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ORIGINAL ARTICLE

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Hydroxytyrosol, a phenolic compound from virgin olive oil, prevents macrophage activation

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Abstract We investigated the effect of hydroxytyrosol (HT), a phenolic compound from virgin olive oil, on inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in J774 murine macrophages stimulated with lipopolysaccharide (LPS). Incubation of cells with LPS caused an increase in iNOS and COX-2 mRNA and protein level as well as ROS generation, which was prevented by HT. In addition, HT blocked the activation of nuclear factor-κB (NF-κB), signal transducer and activator of transcription-1α (STAT-1α) and interferon regulatory factor-1 (IRF-1). These results, showing that HT down-regulates iNOS and COX-2 gene expression by preventing NF-κB, STAT-1α and IRF-1 activation mediated through LPS-induced ROS generation, suggest that it may represent a non-toxic agent for the control of pro-inflammatory genes.

Keywords Hydroxytyrosol · NF-κB · IRF-1 · STAT-1α · iNOS · COX-2

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Introduction

Numerous epidemiological data have demonstrated an association between a diet rich in antioxidants, such as the "Mediterranean diet," and a lower incidence of several pathologies (Keys et al. 1986; De Lorgeril et al. 1999). Although the protective effect of such a diet is likely to be multifactorial, there is consistent evidence for an antioxidant activity of some selected polyphenolic compounds from extra virgin olive oil (Owen et al. 2000a,b; Tuck and Hayball 2002; Visioli et al. 2002). Recently it has been shown that virgin olive oil with a high content of polyphenolic compounds has protective effects on carrageenin-induced oedema and adjuvant arthritis in rat (Martínez-Domínguez et al. 2001); nevertheless, the molecular mechanisms have not been elucidated. Hydroxytyrosol (HT), present in the phenolic fraction of virgin olive oil in a concentration range of 10–300 ppm, is abundant in naturally fermented table olives and can be also recovered from virgin olive oil by-products (olive pomace, olive waste water; Boskou 1996). At nutritionally relevant concentrations, HT possesses a marked antioxidant activity and is a good radical scavenger (Deiana et al. 1999; Manna et al. 1999). HT in vitro prevents LDL oxidation (Salami et al. 1995), platelet aggregation (Petroni et al. 1995) and inhibits 5- and 12-lipoxygenase (Kohyama et al. 1997; De la Puerta et al. 1999). A recent report indicates that HT and other phenolic antioxidants reduce vascular cell adhesion molecule-1 mRNA expression by blocking the activation of transcription factors nuclear factor-kappaB (NF-κB) and activator protein-1 (Carluccio et al. 2003). It has been demonstrated that natural and synthetic antioxidants inhibit pro-inflammatory gene expression regulated by transcription factors, including NF-κB, signal transducer and activator of transcription-1α (STAT-1α) and interferon regulatory factor-1 (IRF-1; Hecker et al. 1996; Epinat and Gilmore 1999; Faure et al. 1999; Kim et al. 2003; Lee et al. 2003). These transcription factors are dependent on the intracellular redox state (Pahl 1999; Ramana et al. 2000; Kröger et al. 2002) and can cooperate in order to

promote synergistically transcriptional activity (Ohmori and Hamilton 1993; Kinugawa et al. 1997). The inducible enzymes nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are mediators of the inflammatory response (D'Acquisto et al. 2000a) and implicated in the pathogenesis of several diseases, including human gastrointestinal cancers (Lala and Chakraborty 2001; Zha et al. 2004). The promoter regions of iNOS and COX-2 contain consensus sequences for NF- κB, STAT-1α and IRF-1 (Fletcher et al. 1992; Lowenstein et al. 1993; Sirois et al. 1993; Kamijo et al. 1994; Kosaka et al. 1994; Gao et al. 1997). In the present study, we show that HT inhibits iNOS and COX-2 gene expression in lipopolysaccharide (LPS)-stimulated J774 macrophages by preventing the activation of NF-κB, STAT-1α and IRF-1.

Materials and methods

Hydroxytyrosol The (3,4-dihydroxyphenyl) ethanol (hydroxytyrosol, HT) was obtained by acid hydrolysis of oleuropein glucoside (Extrasynthese Z.I., Lyon Nord, France) according to the procedure described by Owen et al. (2000a, b). Acid hydrolysis was achieved by dissolving 100 mg of the oleuropein in 1 l of 1 mol/l H_2SO_4 and incubating at 37°C for 3 h. The hydrolysate was extracted twice with 250 ml of ethyl acetate and dried over anhydrous sodium sulphate; the solvent was removed under reduced pressure. The purity of HT was checked by HPLC analysis (Sacchi et al. 2002). The residue was dissolved in ethanol and stored at 4°C until the tests.

Cell culture The murine monocyte/macrophage cell line J774 (American Tissue Culture Catalogue T1B) was cultured at 37°C in humidified 5% CO_2 /95% air in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum, 2 mM glutamine, 100 UI/ml penicillin, 100 μg/ml streptomycin, 25 mM Hepes and 5 mM sodium pyruvate. The cells were plated in 24-culture wells at a density of 2.5×10^5 cells/ml/well or 10 cm diameter culture dishes at a density of 3×10^6 cells/ml/dish and allowed to adhere for 2 h. Thereafter, the medium was replaced with fresh medium and cells were stimulated with LPS (1 μg/ml). HT (50, 100 and 200 μM) or pyrrolidine dithiocarbamate (PDTC; 10 μM), a synthetic antioxidant used as a reference drug, were added to the cells 10 min before LPS challenge. In some experiments HT (200 μM) or PDTC (10 μM) were added 12 h after LPS challenge. The cell viability was assessed by using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) conversion assay (D'Acquisto et al. 2001). Cytosolic and nuclear extracts of macrophages stimulated for 24 h with LPS (1 μg/ml) in the presence or absence of HT (200 μM) or PDTC (10 μM) were prepared as previously described (D'Acquisto et al. 2001). Protein concentration was determined by Bio-Rad (Milan, Italy) protein assay kit.

