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Anti-inflammatory effect of oleuropein on microglia through regulation of Drp1-dependent mitochondrial fission

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ABSTRACT

Oleuropein is a primary phenolic compound found in olive leaf and *Fraxinus rhynchophylla*. Here, we investigated the impact of oleuropein on LPS-induced BV-2 microglial cells. Oleuropein suppressed the LPS-induced increase in pro-inflammatory mediators, such as nitric oxide, and pro-inflammatory cytokines, via inhibition of ERK/p38/NF-κB activation and reactive oxygen species (ROS) generation. Furthermore, it suppressed LPS-induced excessive mitochondrial fission, which regulates mitochondrial ROS generation and pro-inflammatory response by diminishing Drp1 dephosphorylation. Collectively, we demonstrated that oleuropein suppresses pro-inflammatory response of microglia by inhibiting Drp1-dependent mitochondrial fission. Our findings suggest a potential role of oleuropein in microglial inflammation-mediated neurodegenerative disorders.

1. Introduction

Fraxinus rhynchophylla is a traditional medicinal plant widely used in Asia. Its extract has been used as an anti-bacterial, analgesic, and anti-inflammatory agent (BK, 2001). In addition, diuretic, anti-coagulant, and anti-allergic effects of *F. rhynchophylla* have been reported in pharmacological studies (BK, 2001, Kim et al., 1999). Oleuropein is one of the major bioactive components of *F. rhynchophylla* and has a basic phenylethanoid structure characterized by a phenethyl alcohol structure-benzene rings linked through an alcohol group. Oleuropein and its derivatives have a variety of beneficial properties, including antioxidant, anti-microbial, anti-carcinogenic, and anti-viral properties, both in vivo and in vitro (Omar, 2010, Sudjana et al., 2009, Visioli and Galli, 2002).

Microglia account for approximately 20% of the total glia population and are the forefront immune defense system in the brain (Kreutzberg, 1995). They are not only the most reactive sensors of threats by various pathogens but also scavenge for plaques, damaged neurons, and infectious agents in the central nervous system (Gehrmann et al., 1995). However, consistently activated microglia can exert neurotoxic effects through the excessive production of cytotoxic molecules, such as nitric oxide (NO), and pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 (Echeverria et al., 2009, Gonzalez-Scarano and Baltuch, 1999, Hoang et al., 2009). Therefore, modulation of microglial cell activation is important for maintaining neuronal function.

Microglia is activated in response to various pathological stimuli (Ransohoff and Perry, 2009). Lipopolysaccharide (LPS) is well known as the main endotoxin component of gram-negative bacteria and triggers production of pro-inflammatory factors by stimulating toll-like receptor 4 (TLR4) (Pawate et al., 2004). LPS-induced TLR4 activation triggers downstream signaling molecules, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK), which are involved in the signaling cascades leading to the abundant expression of pro-inflammatory molecules (Arthur and Ley, 2013, Hayden and Ghosh, 2011).

Abbreviations: NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase.

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Mitochondria are highly dynamic organelles that constantly undergo fission and fusion. Dynamic mitochondrial morphology changes are closely associated diverse cellular processes (Liesa et al., 2009). Mitochondrial fission is regulated by dynamin-related protein 1 (Drp1), whereas fusion is regulated by mitofusin 1, mitofusin 2, and optic atrophy 1 (Liesa et al., 2009). Impaired balance of mitochondrial morphology in neuronal cells has been implicated in many neurodegenerative pathological processes (Knott et al., 2008). Growing evidence, including our research, demonstrates that an increase in mitochondrial fission is induced by immune stimulation via dephosphorylation of Drp1 at serine 637(S637) residue and that it is associated with the regulation of inflammatory responses (Park et al., 2013, Roth et al., 2014, Ye et al., 2015). Therefore, a number of pharmacological reagents are required to regulate the mitochondrial dynamics in immune cells.

Here, we investigated the anti-inflammatory property of oleuropein in LPS-induced activated BV-2 microglial cells. Oleuropein effectively controls the LPS-induced increase in production of pro-inflammatory mediators, such as NO, inducible NO synthase (iNOS), cyclooxygenase (Cox)-2, TNF-α, IL-1β, and IL-6, through regulation of MAPK and NF-κB signaling. In addition, oleuropein modulates LPS-induced excessive mitochondrial fission by blocking dephosphorylation of Drp1(S637). Our findings propose a role for oleuropein in controlling microglial cell activation and as a potential drug candidate for inflammation-mediated neurodegenerative disorders.

