Food and Chemical Toxicology 62 (2013) 172–178

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Tyrosol exhibits negative regulatory effects on LPS response and endotoxemia

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ARTICLE INFO

Article history:
Received 16 April 2013
Accepted 14 August 2013
Available online 27 August 2013

Keywords:
Tyrosol
Cytokines
Endotoxemia
TNF-α
NF-κB
MAPKs

ABSTRACT

Tyrosol, a phenolic compound, was isolated from wine, olive oil and other plant-derived products. In the present study, we first investigated the negative regulatory effects of tyrosol on cytokine production by lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages in vitro, and the results showed that tyrosol reduced tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) secretion. This inspired us to further study the effects of tyrosol in vivo. Tyrosol significantly attenuated TNF-α, IL-1β and IL-6 production in serum from mice challenged with LPS, and consistent with the results in vitro. In the murine model of endotoxemia, mice were treated with tyrosol prior to or after LPS challenge. The results showed that tyrosol significantly increased mice survival. We further investigated signal transduction ways to determine how tyrosol works. The data revealed that tyrosol shocked LPS-induced mitogen activated protein kinases (MAPKs) and nuclear transcription factor-κB (NF-κB) signal transduction pathways in RAW 264.7 macrophages. These observations indicated that tyrosol exerted negative regulatory effects on LPS response in vitro and in vivo through suppressing NF-κB and p38/ERK MAPK signaling pathways.

1. Introduction

Macrophages are important inflammatory cells involved in the initiation of inflammatory responses (Huo et al., 2012). Lipopolysaccharide (LPS), which is the key component of outer membrane of Gram-negative bacteria, is a potent stimulus that can induce strong inflammatory responses, extensive tissue edema and damage (Wu et al., 2002). LPS-mediated activation of macrophages leads to the production of various cytokines such as TNF-α, IL-1β, IL-6 and other inflammation mediators. The excessive production of these cytokines, especially TNF-α, may result in the systemic inflammatory response syndrome (SIRS), severe tissue damage, and septic shock. Thus, LPS-activated macrophages have typically been used to evaluate the anti-inflammatory effects of various materials (Hewett and Roth, 1993; Parrillo et al., 1990). During the past several decades, the prevalence of inflammatory diseases has become a major public health concern in the United States (Gao et al., 2008; Jiang et al., 2000; Parsons et al., 1989). Several attempts are going on by intervening TNF-α pathway, including protein reagents, such as TNF-α antibody and enbrel, a TNF-α receptor fusion protein (Mutschler et al., 2006); as well as small molecule inhibitor of TNF-α, such as TACE inhibitors (TNF-α converting Enzyme) (Souza et al., 2007). In addition, a few reports indicate that ciprofloxacin inhibits TNF-α production targeted at binding TLR4 without any signaling function, resulting in blocking LPS/TLR pathway, and attenuating TNF-α production. Unfortunately, there are currently few effective adjuvant therapies in clinical use. Therefore, it is very important to search for new anti-inflammation compounds.

Tyrosol (2-(4-hydroxyphenyl) ethanol) (Fig. 1) is a well-known phenolic compound that is mainly present in extra-virgin olive oil and white wine (Covas et al., 2003). It is a kind of natural antioxidant and has scavenging effects on $ONOO^-$ (Puerta et al., 2001) and O_2^- (Bertelli et al., 2002). In addition to antioxidant effects, tyrosol have anti-inflammatory (Giovannini et al., 2002) and neuroprotective (Bu et al., 2007) effects. However, little study has evaluated the mechanisms of tyrosol on LPS induced inflammation and endotoxemia. Thus, the aim of the present study was to investigate the anti-inflammatory effects of tyrosol on LPS-activated pro-inflammatory cytokines production in RAW 264.7 macrophages and mortality rate in mice with endotoxic shock that were treated with tyrosol prior to or after LPS challenge. Our data showed that tyrosol

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http://dx.doi.org/10.1016/j.fct.2013.08.031

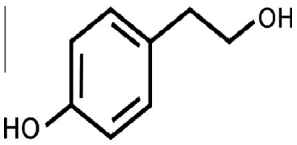

Fig. 1. Chemical structure of tyrosol.

significantly decreases the inflammation in vitro and in vivo, may represent a novel valid compound for inflammation.