PGE_2 and nitrite determination The accumulation of PGE_2 in the culture medium, 24 h after LPS challenge, was measured by an ELISA kit according to the manufacturer's instructions (TEMA Ricerche, Milan, Italy). NO generation was measured as nitrite (NO_2^-, nmol/10^6 cells) accumulated in the incubation medium, 24 h after LPS challenge, using a spectrophotometric assay based on Griess reaction as previously described (D'Acquisto et al. 2000b). The nitrite concentration was determined by reference to a standard curve of sodium nitrite.

Measurement of reactive oxygen species The formation of ROS was evaluated by means of the probe 2′,7′-dichlorofluorescin-diacetate (H_2DCF-DA, Sigma) as described elsewhere (Santamaria et al. 2004). Briefly, J774 cells were grown in DMEM containing 10% (v/v) foetal bovine serum, then were plated at a density of 1.5×10^4 cells/well into 96-well dishes. Cells were allowed to grow for 24 h

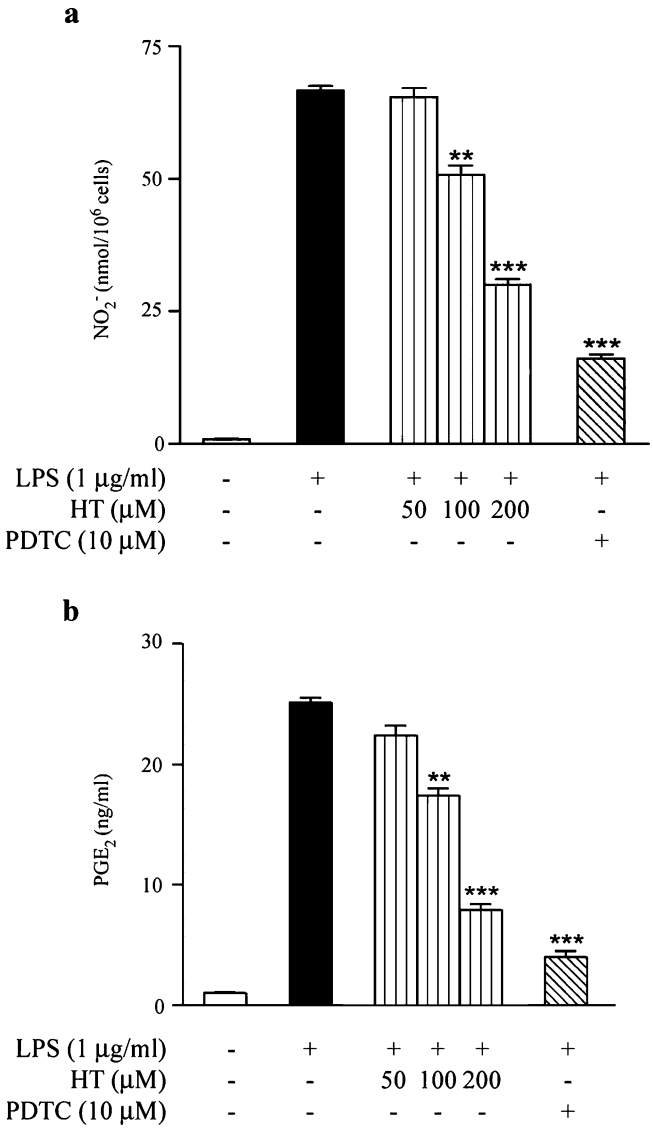


Fig. 1 Effect of hydroxytyrosol (HT; 50, 100 and 200 μM) and pyrrolidine dithiocarbamate PDTC (10 μM) **a** on nitrite and **b** PGE_2 production by J774 macrophages stimulated with lipopolysaccharide (LPS; 1 μg/ml) for 24 h. Data are expressed as mean±SEM of three experiments. $^{**}P<0.01$, $^{***}P<0.001$ vs. LPS alone

and then incubated in the growth medium containing 5 μM H_2DCF-DA for 2 h at 37°C. H_2DCF-DA is a non-fluorescent permeant molecule that passively diffuses into cells, where the acetates are cleaved by intracellular esterases to form H_2DCF and thereby traps it within the cell. In the presence of intracellular ROS, H_2DCF is rapidly oxidised to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Cells were washed twice with PBS buffer; thereafter, the medium was replaced with fresh medium and cells were stimulated with LPS (1 μg/ml) for 24 h. HT (200 μM) or PDTC (10 μM) were added to the cells 10 min before LPS challenge. After treatment, cells were washed twice with phosphate buffer saline (PBS) buffer and plates were placed in a fluorescent microplate reader (Perkin Elmer LS55 Luminescence Spectrometer; Perkin Elmer, Beaconsfield, UK). Fluorescence was monitored using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. In each experiment, fluorescence increase was measured in ten replicate cultures (n=10) for each treatment.