2. Materials and methods

2.1. Isolation of oleuropein from F. rhynchophylla

F. rhynchophylla was obtained from Daegu Oriental Pharmacology Corporation (Daegu, Korea). The specimen was stored at the Natural Products Medicine Laboratory, Kyungpook National University (Daegu, Korea; voucher specimen number KNUNPM-BFR-11-12-001). The dried stem barks of *F. rhynchophylla* (4 kg) were extracted twice with 8 L ethanol (EtOH) for 2 h. The ethanolic extract (510 g) was suspended in distilled water for partition using organic solvents such as dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and normal-butanol (n-BuOH), successively. After concentration by a rotary evaporator, the CH_2Cl_2 soluble fraction (22 g), EtOAc soluble fraction (45 g), and n-BuOH soluble fraction (10 g) were obtained. The EtOAc soluble fraction was applied to a silica gel column chromatography [Ø 5.5 × 83 cm, chloroform ($CHCl_3$):methanol (MeOH) = 100:1–1:1] to yield 9 fractions (Fr. 1–9). Fr. 5 was rechromatographed on a silica gel column (Ø 2.5 × 83 cm, CH_2Cl_2:MeOH = 20:1–1:1) to obtain 6 fractions (Fr. 5-1–5-6). Compound #1 (710 mg) was isolated as fine needles from Fr. 5-3 through recrystallization at 4 °C in the solvent consisted with $CHCl_3$ and MeOH. For structure determination of compound #1, ^1H (500 MHz) and ^{13}C (125 MHz) nuclear magnetic resonance (NMR) was measured (solvent, MeOH-d_4). Finally, compound #1 was identified as oleuropein by comparing the NMR spectral data with those of reference (Takenaka et al., 2000). The purity of oleuropein was confirmed as >91% by high performance liquid chromatography (HPLC) analysis. The HPLC analysis was performed on Agilent 1100 series (Agilent Technologies, Germany) equipped with an evaporative light scattering detector (ELSD, PL-ELS2100, Polymer Laboratories, UK). The evaporator and nebulizer temperature were 70 °C and 50 °C, respectively. The gas flow rate was 1.85 standard liter per minute (SLM). As a stationary phase, a Kintex 5 μm C18 100 Å (4.6 × 100 mm, Phenomenex, CA, USA) was used. The mixture of water (A) and acetonitrile (MeCN, B) was used as a mobile phase with a gradient condition (5–100% B at 0–30 min). The flow rate was 1.0 mL/min. Oleuropein was dissolved in HPLC grade MeOH (500 μg/mL) and 8 μL was injected into the column.

2.2. Cell culture and treatment

BV-2 murine microglial cells were immortalized by infection with v-raf/c-myc recombination retrovirus (Blasi et al., 1990), which was kindly provided by Dr. Jau-Shyong Hong (National Institute of Environmental Health Sciences, NC, USA). BV-2 cells were propagated in Dulbecco's Modified Eagle's Medium (Welgene, Daegu, Korea), containing 10% fetal bovine serum (Thermo Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Welgene), and were maintained at 37 °C in a humidified 5% CO_2 incubator (SANYO, Osaka, Japan). Cells were subcultured at a density of 1×10^5 cells in 6-well plates and were grown for 24 h prior to experiments. Exponentially growing BV-2 cells were pretreated with oleuropein for 1 h, followed by stimulation with 1 μg/mL LPS from *Escherichia coli* serotype O26:B6 (Sigma-Aldrich, St. Louis, MO, USA).

2.3. Plasmid construction

DsRed2-mito gene was obtained from pDsRed2-Mito (Clontech, Palo Alto, CA, USA). The *DsRed2-mito* genes were amplified by PCR using LA Taq polymerase (Takara, Shiga, Japan), cloned into the pLenti6/V5-DEST (Thermo Scientific) using LR clonase (Thermo Scientific).

2.4. Lentivirus-mediated gene transfer and preparation of a stable cell line

Construction of the lentivirus was performed as previously described (Kim et al., 2010). *DsRed2-mito* expressing lentivirus (multiplicity of infection = 5) was transduced in BV-2 cells with 8 μg/mL polybrene (Sigma-Aldrich), which increases the efficiency of lentivirus transduction in mammalian cells. Cells were cultured for 72 h and then lentivirus-transduced BV-2 cells were selected with 4 μg/mL blasticidin (Thermo Scientific) for several days.

2.5. RNA isolation and RT-PCR

Total RNA was isolated from the BV-2 cells using TRI-Solution (Bio Science Technology, Seoul, Korea), according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA using a Reverse Transcription Premix (Bioneer, Daejeon, Korea). PCR was performed using gene-specific primers and the PCR premix (Bioneer). The following PCR primers were used: 5′-TNF-α, 5′-AGTTCTATGGCCCAGACCCT-3′; 3′-TNF-α, 5′-GTGGGTGAGGAGCACGTAGT-3′; 5′-IL-1β, 5′-CGACAAAAACCTGTGGCCT-3′; 3′-IL-1β, 5′-AGGCCACAGGTATTTTGTCG-3; 5′-IL-6, 5′-AGTTGCCTTCTTGGGACTGA-3′; 3′-IL-6, 5′-TTCTGCAAGTGCATCATCGT-3′; 5′-GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′; and 3′-GAPDH, 5′-TCCACCACCCTGTTGCTGTA-3′.

2.6. Protein extraction

Whole cell lysates were prepared using PRO-PREP protein extraction solution (Intron Biotechnology, Seongnam, Korea). Nuclear and cytoplasmic fractionation was performed with the NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Scientific), according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and lamin B were used as loading controls for cytoplasmic and nuclear proteins, respectively.

2.7. Western blot analysis

Equal amounts of whole cell protein, nuclear, mitochondrial, and cytoplasmic lysates were separated by 8–12% SDS-PAGE and were transferred onto nitrocellulose membranes (Pall Corporation, Pensacola, FL, USA). The membranes were blocked by incubation with blocking buffer (Thermo Scientific). They were blotted with the primary antibodies rabbit anti-iNOS, rabbit anti-p-Drp1(S637), rabbit anti-p-IκB, rabbit anti-ERK, rabbit anti-p-ERK, rabbit anti-