Naunyn-Schmied Arch Pharmacol (2009) 379:581–586
DOI 10.1007/s00210-009-0399-7

ORIGINAL ARTICLE

Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX-2 expression in human monocytic cells

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Received: 11 November 2008 / Accepted: 12 January 2009 / Published online: 7 February 2009
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Abstract Hydroxytyrosol (HT), isolated from extra-virgin olive oil, possesses a marked antioxidant activity and is a good radical scavenger. In this study, our aim was to examine the anti-inflammatory mechanism of HT through measuring the inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) expression, TNF-α formation, and nitric oxide (NO) release in lipopolysaccharide (LPS)-induced human monocytic (THP-1) cells. Results showed that HT remarkably suppressed the LPS (1 μg/ml) induction of NO release. It also significantly attenuated the LPS-induced transcription of TNF-α, iNOS, and COX-2 in a dose-dependent manner. Furthermore, it was also found that HT in a concentration-dependent manner inhibited the expression of iNOS and COX-2 in THP-1 cells treated with 1 μg/ml LPS using Western Blot. Taken together, these results suggest that HT exerts anti-inflammatory effects probably through the suppression of COX-2 and iNOS expression.

Keywords Hydroxytyrosol · iNOS · COX-2 · THP-1 cells

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Introduction

Lipopolysaccharide (LPS), which is a component of the cell wall of Gram-negative bacteria, is known to activate a number of cellular signals of human monocytic cells during inflammation and infection (Yin et al. 2005). TNF-α is the primary cytokine induced in this system and the cytokine responsible for the perpetuation of the inflammatory response in monocytes. During the inflammatory process, large amounts of the pro-inflammatory mediators, nitric oxide (NO), and prostaglandin E2 (PGE2) are generated by the inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2; Posadas et al. 2000).

NO is synthesized from L-arginine by nitric oxide synthase (NOS) in various animal cells and tissues. At least three NOSs have been identified, including endothelial NOS (eNOS), neural NOS (nNOS), and inducible NOS (iNOS). The small amount of NO produced by constitutive NOS (cNOS), including eNOS and nNOS, is an important regulator of physical homeostasis, whereas the large amount of NO produced by iNOS has been closely correlated with the pathophysiology of a variety of diseases and inflammation. PGE_2 is generated from arachidonic acid by cyclooxygenase (COX; Vane and Botting 1998), and two distinct isoforms of COX were identified in the early 1990s. COX-1 is constitutively expressed in nearly all tissues and provides PGs to maintain physiological functions like cytoprotection of the stomach and the regulation of renal blood flow (Vane et al. 1998). In contrast, COX-2 is induced in immune cells, such as macrophages and synoviocytes, in response to infection, injury, or other stresses, and produces large amounts of PGs that act to sensitize nociceptors and induce inflammatory states (Needleman and Isakson 1997; Seybold et al. 2003).

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 (Boskou 1996). At nutritionally relevant concentrations, HT possesses a marked antioxidant activity and is a good radical scavenger (De la Puerta 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).

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). In this study, our aim was to investigate the anti-inflammatory mechanism of HT through measuring the iNOS, COX-2 expression, TNF-α formation, and NO release in LPS-induced THP-1 cells.

Materials and methods

Chemicals

The sample of HT was supplied by Eisai Food & Chemical Co, Ltd, Japan. LPS (*Escherichia coli* LPS, serotype 0127: B8), DCFH-DA were bought from Sigma (St. Louis, MO).

Cell culture and treatment

Human monocytic THP-1 cells (American Type Culture Collection, Manassas, VA) were obtained from Peking Union Medical College (Peking, China) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/mL; Gibco), and streptomycin (100 mg/mL; Gibco) at 37°C in a 5% CO_2-humidified incubator. The cells were treated with or without the indicated concentrations of HT (25, 50, and 100 μM) for 10 min before the challenge with LPS (1.0 μg/ml) for 3 h.

