Neuroscience Letters 643 (2017) 131–137

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Krüppel-like factor 4 regulates amyloid-β (Aβ)-induced neuroinflammation in Alzheimer's disease

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HIGHLIGHTS

- Oligomeric Aβ42 increases the Klf4 expression in microglial BV2 cells.
- Gene and protein expressions of Klf4 are increased in brain microglia of AD mice.
- p53 mediates Aβ42-induced expression of Klf4.
- Silence of Klf4 restores Aβ42-induced neuroinflammation.
- Overexpression of Klf4 exacerbates Aβ42-induced neuroinflammation.

ARTICLE INFO

Article history:
Received 1 November 2016
Received in revised form 3 February 2017
Accepted 7 February 2017
Available online 9 February 2017

Keywords:
Alzheimer's disease
Amyloid-β (Aβ)
Krüppel-like factor (KLF) 4
Neuroinflammation
p53

ABSTRACT

Alzheimer's disease (AD), one of the most common neurodegenerative diseases, is characterized by extracellular deposition of amyloid-β (Aβ) peptide, and neuro-inflammatory processes mediated by microglial activation are known to play a pivotal role in AD. However, the expression pattern and function of Krüppel-like factor (KLF) 4 in AD remain unknown. In this study, KLF4 was found to be increased at both the gene and protein levels in response to incubation with oligomeric Aβ42 in a dose-dependent manner in BV2 microglial cells. An in vivo study also displayed that expression of KLF4 in the brains of J20 transgenic AD model mice was increased due to accumulation of Aβ. Mechanistically, activation of p53 resulting from an increase in phosphorylation at ser15 was verified as the mediator of the oligomeric Aβ42-induced expression of KLF4. Subsequent experiments have demonstrated that KLF4 silencing in BV2 cells attenuates oligomeric Aβ42-induced neuroinflammation by ameliorating the release of proinflammatory cytokines, such as tumor necrosis factor-a (TNF-α), interleukin (IL)-1β, IL-6, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). In addition, overexpression of KLF4 promoted oligomeric Aβ42-induced neuroinflammation by exacerbating the release of pro-inflammatory factors. These results suggest a KLF4 plays a potential role in oligomeric Aβ42-induced neurotoxicity and the pathogenesis of AD.

1. Introduction

Plaques formed from the extracellular deposition of amyloid-β (Aβ) peptide are one of the most important hallmarks of Alzheimer's disease (AD) [1]. An increasing number of basic and clinical studies demonstrate that Aβ-mediated inflammation plays a pivotal role in the pathogenesis of AD [2]. In addition, epidemiological studies have proven that administration of nonsteroidal anti-inflammatory drugs (NSAIDs) could reduce the risk of developing AD [3]. Activated microglia has been found in the brains of AD patients [4]. Multiple lines of evidence have proven that exposure of microglia to oligomeric Aβ peptides leads to the production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), inducible nitric oxide syn-

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; Klf 4, Krüppel-like factor 4; TNF-α, tumor necrosis factor-a; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; CNS, central neural system; LPS, lipopolysaccharide; HFIP, 1,1,1,3,3,3- hexafluoro-2-propanol; PBS, phosphate-buffered saline; DMEM, Dulbecco's Modified Eagle's Medium; NaHCO3, sodium bi-carbonate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; PCR, polymerase chain reaction; PFA, paraformaldehyde.

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http://dx.doi.org/10.1016/j.neulet.2017.02.017
0304-3940/

thase (iNOS), cyclooxygenase-2 (COX-2), and so on. Overproduction of these factors is associated with increased levels of reactive oxygen species (ROS) and reactive NO species (RNS), which induce neuronal death in the brain [5]. However, its underlying mechanism needs to be elucidated.

Krüppel-like factor 4 (KLF4) is a member of the KLF family, which makes up a class of zinc finger DNA-binding proteins. KLF family members contain a zinc finger DNA-binding domain at the C-terminus, which binds to either a CACCC element or GC-box, regulating target gene expression in various cells [6]. KLF4 is known as gut-enriched KLF, which was first found in the epithelial lining of the gut [7]. Previous studies have reported that KLF4 can regulate multiple biological functions, including cell growth, proliferation and differentiation [8,9], and that KLF4 plays a key role in regulating inflammation mediated by endothelial cells, epithelial cells, and macrophages [10]. As most of these studies were limited to the peripheral system, there are few studies on its role in the central neural system (CNS). However, a recent study demonstrated that KLF4 is involved in mediating the upregulation of proinflammatory cytokines in microglial cells following lipopolysaccharide (LPS) treatment, suggesting a potential proinflammatory function of KLF4 in the CNS [11]. Moreover, the expression patterns of KLF4 in neurodegenerative diseases remain an enigma. Whether KLF4 is involved in the pathological processes that take place in neurodegenerative diseases, and especially in Alzheimer's disease (AD), remains unknown. In the present study, we investigated the effects of KLF4 in an oligomeric Aβ-induced neurotoxicity model and demonstrated that p53-mediated expression of KLF4 plays an essential role in Aβ-induced inflammation.

