.[12] Our findings are in agreement with other *in vitro* studies where EVOO-isolated polyphenols *i.e.* HT or oleuropein showed strong anti-oxidant effects acting as powerful scavengers of free radicals in a similar range and higher than those tested in our study (50 to 100 μM).[13,14]

On the other hand, excess of NO acts as an intracellular messenger which modulates the formation of endogenous ROS that orchestrate the inflammatory responses.[12,15] It has been reported that increased NO production in activated macrophages is due to increased levels of inducible iNOS expression, which in turn increase the transformation of L-arginine to NO.[16] In the present study we found that exposure of peritoneal macrophages to LPS resulted in a significant increase of nitrite/nitrate levels as an indicator of NO production and an upregulation of iNOS expression and PE and HT treatments significantly were able to inhibit these effects. These results are in accordance with previous reports of Richard *et al.*, (2011) where HT prevented the up-regulation of iNOS. Similar data were obtained by Zhang *et al.*, (2009) in THP-1.[17,18] Thus we suggest that PE and HT may decrease intracellular oxidant stress by direct ROS scavenging including reduced intracellular activation of redox-sensitive genes and subsequent downregulation of iNOS.

In addition, blocking of inflammatory enzymes like COX-2 or mPGES-1 represents important pharmacological tools for the treatment of inflammatory related diseases.[19] mPGES-1 is an efficient downstream enzyme for the production of PGE_2 in LPS-activated macrophages, thus a selective inhibitor of mPGES-1 would be expected to inhibit PGE_2 production induced by inflammation while sparing constitutive PGE_2 production.[20] Moreover, this enzyme is co-localized and functionally coupled with COX-2 which catalyzes the two sequential steps in the biosynthesis of PGs from arachidonic acid playing a critical role in the inflammatory response.[21,22] The treatment of LPS-stimulated murine macrophages with PE, but not with 50 μM of HT, produced down-regulation of both mPGES-1 and COX-2, indicating a potential dual action on these proteins and suggesting an inflammatory activity reduction. As such, the highest concentration used of PE (50 μg mL^{-1}) contents about 50 μM of HT; we might propose that this dual down-regulation implicated more olive oil polyphenols than HT.

MAPKs are a family of serine–threonine kinase enzymes which includes extracellular signal-regulated kinases ERKs-1 and -2, JNKs and p38 MAPKs. These proteins orchestrate the recruitment of gene transcription, protein biosynthesis, cell cycle control, apoptosis, and differentiation and allow cells to respond to oxidative stress and inflammatory stimuli.[23] Similarly, the MAPK pathway is a critical axis essential for both induction and propagation of the inflammatory in the LPS-activated macrophage response.[24] LPS was found to induce the appearance of phosphorylated JNK and p38, in contrast our results demonstrated that JNK and p38 activation were reduced by PE treatment, but not by HT. Although there are a few studies

about the effects of olive oil polyphenols in the MAPK pathway, a recent study by our research group has also demonstrated that a diet made with EVOO and enriched with PE downregulated MAPK activation in an experimental ulcerative colitis model.[25] The fact that we did not find any effect on MAPK regulation after HT treatment while PE exerted a significant inhibition in p38 and JNK-posporilation, even at 25 μg mL^{-1}, may significantly suggest a possible synergic effect between different compounds of PE.

NFκB signalling plays a key role in mediating inflammation and immune response through induction of pro-inflammatory cytokines, chemokines and other proteins. NFκB, as a dimeric transcription factor composed of p65 (RelA), RelB, c-Rel, NFκB1 (p50/p105) or NFκB2 (p52/p100), exists in the cytoplasm as an inactive complex with the inhibitory protein, IκBα. When cells are challenged with pro-inflammatory stimuli, for example LPS, IκBα undergoes phosphorylation and subsequently ubiquitination, allowing NFκB to translocate to the nucleus. Consequently, NFκB binds to κB enhancer elements present in the promoter region of many pro-inflammatory genes, for instance iNOS and COX-2.[26,27] Our data showed that 18 h after LPS-induction PE significantly inhibited the degradation of IκBα and blocked the translocation of p65 into the nuclei. This capacity of regulating NFκB genes was also described by Brunelleschi *et al.* (2007) who found that an EVOO extract particularly rich in phenolic compounds inhibited p50 and p65 NFκB translocation in monocytes and monocyte-derived macrophages (MDM) isolated from healthy volunteers.[28] On the other hand, other research studies have shown that the anti-inflammatory activity of HT is mediated, at least in part, by NFκB signalling concentrations, however higher concentrations than 50 μM were needed. For instance, Maiuri *et al.* (2005) suggested that HT at concentrations of 200 μM might block the NFκB signalling pathway in J774 murine macrophages,[29] as well as, Zhang *et al.* (2009) proposed that 100 μM, but not 50 μM of HT reduced NFκB-p65 nuclear protein expressions in human monocytes THP-1.[18] However, 50 μg mL^{-1} of PE reduced NFκB translocation in LPS-stimulated macrophages suggesting that more polyphenols than HT are involved in the regulation of the NFκB pathway.