MTT test

The tetrazolium dye colorimetric test (MTT test) is used to monitor cell growth indirectly as indicated by the conversion of the tetrazolium salt to the colored product, formazan, the concentration of which can be measured spectrophotometrically (Hansen et al. 1989). Briefly, THP-1 cells were first cultured in 96-well microplates (1.5×10^5 cells/ml), for 50 μl 1640 medium, and then added 50 μl of 0, 6.25, 12.5, 25, 50, 100, 200, 400, 800 μM HT for culturing 24 h. At the end of the incubation, 10 μl of MTT (5 mg/mL) was added to each well and incubation was allowed to continue for a further 1 h. Finally, 100 μl of lysing buffer was added to each well and incubated for another half an hour. The plate was read using a microplate reader (BIO-RAD Model 3550) at a wavelength of 595 nm.

Measurement of NO

NO production was measured as nitrite (a stable metabolite of NO) concentrations using the Griess reagent system following Jiancheng Institute of Biotechnology protocols (Jiancheng Institute of Biotechnology, Nanjing, China). Cells were washed twice with cold PBS and then suspended in 1 ml PBS, and incubated with 10 μl of 2,3-naphthalenediamine DCFH-DA at the concentration of 0.5 mg/ml for an additional 40 min at 37°C in the dark. After incubation, 50 μl of 2.8 N NaOH was added to terminate the reaction. The fluorescent intensity of the cell suspensions was then monitored with a fluorescence spectrophotometer (HITACHI 650-60, Tokyo, Japan, excitation wavelength of 365 nm, emission wavelength of 450 nm). The nitrite concentration was determined by reference to a standard curve of sodium nitrite.

Measurements of TNF-α

The levels of TNF-α were measured by enzyme-linked immunosorbent assay (R&D, Minneapolis, MN). Briefly, THP-1 cells were suspended at a concentration of 2×10^5cells per six wells in fresh medium. After treatments, particulates were removed from cell cultures by centrifugation and supernatants were assayed following the manufacturer's instructions. The standard of TNF-α was diluted into the doses of 1,000, 500, 250, 125, 62.5, 31.25, 15.625, 0 pg/ml, and the OD values were determined as the wave of 492 nm using a microplate reader (BIO-RAD Model 3550) as the same as samples following the manufacturer's instructions. Then, a standard curve of TNF-α was done, by which the TNF-α concentration was determined.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

The cells were harvested and total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using RT-PCR kit (TaKaRa RNA PCR Kit (AMV) Ver. 3.0, TaKaRa, Japan) according to the manufacturer's instruction. The cDNA was amplified by PCR using the specific primer set for TNF-α and GAPDH as an internal control. The sequences of the upstream and downstream primers were as follows: 5′-CAGAGGGAA GAGTCCCCCAG-3′ and 5′-CCTTGG TCTGGTAGGA GACG-3′ for TNF-α; 5′-AAT GGC AAC ATC AGG

TCG GCC ATC ACT-3′ and 5′-GCT GTG TGT CAC AGA AGT CTC GAA CTC-3′ for iNOS ; 5′-GGA GAG ACT ATC AAG ATA GT-3′ and 5′-ATG GTC AGT AGA CTT TTA CA-3′ for COX-2; 5′-ATGAAGATCCT GACCGCGCGT-3′ and 5′-AACGCAGC TCAGTAA CAGTCCG-3′ for β-actin, respectively. For PCR amplification, the following conditions were used: denaturation at 94°C for 1 min, and then 30 cycles of denaturation for 20 s at 97°C, annealing for 20 s at 64°C for TNF-α, 58°C for GAPDH and extension for 20 s at 72°C. The amplified PCR products were separated with 1% agarose gel, stained with ethidium bromide and then photographed under ultraviolet radiation light. Band intensities were quantified using BioImaging systems (UVP, labworksTM, ver 4.6, Upland, CA) and were normalized to those for β-actin.

Western immunoblot analysis

Western blot analysis of COX-2 and iNOS were carried out by employing the respective antibodies. The cells were incubated with HT for 10 min before exposure to LPS (1 μg/ml) for 3 h. After washing twice with PBS (pH 7.4), the cells were added 100 μl cold lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 10 mg/ml aprotinin) and placed on ice for 30 min with intermittent vigorous mixing. The lysates were centrifuged at 10,000×*g* for 30 min, and the supernatant was saved for determination of Western blotting of iNOS and COX-2. Protein contents in the samples were determined using BCA* Protein Assay Reagent (Pierce, Rockford, IL). Twenty micrograms of protein per sample was electrophoresed on a 10% polyacrylamide gel and transferred onto nitrocellulose membranes. Membranes were initially blocked in 10% skimmed milk for 2 h and probed with recombinant, polyclonal anti-iNOS primary antibody (Lab Vision, USA; 1:200) and anti-COX-2 primary antibody (1:200). Secondary antibody was anti-goat IgG, peroxidase conjugated at 1:200 dilution. Antibody-bound proteins were visualized with the chromogenic substrate 3,3-diaminobenzidine tetrahydrochloride in the presence of H_2O_2. The expression of β-actin was used as a control.

