University of Cincinnati

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I, Jie Wang, hereby submit this original work as part of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

It is entitled:
The Study of the Effects of (1S,2E,4R,6R,-7E,11E)-2,7,11-cembratriene-4,6-diol on Microglia Polarization Using an Ischemia in Vitro Model

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This work and its defense approved by:

Committee chair: Jiukuan Hao, Ph.D.

Committee member: Joan Garrett, Ph.D.

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The Study of the Effects of
(1S,2E,4R,6R,-7E,11E)-2,7,11-cembratriene-4,6-diol on
Microglia Polarization Using an Ischemia in Vitro Model

A thesis submitted to the
Graduate School
of the University of Cincinnati
in partial fulfillment of the requirements for the degree of

Master of Science

In the Department of Pharmaceutical Sciences
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by

Jie Wang

B.Sc. Southern Illinois University Carbondale, approved in 2015
March, 2017

Committee Chair: Jiukuan Hao, Ph.D
ABSTRACT

(1S, 2E, 4R, 6R, -7E, 11E)-2, 7, 11-cembratriene-4, 6-diol (4R) is one of the cembranoids found in tobacco leaves. Previous studies have found that 4R protected acute rat hippocampal slices against neurotoxicity induced by N-methyl-D-aspartate (NMDA) and against the toxic organophosphorus compounds paraoxon and diisopropylfluorophosphate (DFP). Furthermore, 4R reduced the infarct size in a rodent ischemic stroke model and neurodegeneration caused by DFP and 6-hydroxydopamine (6-OHDA). The present study expanded our previous study focusing on the effect of 4R in N9 cells (murine-derived microglia cell line) polarization. The results showed that 4R promoted M2 activation of N9 cells and inhibited M1 activation of N9 cells induced by lipopolysaccharide (LPS) or oxygen glucose deprivation (OGD). LPS or OGD-induced M1 activation marker-inducible nitric oxide synthase (iNOS) of N9 cells was attenuated by 1uM of 4R treatment, which was 69.4% and 56.4% of vehicle control, respectively. In addition, 1uM 4R promoted M2 activation marker-Argnase-1 of N9 cells under LPS or OGD conditions, which was 192.7% and 188.0% of vehicle control, respectively. Furthermore, the conditioned medium of 4R-treated post-OGD N9 cells protected neuro2a cells from OGD-induced injury. The cells viability increased by 54.5% by treatment with the conditioned medium of 4R-treated post-OGD N9 cells compared with the conditioned medium from post-OGD N9 cells without 4R. Furthermore, cytokines
release measured by ELISA showed that 4R increased anti-inflammatory cytokine IL-10 production and inhibited pro-inflammatory cytokine TNF-α release from N9 cells induced by OGD.

In conclusion, the present study indicates that 4R exhibits anti-inflammatory properties by modulating polarization of microglia toward M2 subtype.
ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

1.1 Roles of glial cells activation in neuroinflammation

Inflammatory responses in the central nervous system (CNS) exist in many brain and neurodegenerative diseases, such as multiple sclerosis, Alzheimer’s disease, Parkinson’s disease and strokes. Immune activities are considered permissive in the healthy brain for protecting neurons and defensive pathogens [1]. After brain injury, the rapid activation of glial cells acts as brain’s own immune system in response to acute injury. The activated glial cells secret several pro-inflammatory factors, such as chemoattractants, cytokines, and chemokines. These pro-inflammatory factors may cause significant damage to the blood-brain barrier (BBB), and act as signals to recruit peripheral immune cells [2]. In healthy brain, CNS was separated from the circulating blood by BBB, and peripheral immune relative cells do not have access to CNS [3]. However, the compromised BBB become permeable to circulating blood components and peripheral immune cells, then cause infiltration of peripheral leukocytes, which will exacerbate the inflammation in brain and finally cause chronic neuroinflammation and neurodegeneration [4].
1.2 Two polarizations of microglia

Microglia are major glia cells in brain immune system. The “resting” microglia are characterized by the ramified phenotype, and which will be activated and change their morphology into amoeboid-phagocytic microglia once brain injury occurs [5]. Microglia can exhibit both pro-inflammatory and anti-inflammatory properties after activation, depending on the condition levels of the disease and the signals to them [6]. Generally, different activated states of microglia are classified to “classically activated” M1 phenotype and “alternatively activated” M2 phenotype.

