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Resolvin D2 recovers neural injury by suppressing inflammatory mediators expression in lipopolysaccharide-induced Parkinson's disease rat model

Yuguang Tian [a, *], Ying Zhang [a, b, *], Ruoyu Zhang [c], Song Qiao [d], Jing Fan [b]

[a] School of Pharmacy, Guangdong Food and Drug Vocational College, Guangzhou 510520, PR China
[b] Neuroscience Care Unit, The Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Number 88, Jiefang Road, Hangzhou, Zhejiang 310009, PR China
[c] Department of Geriatrics, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Number 88, Jiefang Road, Hangzhou, Zhejiang 310009, PR China
[d] Department of Neurology, Zhejiang Hospital, Number 12, Lingyin Road, Xihu District, Hangzhou, Zhejiang 310009, PR China

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ABSTRACT

Activation of microglial cells have been treated as the main role in the pathogenesis of neuropathic inflammation and neurodegenerative disease, including Parkinson's disease (PD), prion disease and Alzheimer's disease (AD). Resolvin D2 (RvD2) is derived from omega-3 polyunsaturated fatty acid and performs potent anti-inflammatory and pro-resolution effects. Here we investigated the effects of intrathecal injection of RvD2 for substantia nigra pars compacta (SNpc) in vivo and primary microglia in vitro experiment on pro-inflammatory cytokine expression and NF-κB activation in Lipopolysaccharide (LPS)-induced PD rat model. The total of 30 days experimental period were used for the rats' experiment, the LPS-induced inflammation in SNpc increase the expression of NO, iNOS, TNF-α, IL-1, IL-18, IL-6, IL-1β, ROS production, the translocation of NF-κB p65, IκBα, and IKKβ expression in glial cells. After injection of RvD2, the treatment prevented development of behavioral defects and TLR4/NF-κB pathway activation. Therefore, we demonstrated a novel role of RvD2 in treatment of rat PD model and LPS activated microglia inflammation. Given the significant potency of RvD2 and well-known side effects of microglia inflammatory inhibitors, it may represent novel hotspot for treating neurodegenerative disease.

1. Introduction

Parkinson's disease (PD) is a common geriatric neurodegenerative disease, which is mainly pathologically characteristic of the progressive degeneration of nigrostriatal dopaminergic neurons in midbrain and nerve endings. Clinical symptoms include resting tremor, rigidity and bradykinesia [1]. PD incidence is only lower than that of Alzheimer's disease, although individual gene mutations have been linked to some familial PD, the vast majority of PD etiology and pathogenesis remains unclear, therefore PD treatment maintains at the symptomatic level, and there is no completely effective therapeutic measure [2,3]. McGeer et al. [4] have found activated microglial cells in mesencephalic substantia nigra pars compacta (SNpc) of PD patients, then scholars are interested in the activation of microglia as the mechanism underlying neural immune inflammation. Microglial cells are the main immune cells in the brain, and are not evenly distributed in normal brain, most dense in substantia nigra compacta of the midbrain. On one hand, activated microglia releases neurotropic factors and anti-inflammatory cytokines to play a protective role [5–7]; on the other hand, microglia activation generates a large amount of free radicals such as superoxide, intracellular reactive oxygen species, hydrogen peroxide, hydroxyl radical and cytotoxic cytokines, including tumor necrosis factor α, interleukin 1β, nitrogen monoxide and superoxide, these radicals may injury neurons [8,9].

Recently, a novel family of lipid mediators including resolvin E and resolvin D has been reported that they have obvious potency in

Abbreviations: MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-kappa B; TNF-α, tumor necrosis factor α; TLR4, toll-like receptors 4; NO, nitrogen monoxide; SNpc, substantia nigra pars compacta; LPS, lipopolysaccharide; IL-β, interleukin 1β.

* Corresponding authors. School of Pharmacy, Guangdong Food and Drug Vocational College, Guangzhou 510520, PR China.
E-mail addresses: xukang316004@126.com (Y.G. Tian), zhangying310009@126.com (Y. Zhang).