Statistical analysis

Data are presented as means±standard deviation (SD). The statistical significance of differences among groups was performed with one-way analysis of variance (ANOVA), followed by least significant difference (LSD) for multiple comparison, as a post hoc test. The level of significance was set at $p<0.05$ and 0.01 for all statistical analysis.

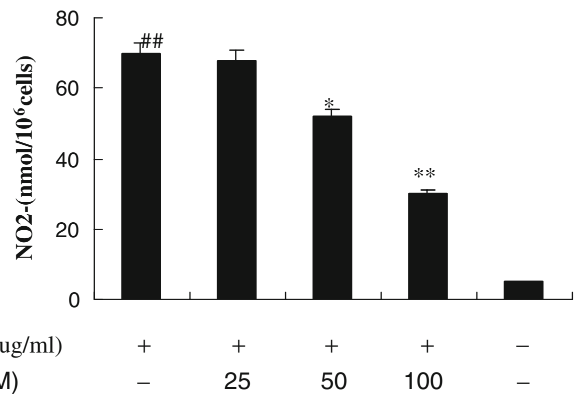

Fig. 1 Effect of HT on the NO level in THP-1 cells stimulated by LPS. Each bar represents mean±S.D. of three independent experiments ($n=3$). *$p<0.05$, **$p<0.01$ significant difference from the group treated with LPS only. $^{\#\#}p<0.01$ significant difference from cells without LPS and HT

Results

Effects of HT on NO production in LPS-stimulated THP-1 cells

Nitrite was used as an indicator of NO production, due to the short half-life of NO. The nitrite concentration in cells was significantly increased in the LPS-only-treated group. As shown in Fig. 1, 50 and 100 μM HT treatment significantly suppressed the increase of NO formation ($p<0.05$ or $p<0.01$).

Effect of HT on the LPS-induced TNF-α secretion and mRNA expression in THP-1 cells

Unstimulated THP-1 cells released low levels of TNF-α in culture supernatants. LPS stimulation for 3 h led to a significant increase in TNF-α secretion ($p<0.01$; Fig. 2).

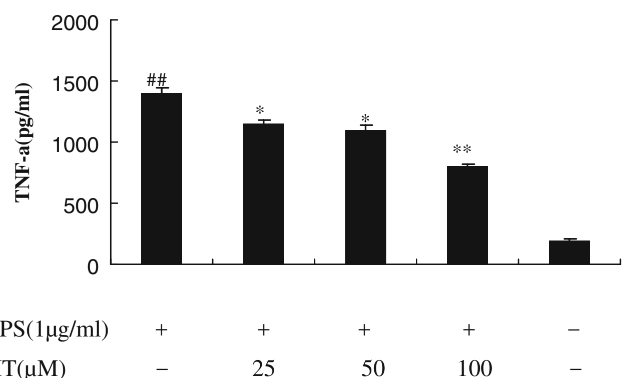

Fig. 2 Effects of HT (25, 50, and 100 μM) on production of TNF-α by THP-1 cells stimulated with LPS (1 μg/ml) for 3 h. Data are expressed as mean±SD of three experiments. *$p<0.05$, **$p<0.01$ vs LPS alone and $^{\#\#}p<0.01$ vs control

