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Influence of olive-derived hydroxytyrosol on the toll-like receptor 4-dependent inflammatory response of mouse peritoneal macrophages

Yohei Takeda [a,1], Vuong N. Bui [a], Kenta Iwasaki [b], Takaaki Kobayashi [b,c], Haruko Ogawa [a,*], Kunitoshi Imai [a]

[a] Research Center for Animal Hygiene and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
[b] Department of Transplant Immunology, Nagoya University School of Medicine, Nagoya, Aichi 466-8550, Japan
[c] Department of Surgery II, Nagoya University School of Medicine, Nagoya, Aichi 466-8550, Japan

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ABSTRACT

Macrophages play important roles in the host innate immune response and are involved in the onset of diseases caused by inflammation. Toll-like receptor 4 (TLR4)-mediated inflammatory responses of macrophages may be associated with diseases such as diabetes and diseases of the cardiovascular system. Hydroxytyrosol (HT) exerts strong antioxidant and anti-inflammatory effects and may be applied in the treatment of inflammatory diseases. In the present study conducted *in vitro*, we investigated the effects of the TLR4-dependent anti-inflammatory effect of HT on peritoneal macrophage of BALB/c mice. We show here that the elevated levels of iNOS gene expression and nitric oxide production induced by lipopolysaccharide (LPS) (0.25 μg/ml) were suppressed by HT (12.5 μg/ml). LPS-dependent NF-κB gene expression and phosphorylation of NF-κB were not affected by HT under these conditions. In contrast, the expression of TNF-α was significantly increased in the presence of LPS and HT. These results suggest that HT suppressed nitric oxide production by decreasing iNOS gene expression through a mechanism independent of the NF-κB signaling pathway. These novel findings suggest that the modulation by HT of the expression of genes involved in inflammation may involve multiple mechanisms.

1. Introduction

Macrophages play important roles in innate immune responses by quickly responding to invading pathogens, leading to their elimination through phagocytosis and the inflammatory response. Recent studies reveal the functional diversities of macrophages, which include two main phenotypes designated M1 and M2 and further emphasize the role of macrophages in the host defense system and the maintenance of homeostasis. In contrast, macrophages are involved in the progression of inflammation-related diseases through a mechanism involving their penetration into tissues and induction of the production of proinflammatory cytokines and nitric oxide (NO) [1].

Toll-like receptor 4 (TLR4), which is a pattern-recognition receptor, recognizes a variety of molecules such as lipopolysaccharide (LPS), which is a component of the outer membrane of Gram-negative bacteria, as well as viral envelope proteins and internal ligands such as intracellular proteins and extracellular matrix components [2]. TLR4 initiates the inflammatory responses of macrophages. Chronic inflammation of adipose tissues and liver is caused by the infiltration of inflammatory macrophages activated by the TLR4 signaling pathway in a mouse model of obesity. Chronic inflammation may induce insulin resistance and dysfunction of islet beta cells, which lead to type 2 diabetes [3,4] and indirectly lead to cardiovascular disorders and inflammatory disorders of the central nervous system [5,6].

$CD14^{+}TLR4^{+}$ macrophages infiltrating the intestinal tract are involved in the pathogenesis of chronic intestinal inflammation in Crohn disease [7]. Moreover, an elevated level of high mobility group box protein-1, an internal ligand of TLR4, induces the production of inflammatory cytokines in a macrophage cell line as well as in the alveolar macrophages of patients with acute lung injury, which indicates an association between the TLR4-dependent production of inflammatory cytokines and pathogenesis [8]. Thus, regulating the excessive inflammatory responses in macrophages through the TLR4 signaling may facilitate to control of the onset of such diseases.

Mediterranean foods, which contain a large amount of olive oil, are associated with the reduction of risk factors for coronary heart

* Corresponding author. Address: Research Center for Animal Hygiene and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan. Fax: +81 155 49 5893.
E-mail address: hogawa@obihiro.ac.jp (H. Ogawa).
[1] Present address: Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido 060-8638, Japan.