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treating neurodegenerative disorders, especially for the disease conditions associated with inflammation [10,11]. Accumulating evidences suggests that omega-3 polyunsaturated fatty acids-derived lipid mediators, such as resolvins, lipoxins, and neuroprotectins produce potent anti-inflammatory and pro-resolution actions in various animal models of inflammation. In the previous research, Xu et al. [11] have reported that resolvins family shows remarkable potency in treating inflammatory pain via central and peripheral actions. Sun et al. [12] and Jin et al. [13] also have indicated that resolvins have the ability to play potent anti-inflammatory and pro-resolution role in animal models of inflammation, as well as the well-known lipoxins and aspirin-triggered lipoxins have similar actions [14–16].

In this regard, we examined whether RvD2 can attenuate LPS-induced inflammatory nerve damage in PD rat model, and further investigated the possible mechanisms involved in cultured microglia activated by LPS in vitro. Our results show that LPS did highly increase the CD11b and Iba-1marker protein expression in glial cells. Also, RvD2 attenuates LPS-induced inflammatory nerve damage in PD rat model by inhibiting the release of inflammatory cytokines and the translocation of TLR4/NF-κB pathway activation in microglial cells.

2. Materials and methods

2.1. Drugs administration

Lipopolysaccharide was obtained from Sigma-Aldrich and resolvin D2 was purchased from Cayman, USA. The RvD2 was prepared in Hanks' buffer (Wisent. bio, Nanjing, China) before it was used. The injection was performed manually within 20 s using a single injection volume of RvD2 per 25, 50 and 100 ng/kg.

2.2. Animals and treatment

200 adult male SD rats weighing 280 g–300 g were purchased from Vital River Laboratory Animals Co., Ltd. (Beijing, China) and housed with a standard 12 h on/off light cycle with food and water in their cages. All rats were randomly separated into 3 groups: (1) the control group; (2) LPS-injected group with model; (3) LPS-injected groups receiving injections with 25, 50 and 100 ng/kg for 3 days; LPS (10 mg/mL, 1.0 uL) was injected into the right part of SNpc following a previously described protocol [17,18]. After LPS injection, rats were continuously treated with RvD2 for 27 days, and the total of 30 days experimental period were used for the rats' experiment. All animal experimental protocols were performed according to the NIH of USA Guide for the care and use of laboratory animals (Publication 85-23, revised 1996).

2.3. Isolation and culture of microglial cells from neonatal rats

The isolation of rat microglial cells from 2-day-old neonatal rats according to the method of Garcia et al. [19] with certain modifications. The cerebral cortex was carefully dissected out, the meninges of cerebral cortices were cautiously separated, and cortices were minced and dissociated with 0.25% trypsin/1 mM EDTA for 20 min at 37 °C. The fragments were washed with cold D-Hank's buffer (GIBCO Corporation, Gaithersburg, MD, USA) and the meninges were gingerly removed. The re-suspended cells were then collected and seeded in uncoated culture flasks containing medium (DMEM/F12 supplemented with 10% FBS, 1×10^5 U/L streptomycin sulfate, pH 7.2, GIBCO Corporation, Gaithersburg, MD, USA) with a concentration 1×10^6/mL at 37 °C, 5% CO_2. Confluent cultures were passaged by trypsinization; microglial cells were isolated by shaking and cultured in 6-well plates, at a density of 2.5×10^5 cells/cm^2. In brief, all the cells are divided into 7 groups. The cells of RvD2 group were incubated in the initial experiments with different concentration of RvD2 (1.25 umol/L, 2.5 umol/L, 5 umol/L, 10 umol/L and 20 umol/L). The LPS group cells will only incubate in vehicle before addition of 100 ng/ml LPS under serum-free condition. Based on the control group, the RvD2+LPS groups will add 100 ng/ml LPS to the cells that have been incubated in RvD2 for 60 min.

