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Research report

1-Phenil-6,7-dihydroxy-isochroman inhibits inflammatory activation of microglia

Anna Rita Togna[a], Valentina Latina[a], Giuliana Trefiletti[a], Marcella Guiso[b], Sabina Moschini[a], Giuseppina I. Togna[a,*]

[a] Department of Physiology and Pharmacology "Vittorio Erspamer", Italy
[b] Department of Chemistry, "Sapienza" University of Rome, P.le Aldo Moro 5, Rome 00185, Italy

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ABSTRACT

Inflammation plays a central role in the pathogenesis of several brain disorders and neuronal injury, and it develops as a consequence of glial cell activation. Activated microglial cells generate potentially damaging nitric oxide, oxygen free radicals, prostanoids, and pro-inflammatory cytokines. Naturally occurring polyphenols have recently received attention for their potential protective effect on neurodegenerative disorders characterized by microglial activation, due to their anti-inflammatory and antioxidant properties.

In the present study, we investigated, using an *in vitro* model of primary microglia, the ability of 1-phenyl-6,7-dihydroxy-isochroman (encoded L 137), a natural polyphenolic compound, to inhibit microglia activation induced by an inflammatory insult.

So, L137 effects (1–100 μM) on production of pro-inflammatory mediators in lipopolysaccharide (LPS)-activated microglial cells were evaluated. The expression of inducible isoforms of nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) as well as of the nuclear transcription Factor-kappa B (NF-κB) was also performed in cellular lysates by Immunoblot.

L137 significantly reduced tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 secretion, as well as nitric oxide (NO) and prostanoids [Thromboxane (TX)B_2, prostaglandin (PG)E_2] production in activated microglial cells. Western blot analyses showed an inhibitory effect of L137 on the iNOS and COX-2 expression, mediated by a modulation of redox-sensitive nuclear transcriptional factor (NF)-κB, known to control a wide array of genes involved in inflammation. In conclusion, this study demonstrate that L137 is able to inhibit the production of pro-inflammatory and neurotoxic mediators by LPS-activated microglial cells thus suggesting L137 as a potential lead compound for drug development for neurodegenerative disorders where microglia-mediated inflammatory responses play an important pathogenic role.

1. Introduction

Neuroinflammation and oxidative damage are common in many neurodegenerative diseases such as Alzheimer's or Parkinson's disease (Jin et al., 2005). Although the mechanisms underlying neurodegenerative diseases remain elusive, accumulating evidence suggests that microglial activation plays a pivotal role in the pathogenesis of neuroinflammatory and degenerative processes (Kim et al., 2004; Dimayuga et al., 2007). Indeed, chronically activated microglia produces several pro-inflammatory and potentially neurotoxic mediators (Mander et al., 2006), including pro-inflammatory cytokines [*e.g.* interleukin (IL)-1β, tumor necrosis factor (TNF)-α)], prostanoids and neurotoxic mediators [prostaglandin E_2 (PGE_2), thromboxane B_2 (TXB_2), and nitric oxide (NO)] (McGeer and McGeer, 2003). An inhibition of microglia activation therefore may be an effective approach to mitigate the progression of neurodegenerative diseases (Zhang et al., 2010a).

Due to their anti-inflammatory and antioxidant properties, naturally occurring polyphenols have recently received attention for a potential protective effect on neurodegenerative disorders characterized by microglial activation (Zhang et al., 2010a,b; Park et al., 2011; Choi et al., 2012).

The present study explores the inhibitory activity of a natural polyphenolic compound, 1-phenyl-6,7-dihydroxy-isochroman (encoded L 137), on LPS-induced microglial activation.

* Corresponding author at: Dipartimento di Fisiologia e Farmacologia "Vittorio Erspamer" Università "Sapienza" di Roma – P.le A. Moro, 5 – 00185 Roma, Italy. Tel.: +39 06 49912905; fax: +39 06 49912363.
E-mail address: giuseppina.togna@uniroma1.it (G.I. Togna).

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This isochroman, an ortho-phenolic compound found by Bianco et al. (2001) in extra-virgin olive oil of the Mediterranean area, is a stable, lipophilic compound that can be also synthesized by a very simple methodology from hydroxytyrosol (Guiso et al., 2001).