<table>
<thead>
<tr>
<th>Phenotype</th>
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<tbody>
<tr>
<td>M1</td>
<td>IFN-γ</td>
<td>Pro-inflammatory, induce M1 phenotype</td>
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<tr>
<td></td>
<td>IL-1β, IL-6, TNF-α</td>
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<tr>
<td>M2</td>
<td>IL-4</td>
<td>Anti-inflammatory, induce M2 phenotype</td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>Anti-inflammatory, inhibit the activity of Caspase-3, up-regulate the level of GSH and NGF</td>
</tr>
<tr>
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<td>TGF-β</td>
<td>Anti-Inflammatory, regeneration, up-regulate the level of Bcl-2 and Bcl-x1</td>
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<tr>
<td></td>
<td>YM1, Arg-1, IGF-1, FIZZ1, HO-1, CD206</td>
<td>Repair and regeneration, Anti-oxidation, Antigen internalization and processing</td>
</tr>
</tbody>
</table>

Figure 1: Identification markers and cytokines of microglia M1 and M2 phenotype [8]

M1 polarization of microglia results in the release of destructive pro-inflammatory factors that are toxic to neurons and inhibit CNS restoration, while M2 polarization
results in the release of anti-inflammatory cytokines to promote debris clearance, wound repair, and neurogenesis in CNS [7]. The identification markers and cytokines of microglia M1 and M2 phenotypes are shown in the figure 1.

*In vitro* study, lipopolysaccharide (LPS) and interferon-γ (IFN-γ) treatment stimulate microglia toward M1 phenotype, while interleukin-4 (IL-4) and interleukin-10 (IL-10) treatment stimulate microglia toward M2 phenotype (figure 2).

![Figure 2](image)

Figure 2: M1 phenotype can be stimulated by LPS or IFNγ treatment, while M2 phenotype can be stimulated by IL4/IL10 treatment [9]

However, it’s important to understand that the “activation” status of microglia is not fixed, there is a dynamically shift between a spectrum of phenotypes. In addition, “resting”
microglia in normal brains are not exact in the central position between inflammatory and anti-inflammatory polarization, they are slightly skewed to M2 polarization, which is critical for preventing microglia from adopting an inflammatory profile, and maintaining brain healthy environment [10]. Microglial activation in brain and neurodegenerative diseases contributes to chronic neuroinflammation, and finally cause neuronal dysfunction, even death. Therefore, microglial activation is being studied as a part of disease pathogenesis [11].

1.3 The relationship between microglia polarization and ischemic stroke

Stroke is 5th leading cause of death in the US, and 87% of them are classified as ischemic, which is resulted from a blockage in the blood vessels supplying blood to the brain. The treatment for stroke is aimed at removing blockage by drugs (thrombolysis) or by surgery (thrombectomy). Currently, the only FDA approved drug for ischemic strokes treatment is tissue plasminogen activator (tPA), which dissolves the clots and restores blood flow to the blood deprived part of brain [12]. tPA improves 10% of chances for patients living without disability when it’s administered within 3 hours of symptom onset. After four and a half hours, tPA loses efficacy in improving outcome [13]. One of the factors that worsen clinical outcomes is believed that inflammatory response following stroke produces a larger number of pro-inflammatory cytokines that can harm neural cells and cause further brain injury [14]. The mechanism of microglial activation following
ischemic stroke plays an important role in processing this inflammation response. The studies by Hu, et. al using murine model of transient focal ischemia suggested that at early stages of ischemic stroke, microglia M2 phenotype dominated at the site of injury for a short period, then gradually transformed into the M1 phenotype. Finally, M1 phenotype dominated the brain. In addition, their studies showed that in vitro ischemic model, M1 microglia promoted OGD-induced neuronal death, while M2 microglia protected post-OGD neurons [15]. Other studies showed that IL-4 or IL-10 gene knockout mice increased infract volume and worsened cognitive performance after ischemia [6]. The accumulation of M2 microglia is essential for continuous neurogenesis after stroke and efficient remyelination in CNS regeneration [16]. Therefore, regulating the M1/M2 phenotype shift at different time points after stroke may alleviate the condition of chronic neuroinflammation and acquire better outcomes in stroke patients.

1.4 Promising therapeutic approaches targeting microglia for strokes

1.4.1 Suppressing microglia activation

The excessive and chronic activation of microglia induced by stroke results in the production of pro-inflammatory cytokines and chemokines such as IL-6, TNF-α and reactive oxygen species (ROS), which may cause secondary injury of neurons and other brain cells. Inhibiting pro-inflammatory cytokines and chemokines release contributes to reduce injury [17]. Therefore, suppressing microglial activation pathways would likely be
effective to reduce brain injury following strokes.

1.4.1.1 Toll-like receptors (TLRs) pathway

Toll-like receptors (TLRs) are signaling receptors that play an important role in the innate immune system. In resting microglia in a healthy CNS, TLRs expression levels are very low. TLRs ligands activate microglial cells, which may mediate different pathways in microglia, and some of them may produce neurotoxic molecules such as pro-inflammatory cytokines [18]. For example, TLR4 is activated in microglia following ischemia, which will lead to substantial brain damage. Therefore, inhibiting the TLR signaling may benefit stroke patients. Previous animal studies showed that TLR4 knockout mice significantly reduced infarct size at 24 h after ischemia [19]. In addition, some TLR antagonists or antibodies such as TAK-242, which blocked TLR4 signaling pathways, and contributed to inhibiting the microglial production of TNF-α, IL-1β, and IL-6 and leading to decrease peripheral inflammatory cells infiltration and neurological deficits in mice [20].