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diseases and stroke, and the results of epidemiological studies show the inverse association between olive oil consumption and the risk of cardiovascular disease [9,10]. Among the small molecules that comprise 2% of the total weight of olive oil, phenolic compounds with antioxidant activity may account for the reduction of risk of cardiovascular diseases [11,12]. Hydroxytyrosol (HT) is one of the small phenolic molecules in olive oil that exerts strong antioxidant activity and acts as an anti-inflammatory, antithrombotic, antitumor, and antimicrobial agent [13]. HT lacks toxicity in rodent studies [14], and it is commercially available in foods and nutritional supplements, which possess anti-inflammatory and antioxidant activities [15]. Elucidating the efficacy of the anti-inflammatory effect of HT may lead to its use to treat diseases caused by inflammation. The present study aimed to evaluate the anti-inflammatory effect of HT in the TLR4-dependent inflammatory response in macrophages, which occurs in a number of diseases.

2. Materials and methods

2.1. Hydroxytyrosol (HT)

HT (>98% purity: Tokyo Chemical Co., Ltd., Tokyo, Japan) was dissolved in ultrapure water (milliQ; Millipore, Billerica, MA) to 500 μg/ml and was stored at −80 °C.

2.2. In vitro culture of mouse peritoneal macrophages and cytotoxicity test

Female BALB/c mice (8–14 weeks of age) were intraperitoneally administered 2 ml of a 4.05% solution of Thioglycolate (Becton Dickinson and company, Franklin Lakes, USA). Four days later, the mice were euthanized, and the peritoneal macrophages were collected in cold phosphate-buffered saline (PBS). The cells were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. To obtain sufficient numbers of cells, macrophages were collected from 2–3 mice and pooled for some experiments. All studies of mice were conducted in compliance with the institutional rules for the care and use of laboratory animals.

The cells were added to 96-well plates (6×10^5 cells per well). Two hours later, the medium was replaced with fresh medium to remove nonadherent cells. Fresh medium containing serially diluted HT (0–12.5 μg/ml) was added to the wells, and the cells were cultured at 37 °C for 24 h. The cytotoxicity of HT was determined by measuring the lactate dehydrogenase released from the cells using a Cytotoxicity Detection kit PLUS (Roche Applied Science, Penzberg, Germany), and the percentage cytotoxicity was calculated.

2.3. Effect of HT on mouse macrophages stimulated with LPS

Mouse peritoneal macrophages were added to 24-well plates ($3–6 \times 10^6$ per well), and nonadherent cells were removed 2 h later. Adherent cells were cultured in medium containing HT (3.13–12.5 μg/ml) with LPS (0.125–0.5 μg/ml) at 37 °C for 24 or 48 h. The cells cultured in the absence of HT, LPS, or both served as controls. The cultured cells were harvested, and the real-time reverse transcription polymerase chain reaction (RRT-PCR) was used to measure the levels of mRNAs encoding proteins involved in inflammation. The NO concentrations of culture supernatants harvested following a 24-h incubation were determined using a Nitric Oxide (total) detection kit (Enzo Life Sciences, New York, USA). For western blotting analysis, the cells were harvested after 15 min to analyze the expression of inhibitor-κBα (IκBα) and phosphorylated nuclear factor-κB (pNF-κB).

2.4. RRT-PCR analysis of expression of the genes encoding inflammation-related proteins and antiviral cytokines

Total RNA was extracted from cells using Isogen II (Nippon Gene, Tokyo, Japan) and were reverse-transcribed using random primers (Invitrogen, Carlsbad, CA) and M-MLV reverse transcriptase (Invitrogen) as follows: 25 °C for 10 min, 37 °C for 60 min, and 60 °C for 10 min. RRT-PCR was performed using the cDNAs and EagleTaq Master Mix with ROX (Roche Applied Science, Germany) with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, USA). The primers and probes (Taqman Gene Expression Assays, Applied Biosystems) are listed in Table 1. The PCR reactions were conducted as follows: 95 °C for 15-s to 10 min, 45 cycles of 95 °C for 15-s, 60 °C for 1 min. The threshold cycle (C_t) was defined as the number of cycles required for the intensity of fluorescence to rise above the threshold value. The C_t values were normalized to 18S rRNA and calculated as the difference relative to the RNA of untreated control cells. The C_t value for samples that were undetectable using RRT-PCR was defined as 45. The results are expressed as the difference in gene expression (relative quantity of template) using the comparative C(T) method [16].

