ISSN 2959-6157

LPS Priming Before Plaque Deposition Activates the Rho/ROCK Pathway to Regulate Microglial M1/M2 Polarization Through the NF-κB Pathway in the 5xFAD Mouse Model of Alzheimer's Disease

Tete Yin*

Upper Senior School, Dulwich College Singapore, Singapore, 658966, Singapore
*Corresponding author email: yintetete@gmail.com

Abstract:

Alzheimer's Disease is a neurodegenerative disease which is characterized by continuous neuroinflammation, beta-amyloid plaques (Aβ) and tau clusters. Microglia is a type of immune cell that resides in the brain and plays a crucial role in disease progression and neuroprotection based on its extent of polarization. This research investigated the effect on microglial polarization caused by LPS priming before plaque deposition through the Rho/ROCK pathway and the NF-κB pathway in a 5xFAD mouse model. It is assumed that LPS priming will activate the Rho/ROCK pathway and then regulate the NF-κB pathway, causing microglial polarization from M1 (pro-inflammatory state) to M2 (inflammatory state). To achieve this goal, the techniques applied include immunohistochemistry, flow cytometry, Western blotting, and RT-qPCR. The expected result is the observation that LPS priming increased Rho/ROCK activation, inhibited the NF-κB pathway, and promoted M2 polarization. This observation indicates the complex relationship between systemic and neuroinflammatory pathways in AD. Moreover, it provides potential therapeutic approaches targeting Rho/ROCK and NF-κB pathways to reduce neuroinflammation and enhance neuroprotection.

Keywords: Alzheimer's Disease, Microglia, LPS Priming, Rho/ROCK Pathway, NF-κB Pathway, M1/M2 Polarization, Neuroinflammation, 5xFAD Mouse Model, Neurodegeneration

1. Introduction

Alzheimer's Disease (AD) is a progressively worsening neurodegenerative disease that has become an increasing concern in this century. AD is characterized by persistent inflammation in the brain, destruction of nerve cells, the presence of tau tangles, and the accumulation of Aβ proteins. Microglia, the immune cells inherent to the central nervous system, play a significant role in brain homeostasis and response to injury or disease. They can "remember" previous inflammatory events through innate immunity memory (IIM), potentially impacting neurodegenerative diseases. Systemic inflammation is linked to AD progression, with microglia displaying unique signatures in AD models and patients. Systemic infections can prime microglia, leading to either protective or harmful responses depending on the timing and strength of the inflammatory stimulus. Previous studies have shown varied outcomes of microglial priming on Aβ burden and neuroinflammation, highlighting the need to understand these processes better. Knowing the interaction between activation/inhibition pathways and microglia polarisation is meaningful in further approaching the potential therapeutic methods for neuroinflammation. This essay explores how LPS (Lipopolysaccharide) priming before deposition activates the Rho/ROCK Pathway and its role in regulating microglial M1/M2 polarisation via the NF-κB Pathway in the 5xFAD Mouse Model of Alzheimer's Disease. We hypothesize that after the LPS induction, microglia polarization will be inhibited from promoted M1 (anti) to M2 (pro) through the NF-kB pathway, which is further inhibited by the upstream Rho/Rock Pathway.

2. Methodology

The 5xFAD mouse model for AD is chosen because of its accelerated amyloid pathology, reproducibility, and genetic control. An accuracy check will be made to eliminate confounding variables, so the genetic background, age, gender, diet and health status are control in the same experimental group. Pathway analysis includes western blotting, immunohistochemistry, flow cytometry, RT-qPCR, and cell lines.

The mouse model used is 5xFAD Mice. We separate the

5xFAD Mice into the control group – mice without LPS priming before plaque deposition, and the experimental group – mice with LPS priming before plaque deposition. The group consists of 60 mice, with 30 mice in each experimental and control group to reduce epistemic uncertainty (uncertainty due to insufficient data). Only male rates were used in the experiment to reduce hormonal effects.

Experiment 1 – validate whether LPS priming before plaque deposition alters microglia polarization

Mice brain tissue was taken for immunofluorescence and immunohistochemical staining, and the number of M1 and M2 microglia was counted. IBA–1 and iNOS serve as markers for identifying M1 microglia. IBA -1 and Arg -1 serve as indicators of M2 microglia.

The number of M1 and M2 microglia is further counted by flow cytometry; the markers are CD86 and CD206, respectively. Then, the RT-qPCR technique is used to detect markers related to M1 and M2 microglia.