1.4.1.2 Matrix metalloproteinase (MMPs) pathway

MMPs are a family of proteases that related to BBB disruption and neuronal injury in ischemia. Resting microglia are activated after the onset of ischemia, which will upregulate MMP-9 expression. Microglia MMPs inhibitors such as minocycline and
doxycycline have been shown to reduce microglial activation under ischemia condition. Recently, the studies suggested that minocycline could inhibit injury to neurons and reduce injury volume after middle cerebral artery occlusion (MCAO) [21].

1.4.2 Shift microglia phenotype from M1 to M2

Simply suppressing microglia activation may result in disrupting the other functions of microglia after ischemia, because activated microglia also play an important role in clearing debris and repair injury of brain [15]. As mentioned above, M1 microglia produce destructive pro-inflammatory mediators, which contribute to the chronic neuroinflammation after ischemia. While, M2 phenotype is neuroprotective. Therefore, inhibiting microglia M1 phenotype and promoting microglia switch to M2 phenotype during chronic inflammation stage after ischemia may improve the brain repair and the outcomes of these patients.

1.5 Neuroprotective and anti-inflammatory activities of cembranoids

Cembranoids are 14-carbon ring diterpenoids that have been isolated from plants, insects and even vertebrates. 4R (figure 3) is one of cembranoids that is extracted from tobacco. Previous studies showed that 4R treatment reduced the infraction volume in the brains of mice and rats after ischemic stroke. In addition, their in vitro studies suggested that 4R reduced OGD neuronal death and inhibited monocytes adhesion to bEND5 cells.
(murine brain-derived endothelia cells) [22]. On the other hand, the pharmacokinetics and metabolism study of 4R demonstrated that 4R could cross the BBB and it was also rapidly metabolized in human [23]. Therefore, 4R could be developed as a neuroprotective drug for preclinical study.

Figure 3: structure of 4R [22]

In addition, some other cembrane-type compounds have been reported as the potential anti-inflammatory agents. For example, previous study demonstrated that some cembranoids from Cultured Soft Corals Sinularia sandensis and Sinularia flexibilis could suppress pro-inflammatory proteins production in LPS-stimulated macrophage-like cell line RAW-264.7 [24].

Microglia are specialized macrophages that exist in CNS [25]. Therefore, microglia
and macrophages share many properties. 4R, as one of the member from cembranoids family, may also has some similar functions with other cembranoids. Moreover, 4R has been shown to increase Akt phosphorylation [22]. Increasing Phosphatidylinositide 3-kinase (PI3K)/Akt signaling by inactivating phosphatase and tensin homologue (PTEN) polarized microglia toward M2 phenotype [37].

Therefore, in this study, we investigated the effects of 4R on microglia polarization under LPS-stimulated/ischemic condition.
CHAPTER 2

AIMS OF THE STUDY

We hypothesized that, except for direct protecting neurons in ischemia, 4R may exhibit anti-inflammatory activities in microglia after ischemia via inhibiting microglia M1 polarization and promoting M2 polarization for neuroprotection.

Our study is processed based on the following steps:

1. to investigate the effects of 4R on normal microglia

2. to investigate the effects of 4R on LPS-stimulated (general neuroinflammation in vitro model) microglia polarization

3. to investigate the effects of 4R on OGD-stimulated (ischemia in vitro model) microglia

   a. the effect of 4R on post-OGD microglia polarization

   b. the effect of 4R-treated post-OGD microglia on neuronal survival

   c. the effect of 4R on post-OGD microglia cytokines release
CHAPTER 3

MATERIALS AND METHODS

3.1 Cell Cultures

Both mouse brain microglial cell line (N9) and mouse neuroblastoma cell line (neuro2a) were kindly provided by Dr. Jiang, University of Cincinnati. The cells were cultured in the Dulbecco’s Modification of Eagle’s Medium (DMEM) (Corning, Manassas, VA) containing 10% v/v fetal bovine serum (FBS) (Atlanta Biological, Lawrenceville, GA), 100U/mL MEM non-essential amino acids (Hyclone Laboratories, South Logan, UT) and 1% v/v Penicillin/Streptomycin (100X) (Corning, Manassas, VA). The cells were incubated at 37°C, 20% O₂, 5% CO₂, and 95% humidity environment. The medium was changed every 2-3 days when the cells grew to about 80% confluence.