2.4. Behavioral analysis

Apo-morphine was used to study the rotational behavior of rats. Rats were placed into cylinders that were attached to a rotameter (Columbus Instruments, Columbus, OH, USA) on the second day after the final RvD2 injection. The rats were allowed to adapt to the testing environment for 15 min and were injected hypodermically with 0.5 mg/kg apo-morphine (Sigma-Aldrich) dissolved in physiological saline. After injection for 5 min, the measurement of rotational activity began and lasted for 30 min under minimal external stimuli. The rotameter recorded the number of full clockwise and counter-clockwise turns the animals performed during the testing period. Counter-clockwise turns were counted as negative turns. Also, the clockwise turns were counted as positive turns. The net number of turns performed during the entire 30 min testing period was counted.

2.5. Tissue processing

For tissue harvest, after the rotational behavior assay, 20 rats were randomly selected from each group for morphological studies. Rats were deeply anesthetized with chloral hydrate. Frozen sections were cut into 35-um-thick sections and processed for immunohistochemistry.

2.6. Immunohistochemistry

Every fifth section of the SN (bregma −4.8 to −6.3 mm) was immunostained for detection of the microglial marker CD11b (1:500; Sigma-Aldrich). After being perforated, all the cell membranes with 0.5% Triton-X 100 and blocked with 2.5% horse serum, then sections were incubated with primary antibodies for 24 h at 4 °C. Then, the antibody was detected using an ABC Elite kit (Vector laboratories, Sigma-Aldrich) with 3,3′- diaminobenzidine (DAB) and nickel enhancement. The average optic density value in the SNpc of each CD11b-stained section was determined using an image analysis system. All sections were coded and examined blindly.

2.7. RNA isolation and quantitative real time-PCR

The total RNA extraction was performed using Trizol reagent (Gibco BRL). 1 μg of total RNA was reverse transcribed using the M-MLV-RT system (Promega). The action was carried out at 42 °C for 1 h and terminated by deactivation of the enzyme at 70 °C for 10 min. qPCR were conducted using SYBR Green (Bio-Rad) in ABI PRISM 7900HT detection systems (Applied Biosystems). Invitrogen coorporation produced all the primers for CD11b, Iba-1, TNF-α, NF-κB p65, iNOS, IL-1, IL-18, IL-6, IκBα, IKKβ, and IL-1β.

2.8. ELISA measurement

Microglial cells culture medium and rats' serum was collected for the ELISA analysis of main pro-inflammatory cytokines. For each reaction in a 96-well plate, 100 μl of medium were used, and ELISA kits (R&D) were performed according to manufacturer's protocol. The microglia were incubated with 100 ng/mL LPS for 24 h, the culture supernatants were harvested and mixed with an equal volume of Griess reagent (Sigma-Aldrich) in 96-well plates at room

temperature for 20 min. Bio-Rad absorbance reader was used to measure the wavelength of 540 nm, and the nitrite (NO) concentrations were confirmed according to standard curve generated by known concentrations of sodium nitrite.

2.9. Western blot analysis

Extraction buffer (Beyotime Company, Jiangsu, China) was used to extract cells proteins from ventral mesencephalon. The microglial cells were lysed in radioimmune precipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail (Roche). Nuclear and cytoplasmic fractionations were performed with Proteo JETTM Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas Life Science) according to manufacturer's protocol. Tissue were homogenized in this buffer using a Fisher model 100 sonic dismembrator and put on ice for 1 h. Equal amounts of protein samples were electrophoresed on SDS-polyacrylamide gels, and then transferred to nitrocellulose filters (Millipore, Bedford, MA, USA) using a semidry blotting apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The transformed membrane was blocked for 1 h and incubated with primary antibodies (Santa, CST and Abcam) at 4 °C overnight. The filters were incubated with TH (1:1000) or GAPDH (1:5000) antibodies. The primary antibodies for microglial cells in plates used were as follows (1:1000): rabbit anti−NF−κB p65, IκBα, IKKβ, MyD88, TLR4 and GAPDH. The membrane was washed three times with TBST for 10 min and incubated with anti-rabbit or anti-mouse IgG-horseradish peroxidase (1:5000,Pierce) at room temperature for 1 h. The signal was visualized using Super signal West Pico chemiluminescent substrate system (Pierce). The density of each band was determined using image software (LI-COR Biosciences, Lincoln, NE, USA).