2. Materials and methods

2.1. Chemicals

Tyrosol (purity > 98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China). Dimethyl sulfoxide (DMSO), LPS (Escherichia coli O55:B5), 3-(4,5-dimethylthiazol-2-y1)-2,5-dipheny-ltetrazolium bromide (MTT), and Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TNF-α, IL-6 and IL-1β ELISA kits were purchased from Biolegend. Dulbeccos modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY). Phospho-specific antibodies for ERK, p38, JNK and IκB as well as antibodies against, IκB, ERK, p38, JNK, β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cy3-conjugated sheep antirabbit IgG, Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H + L) and peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H + L) were purchased from PTG (Chicago, IL, USA).

2.2. Experimental animals

C57BL/6 male mice weighing 18–22 g were purchased from Jilin University Experimental Animal Center and acclimatized for 1 week before use. Rodent laboratory chow and tap water were provided ad libitum and maintained under controlled conditions with a temperature of 24 ± 1 °C, humidity of 40–80%, and a 12-h light/12-h dark cycle. All of the procedures were in strict accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.3. Cell culture

The RAW 264.7 murine macrophage cell line was obtained from the China Cell Line Bank (Beijing, China). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS at 37 °C under a humidified atmosphere of 5% CO_2.

2.4. Cell cytotoxicity assay

Cytotoxicity studies induced by tyrosol were performed by MTT assay. RAW 264.7 cells were mechanically scraped, plated at a density of 4×10^5 cells/mL onto 96-well plates (Costar USA) containing 100 μL of DMEM medium. After overnight incubation, the cells were treated with various concentrations of tyrosol (0–1.5 mM) according to the experimental design. After 20 h, 50 μL of MTT was added to each well and the cells were further incubated for 4 h at 37 °C with 5% CO_2. MTT was removed and cells were lysed with 100 μL/well DMSO. The optical density (OD) values were measured at 570 nm on a microplate reader (TECAN, Austria).

2.5. Cytokine assays in vitro

To determine the effects of tyrosol on cytokine responses from LPS-induced cells, RAW 264.7 cells were plated onto 24-well plates (4×10^5 cells/mL), and incubated in the presence of either 1 μg/mL LPS alone, or LPS plus tyrosol 0.3, 0.6, 1.2 mM for 24 h at 37 °C with 5% CO2. Cell-free supernatants were collected and stored at −20 °C until assayed for cytokines. The concentrations of cytokine TNF-α, IL-1β and IL-6 in the supernatants of RAW 264.7 cells culture were measured by ELISA using commercially available reagents according to the manufacturer's instructions (BioLegend, Inc. Camino Santa Fe, Suite E San Diego, CA, USA).

2.6. RT-PCR Assay for TNF-α, IL-6, and IL-1β mRNA Expression

RAW 264.7 cells (4×105 cells/mL) were incubated in the presence of either 1 μg/mL LPS alone or LPS plus tyrosol 0.3, 0.6, 1.2 mM for 24 h at 37 °C with 5% CO2. Total RNA was isolated using easy-BLUETM kits according to the manufacturer's instructions and stored at −70 °C until use. Briefly, Integrity of RNA was confirmed by agarose gel electrophoresis, and RNA was quantified by spectrophotometric analysis. The PCR mixture was prepared according to the manufacturer's instructions using the following primers: mouse TNF-α, forward 5′-AGCCGATGGGTTGTA-3′ and reverse 5′-ACTTGGGCAGATTGA-3′; mouse IL-6, forward 5′-GAAATCGTGGAAATGAG-3′ and reverse 5′-TAGGTTTGCCGAGTAGA-3′; and IL-1β, forward 5′-GCTTCAGGCAGGCAGTAT-3′ and reverse 5-ACAAACCGCTTTTCCATCT-3′. Each reaction was performed using 2 μg of total RNA, and the thermocycler was programmed for reversetranscription at 60 °C for 45 min, 95 °C for 5 min, and 5 °C for 5 min. The initial denaturation of the cDNA was accomplished at 94 °C for 5 min, followed by 30 amplification cycles, each of which consisted of denaturation at 94 °C for 30 s, 30 s of annealing (51 °C, 53.5 °C, and 54 °C for TNF-α, IL-6, and IL-1β, respectively), and extension at 72 °C for 1 min 30 s. These were followed by a final extension at 72 °C for 10 min. Amplified PCR products were electrophoresed on a 1% TAE agarose gel.