3.2 LPS and 4R preparation

LPS stocking solution was made by dissolving LPS powder (Sigma Aldrich, St. Louis, MO) to phosphate-buffered saline (PBS), and the final concentration of LPS is 20μg/ml in PBS. Diluted LPS stocking solution in cell culture medium by 5:1000 v/v to make 100ng/mL of LPS for N9 cells stimulation.

4R (kindly gift from Dr. Ferchmin, Universidad Central del Caribe) was dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO), the ratio of the total
volume of 4R and DMSO to the volume of cell culture medium is 2:1000, and make sure the final concentrations of 4R in medium are 50nM, 1uM and 8uM, respectively, as indicated.

### 3.3 Ischemia in vitro model

To stimulate ischemic injury, oxygen-glucose deprivation (OGD) was used for N9 and N2a cells. N9 cells and N2a cells in glucose and serum free Roswell Park Memorial Institute (RPMI) medium (Corning, Manassas, VA) were submitted to 1% oxygen incubator for the indicated time and then were returned to 95% air, 5% CO₂ with normal glucose medium for the indicated time. If 4R/DMSO treatment is needed, the treatment was added to the normal glucose medium when cells were returned to 95% air, 5% CO₂.

### 3.4 The protocols of treatment procedures design

3.4.1 The procedures of LPS stimulation and 4R treatment in N9 cells.

N9 cells were seeded into the new 6-wells plate (50000 cells per well) and divided into 6 groups: I) Control; II) DMSO only; III) 4R only; IV) LPS only; V) LPS+DMSO; VI) LPS+4R. The detailed treatment procedures are shown in figure 4. After the treatment, cells were collected for protein expression analysis via western blot.
3.4.2 The procedures of OGD stimulation and 4R treatment in N9 cells

N9 cells were seeded into 6 wells plate (10,000 cells per well) to incubate 24 hours and were divided into 6 groups: 1) Non-OGD; 2) Non-OGD+DMSO; 3) Non-OGD+4R; 4) OGD; 5) OGD+DMSO; 6) OGD+4R. The detailed treatment procedures are shown in figure 5. After the treatment procedures, collected the conditioned media for culturing N2a and determining cytokines release concentration via ELISA assay, and the cells for protein expression analysis via western blot.
3.4.3 The procedures of the transfer of conditioned media from N9 cells to culture N2a cells.

N2a cells were seeded into 96 wells plate (1000 cells per well) and were divided into 2 groups: a) Non-OGD, b) OGD. The conditioned medium from the above N9 cells group 1 was added to Non-OGD N2a cells as control. Then, the conditioned medium that was collected from the above each group of N9 cells was added to 6 groups of OGD-stimulated N2a cells, respectively. The detailed procedures are shown in figure 6. After 24 hours conditioned media treatment, the N2a cells in each well were submitted to MTT assay for cells viabilities analysis.
Figure 6: The procedures of the transfer of conditioned medium from N9 cells to culture N2a cells.

3.5 Western blot

3.4.1 Protein sample preparation

N9 cells after treatment procedures were washed by PBS once. Aspirated the PBS and added RIPA lysis buffer system (contains PMSF, sodium orthovanadate and protease inhibitor cocktail) and 5nM dithiothreitol (DTT) to each well (100 uL per well for 6 wells plate). Cells were scraped and removed from plate to the lysis buffer, and transferred to 1.5mL tubes, put on ice and waited for 30 minutes. Then, centrifuged the samples at 15,000 G for 10 minutes at 4°C and discarded the precipitates.

3.4.2 Bicinchoninic Acid Assay (BCA)

The total protein concentration in each tube was determined by Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL) according to the manufacturer’s protocol. After BCA assay, the same volume of 2X Laemmli sample buffer with 5% v/v 2-mercaptopethanol was added to each sample, and boiled at 100°C for 10 minutes. After
the samples were cooled to room temperature, stored the samples at -20°C for future use.

3.4.3 Western blotting

The Tris-Glycine SDS PAGE Gels and buffers for western blotting were prepared according to the instruction from BIO-RAD. 20ug of each protein sample was loaded onto stacking gel, and electrophoresis was run at 120V for appropriate time until the proteins were separated well. The proteins on gel were then transferred to PVDF membranes (Bio-Rad, Hercules, CA) using semi-dry transfer system at 20V for 70 minutes, and then blocked the membrane for 1 hour at room temperature with 5% (w/v) skim milk powder in 10mM Tris, 100mM NaCl and 0.1% Tween-20 buffer (TBST, PH=7.5). Washed the membrane by TBST and then incubated with desired primary antibody dilute in TBST containing 3% BSA and 0.01% sodium azide overnight at 4°C with mild shaking. The second day, washed the membrane by TBST for 3 times, 10 minutes per time. Then, incubated the membrane with second antibody dilute in the blocking solution for 1 hour at room temperature. After, washed the membrane by TBST for 3 times again. Final, membranes were taken to dark room and chemiluminescence method was used to generate western blot images.

