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Peracetylated hydroxytyrosol, a new hydroxytyrosol derivate, attenuates LPS-induced inflammatory response in murine peritoneal macrophages via regulation of non-canonical inflammasome, Nrf2/HO1 and JAK/STAT signaling pathways

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Abstract

The present study was designed to investigate the anti-inflammatory effects of a new derivative of hydroxytyrosol (HTy), peracetylated hydroxytyrosol (Per-HTy), compared with its parent, HTy, on lipopolysaccharide (LPS)-stimulated murine macrophages as well as potential signaling pathways involved. In particular, we attempted to characterize the role of the inflammasome underlying Per-HTy possible anti-inflammatory effects. Isolated murine peritoneal macrophages were treated with HTy or its derivative in the presence or absence of LPS (5 μg/ml) for 18 h. Cell viability was determined using sulforhodamine B (SRB) assay. Nitric oxide (NO) production was analyzed by Griess method. Production of pro-inflammatory cytokines was evaluated by enzyme-linked immunosorbent assay (ELISA) and inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (STAT3), haem oxigenase 1 (HO1), nuclear factor (erythroid-derived 2)-like 2 (Nrf2) expression and mitogen-activated protein kinases (MAPKs) activation was determined by Western blot. Per-HTy significantly reduced the levels of NO and pro-inflammatory cytokines as well as both COX-2 and iNOS expressions. Furthermore, Per-HTy treatment inhibited STAT3 and increased Nrf2 and HO1 protein levels in murine macrophages exposed to LPS. In addition, Per-HTy anti-inflammatory activity was related with an inhibition of non-canonical nucleotide binding domain (NOD)-like receptor (NLRP3) inflammasome pathways by decreasing pro-inflammatory interleukin (IL)-1β and IL-18 cytokine levels as consequence of regulation of cleaved caspase-11 enzyme. These results support that this new HTy derivative may offer a new promising nutraceutical therapeutic strategy in the management of inflammatory-related pathologies.

Keywords: Hydroxytyrosol; Inflammation; Macrophages; Inflammasome; Nrf2; HO1

1. Introduction

Macrophages are important immune effector cells that contribute not only to injury and infections but also to other types of noxious conditions, changing their functional phenotype to fulfil their roles as sensors, transmitters and responders of inflammation that are required for host defense [1]. These cells can be activated by different stimuli, and in an activated state, they express an excess of inflammatory mediators such as nitric oxide (NO), as well as several pro-inflammatory cytokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-17 [2]. To date, the stimulation of macrophages with lipopolysaccharide (LPS) constitutes an excellent model for the screening and subsequent evaluation of the effects of new candidate drugs.

During the inflammatory response, extracellular LPS is recognized by Toll-like receptor 4, one of the pattern recognition receptors that activates multiple signal transduction pathways such as mitogen-activated protein kinases (MAPKs) and nuclear transcription factor-kappa B (NF-κB) pathways which are involved in the production of inflammatory mediators. The innate immune response is also triggered by intracellular inflammasomes. In fact, inflammasome activation induces pyroptosis and the secretion of pro-inflammatory cytokines such as IL-1β and IL-18 by macrophages [3].

The inflammasome is a cytosolic protein complex that typically contains a nucleotide binding domain (NOD)-like receptors (NLR) member (NLRP1, NLRP3, NLRP6, NLRC4), which serves as a sensor, the adapter protein apoptosis-associated speck-like protein containing a caspase recruit domain (CARD) (ASC) and the cysteine protease caspase-1 [4]. The activated sensor recruits ASC through homophilic interactions of pyrin domains and ASC associates with pro-caspase-1 via caspase activation and recruitment domain (CARD-CARD) interactions, a step needed to induce caspase-1 activation [5]. The activation of caspase-1 results in the cleavage of IL-1β and IL-18 precursors to their mature forms and their eventual secretion [4,6]. Interestingly, recent studies have shown that caspase-11 can also induce caspase-1-dependent maturation and secretion of IL-1β and IL-

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18 as well as caspase-1-independent pyroptosis in response to intracellular LPS derived from cytoplasmic Gram-negative bacteria [7].

Newly accumulating experimental, clinical and epidemiological data have provided support to the traditional beliefs of the beneficial effects provided by olive derivates, specifically its phenolic compounds [8]. In particular, HTy, tyrosol and oleuropein have shown interesting nutraceutical effects as well as very good available safety/toxicity, including antioxidant, antihypertensive, hypoglycemic, hypocholestemic, cardioprotective and anti-inflammatory properties, among others [9,10]. In this sense, our research group has previously demonstrated that a diet elaborated with extra virgin olive oil (EVOO), a kind of oil especially rich on phenolic compounds, and enriched with HTy improved inflammatory processes in a chronic colitis model [11]. Moreover, EVOO diets as well as diets enriched with HTy have shown interesting effects in systemic lupus erythematosus and arthritis models in mice [12,13].