Electrophoretic mobility shift assay Double stranded oligonucleotides containing the NF-κB (5′-CAACGGCAG GGGAATCTCCCTCTCCTT-3′), STAT-1α (5′-CAT GTT ATGCATATTCCTGTAAGTG-3′) and IRF-1 (5′-GG AAG CGAAAATGAAATTGACT-3′) recognition sequences were end-labelled with ^{32}P-γ-ATP. Nuclear extracts containing 5 μg protein were incubated for 15 min with radiolabelled oligonucleotides (2.5–5.0×10^4 cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol. The specificity of the DNA/protein binding was determined by competition reaction in which a 50-fold molar excess of unlabelled wild-type, mutant or Sp-1 oligonucleotide was added to the binding reaction 15 min before the radiolabelled probe. In supershift assay, antibodies reactive to p50, p65, STAT-1α or IRF-1 proteins were added to the reaction mixture 15 min before the addition of the radiolabelled probe. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 1× TBE buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with intensifying screen at −80°C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

Western blot analysis Cytosolic and nuclear fraction proteins were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2 mg/ml of bromophenol) in a ratio of 1:1, boiled for 3 min and centrifuged at 10,000×g for 5 min. Protein concentration was determined and equivalent amounts (30 μg) of each sample were electrophoresed in a 8–12% discontinuous polyacrylamide minigel. The proteins were transferred onto nitro-cellulose membranes, according to the manufacturer's instructions (Protran; Schleicher & Schuell, Dassel, Germany). The membranes were saturated by incubation at room temperature for 2 h with 10% non-fat dry milk in PBS and then incubated with (1:1,000) anti-iNOS, anti-COX-2, anti-p50, anti-p65, anti-STAT-1α and anti-IRF- 1 at 4°C overnight. The membranes were washed three times with 0.1% Tween 20 in PBS and then incubated with anti-rabbit, anti-mouse or anti-goat immunoglobulins coupled to peroxidase (1:1,000; DAKO, Milan, Italy). The immunocomplexes were visualised by the ECL chemiluminescence method (Amersham, Milan, Italy). The

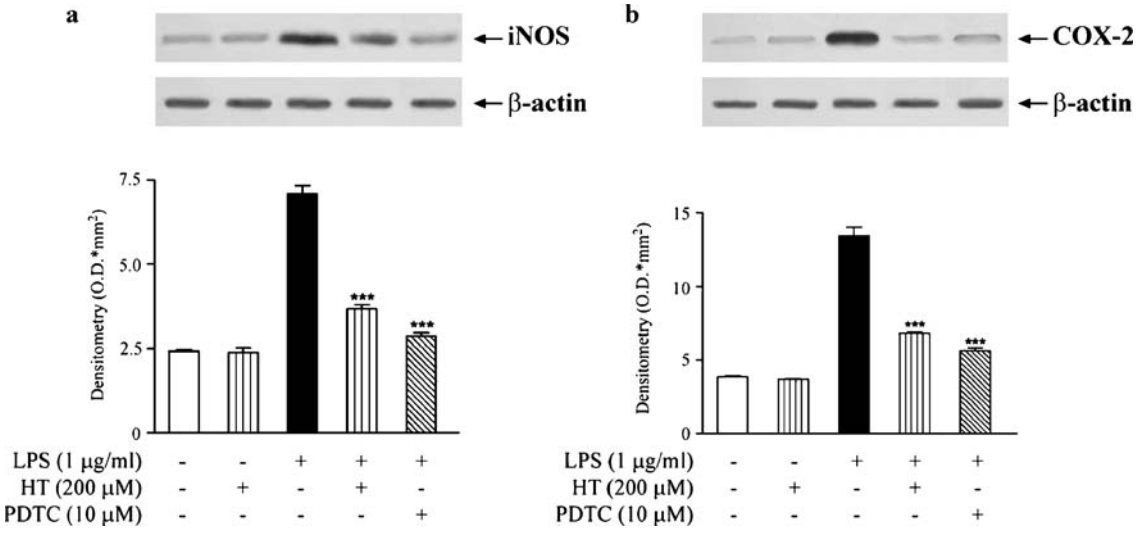


Fig. 2 Representative Western blot of **a** inducible nitric oxide synthase (iNOS) and **b** cyclooxgenase-2 (COX-2) protein expression (*upper panel*) as well as the densitometric analysis (*lower panel*) show the effect of HT (200 μM) and PDTC (10 μM) on iNOS and COX-2 protein expression in J774 macrophages stimulated with LPS (1 μg/ml) for 24 h. β-actin expression is shown as a control. Data in **a** and **b** (*upper panel*) are from a single experiment and are representative of three separate experiments. Data (*lower panel*) are expressed as mean±SEM of three separate experiments. ***P<0.001 vs. LPS alone. *OD* optical density

membranes were stripped and re-probed with β-actin or histone 1 antibody to verify equal loading of proteins. Subsequently, the relative expression of iNOS, COX-2, p50, p65, STAT-1α and IRF-1 proteins in cytosolic and nuclear fraction was quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer and a computer program (Molecular Analyst, IBM).

