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Original article

Peroxiredoxin 5 (Prx5) decreases LPS-induced microglial activation through regulation of Ca^{2+}/calcineurin-Drp1-dependent mitochondrial fission

Junghyung Park [a,b], Hoonsung Choi [c], Bokyung Kim [a,b], Unbin Chae [a,b], Dong Gil Lee [a,b], Sang-Rae Lee [d], Seunghoon Lee [c], Hyun-Shik Lee [a,b], Dong-Seok Lee [a,b,*]

[a] School of Life Sciences, BK21 Plus KNU Creative BioResearch Group, Kyungpook National University, Daegu, Republic of Korea
[b] College of Natural Sciences, Kyungpook National University, Daegu, Republic of Korea
[c] Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, Jeollabuk-do, Republic of Korea
[d] National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Chungcheongbuk-do, Republic of Korea

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ABSTRACT

Microglial activation is a hallmark of neurodegenerative diseases. ROS activates microglia by regulating transcription factors to express pro-inflammatory genes and is associated with disruption of Ca^{2+} homeostasis through thiol redox modulation. Recently, we reported that Prx5 can regulate activation of microglia cells by governing ROS. In addition, LPS leads to excessive mitochondrial fission, and regulation of mitochondrial dynamics involved in a pro-inflammatory response is important for the maintenance of microglial activation. However, the precise relationship among these signals and the role of Prx5 in mitochondrial dynamics and microglial activation is still unknown. In this study, we demonstrated that Ca^{2+}/calcineurin-dependent de-phosphorylation of Drp1 induces mitochondrial fission and regulates mitochondrial ROS production, which influences the expression of pro-inflammatory mediators in LPS-induced microglia cells. Moreover, it is likely that cytosolic and Nox-derived ROS were upstream of mitochondrial fission and mitochondrial ROS generation in activated microglia cells. Prx5 regulates LPS-induced mitochondrial fission through modulation of Ca^{2+}/calcineurin-dependent Drp1 de-phosphorylation by eliminating Nox-derived and cytosolic ROS. Therefore, we suggest that mitochondrial dynamics may be essential for understanding pro-inflammatory responses and that Prx5 may be used as a new therapeutic target to prevent neuroinflammation and neurodegenerative diseases.

1. Introduction

Microglial cells are immune cells resident in the brain that play essential roles in neuronal homeostasis by promoting neurogenesis, cleaning debris, and becoming activated when the brain is challenged with infection or injury. However, consistently activated microglia cells can exert harmful neurotoxic effects through the excessive production of cytotoxic molecules, such as nitric oxide (NO), and pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. Therefore, activation of microglia is regarded as a common and early hallmark of neurodegenerative diseases, and preventing excessive production of pro-inflammatory mediators from microglia attenuate neuronal damage [1–3]. Accordingly, the precise mechanisms that control production of pro-inflammatory molecules in activated microglia may be crucial for neurodegenerative therapy.

Microglia cells become activated in response to a wide variety of pathological stimuli [4]. It has been reported that lipopolysaccharide (LPS), a membrane component of gram-negative bacteria, is a common inflammatory activator of microglia cells via the TLR4 complex that induces the expression of various pro-inflammatory molecules. LPS-induced TLR4 activation activates downstream signaling molecules, including NF-κB and MAPKs, which are involved in signaling cascades leading to the abundant expression of pro-inflammatory molecules [5,6].

Abbreviations: TLR4, Toll-like receptor 4; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; Cys, cysteine; Drp1, dynamin-related protein 1; Fis1, mitochondrial fission 1; Mfn1/2, mitofusin 1 and 2; Opa1, optic atrophy protein 1; Ser, serine; cAMP, Cyclic adenosine monophosphate; PKA, Protein kinase A; NAC, N-acetyl-l-cysteine; TBST, Tris-buffered saline Tween 20; FK506, tacrolimus; A23187, calcimycin; NFAT, nuclear factor of activated T-cells; PGAM5, phosphoglycerate mutase family member 5; Cabin-1, calcineurin-binding protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; MAPK, mitogen-activated protein kinases; IP3R, Inositol trisphosphate receptor; RyR, Ryanodine receptors; MOI, multiplicity of infection

* Corresponding author at: College of Natural Sciences, Kyungpook National University, Daegu, Republic of Korea.
E-mail address: lee1@knu.ac.kr (D.-S. Lee).

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Reactive oxygen species (ROS) actively participate in microglial pathogenesis as secondary messengers capable of modifying gene expression by altering kinase cascades and activating transcription factors, including MAPK and NF-κB, in microglia cells [7–9]. ROS is mainly produced from NADPH oxidase (Nox) and mitochondria in microglia, and these ROS from Nox and mitochondria are involved in microglial immune responses [10,11]. Peroxiredoxins (Prxs) are a family of antioxidant enzymes that eliminate H_2O_2 and are reported to participate in a wide range of cellular functions, such as cell proliferation, differentiation, and apoptosis, by modulating ROS [12,13]. Prx participates in an inflammatory response by regulating the redox signaling pathway. Abnormal expression of Prx subtypes in diverse neurodegenerative disorders and activated microglia cells have been reported [14,15]. Additionally, in an earlier study, Prx5 acts as an inducible inflammatory regulator by modulating the redox signaling pathway in microglia and macrophage cells [16,17]. Prx5 is the only 2-Cys Prx that exhibits atypical biochemical and structural features [18]. Therefore, a precise understanding of the mechanisms of the inducible Prx5 in activated microglia cells is needed.