3.6 Thiazolyl blue tetrazolium bromide reagent (MTT) assay

MTT cell proliferation assay kit (Cayman Chemical Company, Ann Arbor, MI)
was used to perform this assay. According to the manufacturer’s instruction, MTT was dissolved at a concentration of 5 mg/ml in DPBS, then added 20ul per well (96 wells plate) of MTT to N2a cells. Incubated at 37 for 4 hours. The media were then carefully removed from the each well, and precipitated formazan was dissolved in 200µl per well of DMSO (Fisher Scientific, Pittsburg, PA). Shake the plate for 5 minutes, then the absorbance was measured at 560nm wave length minus the background absorbance at 670nm wave length using SPECTRAmax plus384 microplate reader (Molecular Devices, CA).

3.7 The enzyme-linked immunosorbent assay (ELISA)

The cytokine concentrations in conditioned media that were collected from N9 cells were evaluated by using corresponding ELISA Development Kit and based on their guidelines (Pepro Tech Inc, NJ). Briefly, 96-wells ELISA Microplate was coated by capture antibody overnight at room temperature, aspirated the wells and wash each well 4 times by wash buffer (0.05% Tween-20 in PBS). 300ul block buffer (1% BSA in PBS) was added to each well and incubated for 1 hour, aspirated and wash plate 4 times. Then added 100ul conditioned medium per well and incubated for 2 hours, aspirated and wash plate 4 times. Incubated detection antibody for another 2 hours, aspirated and wash plate 4 times. Next, streptavidin-HRP conjugate was added to each well and waited for 30 min, aspirated and wash plate 4 times again. Finally, 100 ul of 3,3’,5,5’-Tetramethylbenzidine
(TMB) (SeraCare company, Gaithersburg, MD) was added to each well for 20 minutes to develop color, and 100ul of 1M hydrochloric acid (Fisher Scientific, Waltham, MA) was then used to stop development. Immediately measured the absorbance at 450nm wave length minus the background absorbance at 620nm wave length using SPECTRAmax plus384 microplate reader (Molecular Devices, CA).

3.8 Statistical analysis

Data are represented as mean ± standard error of the mean. Statistical significant differences among multiple groups were determined by one-way analysis of variance and followed by post hoc test. The threshold of p-value was set at 0.05, p<0.05 was considered statistically significant.
CHAPTER 4

RESULTS

4.1 4R promoted M2 polarization in “resting” N9 cells

To study whether 4R has effect on M2 polarization of resting N9 cells, N9 cells were exposed to 50nM, 1uM, 8uM 4R for 24 hours in FBS free medium, respectively. Then, the Arg-1 expression, a marker of M2 state microglia, was evaluated by western blot. 50nM, 1uM, and 8uM of 4R treatment significantly increased Arg-1 expression to 250.5% ± 65.9, 305.6% ± 52.1, and 284.2% ± 37.2 of control, respectively. Vehicle DMSO didn’t have significant effect on Arg-1 expression, when compared with control group (Figure 7).

![Western blot images of Arg-1 and beta-actin]

<table>
<thead>
<tr>
<th>Treatment</th>
<th>4R</th>
<th>-</th>
<th>50nM</th>
<th>1uM</th>
<th>8uM</th>
</tr>
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<tbody>
<tr>
<td>DMSO</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
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</table>

- Arg-1
- beta-actin
Figure 7: 4R promoted M2 polarization in “resting” N9 cells. Arg-1 expression levels were evaluated by western blot, and the quantification of western blot bands were generated via imageJ software analysis. Mean ± SD, n=4, **p<0.05.

4.2 4R prevented LPS-mediated M1 microglia activation and M2 microglia suppression in N9 cells

Our MTT results suggested that 8uM of 4R treatment in LPS-stimulated N9 cells decreased cells viability to 76.9% ± 15.4, when compared with DMSO vehicle treatment in LPS-stimulated N9 cells. Therefore, we only chose 50uM and 1uM of 4R for the subsequent experiments (Figure 8).

To evaluate whether 4R has effects on LPS-stimulated microglia, 100ng/mL LPS were added to the N9 culture. N9 cells in serum free medium didn’t express microglia M1 marker iNOS, LPS stimulation significantly induced iNOS expression. 50nM of 4R
didn’t show the significant effect on LPS-induced iNOS expression, while 1 uM of 4R treatment inhibited the LPS-induced iNOS expression to 69.4% ± 8.5 of LPS plus DMSO group (Figure 9a). On the other hand, LPS stimulation suppressed Microglia M2 marker Arg-1 expression to 79.3% ± 8.7 of control, both 50nM and 1uM of 4R treatment restored LPS-induced down-regulation of microglia M2 expression to 122.2% ± 10.9, 150.4% ± 22.6 of control, respectively. Vehicle DMSO didn’t have significant effect on iNOS expression (Figure 9b).

