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Pseudoginsenoside-F11 (PF11) exerts anti-neuroinflammatory effects on LPS-activated microglial cells by inhibiting TLR4-mediated TAK1/IKK/NF-κB, MAPKs and Akt signaling pathways

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ABSTRACT

Pseudoginsenoside-F11 (PF11), an ocotillol-type ginsenoside, has been shown to possess significant neuroprotective activity. Since microglia-mediated inflammation is critical for induction of neurodegeneration, this study was designed to investigate the effect of PF11 on activated microglia. PF11 significantly suppressed the release of ROS and proinflammatory mediators induced by LPS in a microglial cell line N9 including NO, PGE_2, IL-1β, IL-6 and TNF-α. Moreover, PF11 inhibited interaction and expression of TLR4 and MyD88 in LPS-activated N9 cells, resulting in an inhibition of the TAK1/IKK/NF-κB signaling pathway. PF11 also inhibited the phosphorylation of Akt and MAPKs induced by LPS in N9 cells. Importantly, PF11 significantly alleviated the death of SH-SY5Y neuroblastoma cells and primary cortical neurons induced by the conditioned-medium from activated microglia. At last, the effect of PF11 on neuroinflammation was confirmed *in vivo*: PF11 mitigated the microglial activation and proinflammatory factors expression obviously in both cortex and hippocampus in mice injected intrahippocampally with LPS. These findings indicate that PF11 exerts anti-neuroinflammatory effects on LPS-activated microglial cells by inhibiting TLR4-mediated TAK1/IKK/NF-κB, MAPKs and Akt signaling pathways, suggesting its therapeutic implication for neurodegenerative disease associated with neuroinflammation.

1. Introduction

Pseudoginsenoside-F11 (PF11), an ocotillol-type ginsenoside contained in *Panax quinquefolium* L., was isolated from leaves of *Panax pseudoginseng* subsp. *Himalaicus* H_{ARA} (Himalayan Panax) (Wang et al., 2011). As a typical member of saponins in ginseng family, PF11 was able to reduce memory impairment in scopolamine-induced dementia (Li et al., 1999) and improved spatial and non-spatial memory in mice treated with morphine or methamphetamine (Hao et al., 2007; Li et al., 2000; Wu et al., 2003). Especially, PF11 could also improve cognitive impairment in mice treated with $Aβ_{1-42}$, as well as APP/PS1 mice through its inhibitory effect on amyloidogenesis, oxidative stress and beneficial effects on neuronal functions (Wang et al., 2013). Therefore, PF11 is a promising candidate for the development of neuroprotective drugs.

Microglia, the resident macrophage-like cells in the brain, have been proposed to play a pivotal role in the immune surveillance of the central nervous system (CNS) (Lue et al., 2010). Furthermore, lines of evidence showed that microglia-mediated neuroinflammation

Abbreviations: PF11, pseudoginsenoside-F11; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; IL, interleukin; PGE_2, prostaglandin E_2; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; TLR4, toll-like receptor 4; MyD88, myeloid differentiation factor 88; TRAF6, tumor necrosis factor receptor-associated factor 6; IRAK1, interleukin-1 (IL-1) receptor–associated kinase 1; TAK1, transforming growth factor β activated kinase 1; IKK, IκB kinases; NF-κB, nuclear factor κB; ERK1/2, extracellular signal regulated kinase; JNK, c-Jun N-terminal protein kinase; PI3K, phosphatidylinositol 3-kinase; NADPH, nicotinamide adenine dinucleotide phosphate; AD, Alzheimer's disease; PD, Parkinson's disease.

