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Research article

Proteomic analysis of the effects of Nur77 on lipopolysaccharide-induced microglial activation

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HIGHLIGHTS

- Microglia are critical players of immune response in central nervous system.
- Nur77 have a role on regulating activating state in microglia.
- Nur77 could be the upstream protein of vav1 and ERK1/2 signaling pathway.

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ABSTRACT

Microglia are critical components of the immune response in the central nervous system. Our study aims to explore potential role of Nur77 in lipopolysaccharide (LPS)-induced microglial activation. Primary wild-type and Nur77$^{-/-}$ microglia were stimulated with LPS and protein extracts were detected via mass spectrometry. Q-PCR and western blotting were performed to validate candidate proteins. A total of 2004 proteins were identified, with 749 and 677 significantly differentially expressed proteins in wild-type and Nur77$^{-/-}$ microglia in resting and activated states, respectively. Signaling pathway analysis showed that significantly differentially expressed proteins in LPS-treated Nur77$^{-/-}$ microglia were present in important signaling pathways of microglial activation, including the Toll-like receptor signaling pathway, the MAPK signaling pathway, FcγR-mediated phagocytosis, and chemokine signaling pathways. Furthermore, we found that Nur77 could be the upstream protein of the vav1 and ERK1/2 signaling pathway. This study provided new insights into the understanding the mechanisms of the effects of Nur77 on LPS-activated microglia.

1. Introduction

Microglia are resident immune cells in the central nervous system (CNS), and they comprise 10–15% of all glial cells [1]. Microglia have a hematopoietic origin; they originate from the yolk sac and proliferate in the CNS [2,3]. Under physiological conditions, microglia actively monitor the surrounding microenvironment [4]. Under pathological conditions, microglia undergo phenotypic transformation to an activated state and participate in the development of many diseases, such as Alzheimer's disease, Parkinson's disease, stroke and intracerebral hemorrhage [5–8]. However, activated microglia are a double-edged sword. On the one hand, microglia exacerbate tissue injury by producing inflammatory cytokines and cytotoxic mediators. On the other hand, microglia also promote tissue repair and remodeling by clearing debris and releasing anti-inflammation cytokines and growth factors. Nevertheless, the molecular mechanisms of microglial activation remain to be elaborated.

Nur77 is an orphan nuclear receptor; it belongs to the orphan nuclear subfamily 4A within the steroid/thyroid receptor family, which also comprises Nurr1 and NOR-1 [9]. Although Nur77 was initially identified as a key regulator of apoptosis, metabolism, and cell cycle mediation, recent studies highlighted a role of Nur77 in the regulation of the active state in periph-

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eral macrophages. The bone marrow-specific deficiency of Nur77 resulted in altered macrophage polarization with the enhanced expression of interleukin-12, interferon γ, and stromal cell-derived factor-1α (SDF-1α) in response to lipopolysaccharide (LPS) [10]. Moreover, mice deficient in Nur77 showed a more pro-inflammatory phenotype of macrophages, which may explain the enhanced development of atherosclerosis [11].

Nur77 altered the response of peripheral macrophages to LPS [10]. LPS is a major component of the outer membrane of Gram-negative bacteria, and LPS-stimulated microglia have been used as a common model to explore microglial activation in vitro. Once activated by LPS, microglia could promote the release of pro-inflammation factors and chemokines release and clear debris [12–15].

Microglia are often referred to as the macrophages of the CNS, however, the effects of Nur77 on LPS-induced microglial activation has not yet been investigated and was the subject of current study. We performed a mass-spectrometry-based proteomic analysis with primary microglia to investigate whether Nur77 plays a role in LPS-induced microglial activation and to examine its potential mechanisms. We found that Nur77 altered the expression of many proteins in LPS activated microglia, including those involved in microglial inflammation, phagocytosis, and migration signaling pathways. Moreover, both vav1 and ERK1/2, which could be induced by LPS stimulation, appear to be associated with the effect of Nur77 on microglial activation.

2. Materials and methods

2.1. Mice

Nur77$^{-/-}$ mice were purchased from Jackson laboratory. They were housed in a pathogen-free environment with a 12-h/12-h light-dark cycle with free access to food and water at the Laboratory Animal Center of Drum Tower hospital.