2.5. Western blotting analysis

Lysates of the cells harvested after 24 h were electrophoresed through a 10% sodium dodecyl sulfate polyacrylamide gel, and the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membranes were blocked with PBS containing 5% skim milk and then incubated with the antibodies provided in the NF-κB Pathway Sampler Kit (Cell Signaling Technology, Danvers, USA). Antigen–antibody complexes bound to the membrane were detected using the ECL Western blotting analysis system (GE healthcare, Buckinghamshire, UK) and analyzed with an LAS-3000 (Fujifilm). Chemiluminescence intensity was measured using Science Lab 2005 Multi Gauge ver. 3.0 (Fujifilm, Tokyo, Japan). As an internal control, β-actin was detected using the anti-β-Actin HRP-DirecT kit (Medical & Biological Laboratories, Aichi, Japan), and the relative ratios of the levels of NF-κB pathway proteins were calculated.

2.6. Statistical analysis

Results are presented as the mean ± standard error of the mean (S.E). Statistical analysis was performed using the Student t test. Statistical values of $P < 0.05$ were considered significantly different.

Table 1
Probe and primer sets (Taqman MGB, Applied Biosystems) used in real-time RT-PCR.

Gene name	Product number
iNOS (nitric oxide synthase 2, inducible)	Mm00440502_m1
IL-10 (interleukin 10)	Mm00439614_m1
Ptgs2 (prostaglandin-endoperoxide synthase 2) (COX-2)	Mm00478374_m1
TNF-α (tumor necrosis factor alpha)	Mm00443258_m1
IL-6 (interleukin 6)	Mm00446190_m1
IL-12 (interleukin 12 alpha)	Mm00434165_m1
NF-κB (Nuclear factor of kappa light polypeptide gene enhancer in B-cells)	Mm00476361_m1
Eukaryotic 18S rRNA (Internal control)	ABI433860F