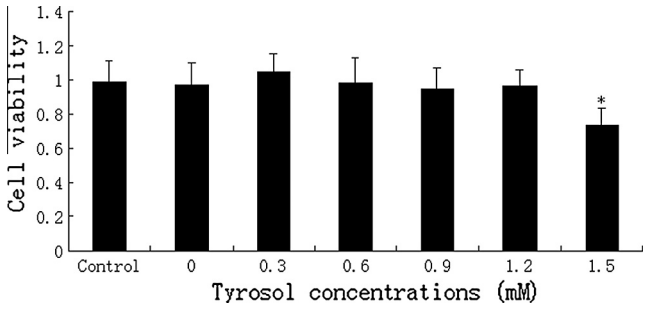

Fig. 2. Effects of tyrosol on the viability of RAW264.7 cells. RAW 264.7 cells were incubated in the presence or absence of tyrosol (0–1.5 mM) and LPS (1 μg/ml) for 24 h. Cell viability was determined by MTT assay. Data are presented as means ± SD of three independent experiments. $^*P < 0.05$ vs control.

2.7. Cytokine assays in vivo

Cytokine concentrations in vivo were measured in mice serum. Tyrosol (160 mg/kg) was given with an intraperitoneal injection (ip). Control mice received an equal volume of vehicle instead of tyrosol. One hour later, all mice received LPS (30 mg/kg) by intraperitoneal injection (ip). Serum was separated from clotted blood at 0, 1, 3, 6 and 12 h following administration of intraperitoneal LPS. Serum was stored at −70 °C and concentrations of cytokine TNF-α, IL-1β and IL-6 were measured by ELISA using commercially available reagents according to the manufacturer's instructions (Lu et al., 2011).

2.8. Murine model of LPS-induced endotoxemia

C57BL/6 mice were challenged in groups of four with LPS (dose range: 10–40 mg/kg) by ip. Mice were observed on mortality for 6 days and twice a day. The items included feeding, activity and grooming (smooth and shiny coats vs dull and ruffled coats). LPS concentration that induced 80–90% lethal was used as working solution in the next step. To evaluate the effects of tyrosol on endotoxemia induced by LPS, mice were divided into five groups (containing 20 mice each): a LPS control group, a low dosage group, a middle dosage group, a high dosage group and a control group. The groups were ip with saline, 40, 80, 160 mg/kg tyrosol or saline, respectively, and then injected intraperitoneally with 30 mg/kg LPS or saline 1 h after administration of tyrosol. Survival rate in each group was assessed every 12 h for 6 days. To further observe the effects of tyrosol on endotoxemia, mice were administered tyrosol (160 mg/kg) at 0, 1 or 4 h after LPS challenge, respectively. Mice in control and LPS groups were only given saline or LPS respectively.

2.9. Immunocytochemical analysis

RAW 264.7 cells (4×105 cells/mL), cultured on glass coverslips, which were plated onto 24-well plates for 24 h, were pretreated with 0.3, 0.6, 1.2 mM tyrosol1 h prior to treatment with 1 ug/mL LPS for 1 h in a 37 °C, 5% CO2 incubator. Glass coverslips were washed with 0.01 M PBS and fixed in 4% formaldehyde for 30 min at room temperature; detergent extraction was performed with 3% TritonX-100 for 10 min at room temperature. Coverslips were saturated with PBS containing 5% bovine serum albumin for 30 min at room temperature and processed for immunofluorescence with rabbit anti-NF-κB/p65 polyclonal antibodies followed by Cy3-conjugated sheep antirabbit IgG. Finally, coverslips were mounted on slides. Fluorescence signals were analyzed by Fluoview microscopy (OLYMPUS, Japan).

2.10. Western blot analysis

In brief, RAW 264.7 cells (4×10^5 cells/mL), cultured in 6-well plates for 24 h, were pretreated with 0.3, 0.6, 1.2 mM of tyrosol 1 h prior to treatment with 1 μg/mL LPS for 30 min in a 37 °C, 5% CO_2 incubator. Then, cytoplasmic protein were lysed and extracted according to manufacturer's instructions. These protein extracts were used for Western blot analysis and protein concentrations were determined using a BCA™ protein assay kit. Equal amounts of protein were loaded into