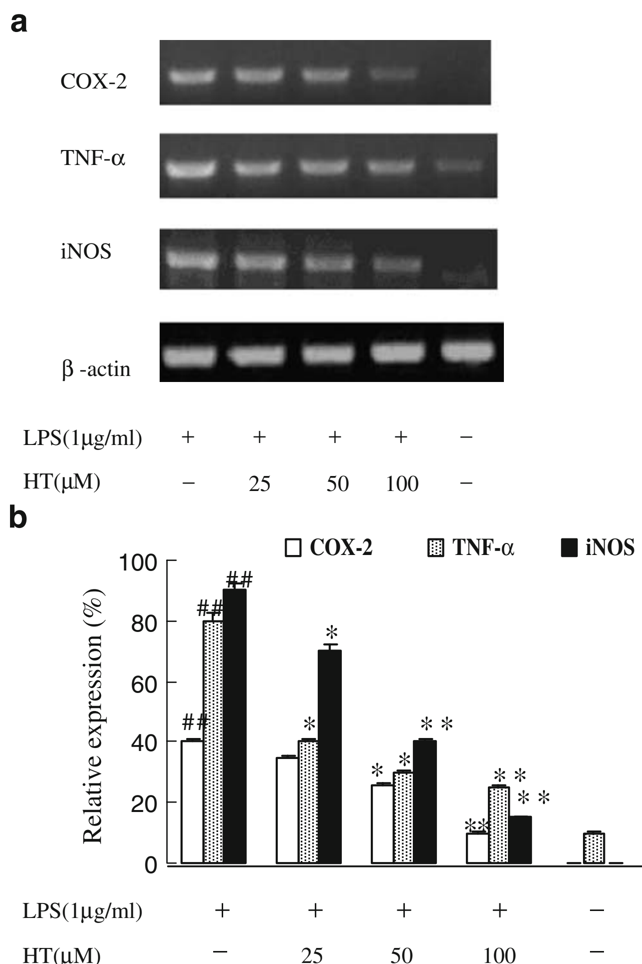

Fig. 3 RT-PCR analysis of iNOS, COX-2, and TNF-α expression (**a**) and the relative expression of iNOS, COX-2, and TNF-α as percent of β-actin (**b**) show the effects of HT (25, 50, and 100 μM) on iNOS (*black bars*), COX-2 (*white bars*), and TNF-α (*light bars*) mRNA expression in THP-1 cells stimulated with LPS (1 μg/ml) for 3 h. After reverse transcription, iNOS, COX-2, and TNF-α mRNA levels were analyzed by PCR followed by agarose gel electrophoresis of the PCR products and ethidium bromide staining. β-actin mRNA levels were used as a control (**a**). Relative expression of iNOS, COX-2, and TNF-α were expressed as percent of β-actin (**b**). $*p<0.05$ and $**p<0.01$ vs LPS alone and $^{\#\#}p<0.01$ vs control

HT-preincubation was able to reduce LPS-induced TNF-α production in a concentration-dependent manner. In addition, to explore whether the reduced level TNF-α secretion observed in cells treated with HT could be attributed to a reduced gene transcription, we analyzed the expression TNF-α mRNA. Stimulation of THP-1 cells with LPS (1 μg/ml) for 3 h resulted in a significantly higher level of TNF-α mRNA compared with control, untreated cells. Treatment of cells with HT (25, 50, and 100 μM) inhibited significantly TNF-α mRNA levels in a concentration-dependent manner (Fig. 3). All of HT did not affect cell viability (>90%; data not shown).

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

The stimulation of THP-1 cells with LPS (1 μg/ml) for 12 h resulted in a significantly high level of iNOS and COX-2 protein expression compared with control, untreated cells. Treatment of cells with HT (50 and 100 μM) reduced iNOS and COX-2 protein expression in a concentration-dependent manner (Fig. 4). In addition, to explore whether the reduced level either of iNOS or COX-2 protein observed in cells treated with HT could be attributed to a reduced gene transcription, we analyzed the expression either of iNOS and COX-2 mRNA. Stimulation of THP-1 cells with LPS (1 μg/ml) for 3 h resulted in a significantly higher level of iNOS and COX-2 mRNA compared with control, untreated cells. Treatment of cells with HT (25, 50, and 100 μM) inhibited significantly both iNOS and COX-2 mRNA levels in a concentration-dependent manner (Fig. 3). All of HT did not affect cell viability (>90%; data not shown).