Acylation of phenolic compounds may bring beneficial properties to these molecules, such as cell membrane penetration, improved bioavailability and enhanced antitumor, anti-inflammatory and antimicrobial activities [14–17].

Recently, we have evaluated the effects EVOO phenols, hydroxytyrosyl acetate and 3,4- dihydroxyphenylglycol, as well as two new acyl derivatives of 3,4-dihydroxyphenylglycol, 4-(1,2 -di(butanoyloxy)ethyl)benzene-1,2-diol and 4-(1,2-di(lauroyloxy)ethyl) benzene-1,2-diol, on LPS-stimulated murine peritoneal macrophages in comparison with HTy. The diacylated compounds showed worse anti-inflammatory activity than the parent dihydroxyphenylglycol. We concluded that hydroxytyrosyl acetate and 3,4-dihydroxyphenylglycol, phenolic derivatives with better hydrophilic/lipophilic balance, could play an important role in the anti-inflammatory effect of EVOO [18].

Taking this background into account, the present study was designed to investigate the anti-inflammatory effects of a new derivative of HTy, Per-HTy, compared with its parent, HTy, on LPS-stimulated murine macrophages. We evaluated NO and pro-inflammatory cytokines production, pro-inflammatory enzymes (iNOS and COX-2) protein expression as well as potential signaling pathways involved. In particular, we attempted to characterize the role of the inflammasome underlying Per-HTy possible anti-inflammatory effects.

2. Materials and methods

2.1. Preparation of Per-HTy

HTy was obtained from olive mill wastewaters using the procedure described by Fernández-Bolaños et al. [19]. To a solution of HTy (860 mg, 5.6 mmoL) in pyridine anhydrous (4 ml, 47.7 mmoL), Ac_2O (4 mL, 40.7 mmoL) was added at 5°C. After standing overnight at room temperature, the mixture was poured into ice water (20 ml) and extracted with CH_2Cl_2 (100 ml). The organic phase was washed with 1 M HCl (3×50 ml) and then with satd. aq. $NaHCO_3$ (2×30 ml) and water, dried over $MgSO_4$ and concentrated to dryness to give a brown syrup. Purification by silica gel column chromatography (EtOAc-cyclohexane 1:10) gave Per-HTy as a colorless syrup. The ^1H, ^{13}C NMR data were in accordance with the literature [20].

HTy and its derivate used in the cell experiments were always freshly prepared as stock solutions in dimethylsulfoxide (DMSO) (Panreac, Barcelona, Spain) and diluted to the desired concentration in the culture medium. The final concentration of DMSO in the culture medium was ≤1% in all experiments, and it had not significantly influenced cell response (data not shown).

2.2. Per-HTy lipophilicity and cell membrane permeability

ClogP has been widely used to estimate the lipophilicity of organic molecules and the cell membrane permeability [21]. The logP of a compound, the logarithm of its partition coefficient between *n*-octanol and water, can be calculated with the use of ChemBioDraw Ultra 14.0 software (Perkin-Elmer Informatics Waltham, MA, USA) [22].

2.3. Animals

Twenty 8–10-week-old female Swiss mice (20–30 g) were provided by Harlan Interfauna Ibérica (Barcelona, Spain), randomly placed in cages (5 mice/cage) and used as sources of peritoneal macrophages. Mice were maintained under constant conditions of temperature (20°C–25°C) and humidity (40%–60%) with a 12-h light/dark cycle and fed standard rodent chow (Panlab A04, Seville, Spain) and water *ad libitum* throughout the whole experiment in our Animal Laboratory Center (Faculty of Pharmacy, University of Seville, Spain). Mice were intraperitoneally injected with 1 ml of sterile thioglycollate medium (3.8% w/v) (BD Difco, Le Pont de Claix, France). After thioglycollate injection, behavioral observation, water and food consumptions, loss of body weight and survival were daily monitored until sacrifice. After 3 days, mice were euthanized in the laboratory surgical area by CO_2 exposure. All animal care and experimental procedures complied with the Guidelines of the European Union regarding animal experimentation (Directive of the European Counsel 2010/630/EU) and followed a protocol approved by the Animal Ethics Committee of the University of Seville.