Reverse transcription-polymerase chain reaction J774 cells were plated in a six-well plate at a density of 3×10^6/well, where they were allowed to adhere for 2 h. Thereafter, the cells were treated with 200 μM HT or 10 μM PDTC and, 10 min after treatment, were stimulated with 1 μg/ml LPS for 6 h. Total RNA extraction, by using TRIzol (Invitrogen, Milan, Italy) was performed as described elsewhere (Ialenti at al. 2005). The levels of COX2 and iNOS mRNA were evaluated by using PCR amplification of reverse-transcribed mRNA. The housekeeping gene β-actin was used as an internal control. Five micrograms of total RNA was reverse-transcribed into cDNA by using oligo $(dT)_{12–18}$ primer (Invitrogen) and MMLV-Reverse Transcriptase (Invitrogen). One microlitre of cDNA was amplified by PCR using Taq Polymerase (Invitrogen) according to the manufacturer's instructions. The primers were: COX-2: sense 5′-CCGGGTTGCTGGGGGAAGA-3′, antisense 5′-GTGGCTGTTTTGGTAGGCTGTGGA-3′; iNOS: sense 5′-TGGGAATGGAGACTGTCCCAG3′, antisense: 5′-GG GATCTGAATGTGATGTTTG-3′; β-actin: sense 5′-AT GAAGATCCTGACCGCGCGT-3′, antisense: 5′-AACGC AGCTCAGTAACAGTCCG-3′. The amplified fragments were 479 bp, 305 bp and 584 bp respectively. The PCR reaction was performed under the following conditions: a first cycle of denaturation at 94°C for 1 min 40 s, then 25 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 40 s, extension at 72°C for 1 min and one additional cycle of extension at 72°C for 8 min. The PCR products were run on a 1% agarose gel and visualised by ethidium bromide staining.

Statistics Results were expressed as the mean±SEM of *n* experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA) and Bonferroni-corrected *P* value for multiple comparison test. The level of statistical significance was defined as $P<0.05$.

Reagents Dulbecco's Modified Eagle's Medium, foetal bovine serum, glutamine, penicillin, streptomycin, Hepes, sodium pyruvate and PBS were from BioWhittaker (Caravaggio, Italy). ^{32}P-γATP was from Amersham. Poly dI-dC was from Boehringer-Mannheim (Milan, Italy). Anti-iNOS antibody was from Pharmingen (BD Biosciences, Milan, Italy). Anti-COX-2, anti-p50, anti-p65, anti-STAT-1α, anti-IRF-1 and histone 1 antibodies were from Santa Cruz (Milan, Italy). Anti-β-actin antibody was from Oncogene (Milan, Italy). Non-fat dry milk was from Bio-Rad. Oligonucleotide synthesis was performed to our specifications by Tib Molbiol (Boehringer-Mannheim, Genova, Italy). Sulphuric acid was from Carlo Erba Reagents (Milan, Italy). Ethanol and ethyl acetate were from

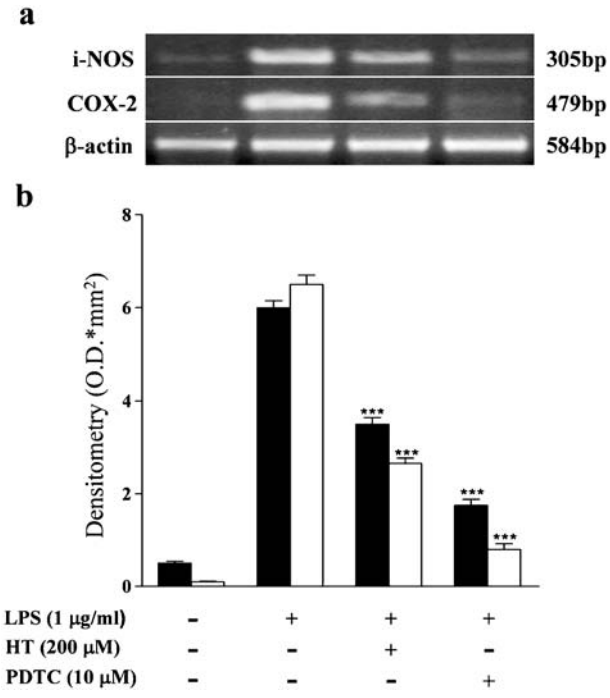
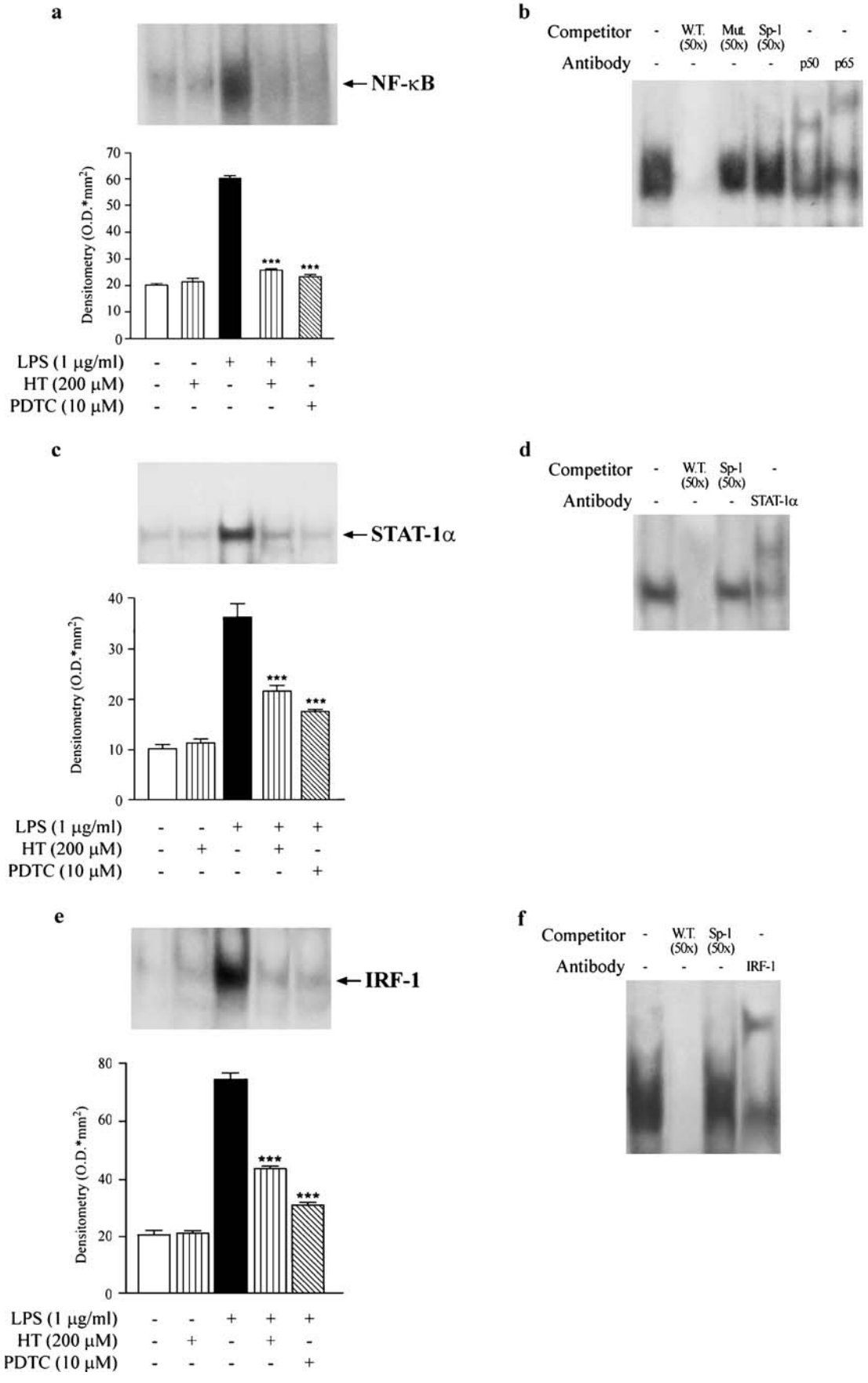