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contribute to the pathology of both acute pathologies such as stroke, traumatic brain injury and chronic neurodegenerative diseases (Gu et al., 2012; Hagberg et al., 2012; Wu et al., 2009). Although the etiology of these diseases is complicated, brain inflammation plays an important role, and it has been confirmed that blocking inflammation could either delay onset or alleviate the symptoms of these diseases (Brown and Neher, 2010). Enzymes associated with inflammation, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), have been identified postmortem in AD and PD brains (Collins et al., 2012; Zilka et al., 2012). When subjected to abnormal stimulation, microglia become activated, with the release of proinflammatory cytokines (Choi et al., 2011; Siopi et al., 2013) and reaction oxygen species (ROS) due to catalysis by NADPH oxidase, which further aggravate neuroinflammatory injury (Dohi et al., 2010). Toll like receptor 4 (TLR4), an important Pattern Recognition Receptor (PRR) is expressed by microglial cells (Okun et al., 2009) and is responsible for inflammatory cascade in microglia upon binding with lipopolysaccharide (LPS) (Verstak et al., 2009). Thus, inhibiting the activation of microglia mediated by TLR4 in inflammatory responses represents a potentially neuroprotective treatment strategy.

Until now, the effect of PF11 on microglia-mediated neuroinflammation has not been investigated. This study was designed to investigate the capability of PF11 on LPS-induced microglial activation *in vitro* and *in vivo*. Here, we report that PF11 is able to inhibit the activation of microglia by inhibiting TLR4-mediated TAK1/IKK/NF-κB, MAPKs and Akt signaling pathways which resulting in neuron protection.

2. Materials and methods

2.1. Reagents and antibodies

Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagel Medium (DMEM), Neurobasal Medium, B27 supplement, fetal bovine serum (FBS) and 0.25% trypsin were purchased from Gibco BRL (Grand Island, NY, USA). Lipopolysaccharide (LPS) from *Escherichia coli* 026:B6, 2′,7′-dichlorodihydrofluororescein diacetate (DCF-DA), Nitrotetrazolium Blue chloride (NBT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), minocycline hydrochloride (MINO), dephenylene iodonium (DPI), N-acetyl-Lcysteine (NAc) and pyrrolidine dithiocarbamate (PDTC), poly-L-Lysine hydrobromide, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and normal horse serum were purchased from Sigma Chemical Co. (St. Louis, MO, USA); LPS-RS (LPS-derived from the photosynthetic bacterium *Rhodobacter sphaeroides*) was from InvivoGen (SanDiego, CA, USA); Trizol reagent was from Invitrogen Co. (Carlsbad, CA, USA); RevertAid™ First Strand cDNA Synthesis Kit was from Fermentas (Burlington, Ontario, Canada); Taq polymerase was from the Takara Biotechnology (Dalian, China). TNF-α, IL-1β, IL-6 and PGE_2 ELISA kits were from R&D Systems (Abingdon, UK); Antibodies see Table 1.

Table 1
Primary antibodies used in this study.

Primary antibody	Dilution	Source
Toll-like Receptor 4 Antibody (Rodent Specific)	1:1000	Cell Signaling Technology (CST)
IKKβ (2C8) Rabbit Antibody	1:1000	
IKKα Rabbit Antibody	1:1000	
TAK1 Rabbit Antibody	1:1000	
Phospho-IKKα/β (Ser176/180) (16A6) Rabbit Antibody	1:500	
Phospho-TAK1 (Thr184/187) (90C7) Rabbit Antibody	1:500	
iNOS Rabbit Antibody	1:1000	
COX-2 Rabbit Antibody	1:1000	
p38 MAPK Rabbit Antibody	1:1000	
SAPK/JNK (56G8) Rabbit Antibody	1:1000	
p44/42 MAPK (Erk1/2) (137F5) Rabbit Antibody	1:1000	
Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP™ Rabbit Antibody	1:1000	
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) XP™ Rabbit Antibody	1:2000	
Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit Antibody	1:1000	
p-Akt (S473) Rabbit Antibody	1:500	
Akt Rabbit Antibody	1:1000	
Anti Iba1 Rabbit Antibody	1:1000	Wako
NF-κB p65 Rabbit Antibody	1:1000	Santa Cruz
MyD88 (HFL-296) Rabbit Antibody	1:500	
NF-κB p50(E-10) Mouse Antibody	1:1000	
β-actin Mouse Antibody	1:500	
Na+/K + -ATPase α(H-3) Mouse Antibody	1:1000	
HDAC1 (10E2) Mouse Antibody	1:1000	CST
Phospho-IκBα (Ser32/36) (5A5) Mouse Antibody	1:1000	
IκBα (L35A5) Mouse mAb (Amino-terminal Antigen)	1:1000	
MAP2 Antibody	1:1000	Millipore