Together these data suggest that the total phenolic fraction extracted from EVOO may exert differential inhibitory effects in comparison with its isolated compounds, HT in this case, on the inflammatory response induced by LPS on murine macrophages.

Experimental

Extraction and chemical characterization of EVOO–polyphenol extract

EVOO (*Olea europaea* L, Picual variety, Aceite de las Valdesas, Córdoba, Spain) batch number 10/32 was used as the matrix to carry out PE. PE was obtained as Vazquez Roncero *et al.*, (1997)[30] described with some modifications. Fifty grams of oil was extracted with methanol : water (80 : 20, v/v, 125 mL). The mixture was centrifuged at 5000*g* for 1 min and sonicated for 15 min. After decantation, the methanolic extract was concentrated in a vacuum under a stream of nitrogen at <35 °C until it reached a syrupy consistency; finally it was lyophilized and stored at −80 °C until use. Quantitative and qualitative analyses of PE were performed according to COI/T20/29doc (International Olive Council) for olive oil based on direct extraction of the phenolic minor polar compounds from olive oil by means of a methanol solution and subsequent quantification by high-performance ternary gradient liquid chromatography (HPLC). After direct extraction of the phenolic minor polar compounds by means of a methanol solution, an aliquot of the supernatant phase was taken and filtered through a 0.45 mm PVDF filter, injected into the HPLC system equipped with a C18 reverse-phase column (4.6 mm × 25 cm), a type Spherisorb ODS-2 5 mm, 100 Å, with a spectrophotometric UV detector at 280 nm and an integrator. The content of the phenols was expressed in mg kg^{-1} of oil and was calculated by measuring the sum of the areas of the related chromatographic peaks. The composition of the isolated PE is detailed in Table 1.

Animals

Male swiss mice (Harlan Interfauna Ibérica, Barcelona, Spain) weighing 20–30 g were placed in cages and maintained under constant conditions of temperature (20–25 °C) and humidity (40–60%) with a 12 h light/dark cycle and fed standard rodent chow (Panlab A04, Panlab, Seville, Spain) and water *ad libitum* throughout the experiment. All experiments were in accordance with the recommendations of the European Union (Directive of the European Counsel) regarding animal experimentation and followed a protocol observed by the Animal Ethics Committee of the University of Seville (approval no.: 86/609/EEC, 24 November 1986).

Isolation and culture of murine peritoneal macrophages

Mice were injected intraperitoneally with 1 mL of sterile thioglycollate medium (10% w/v) (Scharlau, Barcelone, Spain). After 3 days, murine peritoneal macrophages were isolated as described previously by Cárdeno *et al.* (2013).[31] Cells were treated with 5 μg mL^{-1} LPS from *E. coli* (Sigma-Aldrich, St Louis, MO, US) in the presence or absence of PE, HT, or dexamethasone for 18 h. HT (99% pure) was purchased from Extrasynthese (Genay, France) and dexamethasone (99% pure) from Sigma-Aldrich (St Louis, MO, US). Stock solutions were always freshly prepared in dimethylsulfoxide (DMSO) (Panreac, Barcelone, Spain) and diluted to a desired concentration directly in the culture medium. The final concentration of DMSO (Panreac, Barcelona, Spain) in all experiments was always ≤1% and it had not significantly influenced the cell response.

Cell viability

Cells seeded in 96-well plates (1×10^5 cells per well) were incubated in the presence or absence of different PE concentrations for 18 h. At the end of the exposure time, the effect on cell growth/viability was analyzed by sulforhodamine B (SRB) assay. Cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells).[31]

Table 1 Composition of the isolated EVOO polyphenolic extract using COI/T20/29doc