2.2. Primary microglia culture and lipopolysaccharide treatment

Primary microglia were obtained as previously described [16]. Briefly, C57BL/6J and Nur77$^{-/-}$ neonatal pups (P0-P1) were used for primary microglia cultures. After removing meninges and visible blood vessels, the brains were digested with 0.25% trypsin in a 37 °C incubator for 10 min and then the same amount of DMEM supplemented with 10% FBS and 100 μg/ml streptomycin was used to terminate digestion. The cell suspension was seeded in 75-cm^2 flasks and maintained at 37 °C in a humidified incubator with 5% CO_2. Culture medium was changed every three days. After 10–12 days, loosely attached microglia were harvested from medium by shaking flasks for 10 min. The obtained microglia were then plated into 6-well plates at a density of 1×10^6 cells/well and 12-well plates at a density of 5×10^5 cells/well. A total of approximately 108 newborn mice including 54 WT pups and 54 Nur77$^{-/-}$ pups were sacrificed for microglial culture. Each experiment was repeated three times. The purity of the primary microglia cells was more than 95% as determined by Iba-1 immunocytochemical staining, consistance with our previous report [17]. A total of 48 h after plating, microglial cells were washed with PBS twice then stimulated with 0.5 μg/ml LPS (Sigma, USA) in culture medium for 30 min and 24 h in different experiments. For mass spectrometry, we prepared the microglial proteins from three independent microglia cell culture experiments.

2.3. Protein preparation and HPLC–MS/MS analysis

Microglial cells were washed with PBS twice and then lysed in RIPA lysis buffer supplemented with 1% PMSF (vol/vol) on ice. After centrifugation, the supernatant was collected. Total protein concentration was determined with a Bicinchoninic Acid assay (Beyotime biotechnology, China). Samples of supernatant that contained 100 μg of protein were loaded for SDS-polyacrylamide gel electrophoresis. When protein was separated in separation gels, the gels were fixed and stained with colloidal Coomassie Blue G-250 for 4 h while shaking. Each gel lane was sliced into 12 equal-sized parts following destaining in ultrapure H_2O and each part was cut into smaller pieces and collected in an Eppendorf tube. The gel pieces were destained with 50% acetonitrile in 50 mM ammonium bicarbonate, dehydrated in 100% acetonitrile and rehydrated in 50 mM ammonium bicarbonate. The destaining cycle was repeated once. Then, gel pieces were dehydrated in 100% acetonitrile, dried in a SpeedVac, and incubated with trypsin solution that contained 10 μg/ml trypsin (sequence grade; Promega, USA) in 50 mM ammonium bicarbonate overnight at 37 °C. Peptides from the gel pieces were extracted twice with 200 μL of 50% acetonitrile in 0.1% Trifluoroacetic acid and dried in a SpeedVac. Mass spectrometric analysis was performed with an AB SCIEX TripleTOF 5600 mass spectrometer (AB SCIEX, Framingham, MA, USA), coupled with an online HPLC system.

2.4. Proteomic data analysis

Protein identifications were assigned through MaxQuant (version 1.50) with the UniProt *Mus musculus* database. Carbamidomethylation of cysteine was set as a fixed modification while the oxidation of methionine and N-terminal acetylation were set as variable modifications. Trypsin was designated a digestion protease with up to two missed cleavages allowed. The false discovery rate of both peptides and proteins was set within 1%. Differentially expressed proteins were present at a ratio of more 1.5-fold or less than 0.66-fold. Gene ontology functional classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed with the DAVID website (https://david.ncifcrf.gov). Interactions between differentially expressed proteins were evaluated with the STRING website (http://string-db.org). A heat map was constructed with Mev software.

2.5. Quantitative-polymerase chain reaction

Total RNA was extracted from primary microglia using Trizol reagent (Invitrogen, Camarillo, CA). RNA was then reverse transcribed into cDNA with a Prime Script RT reagent kit (Takara, Clontech, USA). Quantitative PCR was performed with SYBR Green (Takara, Clontech, USA) according to the manufacturer's instructions. Each sample was performed in triplicates, while non-reversed RNA and water served as negative controls. The temperature profiles were one cycle at 95 °C for 30 s, 40 cycles of 95 °C (5 s) and 60 °C(34 s), which were performed using the ABI 7500 PCR instrument (Applied Biosystems, Foster City, CA, USA). The vav1 and GAPDH primer sequences were as follows:

vav1 forward primer, 5′-TGTGAGAAGTTCGGCCTCAAG-3′;
vav1 reverse primer, 5′- CAGAGCAGACAGGGTGTAGAT-3′;
gapdh forward primer, 5′-GCCAAGGCTGTGGGCAAGGT-3′;
gapdh reverse primer, 5′- TCTCCAGGCGGCACGTCAGA-3′.

2.6. Western blot

The microglial cells were treated as described above. Protein extraction and quantification have been mentioned above. Equal amounts (40 μg) of total protein samples were separated by SDS-PAGE, and transferred to PVDF membranes. Then, the membranes were incubated with a vav1 antibody (1:500, cat: #4657, Lot:1, CST, USA), ERK1/2 antibody (1:1000, cat: #4695, Lot:14, CST, USA) and p-ERK1/2 antibody (1:1000, cat: #4370, Lot:15,