2.10. ROS measurement

Non-fluorescent dye, 2′, 7′-dichlorofluorescein diacetate (Sigma-Aldrich) was used to measure the presence of intracellular ROS of microglial cells. Cells were incubated in 96-well plates and pre-treated with RvD2 for 2 h before 100 ng/mL LPS or normal medium was added. After 2 h, the cultures were washed with PBS and loaded with 10 nM of DCFH-DA in serum-free DMEM/F12 for 30 min at 37 °C. The cells on 96-well plates were observed by fluorescence microscopy. The fluorescence intensity of the 96-well plates was measured on a fluorescent plate reader (Molecular Device, Sunnyvale, CA, USA) at 485 nm for excitation and 530 nm for emission.

2.11. Statistical analysis

All data are expressed as means ± SEM. Treated cells and tissue and the corresponding controls were compared using Graph Pad PRISM (version 6.0; Graph Pad Software) by a one-way ANOVA with Dunnet's least significant difference tests. Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. Resolvin D2 inhibited LPS-induced the activation of glial cells and development of behavioral defects

In LPS-treated animals, rats performed more apo-morphine-induced rotational cycles (59.8 ± 26.5-turns) and rotational cycles of sham animals were only 3.3 ± 2.1-turns/30 min. In contrast, rats treated with 25, 50 and 100 ng/kg RvD2 showed significantly decrease in the numbers of apo-morphine-induced rotational cycles