2. Materials and methods

2.1. Preparation of Aβ42 oligomer and cell treatment

Aβ42 peptide was used to prepare oligomeric amyloid-β as previously described [12]. Briefly, solid Aβ42 was dissolved in 1,1,1,3,3,3- Hexafluoro-2-propanol (HFIP, Sigma), until the solution was clear and colorless. HFIP was evaporated overnight under a fume hood. Dried peptide films were stored in −20 °C. Immediately before use, clear peptide film treated with HFIP was dissolved in DMSO to make 5 mM stock followed by sonication for 10 min. Freshly re-suspended Aβ42 was diluted in phosphate-buffered saline (PBS) to a final concentration of 100 μM. After centrifuging for 15 s, the final Aβ42 oligomer-enriched solution was used immediately. BV-2 cells were stimulated by Aβ42 at different concentrations (2, 5 and 10 μM) in a CO_2 incubator at 37 °C. Aβ42 peptide oligomerization was assessed by Tris-Tricine SDS-PAGE and western blot analysis as previously described [13].

2.2. Cell culture and transfection

Murine microglial BV-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic (penicillin and streptomycin). Human KLF4 lentiviral vectors were from Qiagen, USA. Briefly, lentiviruses were generated in HEK 293T cells and titrated into BV-2 cells to overexpress KLF4. Non-specific or KLF4-specific siRNA (100 nM) (Dharmacon, USA) was transfected into BV-2 cells using Lipofectamine RNAiMAX (Invitrogen, USA).

2.3. Animals

AD transgenic J20 mice, carrying the Swedish and Indiana APP human mutations, were purchased from Jackson Laboratory (JAX Stock No. 006293: B6.Cg-Tg (PDGFB-APPSwInd) 20L ms/2Mmjax), USA. The J20 line was generated as previously described [14]. Briefly, mice were maintained by heterozygous crosses with C57BL/6J mice. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Central South University (Changsha, China).

2.4. Western blot analysis

Tissue and cell lysates were made using RIPA buffer. Total protein lysates (20 μg of proteins) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Sigma, USA). After blocking with 5% fat-free milk at room temperature (RT) for 1 h, membranes were sequentially incubated with primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. The blots were then visualized with an ECL chemiluminescence detection system (Bio-Rad, USA) in accordance with the manufacturer's instructions. The following antibodies were used: rabbit anti-KLF4 (1:1000, Chemicon International, USA), mouse anti-p-p53 (1:1000, Cell signaling, USA), mouse anti-β-actin (1:5000, Santa Cruz, USA).

2.5. Real-time polymerase chain reaction (PCR)

Total RNA was extracted from BV-2 cells and mouse brain tissues using Trizol reagent. The resulting RNA (1 mg) was reverse transcribed into cDNA using a reverse transcription (RT) cDNA synthesis kit (Invitrogen, USA). The cDNA was then diluted 5 times and analyzed via quantitative real-time PCR using SYBR Green qPCR Master Mix. The following primers were used: Mouse KLF4, 5′-CAAGTCCCGCCGCTCCATTACCAA-3′ (forward) and 5′-CCACAGCCGTCCCAGTCACAGTGG-3′ (reverse); Mouse GAPDH, 5′-TGTGTCCGTCGtGGATCTGA-3′ (forward) and 5′-CCTGCTTCACCACCTTCTTGA-3′ (reverse).

2.6. Enzyme linked immunosorbent assay (ELISA) of TNF-α, IL-1β, IL-6, COX-2 and iNOS

BV-2 microglial cells were plated into 96-well microtiter plates at a density of 3×10^5 cells/ml. After the indicated transfection and treatment, the concentrations of TNF-α, IL-1β, IL-6, COX-2, and iNOS (R&D Systems, USA) in the culture medium were measured using ELISA kits according to the manufacturer's protocol.

2.7. Immunofluorescence microscopy

Protein expression of KLF4 in the brain microglia of AD model J20 mice was determined by double-fluorescence staining of KLF4/GFAP. GFAP was used as a specific microglia marker. Briefly, the brains of wild-type and J20 mice were fixed with 4% paraformaldehyde (PFA) for 24 h at RT. Brain tissues were equilibrated overnight in 40% sucrose and then cut into 8 μm coronal sections with a freezing microtome (Leica, Germany). Brain cryosections were fixed in 4% paraformaldehyde for 10 min at RT. Then, brain sections were washed and blocked for 30 min with 5% BSA in PBS-T (0.1 M PBS containing 0.2% Tween 20). The slides were sequentially incubated overnight at 4 °C with rabbit anti-KLF4 (1:1000, Chemicon International, USA)/mouse anti-GFAP (Cell signaling, USA) antibodies and Alexa 488- or 594-conjugated secondary antibodies for 1 h at RT. Fluorescence signals were observed and captured with a laser scanning confocal microscope (FV10i; Olympus, Japan).

2.8. Statistical analysis

Each experiment was repeated at least three times, and the data are expressed as means ± SD (standard deviation). Statistical anal-