Discussion

The present study demonstrated that HT is an effective inhibitor of LPS-induced NO generation, TNF-α secre-

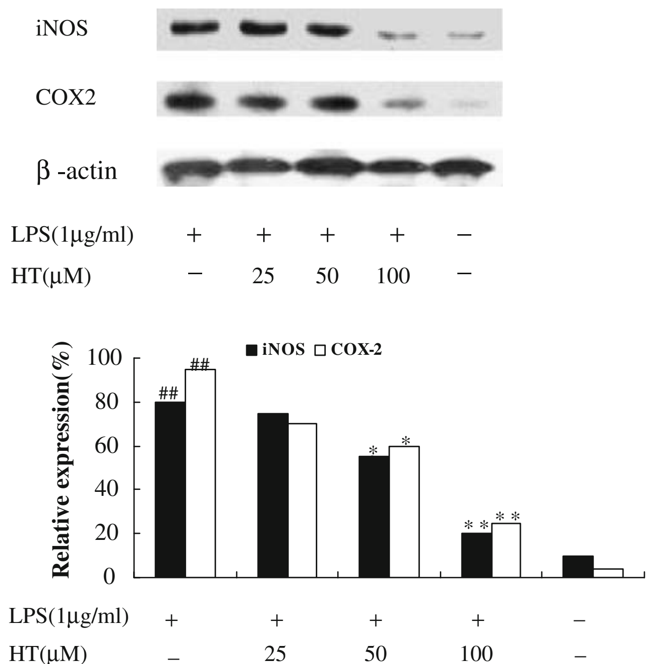

Fig. 4 Representative Western blot of inducible iNOS and COX-2 protein (*upper panel*) as well as the relative expression as percent of β-actin (*lower panel*) show the effect of HT (0, 25, 50, and 100 μM) on iNOS and COX-2 protein expression in THP-1 cells stimulated with LPS (1 μg/ml) for 3 h. $*p<0.05$ and $**p<0.01$ vs LPS alone and $^{\#\#}P<0.01$ vs control

tion and mRNA expression, and iNOS and COX-2 expression in THP-1 cells. Inflammation is a complex process, which involves numerous mediators of cellular and plasma origins. Studies indicated that virgin olive oil and hydrolyzed olive vegetation water (Bitler et al. 2005) exhibited anti-inflammatory activities in human THP-1 cells. In this study, we showed that HT possessed potent anti-inflammatory activity on the same cells.

The pro-inflammatory cytokines, prostaglandins, and NO produced by activated macrophages play critical roles in inflammatory diseases such as sepsis and arthritis (Szabo 1998; Martel-Pelletier et al. 2003). Hence, the inhibition of pro-inflammatory cytokines or iNOS and COX-2 expressions in inflammatory cells, offers us a new therapeutic strategy for the treatment of inflammation (Surh et al. 2001). In the present study, it was found that HT inhibits COX-2 and iNOS expressions in THP-1 cells, and that it probably acts at the transcriptional level, as evidenced by dose-dependent reductions in their mRNA levels. The inhibition of the LPS-stimulated expressions of these molecules in THP-1 cells by HT was not due to HT cytotoxicity, as assessed by MTT assay (data not shown) and the expression of the housekeeping gene, β-actin.

TNF-α is the primary cytokine induced in LPS-induced THP-1 cells and the cytokine responsible for the perpetuation of the inflammatory response in monocytes. Thus, drugs that inhibit TNF-α production may play an important role in the control of inflammation (Hart et al. 2000; Juergens et al. 2004). Studies have indicated that other simple and polyphenol-inhibited cytokines TNF-α production in LPS-stimulated cells (Lin and Lin 1997; Yang et al. 2001). In this study, HT showed inhibitory effect on TNF-α expression in the same cells induced by LPS. This finding further supports that HT possesses potent anti-inflammatory activity.

In conclusion, we have demonstrated that HT possesses potent anti-inflammatory activity. It prevented the cytokines formation, NO generation, and iNOS and COX-2 expression in LPS-treated THP-1 cells. These findings suggest that HT exerts anti-inflammatory effects probably through the suppression of COX-2 and iNOS expression. It has to be noted that this in vitro study does not necessarily indicate respective in vivo effects, especially since the concentrations used are far higher than are achievable in vivo. However, it may contribute to a better understanding of the possible biological action(s) of hydroxytyrosol.

Acknowledgments The authors are grateful to Ling Li and Shen Lv in Central Laboratory of second affiliated hospital of Dalian Medical University for instrumental assistance. This work was supported by the National Natural Science Foundation of China (30771820).

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