Phenol name	PE composition ppm (mg kg^{-1})	μg phenol (50 μg PE)	μM phenol (50 μg PE per mL)
Hydroxytyrosol	45.00	6.43	41.71
Tyrosol	44.40	6.34	45.94
Vanillic acid	6.10	0.87	5.17
P-coumaric acid	4.00	0.57	3.47
Decarboxymethyl oleuropein aglycone (dialdehyde)	23.04	3.34	10.43
Tyrosyl acetate	6.40	0.91	5.05
Decarboxymethyl ligstroside aglycone (dialdehyde)	24.50	3.50	11.51
Pinoresinol	15.70	2.24	6.25
Cinnamic acid	6.80	0.97	6.54
Acetoxy-pinoresinol	18.40	2.63	6.32
Oleuropein aglycone, aldehyde form	88.20	12.60	39.37
Ligstroside aglycone, dialdehyde form	56.60	8.09	26.61
Luteolin	8.70	1.24	4.33
Apigenin	1.70	0.24	0.89
Total phenols expressed in tyrosol	350 ppm		

Measurement of intracellular ROS

Intracellular ROS production was measured using 2,7-dichlorfluorescein-diacetate (DCFH-DA). DCFH-DA penetrates into the cells and is hydrolyzed by intracellular esterases to the nonfluorescent 2,7-dichlorofluorescein (DCFH), which can be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of ROS. Peritoneal macrophages were seeded at 1×10^6 cells per well in 24-well plates and incubated in the absence or presence of EVOO's PE or HT and 30 min later, cells were stimulated with LPS for 18 h. The fluorescence intensity was measured as described previously by Cárdeno *et al.* (2013).[31] After the incubation time, cells were treated with 10 μM DCFH-DA at 37 °C for 30 min and washed twice with PBS. The fluorescence intensity was measured with a plate reader (BioTek, Bad Friedrichshall, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The results were expressed as the intracellular ROS production percentage compared with LPS–DMSO control cells. H_2O_2 (100 μM) (30% pure) (Panreac, Barcelone, Spain) was used as the pro-oxidant positive control.

Measurement of nitrite production

Cells in a 24-well plate were untreated or treated with selected concentrations of PE, HT or dexamethasone and 30 min later they were stimulated with LPS for 18 h. The amount of nitrite, as an index of NO generation, was determined as described previously by Cárdeno *et al.* (2013).[31] The results were expressed as the nitrite production percentage compared with LPS control cells (stimulated untreated cells). 1 μM dexamethasone 99% (Sigma, St Louis, MO, USA) was used as the positive control.

Isolation of proteins and immunoblotting detection

Cells (1×10^6 cells per mL) were untreated or treated with selected concentrations of PE, HT or dexamethasone, and stimulated with LPS for 18 h. After incubation, the protein concentration was determined following Bradford's colorimetric method.[32] Aliquots of supernatant containing an equal amount of protein (20 μg) were evaluated to determine COX-2 iNOS, mPGES-1, IκBα, p65, pJNK, JNK, pp38, and p38 proteins by Western blot as described by Cardeno *et al.* (2014).[31] The signals were analyzed and quantified by Image Processing and Analysis in Java (Image J, Softonic). 1 μM dexamethasone 99% (Sigma, St Louis, MO, USA) was used as the positive control.

Statistical analysis

All values in the figures and text are expressed as arithmetic mean value ± standard error (S.E.M). Data were evaluated with GraphPad Prism® Version 5.01 software. Comparison was done using one-way analysis of variance (ANOVA) followed by Tukey or Dunnett's test when appropriate. P values of <0.05 were considered statistically significant. The figures shown are representative of at least three different experiments performed on different days.

Conclusions

In conclusion, this study establishes for the first time that the total phenolic fraction extracted from EVOO (PE) inhibits LPS-induced oxidative stress and inflammatory responses *via* direct downregulation of NO and ROS generation. These protective effects seem to be due to downregulation of iNOS, mPGES-1 and COX-2 expression *via* inhibition of MAPK activation and NFκB signalling pathways.

Furthermore, our results suggest that HT plays an important role in the PE anti-oxidant and anti-inflammatory effects. Nevertheless other minor bioactive compounds present in PE such as oleuropein aglycone, tyrosol, pinoresinol or oleocanthal among others might contribute in synergy. In fact, previous reports have described olive oil polyphenols as a kind of natural product with antioxidant and anti-inflammatory properties.[33–36]

Acknowledgements

This work was supported by funds from the Spanish Ministerio de Ciencia e Innovación (AGL 2008-02475, AGL 2011-26949) and Junta de Andalucía (P-10AGR-6609; Proyecto de Excelencia). Authors gratefully acknowledge support from Postgraduate National of FPU and Inncorpora-Programa Torres Quevedo (INNCORPORA-PTQ) from Ministerio de Economía y Competitividad and Social European Fund. The authors also appreciate the assistance of the Center for Technology and Innovation Research, University of Seville (CITIUS). The authors declare no conflict of interest.

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