Fig. 3 **a** Representative PCR of iNOS and COX-2 and **b** the densitometric analysis show the effect of HT (200 μM) and PDTC (10 μM) on iNOS (*black bars*) and COX-2 (*white bars*) mRNA expression in J774 macrophages stimulated with LPS (1 μg/ml) for 6 h. iNOS and COX-2 mRNA levels were normalised to β-actin mRNA. Data in **a** are from a single experiment and are representative of three separate experiments. Data in **b** are expressed as mean±SEM of three separate experiments. ***$P<0.001$ vs. LPS alone

Fig. 4 Representative electrophoretic mobility shift assay (EMSA) ▶ of **a** nuclear factor-kappaB (NF-κB), **c** signal transducer and activator of transcription-1α (STAT-1α), **e** interferon regulatory factor-1 (IRF-1; *upper panel*) and the densitometric analysis (*lower panel*) show the effect of HT (200 μM) and PDTC (10 μM) on NF-κB, STAT-1α, IRF-1/DNA binding activity in J774 macrophages stimulated with LPS (1 μg/ml) for 24 h. Data in **a**, **c**, **e** are from a single experiment and are representative of three separate experiments. Data (*lower panel*) are expressed as mean±SEM of three separate experiments. ***$P<0.001$ vs. LPS alone. **b** In competition reaction nuclear extracts from LPS-treated J774 macrophages were incubated with radiolabelled NF-κB probe in the absence or presence of: identical but unlabelled oligonucleotides (W. T., 50×), mutated non-functional NF-κB probe (Mut., 50×) or unlabelled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50×). In supershift experiments nuclear extracts were incubated with antibodies against p50 and p65 15 min before incubation with radiolabelled NF-κB probe. **d** In competition reaction nuclear extracts from LPS-treated J774 macrophages were incubated with radiolabelled STAT-1α probe in the absence or presence of: W.T. (50×) or unlabelled oligonucleotide containing the consensus sequence for Sp-1 (50×). In supershift experiments nuclear extracts were incubated with antibody against STAT-1α 15 min before incubation with radiolabelled STAT-1α probe. **f** In competition reaction nuclear extracts from LPS-treated J774 macrophages were incubated with radiolabelled IRF-1 probe in the absence or presence of: identical but unlabelled oligonucleotides W. T. (50×) or unlabelled oligonucleotide containing the consensus sequence for Sp-1 (50×). In supershift experiments nuclear extracts were incubated with antibody against IRF-1 15 min before incubation with radiolabelled IRF-1 probe. Data in **b**, **d**, **f** are from a single experiment and are representative of three separate experiments


a
← NF-κB
Densitometry (O.D.*mm²)
70
60
50
40
30
20
10
0
***
***
LPS (1 μg/ml) - - + + +
HT (200 μM) - + - + -
PDTC (10 μM) - - - - +
b
Competitor - W.T. (50x) Mut. (50x) Sp-1 (50x) - -
Antibody - - - - p50 p65
c
← STAT-1α
Densitometry (O.D.*mm²)
40
30
20
10
0
***
***
LPS (1 μg/ml) - - + + +
HT (200 μM) - + - + -
PDTC (10 μM) - - - - +
d
Competitor - W.T. (50x) Sp-1 (50x) -
Antibody - - - STAT-1α
e
← IRF-1
Densitometry (O.D.*mm²)
80
60
40
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LPS (1 μg/ml) - - + + +
HT (200 μM) - + - + -
PDTC (10 μM) - - - - +
f
Competitor - W.T. (50x) Sp-1 (50x) -
Antibody - - - IRF-1

Labscan Ltd. (Dublin, Ireland). LPS (from *S. Thyphosa*) and all the other reagents were from Sigma (Milan, Italy).