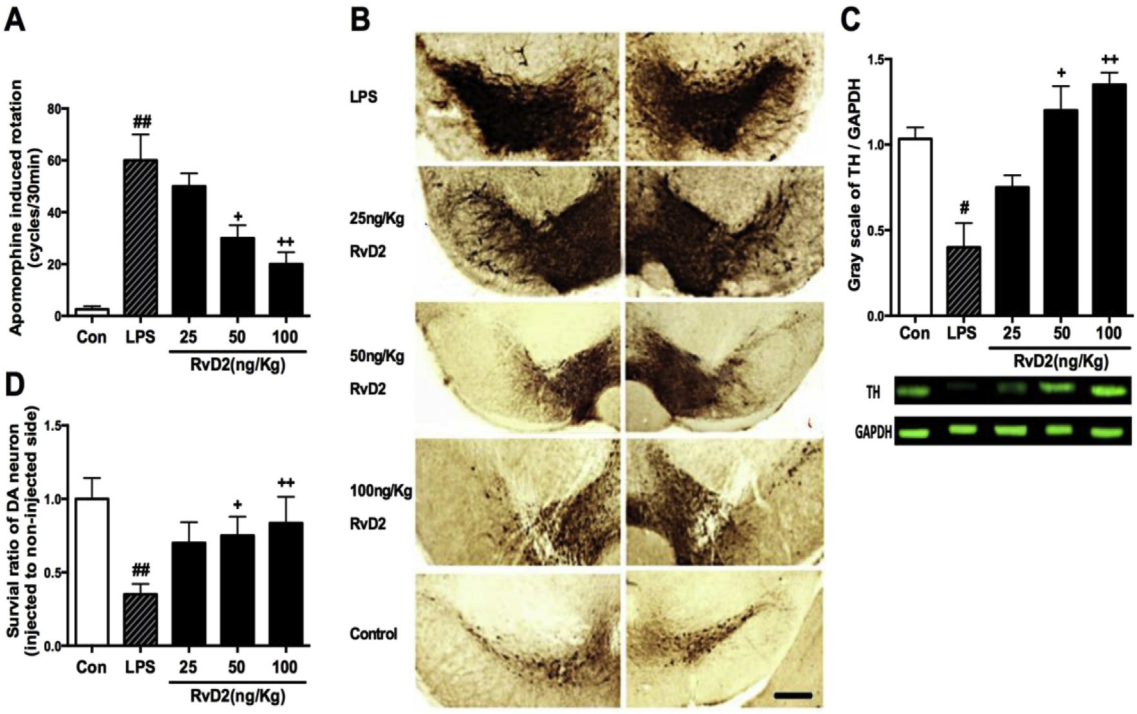

Fig. 1. Resolvin D2 inhibited LPS-induced development of behavioral defects. (A) Before LPS injection, rats were pre-treated with different concentration of RvD2 (25, 50 and 100 ng/kg) or vehicle 3 days and after LPS injection, rats were continuously treated with RvD2 for 27 days (30 days in total). On the second day after the final RvD2 injection, rats were injected hypodermically with apo-morphine (0.5 mg/kg) to induce rotational behavior. The total numbers of turns were counted using a computerized rotameter for 30 min, with the numbers regarded as an index of the degree of lesion. (B) SNpc photomicrographs with staining of both the non-injected and injected sides using an antibody against tyrosine hydroxylase (TH). (C) Western blot analysis shows that treatment of RvD2 inhibited the LPS-induced increase of TH protein expression in the SNpc on the injected sides. (D) Survival TH-positive neurons ratio on the injected side to the non-injected side in the SNpc. All data are shown as means ± SEM. #$p < 0.05$ or ##$p < 0.01$ compared with the control group, +$p < 0.05$ or ++$p < 0.01$ compared with the LPS group.

to about 24.4 ± 26.9-turns/30 min, 15.3 ± 10.19-turns/30 min and 10.4 ± 5.6-turns/30 min (Fig. 1A and B). Here we also used immunoblotting to analyze the TH protein expression of SNpc (Fig. 1C). On the injected side, LPS treatment markedly reduced the levels of TH protein compared with the control group. Fig. 1D showed that the ratio of non-injected side of dopaminergic neuron survival on the LPS-injected side. The injection of LPS decreased the number of TH-positive neurons to 0.3 ± 0.1. The survival ratio of TH-positive neurons of the SNpc was stored to 0.6 ± 0.2, 0.8 ± 0.1 and 0.9 ± 0.2 of the side in LPS-injected rats treated with RvD2. These results suggested that RvD2 treatment not only retained TH expression of LPS-treated animals but also inhibited glial cells activation.

3.2. Resolvin D2 inhibited LPS-induced microglia activation

Here we used CD11b to study microglial activation and morphological changes during neurodegenerative inflammation. Immunocytochemical staining of CD11b (Fig. 2A) showed that different concentrations of RvD2 impact on the cells in the SNpc. After LPS injection, activated microglia were easily identifiable throughout the SNpc by their processes and more rounded cell bodies. In contrast, pre-treatment with RvD2 inhibited the activation of microglia, with more microglia performing the ramified morphology than in the vehicle group. Fig. 2B suggested that the average optical density (OD) value was confirmed to stand for the level of CD11b expression in the SNpc. The CD11b content increased significantly in the LPS-injected vehicle group. After treatment with 25, 50 and 100 ng/kg RvD2, the CD11b content of the SNpc was decreased by about 19%, 25% and 43%.

3.3. Resolvin D2 inhibited LPS induced inflammatory signal pathway

The microglial cells were treated with 100 ng/mL LPS for 24 h and compared with control group. ELISA method was used to analyze the inflammatory factors protein. Western blot was used to analyze the protein expression of TLR4, MyD88, IκBα, IKKβ, and NF-κB p65 (Fig. 2C–I). The ELISA results showed that the concentration of IL-18, IL-6, NO, TNF-α and IL-1β in cytoplasm (Fig. 3A1–A5) and serum (Fig. 3B1–B5) with LPS treated increased