In a previous study, we demonstrated L137 to be effective as a free-radical scavenger and capable of inhibiting platelet aggregation and thromboxane release evoked by agonists inducing reactive oxygen species-mediated platelet activation (Togna et al., 2003). Moreover, L137 also elicited an anti-inflammatory activity by reducing (as demonstrated) the production of NO and other pro-inflammatory mediators in human monocytes primed with LPS (Trefiletti et al., 2011). LPS stimulation leads to a cascade of intracellular signaling events resulting in the production and secretion of inflammatory mediators that constitute the pro-inflammatory response (Lu et al., 2007; Gong et al., 2008).

Based on these results, here we verified the effect of L137 on pro-inflammatory cytokine production [tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6], nitric oxide (NO) and prostanoids [Thromboxane (TX)B_2, Prostaglandin (PG)E_2] in lipopolysaccharide (LPS)-activated microglial cells. The L137 effect on the protein expression of the inducible form of nitric oxide synthase (iNOS) and cyclooxygenase (COX-2), responsible for elevated level of NO and prostanoids, respectively, as well as on Nuclear Factor-kappa B (NF-κB) activation LPS-induced was also evaluated.

2. Materials and methods

2.1. Chemicals and reagents

Purified L137 was synthesized in our laboratory by reaction between hydroxytyrosol and benzaldehyde under very mild conditions (Guiso et al., 2001).

Dulbecco's MEM, and D-MEM/F12 media, trypsin, penicillin and streptomycin, FCS (fetal calf serum) were purchased from Invitrogen (Paisley, Scotland).

DNAse I, LPS from *Escherichia coli* (serotype 026:B6), antibody against β-actin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Polyclonal anti-iNOS and anti-COX-2 antibody were from Cayman Chemicals (Ann Arbor, MI, USA). Polyclonal anti-COX-1 and anti-NFκB was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibody anti-Iba-1 was from Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan). Western blot enhanced chemiluminescence detection system was from Bio-Rad Laboratory (Hercules, CA, USA). Elisa kits for rat IL-1β, IL-6 were purchased from R&D System (Minneapolis, MN, USA) and TNF-α kit was from IBL (Hamburg, Germany). Stock solution of LPS (1 mg/ml) was prepared in medium culture.

2.2. Cell cultures

All the animal-related procedures were conducted in accordance with European Communities Council Directive no 86/609/EEC.

Microglial cells were obtained from the cerebral cortex of 1- or 2-day old decapitated rats. The brains were removed under aseptic conditions, the meninges were carefully removed and the cortex dissected. The tissue was cut into small fragments and digested for 20 min at 37 °C with trypsin (0.125%) in PBS without Ca^{2+} and Mg^{2+} (PBSw/o), with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), and for further 5 min at 37 °C in the same medium as above, supplemented with 50 KU/ml Dnase I.

Cells were plated at a density of 4.5×10^4 cells/cm^2 in T75 flasks in 10 ml D-MEM supplemented with 10% FCS and antibiotics. The medium was changed within 24 h, and then twice a week. After 10–14 days from dissection, microglia was detached from the astrocyte monolayer by shaking in the same culture medium; the supernatant was centrifuged (1 500 × *g* for 5 min) and the cells re-suspended in D-MEM/F12 supplemented with 10% FCS and antibiotics as above. Thereafter, the cells were placed in 24-well plastic plates at a density of 4×10^5 cells/ml, incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and allowed to adhere. After 6 h the medium was replaced with 1 ml of fresh medium. On the basis of our previous results on anti-inflammatory effect of L137 on human monocytes, L137, dissolved in Tris–HCl buffer solution (pH 7.8), was assayed at concentrations of 1, 10 and 100 μM and added to the microglial cultures 30 min before stimulus (LPS 10 ng/ml) for 24 h incubation. Supernatants were then collected, centrifuged and storaged at −80 °C until tested.

Purity of microglial cell populations (>98%) was verified by staining with IBA-1 (1:1 000) antibody.