2.4. Isolation and culture of murine peritoneal macrophages

Peritoneal cells were obtained following the protocol described by Aparicio-Soto et al. [18] Peritoneal harvested cells were washed and resuspended in RPMI 1640 medium (PAA, Pasching, Austria) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAA, Pasching, Austria), L-glutamine (2 mM), glucose (4.5 g/L) and HEPES buffer (10 mM) in the presence of 100 mg/ml streptomycin and 100 U/ml penicillin (PAA, Pasching, Austria) and then seeded in culture plates (1×10^6 cells/ml) for 2 h at 37°C in a 5% CO_2 humidified atmosphere. After 2 h, nonadherent cells were removed by washing with PBS and fresh RPMI 1640 medium without FCS containing at 50 μM of HTy or 50, 25, 12.5 μM of Per-HTy were added. After 30 min, cells were stimulated with 5 μg/ml of LPS from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO, USA) during 18 h in the absence or presence of each compound in the study.

2.5. Cell viability

Cells seeded in 96-well plates (1×10^5 cells/well) were incubated in presence or absence of HTy or Per-HTy for 18 h. At the end of the exposure time, cell viability was analyzed by sulforhodamine B (SRB) assay [23]. After incubation time, adherent cell cultures were fixed *in situ* by adding 50 μl of 50% (w/v) cold trichloroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 60 min at 4°C. The supernatant was discarded, and plates were washed five times with deionized water and dried. Fifty microliters of SRB (Sigma-Aldrich, St. Louis, MO, USA) solution (0.4% w/v) in 1% acetic acid (Panreac, Barcelona, Spain) was added to each well and incubated for 30 min at room temperature. Plates were washed five times with 1% acetic acid. Then, plates were air dried, 100 μl per well of 10 mmol/L Tris base pH 10.5 (Sigma-Aldrich, St. Louis, MO, USA) was added, and the absorbance of each well was read on an enzyme-linked immunosorbent assay (ELISA) reader at 510 nm (BioTek, Bad Friedrichshall, Germany). Finally, cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells).

2.6. Measurement of nitrites production

Cell supernatants were transferred into a 96-well assay plate, mixed with Griess reagent (Sigma- Aldrich, St. Louis, MO, USA) and incubated for 15 min at room temperature. The amount of nitrite as an index of NO generation was determined and obtained by extrapolation from a standard curve with sodium nitrite. The absorbance at 540 nm was measured by an ELISA reader (BioTek, Bad Friedrichshall, Germany). Results were expressed as the nitrite production percentage compared with DMSO-LPS cells (stimulated untreated cells).

2.7. Enzyme-linked immunosorbent assay

Supernatants were collected after 18 h and processed by IL-1β and TNF-α (R&D System, Minneapolis, MN, USA), IL-6 and interferon (IFN)-γ (Diaclone, Besancon Cedex, France) and IL-17 ELISA kits (Peprotech, London, UK) according to manufacturers' instructions. Quantification was performed as the manufacturers' instruction, and the results were expressed in pg/ml or ng/ml.

2.8. Isolation of proteins and immunoblotting detection

After 18 h, cells were rinsed, scraped off and collected in ice-cold PBS containing a cocktail of protease and phosphatase inhibitors and processed as described by Cárdeno et al. [24] in order to isolate proteins. Protein concentration was measured for each sample using a protein assay reagent (BioRad, CA, USA) according to the Bradford's method and using γ-globulin as a standard [25]. Aliquots of supernatant containing equal amount of protein (25 μg) were separated on 10% acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred into a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-COX-2 and rabbit anti-iNOS (Cayman, Ann Arbor, MI, USA) (1:2500 and 1:1000, respectively), mouse anti-pJNK, rabbit anti-JNK, mouse anti-pp38, rabbit anti-p38 (Cayman Chemical, Ann Arbor, MI, USA) (1:1000), rabbit anti-heme oxigenase (HO) 1 and rabbit anti- nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Cayman Chemical, Ann Arbor, MI, USA) (1:500), mouse polyclonal anti-p-signal transducer and activator of transcription (STAT)3 (Cell Signaling Technology, Danvers, MA, USA) (1:200), mouse anti-NLRP-3, mouse anti-ASC and rabbit anti-Caspase-1 (Cell Signalling Technology, Danvers, MA, USA) (1:500, 1:100, 1:400, respectively), rabbit anti-Caspase-11 (Novus Biologicals, Littleton, CO, USA) (1:500) and rabbit anti-IL-18 (Abcam, Cambridge, UK) (1:500) overnight at 4°C. After