Figure 8: N9 cells viabilities after LPS and 4R treatment. Cells viabilities were evaluated by MTT assay. Mean ± SD, n=3, **p<0.05.
- iNOS (top band)
- β-actin

<table>
<thead>
<tr>
<th></th>
<th>100ng/mL</th>
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<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>DMSO</td>
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(a)
Figure 9. 4R prevented LPS-mediated M1 microglia activation and M2 microglia suppression in N9 cells. (a) iNOS expression (b) Arg-1 expression. iNOS and Arg-1 expression levels were evaluated by western blot, and the quantification of western blot bands were generated via imageJ software analysis, Mean ± SD, n=3, **p<0.05.
4.3 4R attenuated 1h OGD-induced M1 polarization, and promoted M2 polarization in N9 cells

To evaluate the effect of 4R on microglia polarization after ischemia, N9 cells were submitted to OGD condition for 1h. 1h OGD stimulation significantly increased M1 marker iNOS expression in N9 cells to 311.5% ± 24.7 of control group, the OGD-stimulated iNOS expression was then inhibited after 1uM of 4R treatment, which is reduced to 168.5% ± 50.3 of control. iNOS expression wasn’t significantly affected by DMSO vehicle treatment, which is 279.2% ± 42.6 of control (Figure 10a). On the other hand, M2 marker Arg-1 expression wasn’t affected by OGD stimulation, which is 99.7% ± 11.8 of control group, while 1uM of 4R treatment increased Arg-1 expression by 88.0% ± 9.9 after OGD stimulation, when compared to DMSO vehicle treatment after OGD stimulation. DMSO vehicle didn’t have effect on Arg-1 expression (Figure 10b).
(a)

- Arg-1

- Tublin

<table>
<thead>
<tr>
<th>Treatment</th>
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Figure 10: 4R attenuated OGD-induced M1 expression, also increased M2 expression under both normal and ischemic condition in N9 cells. (a) M1 marker iNOS expression; (b) M2 marker Arg-1 expression. iNOS and Arg-1 expression levels were evaluated by western blot, and the quantification of western blot bands were generated via imageJ software analysis, Mean ± SD, n=3, **p<0.05.

4.4 Phosphorylation of NF-kb was involved in the 4R-mediated polarization in N9 cells

To study whether phosphorylation of NF-kb was involved in the 4R-mediated effects on microglia, phosphorylation NF-kb expression levels in the N9 cells were evaluated by western blot. In LPS stimulation experiments, 100ng/mL LPS treatment significantly increased NF-kb phosphorylation levels to 364.4% ± 32.8 of control. Vehicle DMSO and 50 nM of 4R didn’t have significant effect on NF-kb phosphorylation
in N9 cells. 1uM of 4R treatment suppressed the LPS-induced NF-kb phosphorylation to 145.5% ± 32.0 of control in N9 cells (Figure 11a). Similar with LPS stimulation, NF-kb phosphorylation levels were increased to 238.4% ± 65.4 of control after 1h OGD stimulation, which were attenuated to 109.9% ± 27.8 of control when 1uM of 4R was applied to the medium during recovery period after 1h OGD. In normal N9 cells, 4R treatment didn’t affect the NF-kb phosphorylation that was indicated by 114.1% ± 33.4 of control. Vehicle DMSO also has no significantly effect on NF-kb phosphorylation in both normal and post-OGD N9 cells (Figure 11b).

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(a)

Phosphorylation of NF-κb % of control

ctr  LPS  LPS+DMSO  LPS+50nM 4R  LPS+1μM 4R

**

-p-NF-κb, p65

-NF-κb, p65

-Tublin

OGD  -  -  -  1h  1h  1h

4R   -  -  1μM - -  1μM

DMSO - + - - + -
Figure 11: Phosphorylation of NF-kb was involved in the 4R-mediated polarization in N9 cells. (a) NF-kb phosphorylation levels of N9 cells in LPS stimulation model; (b) NF-kb phosphorylation levels of N9 cells in OGD stimulation model. NF-kb phosphorylation levels were evaluated by western blot, and the quantification of western blot bands were generated via imageJ software analysis, Mean ± SD, n=3, **p<0.05.