2.3. Nitrite quantification

The production of NO was measured as accumulated nitrite (NO_2^-), one of the stable end products of NO, by a Griess reaction. Briefly, a volume of conditioned culture medium from each sample was mixed with the same volume of the Griess reagent (50 mM sulfanylamide, 5% H_3PO_4 and 3 mM N-(1-naphthyl) ethylenediamine dihydrocloride). The amount of NO_2^- was measured through the determination of A_{540} nm in a microplate spectrophotometer. The nitric concentration was determined from a sodium nitrite standard curve (0.7–50 μM) of $NaNO_2$.

2.4. PGE_2 and TXA_2 determination

PGE_2 and TXB_2 (the stable breakdown product of TXA_2) concentrations were determined by RIA (Patrignani et al., 1994). The least detectable concentration was 2 pg/ml for both assays.

In a set of experiments, the effect of L137 on prostanoid production was evaluated in microglial cultures treated with NS-398, a selective COX-2 inhibitor. NS-398 (10 μM) was added to culture 20 min before the LPS stimulation.

2.5. IL-1β, IL-6, TNF-α determination

Cells were treated with L137 for 30 min, then stimulated for 24 h with 10 ng/ml LPS for TNF-α and IL-6 or 1 μg/ml LPS for IL-1β determination (Chao et al., 1992). The supernatants were then withdrawn and IL-1β, IL-6, and TNF-α concentrations were assayed in the medium by using specific enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. All measurements were run at least in duplicate for each sample.

2.6. Cell viability

Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2yl)-2,5 diphenyltetrazolium bromide) assay. MTT was converted in living cells to formazan. Cells, seeded in triplicate at the density of 8×10^4 cells/well on 96 well plate, were treated with 1, 10 and 100 μM L137 with or without 10 ng/ml LPS. After 24 h incubation, MTT (0.5 mg/ml) was added to each well for 4 h, then culture media were discarded and dimethyl sulfoxide was added to dissolve formazan dye. The optical density was measured at 570 nm.

2.7. Western blot analysis of iNOS, COX-1, COX-2 and NF-κB protein expression

Microglial cells were washed with PBS three times, placed at 4 °C and lysed for 10 min in an ice-cold lysis buffer (10 mM Tris–HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA; 50 mM sodium fluoride; 1% Triton X-100; 10% glycerol; 1 mM sodium orthovanadate; 1 mM phenylmethanesulfonyl fluoride; 25 mM glycerol-2-phosphate; 10 μg/ml aprotinin and 1 μg/ml leupeptin). Lysates were then centrifuged at 1000 × *g* for 10 min a 4 °C. Equal amounts of protein (30 μg) were electrophoresed on a 10% SDS-polyacrylamide gel (Mini-PROTEAN II apparatus, Bio-Rad Laboratories) before being transferred onto nitrocellulose membranes with the Mini-Trans blot system (Bio-Rad Laboratories). Membrane was blocked with 5% non-fat milk and incubated overnight at 4 °C with anti-iNOS polyclonal antibody (1:1 000) for iNOS detection. For western blot analysis of COX-1, COX-2 membranes were incubated, overnight at 4 °C, with a polyclonal anti-COX-1 (1:250) or with a polyclonal anti-COX-2 (1:100), respectively. For western blot analysis of NF-κB membranes were incubated, overnight at 4 °C, with a polyclonal anti-NF-κB primary antibody (1:500).

Membranes were then washed and incubated with a peroxidase-coniugated anti-rabbit secondary antibody (1:1000) for 1 h at room temperature using chemiluminescence (ECL) reagents for detection. To ensure equal protein loading, the membranes were separately probed for β-actin protein. Scanning densitometry was performed using the ImageJ 1.47 program, and signal density was normalized to β-actin density.

2.8. Protein assay

The total protein content of cell cultures was assessed using the Bradford method. Briefly, cells were lysed and centrifuged at 12,000 × *g* for 3 min. The quantity of protein was estimated by determining the amount of dye in the blue ionic form by measuring the absorbance of the solution at 595 nm in a visible-light spectrophotometer. The amount of protein was measured by extrapolation from a standard curve using BSA as standard (0.14–2 mg/ml).

2.9. Statistical analysis

Data are presented as means ± standard deviation (SD). The statistical significance of differences among groups was performed with one-way ANOVA, followed by *post hoc* Dunnett's test. The level of significance was set at $P < 0.05$.