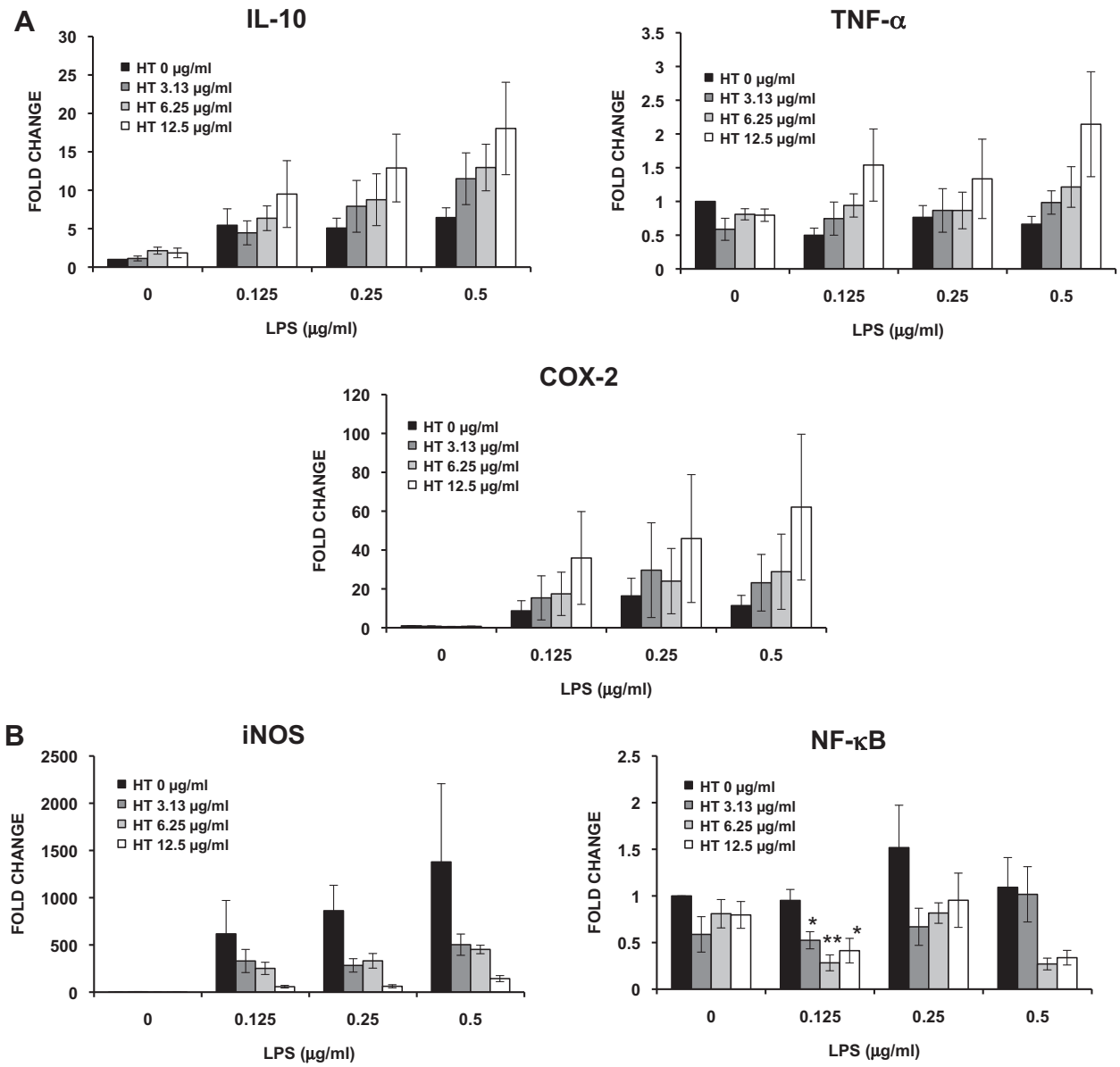

Fig. 1. Analysis of the expression of the IL-10, COX-2, and TNF-α genes (A) and iNOS and NF-κB genes (B) in mouse peritoneal macrophages cultured with LPS (0–0.5 μg/ml) and HT (0–12.5 μg/ml) for 48 h. Mean ± S.E $^{*}P < 0.05$, $^{**}P < 0.001$ vs. HT, 0 μg/ml ($n = 4$).

3. Results

3.1. Expression by mouse macrophages of genes involved in inflammation

Mouse macrophages were treated with HT (0–12.5 μg/ml) and LPS (0–0.5 μg/ml) for 48 h. The expression of IL-10, TNF-α, and COX-2 genes increased as a function of increasing HT concentrations (3.13, 6.25, and 12.5 μg/ml) in the presence of 0.125 and 0.5 μg/ml LPS, although LPS alone had no effect (Fig. 1A). In contrast, iNOS gene expression increased in the presence of LPS alone in a dose-dependent manner, and the elevated levels of iNOS were reduced as a function of increasing HT concentration. The expression of the NF-κB gene was not affected by any concentration of LPS; however, NF-κB gene expression was significantly inhibited by HT only in the presence of 0.125 μg/ml of LPS. At higher concentrations of LPS, NF-κB gene expression was not significantly altered by the presence of HT (Fig. 1B). In the absence of LPS, HT (3.13–12.5 μg/ml) alone did not alter the expression of any of the genes tested (Fig. 1A and B).

The next experiment was conducted using 0.25 μg/ml of LPS and 12.5 μg/ml of HT. LPS (0.25 μg/ml) alone increased IL-10 gene expression by 7-fold compared with the control, and the addition of HT (12.5 μg/ml) further increased expression. The expression of the TNF-α gene was also significantly increased in the presence of LPS and HT, although LPS alone had no effect. The expression levels of the COX-2, IL-6, and IL-12 genes were elevated in the presence of LPS, but the addition of HT had no significant effect. In contrast, the expression of the iNOS gene was significantly elevated in the presence of LPS compared with the control. Addition of HT significantly suppressed the elevation of iNOS gene expression induced by LPS. A significant change in NF-κB gene expression was not detected under any condition. HT alone did not significantly alter the expression of any of the genes tested (Fig. 2). HT was not cytotoxic between 1.56–12.5 μg/ml (Fig. 3A).

3.2. NO concentration in the culture medium of mouse macrophages

LPS (0.25 μg/ml) increased the NO concentration in supernatants of mouse macrophage cultures, and the increased level of NO decreased in the presence of HT (12.5 μg/ml). HT alone did not affect the concentration of NO (Fig. 3B).

3.3. Expression of NF-κB-related proteins

Western blotting was used to determine the levels of expression of IκBα and pNF-κB in cells cultured for 15 min in the presence of LPS, HT, or both. LPS (0.25 μg/ml) decreased IκBα expression, and increased pNF-κB expression. Addition of HT (12.5 μg/ml) had no effect on the LPS-induced elevation of IκBα and pNF-κB expression. HT alone had no effect on the expression of either protein (Fig. 3C).