2.2. Microglial cell culture

The murine microglia cell line N9 was a kind gift from Dr. P. Ricciardi-Castagnoli (Universita Degli Studi di Milano-Bicocca, Milan, Italy). The cells were similar to primary microglia in producing NO and various cytokines after stimulation. N9 cells were cultured in IMDM supplemented with 5% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in humidified 5% CO_2 (Iribarren et al., 2005).

2.3. Animals

Thirty-six male C57BL/6 mice were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The animals were housed under conventional conditions at 22 °C, 50–60% humidity and a 12 h light/12 h dark cycle. Mice were provided free access to food and water. All experiments were conducted to minimize the suffering of animals caused by any procedures according to the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China.

2.4. PF11 treatments

PF11 (Fig. 1A) was isolated from the aerial parts of *Panax quinquefolium* L. by Department of Chemistry for Nature Products of Shenyang Pharmaceutical University (China). The purity of PF11 detected with HPLC was more than 98%.

2.4.1. Treatment procedures of PF11 in vitro

PF11 was initially dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate-buffered saline (PBS). The highest concentration of DMSO present in experimental conditions (0.1%) was not toxic to the cells. The endotoxin content of purified PF11 was less than 0.2 EU/mg. To determine interactions of TLR4 with MyD88 or TAK1 by immunoprecipitation, N9 microglial cells were pretreated with PF11 for 2 h, and then were exposed to LPS for 10 min. For determination phosphorylation of IKKα/β, TAK1 by Western blot, N9 microglial cells were pretreated with PF11 for 2 h, and then were exposed to LPS for 15 min. To detect phosphorylation of MAPKs, NF-κB and IκB-α by Western blot, and nuclear translocation of NF-κB p65 by High Content Screening System, N9 microglial cells were pretreated with PF11 for 2 h, and then were exposed to LPS for 30 min. For detection of mRNA expression of proinflammatory factors by RT-PCR and ROS production, N9 microglial cells were pretreated with PF11 for 2 h, and then were exposed to LPS for 6 h. In NADPH oxidase activity assay, N9 microglial cells were pretreated with PF11 for 2 h, and then were exposed to LPS for 12 h. To investigate NO production by Griess reaction, proinflammatory factors release by ELISA, and expression of TLR4, MyD88, iNOS and COX-2 by western blot, N9 microglial cells were pretreated with PF11 for 2 h, and then were exposed to LPS for 24 h. Generally, the MTT reduction assay was performed after treatment with LPS for 24 h.

2.4.2. Treatment procedures of PF11 in vivo

The mice were divided into three groups randomly ($n = 12$ in each group): (1) saline group; (2) LPS group; and (3) PF11 8 mg/kg group (Hao et al., 2007; Wang et al., 2013; Wu et al., 2003). Each mouse in the PF11 8 mg/kg group was treated with PF11 at a dose of 8 mg/kg orally once daily for 21 days, while each mouse in the saline group and LPS group received an equal volume of saline. On day 8, the mice in the LPS group and PF11 8 mg/kg group were slowly injected with LPS 40 μg into hippocampus bilaterally (1 μl on each site over 5 min) using the following coordinates: AP -2.4 mm, ML ±2.2 mm and DV -2.4 mm from the bregma (Paxinos and Franklin, 2001), the mice of saline group were injected with equal volume of saline. When the injection was finished, the needle was left in situ for 5 min to avoid reflux along the injection track before withdraw.