Recently, amounting reports have demonstrated that mitochondria are highly dynamic organelles that constantly undergo fission and fusion. Mitochondrial fission and fusion are associated with a wide range of cellular processes, participating in the maintenance of mitochondrial functions. Mitochondrial fission is regulated by Drp1 and Fis1, and fusion is regulated by Mfn1, Mfn2, and Opa1 [19]. Imbalanced mitochondrial dynamics in neuronal cells have been implicated in various neurodegenerative pathological processes [20,21]. Specially, growing evidence, including from our research, demonstrate that mitochondrial fission is increased by immune-stimulation in a de-phosphorylation of Drp1-dependent manner, and that it is related to inflammatory responses [22–25]. However, details of the regulation of mitochondrial dynamics in activated microglial cells are not fully understood.

Drp1 activity is regulated by phosphorylation at the 637th serine residue in Drp1. This residue is recognized to be phosphorylated by cAMP-dependent PKA and de-phosphorylated by calcineurin [26,27]. In particular, calcineurin is known to calcium (Ca^{2+})/calmodulin (CaM)-dependent serine/threonine phosphatase that belong to the family of protein phosphatase 2B [28]. Calcineurin activity is regulated by interactions of the Ca^{2+}/CaM complex and heterodimerization of calcineurin subunit A (CnA) and B, which is tightly controlled by cellular conditions, such as Ca^{2+} concentration [28]. The CnA contains a catalytic domain, the binding site for calmodulin, and a C-terminal autoinhibitory domain. Calcineurin activation can also be induced by permanent cleavage of the autoinhibitory domain by calpain, a Ca^{2+}-dependent cysteine protease [29,30]. In addition, calcineurin, as well as Ca^{2+}/CaM and the calpain signaling pathway, are considered to be important mediators in a variety of immune responses [31–34]. However, the precise relationship among these signals and their roles in mitochondrial dynamics in an innate immune response are still unknown.

Therefore, in the present study, we showed how mitochondrial fission activated by LPS is related to a microglial pro-inflammatory response and is modulated by variety of upstream signals, including Ca^{2+}/CaM and the calpain signaling pathway. We also investigated the molecular mechanism of anti-inflammatory Prx5 in activated microglia cells on mitochondrial dynamics and inflammatory responses.

2. Materials and methods

2.1. Cell culture and treatment

BV-2 murine microglial cells were immortalized by infection with v-raf/c-myc recombination retrovirus [35], which were kindly provided by Dr. Jau-Shyong Hong (National Institute of Environmental Health Sciences, NC, USA). BV-2 cells were propagated in DMEM (Welgene, Daegu, Korea) containing 10% FBS (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Welgene) and maintained at 37 °C in a humidified 5% CO_2 incubator (SANYO, Osaka, Japan). Cell were sub-cultured at a density of 1×10^5 cells in 6-well plates and grown for 24 h prior to experiments. Exponentially growing BV-2 cells were pre-treated with NAC, CsA, FK506, ALLN, apocynin (Sigma-Aldrich, St. Louis, MO, USA), BAPTA-AM (Invitrogen, Carlsbad, CA, USA), W13, or mito-TEMPO (Calbiochem) for 1 h, followed by stimulation with 1 μg/mL of LPS from *E. coli* serotype O26:B6 (Sigma-Aldrich).

2.2. Primary microglia cell culture

The primary microglia enrichment was performed as previously describe [36]. Whole brains of 1–2 day C57BL/6 wild-type mice were stripped of the meninges, mechanically dissociated, and filtered through a 70 μm nylon mesh (Falcon, Becton Dickinson, NJ, USA) to recover isolated cells. The cells were harvested by centrifugation at $700 \times g$ for 5 min, transferred to culture dishes coated with poly-L-lysine (Sigma-Aldrich), and cultivated in DMEM containing 10% FBS at 37 °C in 5% CO_2 incubator for 14 day. Mild trypsinization was performed by a 25% diluted 0.25% trypsin in DMEM. After 15 min, upper layer of glia-astrocyte mix cell layer was removed, whereas a number of cells remained attached to the bottom of the well. Attentively removed supernatant and washed the well with PBS. After then 0.25% trypsin solution was treated for harvest of attached cells to the bottom. The cells were harvested by centrifugation at $700 \times g$ for 10 min, transferred to culture dishes coated with poly-L-lysine. C57BL/6 wild-type mice were maintained in a pathogen-free facility at Korea Research Institute of Bioscience and Biotechnology (KRIBB) and were conducted in accordance with the National Institute of Health Guidance for the Care and Use of Laboratory Animals and were approved by the KRIBB Institutional Animal Care and Use Committee. Prx5 lentivirus (MOI=5) was transduced in primary microglia cells with 4 μg/mL of Polybrene (Sigma-Aldrich), which increases the efficiency of lentivirus infection in mammalian cells.

2.3. Plasmid cloning and mutation

The DsRed2-mito gene was obtained from pDsRed2-Mito (Clontech, Palo Alto, CA, USA), and the Prx5 gene was kindly provided by Dr. Tae-Hoon Lee (Chonnam National University, Gwangju, Korea). The C48S mutation of the Prx5 gene was introduced using a site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The following primers were used for mutation: sense-Prx5 (C48S), 5′-CCTGGCTTGGATCAACGCCCCCCGGAAAG-3′, antisense-Prx5 (C48S), 5′-CTTTCCGGGGGGCGTTGATCCAAGCCAGG-3′. The DsRed2-mito, Prx5-WT, and Prx5-C48S genes were amplified by PCR using LA Taq polymerase (Takara, Shiga, Japan) and cloned into the pCR8/GW/TOPO vector (Invitrogen), and then each gene were inserted into the pLenti6.3/V5-DEST or pLenti7.3/V5-DEST vector (Invitrogen) using LR clonase (Invitrogen).

2.4. Lentiviral vector-mediated gene transfer and preparation of stable cell lines

Construction of the lentivirus was performed as previously described [37]. The lentiviral vector (MOI=5) was transduced in