4. Discussion

In the present study, iNOS gene expression and NO production by mouse peritoneal macrophages treated with LPS were suppressed by HT. These results are consistent with those of previous studies of human macrophages or a mouse monocyte/macrophage cell line [17,18], suggesting that HT acts upstream of the transcription of the iNOS gene. The expression of iNOS by macrophages is induced by LPS, IFN-γ, and inflammatory cytokines, and the elevated level of iNOS results in the production of NO. Although iNOS-induced NO production contributes to the host's response against pathogens, it also exerts a cytotoxic effect involved in the pathogenesis of chronic inflammatory diseases and tissue damage caused by inflammation [19]. The symptoms of mice with arthritis and inflammatory respiratory diseases such as asthma are alleviated by an iNOS inhibitor [20,21], and mortality due to endotoxic shock is reduced in mice with loss-of-function mutations in the iNOS gene [22]. These results suggest that iNOS-mediated NO production is involved in the exacerbation of inflammatory diseases. Therefore, the suppressive effect of HT on iNOS-mediated NO production may control such diseases.

In mouse and human macrophages, iNOS induction is mediated by signal transduction through the NF-κB pathway as well as by the signal transducer and activator of transcription 1 (STAT1) pathway. NF-κB is activated by various stimuli, including LPS, and STAT1 is activated by IFN-γ or LPS. These molecules activate the transcription of the iNOS gene [23–25]. TLR4 activation by LPS activates two downstream pathways, which induce the translocation