Results

Effect of HT on LPS-induced iNOS and COX-2 expression

The stimulation of J774 macrophages with LPS (1 μg/ml) for 24 h resulted in an accumulation of nitrite (66.7±0.8 nmol/10^6 cells; n=3) and PGE_2 (25.1±0.9 ng/ml; n=3) in the medium, compared with unstimulated cells (0.8±0.11 nmol/10^6 cells, n=3; 1.0±0.7 ng/ml, n=3). Treatment of cells with HT (50, 100, 200 μM) or PDTC (10 μM) reduced, in a concentration-dependent manner, nitrite (by 23.9±1.8%, 54.87±1.1% and 76.0±0.8% respectively; n=3) and PGE_2 (by 30.7±0.6%, 68.5±0.5% and 84.1±0.5% respectively; n=3) production (Fig. 1a,b respectively). The addition of HT (200 μM) or PDTC (10 μM) to the cells 12 h after LPS challenge did not reduce nitrite (65.3±0.6 nmol/10^6 cells and 64.9±0.3 nmol/10^6 cells respectively; n=3) and PGE_2 (24.5±0.6 ng/ml and 24.2±0.8 ng/ml respectively; n=3) production at 24 h. Moreover, upon stimulation with LPS (1 μg/ml) for 24 h the cells expressed a significantly high level of iNOS and COX-2 protein expression compared with control, untreated cells. Treatment of cells with HT (200 μM) or PDTC (10 μM) reduced iNOS (by 49.9±0.12% and 63.7±0.1% respectively; n=3) and COX-2 (by 53.5±0.09% and 61.6±0.2% respectively; n=3) protein expression (Fig. 2a,b). In addition, to explore whether the reduced level either of iNOS or COX-2 protein observed in cells treated with HT or PDTC could be attributed to a reduced gene transcription, we analysed the