4.5 Conditioned medium from OGD-stimulated N9 cells exacerbated post-OGD N2a cells survival, while 4R treatment in OGD-stimulated N9 cells restored post-OGD N2a cells survival

To verify whether the effect of 4R on microglia polarization could protect neurons after ischemia, a conditioned medium transfer system was applied in this experiment. N2a cells were submitted to OGD for 2h. Then, the conditioned medium from N9 cell culture system was transferred to N2a cell culture system, and incubated for 24 h. Finally,
N2a cells viabilities were determined by MTT assay. 2h OGD stimulation (group 2) decreased N2a viabilities to 70.3% ± 2.0, when compared to normal N2a cells (group 1). The conditioned medium from N9 cells under normal condition with 1uM of 4R treatment (group 4) didn’t affect post-OGD N2a viabilities (71.3% ± 2.6 of group 1), when compared with the medium from the normal N9 cells with only vehicle DMSO treatment (group 3), in which, the N2a cells viability is 68.6% ± 4.4 of group 1. The conditioned medium from 1 h OGD-stimulated N9 cells (group 5) further exacerbated post-OGD N2a viabilities to 35.2% ± 5.9 of group 1. However, after 1 uM of 4R treatment in OGD-stimulated N9 cells (group 7), the conditioned medium restored the post-OGD N2a cells viabilities to 52.8% ± 2.6 of group 1 (Figure 12).

![MTT results](image)

Figure 12. 4R treatment in post-ischemia microglia protected neuronal survival after ischemia. (1) conditioned medium from normal N9 cells culture normal N2a cells; (2) conditioned medium from normal N9 cells culture post-OGD N2a cells; (3) conditioned medium from DMSO-treated N9 cells culture post-OGD N2a cells; (4) conditioned
medium from 4R-treated N9 cells culture post-OGD N2a cells; (5) conditioned medium from post-OGD N9 cells culture post-OGD N2a cells; (6) conditioned medium from DMSO-treated post-OGD N9 cells culture post-OGD N2a cells; (7) conditioned medium from 4R-treated post-OGD N9 cells culture post-OGD N2a cells. N2a cells viabilities were evaluated by MTT assay. Mean ± SD, n=3, **p<0.05.

4.6 4R treatment inhibited OGD-induced pro-inflammatory cytokine release, while increased anti-inflammatory cytokine release in N9 cells.

To understand how the conditioned medium from N9 cells affect N2a cells viabilities, ELLISA assay was performed to evaluate the concentration of TNF-α and IL-10 in the conditioned medium from N9 cells. 1uM of 4R treatment in N9 cells under normal condition didn’t affect their TNF-α release, when compared with vehicle DMSO treatment. 1h OGD stimulation significantly increased TNF-α release in N9 cells to 203.4% ± 30.6 of control. Vehicle DMSO didn’t affect TNF-α release in post-OGD N9 cells. The OGD-induced TNF-α release in N9 cells was attenuated after 1uM of 4R treatment, which is 156.1% ± 5.8 of control (Figure 13a). Vehicle DMSO didn’t show significant effect on IL-10 release (117.0% ± 14.9 of control) in N9 cells under normal condition. IL-10 release in N9 cells under normal condition was increased to 223.8% ± 13.3 of control after 1uM of 4R treatment. 1h OGD stimulation didn’t have significantly change when compared with control. However, 4R treatment in OGD-stimulated N9 cells significantly increased IL-10 release to 329.4% ± 30.4 of control, when compared with vehicle DMSO treatment. Here, DMSO also didn’t have significant effect on IL-10 release in OGD-stimulated N9 cells (Figure 13b).
Figure 13. 4R treatment inhibited OGD-induced pro-inflammatory cytokine release, while increased anti-inflammatory cytokine release in N9 cells. (a) TNF-α concentration in conditioned medium; (b) IL-10 concentration in conditioned medium. Cytokines concentrations were determined by ELLISA assay. Mean ± SD, n=3, **p<0.05.
CHAPTER 5

DISCUSSION

Previous studies indicated that M1 microglia promoted the phagocytosis of viable neurons during post-ischemia, while, M2 microglia contributed to debris clearing and tissue repairing and neuronal survival [26], which suggested high ratio of M1/M2 microglia may be one of the factors that contribute to exacerbating post-ischemic injury [8]. In this study, we found that both LPS and 1h OGD stimulation induced microglia M1 marker expression, while, LPS stimulation suppressed M2 state marker expression, but 1h OGD didn’t show significant effect on microglia M2 polarization in N9 cells. Previous study showed that in response to ischemia, different from a constant increase of M1 microglia expression, mice microglia expressed high levels of M2 genes at beginning, then gradually decreased to basal level [27], which revealed that the expression level of microglia M2 phenotype may have a potential relation with ischemic stimulation severity and recovery time. Although 1h OGD didn’t downregulate M2 polarization, since it induced M1 polarization, therefore, the overall ratio of M1/M2 in microglia after 1h OGD treatment was higher than the ratio in normal condition. Consequently, our study found that the conditioned medium from post-OGD microglia significantly promoted OGD-neuronal death when comparing with the conditioned medium from normal microglia, which partially supported that high ratio of M1/M2 exacerbated injuries in
ischemic patients.