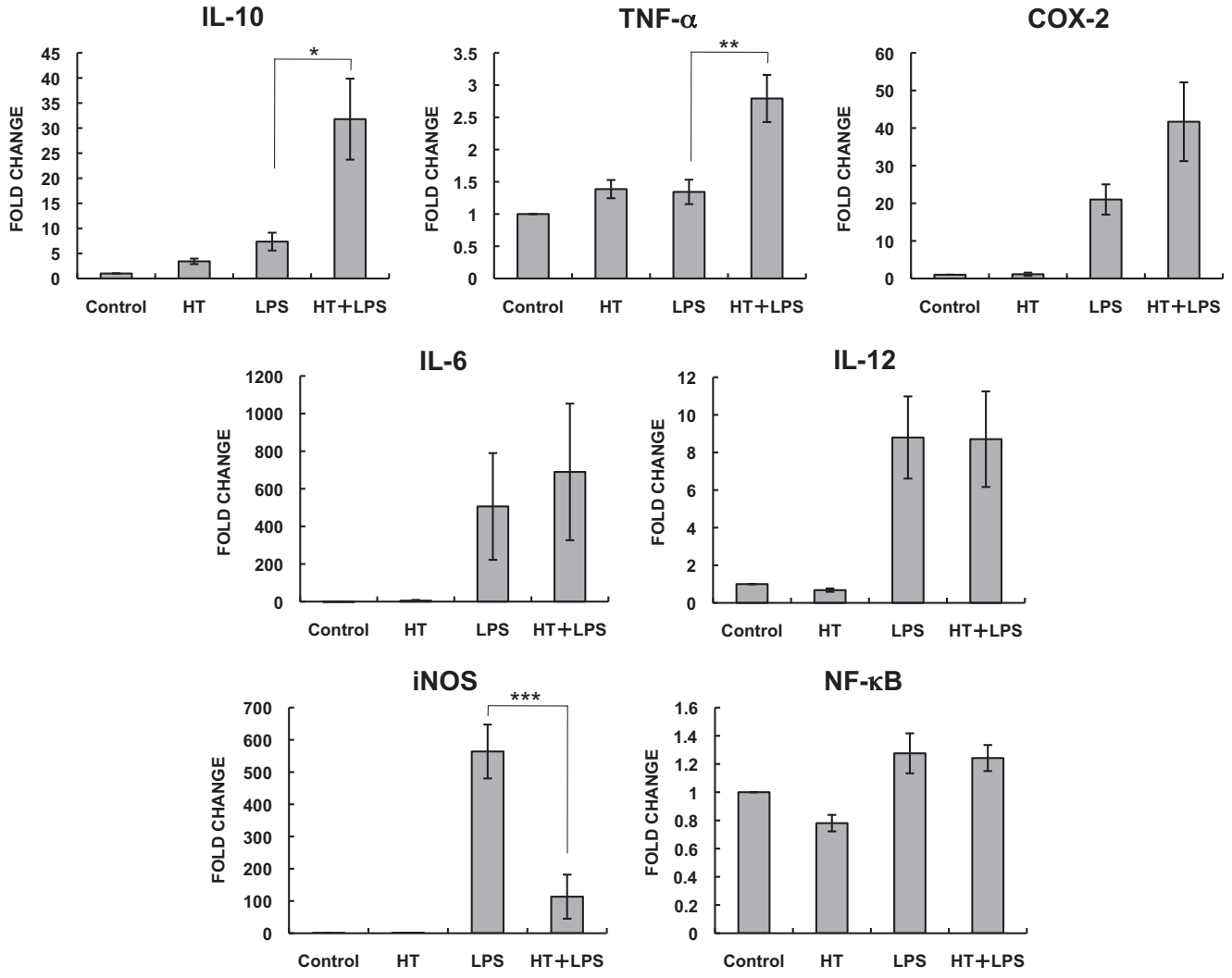

Fig. 2. Analysis of the expression of the IL-10, TNF-α, COX-2, IL-6, IL-12, iNOS and NF-κB genes in cultures of mouse peritoneal macrophages treated with LPS (0.25 μg/ml) and HT (12.5 μg/ml) for 24 h. Mean ± S.E $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$ ($n = 8$).

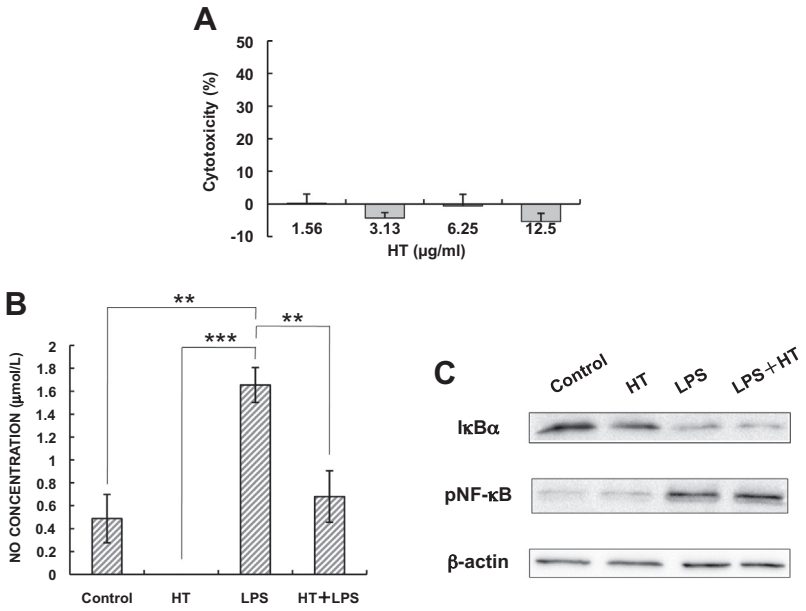

Fig. 3. (A) Analysis of the cytotoxicity of HT. Mouse peritoneal macrophages were cultured with HT (0–12.5 μg/ml) for 24 h. Mean ± S.E ($n = 3$). (B) NO production by mouse peritoneal macrophages cultured with LPS (0.25 μg/ml) and HT (12.5 μg/ml) for 48 h. Mean ± S.E $^{**}P < 0.001$, $^{***}P < 0.0001$ ($n = 5$). (C) Expression of IκBα and phosphorylated-NF-κB (pNF-κB) by mouse peritoneal macrophages stimulated with LPS (0.25 μg/ml) and HT (12.5 μg/ml). Western blotting analysis was used to determine the levels of protein expression of cells treated for 15 min. Representative results of triplicate experiments are shown.

of NF-κB to the nucleus. Following the activation of TLR4 on the cell surface, the myeloid differentiation factor 88 adaptor molecule is recruited and causes the degradation of IκB and translocation of NF-κB to the nucleus. In contrast, activation of TLR4 in endosomes is followed by activation of the toll-like receptor adaptor molecule 1 (TICAM1/TRIF) and then the translocation of NF-κB to the nucleus. TLR4 activation caused by LPS also induces STAT1 activation through the p38 mitogen-activated protein kinase (MAPK) signaling cascade. NF-κB promotes the expression of genes encoding inflammation-related proteins such as TNF-α, IL-6, COX-2, and iNOS [24,26,27]. In the present study, expression of TNF-α and COX-2 genes increased as a function of HT concentration in the presence of LPS 48 h after addition of LPS (Fig. 1A). Further, 24 h after LPS treatment, the expression of the COX-2 and IL-6 genes increased, and the expression of the TNF-α gene significantly increased (Fig. 2). These results are inconsistent with those of previous studies of human and mouse macrophages, which show that HT suppressed LPS-stimulated NF-κB activation as well as the expression of inflammation-related genes such as TNF-α and COX-2 [17,18,28]. For example, Maiuri et al. [17] reported that HT suppresses LPS-induced NF-κB activation as well as the LPS-induced activation of STAT1 and its downstream signaling molecule interferon regulating factor 1 (IRF1), which also induces iNOS gene expression. They concluded that HT downregulates iNOS and COX-2 gene expression by preventing LPS-induced activation of NF-κB, STAT-1, and IRF-1. In the present study, HT suppressed LPS-activated iNOS gene expression and enhanced LPS-activated TNF-α, IL-6, and COX-2 gene expression. These results suggest that suppression of iNOS gene expression by HT is not mediated by the NF-κB pathway but possibly through others such as the STAT1 pathway.