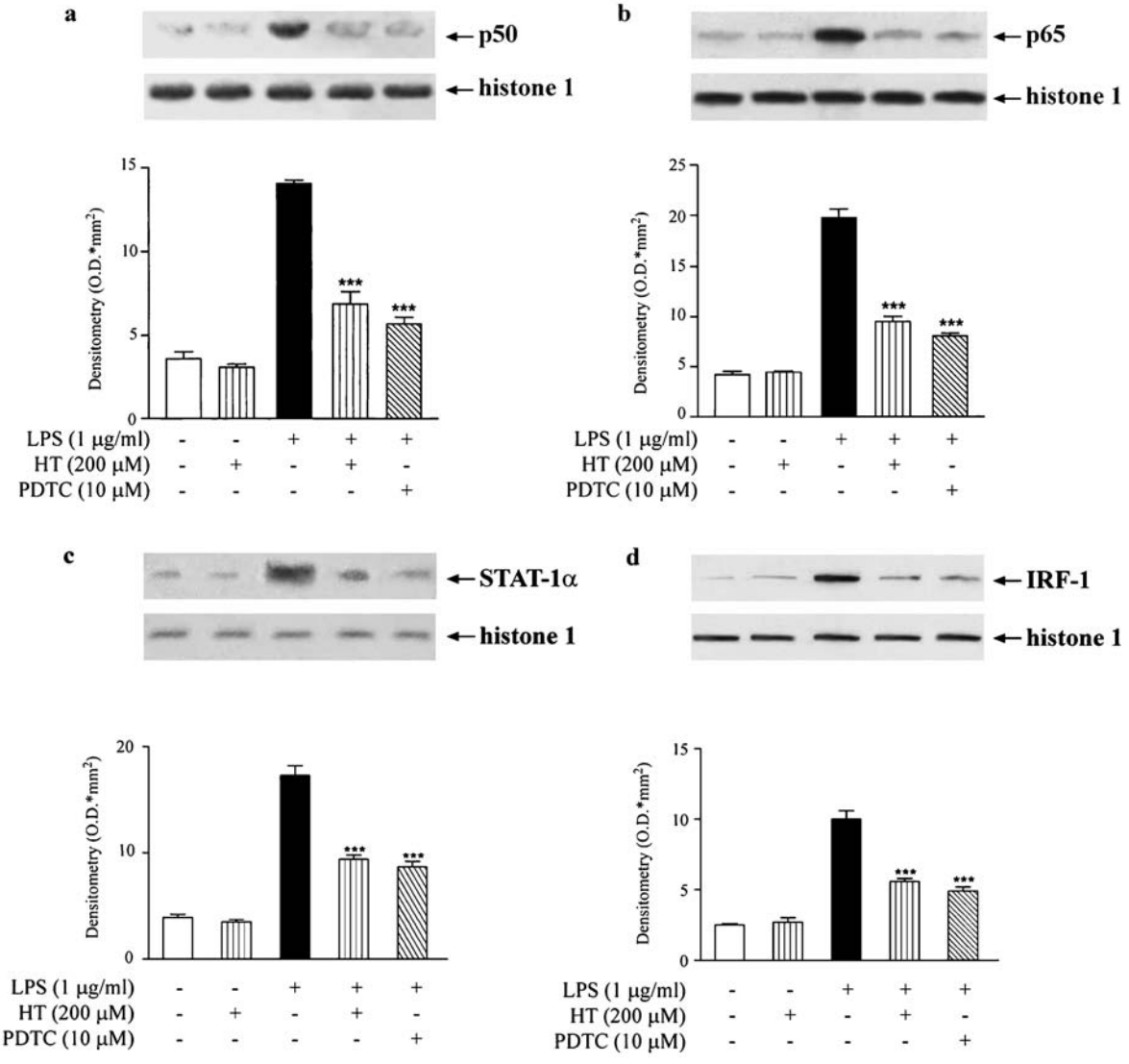

Fig. 5 Representative Western blot of **a** p50, **b** p65, **c** STAT-1α, **d** IRF-1 (*upper panel*) and the densitometric analysis (*lower panel*) show the effect of HT (200 μM) or PDTC (10 μM) on LPS-induced p50, p65, STAT-1α and IRF-1 nuclear translocation in J774 macrophages. Data in **a**–**d** are from a single experiment and are representative of three separate experiments. Histone 1 expression is shown as a control. Data (*lower panel*) are expressed as mean±SEM of three separate experiments. ***P<0.001 vs. LPS alone

expression either of iNOS and COX-2 mRNA. Stimulation of J774 cells with LPS (1 μg/ml) for 6 h resulted in a significantly higher level of iNOS and COX-2 mRNA compared with control, untreated cells. Treatment of cells with HT (200 μM) inhibited significantly both iNOS and COX-2 mRNA levels by 41% and 60% respectively. Similarly, treatment of cells with PDTC (10 μM) inhibited both iNOS and COX-2 mRNA expression by 70% and 88% respectively (Fig. 3a,b). HT and PDTC did not affect cell viability (>90%; data not shown).

Effect of HT on NF-κB, STAT-1α and IRF-1 activation

The effects of HT (200 μM) or PDTC (10 μM) on NF-κB, STAT-1α and IRF-1/DNA binding activity in J774 macrophages stimulated with LPS (1 μg/ml) for 24 h were tested by electrophoretic mobility shift assay (EMSA). A low basal level of NF-κB, STAT-1α and IRF-1/DNA binding activity was detected in nuclear proteins from unstimulated macrophages. Conversely, a retarded band was clearly detected following stimulation with LPS (1 μg/ml). Treatment of cells with HT (200 μM) or PDTC (10 μM) caused a significant reduction in LPS-induced activation of NF-κB (by 57.09±0.48% and 61.26±0.72%), STAT-1α (by 40.17±1.1% and 51.47±0.38%) and IRF-1 (by 41.56± 0.13% and 58.7±1.04%; Fig. 4a,c,e). The composition of NF-κB, STAT-1α and IRF-1 complexes activated by LPS was determined by competition and supershift experiments. In the competition reaction the specificity of the NF-κB/DNA binding complex was demonstrated by the complete displacement of NF-κB/DNA binding in the presence of a 50-fold molar excess of unlabelled NF-κB. In contrast a 50-fold molar excess of unlabelled mutated NF-κB probe (Mut, 50×) or Sp-1 oligonucleotide (Sp-1, 50×) had no effect on this DNA-binding activity. Addition of either anti-p50 and anti-p65 antibodies to the binding reaction clearly gave rise to a characteristic supershift of the retarded complex, suggesting that the NF-κB complex contained p50 and p65 dimers (Fig. 4b). In the competition reaction the specificity of STAT-1α and IRF-1/DNA binding complex was demonstrated by the complete displacement of protein/DNA binding in the presence of a 50-fold molar excess of unlabelled STAT-1α and IRF-1 probe respectively. In contrast, a 50-fold molar excess of unlabeled Sp-1 oligonucleotide had no effect on DNA-binding activity. Addition of anti-STAT-1α or anti-IRF-1 antibody to the binding reaction clearly gave rise to a characteristic supershift of the retarded complex respectively (Fig. 4d,f). Moreover, in LPS-stimulated cells the nuclear level of p50, p65, STAT-1α and IRF-1 was increased compared with untreated macrophages. Treatment of cells with HT (200 μM) or PDTC (10 μM) reduced the band intensity of p50 (by 50.68±0.7% and 59.29± 0.4%), p65 (by 51.82± 0.5% and 59.4±0.3%), STAT-1α (by 45.67±0.4% and 49.71±0.5%) and IRF-1 (by 44.06± 0.2% and 51.05±0.3%; Fig. 5a–d).