4R is one of the cembranoids found in tobacco leaves, and a growing list of in vitro and in vivo studies suggested that 4R work with nicotinic acetylcholine receptors (AChRs) to exhibit neuroprotective and anti-inflammatory properties in many CNS diseases [29]. In addition, 4R has been shown to protected acute rat hippocampal slices against NMDA and DFP [39, 40]. 4R also exhibited protective activities in a rodent ischemic stroke model and neurodegeneration caused by DFP [22, 41]. On the other hand, many cembranoids have been proven anti-inflammatory effects in macrophages and hepatic tumor cells [24, 36]. As a member of cembranoids family, 4R may share functional similarities with other cembranoids. Combining these information, we hypothesized that except for direct protecting neurons from ischemia, 4R may have anti-inflammatory effect on microglia to protect neurons after ischemia. Our study showed that 4R played dual roles in LPS/OGD-stimulated N9 cells, including inhibiting the microglia M1 phenotype activation and promoting the M2 phenotype activation of N9 cells. And the effects of 4R on microglia polarization eventually contributed to post-OGD N2a cells survival.

NF-kb is a transcription factor that plays an important role in regulating inflammation process. Suppressing NF-kb phosphorylation could be a promising therapeutic targeting goal for chronic inflammatory diseases [30]. Previous studies
indicated that NF-kb activation is promoted in M1 macrophage and inhibited in M2 macrophage [35]. In addition, 4R upregulated Akt phosphorylation to protect neurons from ischemia [22], and activation PI3K/Akt upregulated the specific endogenous NF-kb inhibitor - IkBa expression [38]. Since this study has shown that 4R promoted M2 polarization and inhibited M1 polarization in LPS/OGD-stimulated N9 cells, we hypothesized that 4R may associate with NF-kb phosphorylation in microglia polarization. Our results demonstrated that 4R inhibited LPS/OGD-stimulated NF-kb phosphorylation in N9 cells. In cerebral ischemia, NF-kb signal cascade regulated the production of pro-inflammatory mediators including IL-1, IL-2, IL-6, TNF-α, iNOS and COX-2 [31]. Therefore, inhibiting NF-kb activation may decrease the release of pro-inflammatory mediators. Our ELISA results showed that 4R treatment reduced the OGD-induced pro-inflammatory cytokines TNF-α release. TNF-α is considered to cause neuronal excitotoxic death by increasing glutamate production [32]. This may be one of the reasons that the conditioned medium from post-OGD N9 cells with 4R treatment can promote post-OGD neuronal survival. Our ELISA results also showed that 4R treatment increased the anti-inflammatory cytokines IL-10 secretion in microglia both under normal and OGD condition, which was consistent with the microglia M2 marker-Arg-1 expression levels. IL-10 has been shown to reduce rat brain injury following ischemic stroke [33]. Therefore, upregulating microglia M2 expression and anti-inflammatory
cytokines production may be another role of 4R to protect neurons after ischemia.

Interestingly, although 4R treatment increased microglial anti-inflammatory cytokines release under normal condition, the conditioned medium from 4R-treated normal microglia didn’t increase post-OGD neuronal survival compared to the medium from normal microglia without 4R treatment. The assumption that under normal conditions, microglia are skewed toward a protective, anti-inflammatory phenotype may be able to explain this phenomenon, because resting microglia also secret some neuro-supportive cytokines such as IGF-1 and BDNF. Thus, the conditioned media from “resting” and 4R treated microglia both should exhibit neuroprotective properties [10].

However, some questions still remain and need to be answered in the future study. For example, first, are there any differences between N9 cell line and primary microglia/in vivo? The N9 microglia is derived from mouse brain, but they are genetic modified. N9 microglia cells may develop some unique properties. For example, N9 cells are tested negative for some microglia cell surface markers, such as GFAP, A2B5, and Gal-C [34]. In addition, in vivo, many other factors may affect the activities of 4R. Therefore, our results should be verified in primary microglia and in vivo. Second, how can we prove that the effects of 4R on microglia polarization relate to its function of inhibiting NF-kb signaling? Our current study only indicated that 4R inhibited microglia NF-kb activation after ischemia. However, it needs to be specified that whether 4R
affects microglia polarization via inhibiting NF-kb activation. Moreover, although 4R promoted M2 polarization and increased anti-inflammatory cytokine IL-10 release in microglia under normal condition, our results didn’t show that 4R had significant effect on NF-kb activation of microglia under normal condition. Therefore, some other signaling pathways might exist to mediate the function of 4R on microglia M2 polarization.
CHAPTER 6

CONCLUSION

In summary, our findings suggested that 4R plays dual roles in OGD-stimulated N9 cells, including inhibiting the microglia M1 phenotype activation and promoting the M2 phenotype activation of N9 cells, as well as increasing anti-inflammatory cytokines release, and decreasing pro-inflammatory cytokines release in N9 cells, which may contribute to its protective function on post-OGD N2a cells. NF-kb signaling may associated with the function of 4R on microglia polarization.
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