NF-κB gene expression was significantly inhibited by HT (3.13–12.5 μg/ml) in the presence of 0.125 μg/ml of LPS after 48 h (Fig. 1B). To further determine the influence of HT on the NF-κB signaling pathway, the level of NF-κB gene expression was analyzed in the presence of 0.25 μg/ml of LPS and 12.5 μg/ml of HT at 24 h. LPS alone and LPS in the presence of HT did not alter the level of NF-κB gene expression, although HT significantly suppressed the LPS-induced expression of the iNOS gene (Fig. 2). Western blotting analysis also showed that HT did not alter the expression of pNF-κB (Fig. 3C). In contrast, the addition of HT together with LPS, but not HT alone, induced a significant increase in the expression of the TNF-α gene. The increases in COX2 and IL-6 gene expression were not significant (Fig. 2). These results suggest that the NF-κB signaling pathway is not involved in the modulation of the expression of genes related to inflammation under the experimental conditions used in the present study.

Maiuri et al. [17] studied J774 macrophages, in contrast to the peritoneal macrophages used here, although the cells were treated for 24 h in both studies. Further, the lower HT concentrations used here (80 μM vs. 200 μM) may account for the differences between the two studies. Moreover, Mairui et al. [17] showed that LPS-dependent PGE2 and nitrite production by J774 macrophages is not affected by 50 μM HT. Zhang et al. [18] showed that the regulation by HT of LPS-dependent expression of TNF-α and COX2 genes is concentration-dependent in THP-1 cells, and the suppressive effect of HT on LPS-stimulated COX-2 gene expression occurred at concentrations $\geqslant$50 μM. Further, HT suppressed the translocation of NF-κB to the nucleus in the presence of LPS in THP-1 cells at 100 μM but not 50 μM [28]. Thus, 12.5 μg/ml is likely lower than the threshold concentration of HT required to suppress the NF-κB-mediated inflammatory response. Note that 12.5 μg/ml HT unexpectedly elevated the level of inflammation-related cytokine gene expression here. However, there were differences in the times that samples were taken after LPS was added between the previous (a few hours) and current studies (24 or 48 h). A time-course study is required to further elucidate the mode of action of HT in regulating inflammation.

In the present study, HT increased the LPS-dependent expression of the IL-10 gene. IL-10 prevents an excessive inflammatory response by suppressing the production of inflammatory cytokines and antigen presentation. IL-10 expression is induced by LPS as well as by type I IFN or inflammatory cytokines. IL-10 production by macrophages treated with LPS is mediated by the signaling pathways as follows: (1) the p38 MAPK pathway acts downstream of TLR4, and (2) the STAT3 pathway is induced by type I IFN

produced via the TICAM1 pathway [29–31]. Extra-virgin olive oil containing HT suppresses the phosphorylation of p38 MAPK and increases IL-10 expression in colon tissue in a mouse model of colitis [32], and HT suppresses STAT3 gene expression in a human erythroleukemia cell line [33]. However, the effect of HT on IL-10 expression by LPS-stimulated macrophage has not been studied in detail. Further studies to elucidate the intracellular signaling pathway involved in HT-mediated IL-10 production demonstrated here likely will provide a better understanding of the anti-inflammatory effects of HT.

In contrast to the results of other studies, we show here that HT may suppress NO production through a mechanism independent of the NF-κB signaling pathway inhibition, and we show further that the expression of inflammation-related genes was increased by HT. Although HT acts as an anti-inflammatory agent, it may act through multiple mechanisms. Therefore, further studies are required to define the unique properties HT and to determine whether it will serve as an efficacious treatment of inflammatory diseases.

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