Experiment 2 – demonstrate whether LPS priming inhibits the Rho/ROCK pathway

Our objective is to use western blotting to assess the activation of the Rho/ROCK pathway by analyzing specific markers, including ROCK1, ROCK, 2p-MYPT1/t-MYPT1, and MLC(myosin light chain).

Procedure:

1. Cultivate microglia cells in Eagle cell culture and keep the culture in a humidified atmosphere with 5% CO_2 at 37 degrees.

2. As long as the cell reach approximately 70-80% confluence, treat them with LPS. At the same time, treat the control group with an equal volume of PBS (phosphate – buffered saline) without LPS.

3. After LPS treatment, wash the microglia cells with cold PBS twice to remove residual media and unattached cells. Lyse the cells with RIPA buffer (radioimmunoprecipitation assay buffer) to prevent protein degeneration and dephosphorylation.

4. Collect the cell lysates and centrifuge at 14000 rpm for a duration of 15 minutes at a temperature of 4 degrees Celsius in order to eliminate any cellular waste material.

5. Test the protein concentration of the supernatant with a BCA protein assay kit.

6. Prepare the appropriate concentration of SDS-PAGE gel according to the molecular weight of the target protein.

7. To prevent non-specific binding, obstruct the membrane with a solution of 5% non-fat dried milk in TBS-T for a duration of one hour. Incubate the membrane at 4 degrees with primary antibodies specific to the targets (ROCK1, ROCK, 2p-MYPT1, t-MYPT1, and MLC) overnight.

8. Following the washing of the membrane with TBS – T, proceed to incubate it with suitable HRP-conjugated secondary antibodies for one hour. Then use an ECL detection system to visualize the protein bands and capture the images using a gel documentation system.

9. Quantify the intensity of the protein bands for ROCK1, ROCK, 2p-MYPT1, t-MYPT1, p-MLC, MLC, and the loading control using image analysis software. Make a statistical test to determine the level of significance of the statistical difference between the experimental group and the control group.

Experiment 3 – NF-κB pathway inhibition

To investigate the inhibition of the NF-κB pathway inhibition in the brain, we will use multiple techniques, including western blotting, immunohistochemistry, RT-qPCR, and flow cytometry.

Western blotting is used to measure levels of NF-κB and phosphorylated NF-κB (p-NF-κB p65).

Procedure:

1. Extract proteins from brain tissue samples.
2. Perform SDS-PAGE and transfer to membrane.
3. Block membrane and incubate with primary antibodies (anti-NF-κB p65, anti-phospho-NF-κB p65).
4. Incubate with HRP-conjugated secondary antibodies and detect using ECL.

Immunohistochemistry is used to visualize NF-κB localization in brain sections.

Procedure:

1. Fix and section brain tissues.
2. Stain with primary antibodies.
3. Incubate with fluorescently labelled secondary antibodies and visualize using a fluorescence microscope.

RT-qPCR is used to measure mRNA expression levels of NF-κB target genes.

Procedure:

1. Extract RNA from brain tissue samples.
2. Convert RNA to cDNA and perform qPCR.
3. Use specific primers for NF-κB target genes, including IL-6, TNF-α, IL-1β, IL-10, and Arg1.

Flow Cytometry is used to quantify M1 and M2 microglial populations.

Procedure:

1. Isolate microglia from brain tissues.
2. Stain cells with antibodies against M1 markers (CD86) and M2 markers (CD206).
3. Analyze stained cells using a flow cytometer.

Expected result

Result 1

The immunofluorescence and immunohistochemical staining indicate a reduction in the quantity of IBA-1 and iNOS-positive cells, and an increase in the quantity of

IBA-1 and Arg-1-positive cells. Flow cytometry revealed a reduction in the quantity of CD86-positive cells and an increase in the quantity of CD206-positive cells. The RT-qPCR data is expected to demonstrate a decline in M1 microglia markers and an elevation in M2 microglia markers. Hence, it may be inferred that the experimental group exhibited a decrease in the proportion of M1 microglia and an increase in the proportion of M2 microglia, as compared to the control group.

Result 2

The expression of Rho/ROCK pathway-related proteins increased, and the p-MYPT1/t-MYPT1 ratios increased. Experiments showed that the Rho/ROCK pathway was inhibited.