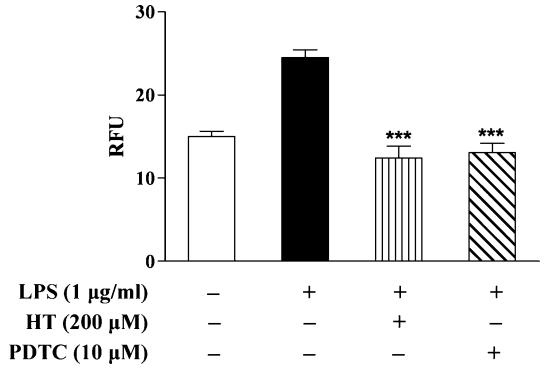

Fig. 6 Effect of HT (200 μM) and PDTC (10 μM) on intracellular ROS production in J774 macrophages stimulated with LPS (1 μg/ml) for 24 h. Data are expressed as means±SEM of three independent experiments (n=10). ***P<0.001 vs. LPS-stimulated J774 macrophages. *RFU* relative fluorescence units

Effect of HT and PDTC on intracellular ROS production

To clarify whether the inhibitory effect of HT on NF-κB, STAT-1α and IRF-1 activation as well as iNOS and COX-2 expression was mediated through the inhibition of ROS generation induced by LPS, we measured intracellular ROS production in J774 macrophages stimulated with LPS (1 μg/ml) for 24 h in the presence or absence of HT (200 μM) or PDTC (10 μM). Exposure of J774 macrophages to LPS for 24 h resulted in an increased intracellular ROS production compared with unstimulated cells. In the presence of HT as well as PDTC, the generation of ROS by macrophages was significantly reduced by 51% and 53% respectively (Fig. 6).

Discussion

The results of the present study show that HT, a strong antioxidant compound from extra virgin olive oil, inhibits iNOS and COX-2 expression in LPS-stimulated J774 cells at the transcriptional level by preventing the activation of NF-κB, STAT-1α and IRF-1. First we found that HT reduced in a concentration-dependent manner nitrite and PGE_2 production that was not a consequence of direct inhibition either of iNOS and COX-2 activity. In fact, HT, when added to the cells 12 h after LPS challenge, failed to inhibit nitrite or PGE_2 production, suggesting that HT and PDTC are not able to inhibit both enzymes once they have been expressed. Interestingly, inhibition of LPS-mediated nitrite and PGE_2 production by HT was correlated and quantitatively comparable with a reduction either of iNOS or COX-2 protein and mRNA levels. It has been reported that NF-κB, STAT-1α and IRF-1 are essential in the regulation of iNOS and COX-2 expression after LPS or cytokine challenge (Kamijo et al. 1994; Gao et al. 1997; Kinugawa et al. 1997; D'Acquisto et al. 2000b; Ohmori and Hamilton 2001; Zhang et al. 2002; Kim et al. 2003). Our data also show that HT inhibited the LPS-induced

NF-κB, STAT-1α and IRF-1/DNA binding activity in J774 cells. Moreover, PDTC, a synthetic antioxidant able to inhibit NF-κB, STAT-1α and IRF-1 activation (D'Acquisto et al. 2000b; Faure et al. 1999; Lee et al. 2003) and a reference drug used in this study, showed the same profile of activity as HT. Growing bodies of evidence report that the inflammation is a critical component of tumour development and progression (Coussens and Werb 2002; Marx 2004). In particular, it has been shown that iNOS and COX-2 induction contribute to promoting the neoplastic process (Lala and Chakraborty 2001; Zha et al. 2004); nevertheless, the mechanisms are not clear. Several studies have been devoted to the development of new molecules that are inhibitors of the enzymatic activity of either iNOS or COX-2. However, an alternative approach is to find new agents that can prevent expression of the respective gene coding for these enzymes. Furthermore, it is now becoming clear that many of the important anti-inflammatory agents, including salicylates and glucocorticoids, share the ability to inhibit transcription factor activation and thus a large variety of pro-inflammatory genes (Kopp and Ghosh 1994; Hu et al. 2003). Natural and synthetic antioxidants have been reported to have anti-inflammatory properties; a likely target for these compounds seems to be the signal transduction cascade leading to the activation of transcription factors (Hecker et al. 1996; Epinat and Gilmore 1999; Faure et al. 1999; D'Acquisto et al. 2000b; Carluccio et al. 2003; Kim et al. 2003; Lee et al. 2003). Our findings indicate that HT inhibits iNOS and COX-2 gene expression by preventing NF-κB, STAT-1α and IRF-1 activation. LPS is a potent macrophage-activating stimulus that appears to initiate an ordered recruitment of adaptor molecules and tyrosine, serine/threonine kinases leading to the transcriptional activation of many genes (Lowenstein et al. 1993; Ohmori and Hamilton 1993; Kinugawa et al. 1997; Zhang et al. 2002; Kim et al. 2003). Indeed, NF-κB, STAT-1α and IRF-1 activation are dependent on the intracellular redox state (Pahl 1999; Ramana et al. 2000; Kröger et al. 2002). In our study, HT effectively reduced the LPS-induced ROS formation that was correlated to the inhibition of NF-κB, STAT-1α and IRF-1 activation at 24 h. Although we did not investigate the precise mechanism by which HT prevents the activation of three transcription factors in LPS-stimulated J774 macrophages, this effect is likely to be correlated to its antioxidant property. Furthermore, since the inhibitory effects elicited by HT were observed when it was added to cells prior to LPS exposure, but not after LPS challenge, it is possible to suggest that antioxidant HT acts at an early step of the LPS-induced signalling cascade leading to NF-κB, STAT-1α and IRF-1 activation. Further studies are necessary to verify this hypothesis and evaluate the potential anti-inflammatory activity of HT. Our results suggest that HT, by preventing NF-κB, STAT-1α and IRF-1 activation, may represent a potential non-toxic agent for the control of inflammation and ultimately play a key role as a component of virgin olive oil in cancer-preventing action.

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