Result 3

If the western blotting shows a decrease of p-NF-κB p65 relative to total NF-κB p65, then the NF-κB pathway is inhibited, and vice versa. The immunohistochemistry shows a reduced nuclear localization of p-NF-κB p65 compared to control samples, indicating decreased activation. If the NF-κB pathway is inhibited, the RT – qPCR results may show decreased mRNA levels of NF-κB target genes (IL-6, TNF-α, IL-1β) and possibly increased levels of anti-inflammatory genes (IL-10, Arg1). Therefore, the expected flow cytometry result is that NF- κB is inhibited, shown by an increased proportion of M2 microglia compared to M1 microglia.

3. Conclusion

In conclusion, our findings suggest that LPS priming prior to plaque deposition activates the Rho/ROCK pathway, which in turn regulates the M1/M2 polarisation of microglia through the NF-κB pathway. LPS priming promotes the polarisation of microglia from the pro-inflammatory M1 type to the anti-inflammatory M2 type. This study highlights the intricate interplay between systemic inflammation and neuroinflammatory responses. Comprehending these molecular mechanisms offers vital insights into prospective therapeutic options that focus on Rho/ROCK and NF-κB pathways to reduce neuroinflammation and promote neuroprotection in AD.

Future studies should focus on further elucidating the specific molecular interactions and regulatory networks involved in these pathways. In addition, exploring the therapeutic potential of modulating these pathways at different stages of AD and other neurodegenerative diseases may lead to novel interventions aimed at reducing inflammation and promoting neuronal health.

References

Chen, Jingkao, et al. “Inhibition of AGEs/RAGE/Rho/ROCK Pathway Suppresses Non-specific Neuroinflammation by Regulating BV2 Microglial M1/M2 Polarization through the NF-κB Pathway.” *Journal of Neuroimmunology*, vol. 305, Apr. 2017, pp. 108-14. *PubMed*, https://doi.org/10.1016/j.jneuroim.2017.02.010. Accessed 13 July 2024.

Guo, Shenrui, et al. “Microglia Polarization from M1 to M2 in Neurodegenerative Diseases.” *Frontiers in Aging Neuroscience*, vol. 14, 16 Feb. 2022. *PubMed*, https://doi.org/10.3389/fnagi.2022.815347.

Hoff, Florian. “How to Prepare Your Specimen for Immunofluorescence Microscopy.” *Feica*, 10 Jan. 2022, www.leica-microsystems.com/science-lab/life-science/how-to-prepare-specimen-for-immunofluorescence-microscopy/. Accessed 12 July 2024.

Kitazawa, Masashi, et al. “Lipopolysaccharide-Induced Inflammation Exacerbates Tau Pathology by a Cyclin-Dependent Kinase 5-Mediated Pathway in a Transgenic Model of Alzheimer’s Disease.” *The Journal of Neuroscience*, vol. 25, no. 39, 28 Sept. 2005, pp. 8843-53. *PubMed*, https://doi.org/10.1523/jneurosci.2868-05.2005. Accessed 13 July 2024.

Liu, Ting, et al. “NF-κB Signaling in Inflammation.” *Signal Transduction and Targeted Therapy*, vol. 2, no. 1, 14 July 2017. *Nature*, https://doi.org/10.1038/sigtrans.2017.23. Accessed 13 July 2024.

Sudwarts, Ari, and Gopal Thinakaran. “Alzheimer’s Genes in Microglia: A Risk worth Investigating.” *Molecular Neurodegeneration*, vol. 18, no. 1, 20 Nov. 2023. *ResearchGate*, https://doi.org/10.1186/s13024-023-00679-4. Accessed 13 July 2024.

Yang, Yiyi, et al. “LPS Priming before Plaque Deposition Impedes Microglial Activation and Restrains Aβ Pathology in the 5xFAD Mouse Model of Alzheimer’s Disease.” *Brain, Behavior, and Immunity*, vol. 113, Oct. 2023, pp. 228-47. *PubMed*, https://doi.org/10.1016/j.bbi.2023.07.006. Accessed 13 July 2024.

Zhao, Kaiyue, et al. “The MiR-25802/KLF4/NF-κB Signaling Axis Regulates Microglia-mediated Neuroinflammation in Alzheimer’s Disease.” *Brain, Behavior, and Immunity*, vol. 118, May 2024, pp. 31-48. *PubMed*, https://doi.org/10.1016/j.bbi.2024.02.016.

---. “The MiR-25802/KLF4/NF-κB Signaling Axis Regulates Microglia-mediated Neuroinflammation in Alzheimer’s Disease.” *Brain, Behavior, and Immunity*, vol. 118, May 2024, pp. 31-48. *PubMed*, https://doi.org/10.1016/j.bbi.2024.02.016.