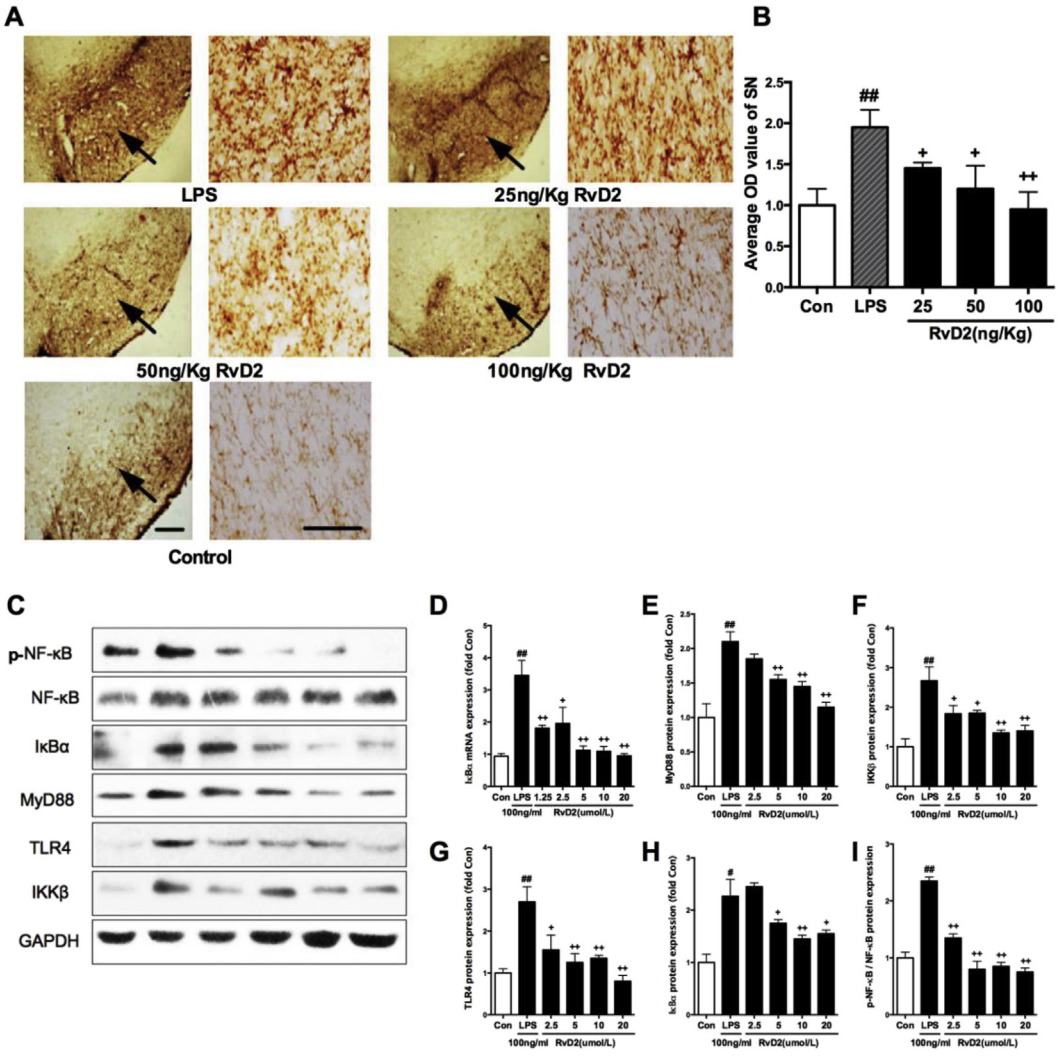

Fig. 2. Resolvin D2 inhibited LPS-induced microglia activation and inflammatory signal pathway protein production. (A) CD11b was detected by immunohistochemical staining to identify microglia in the SNpc. Treatment with RvD2 significantly inhibited the activation of microglia, with more microglia performing the ramified morphology than in the vehicle group. (B) Effects of RvD2 treatment on the average optical density of CD11b in the SNpc. (C)–(I) Microglia were incubated in 2.5, 5, 10, 20 umol/L RvD2 and then incubated in a medium containing 100 ng/mL LPS for 24 h. Western blot analysis shows that treatment of RvD2 inhibited the LPS-induced increase of TLR4, MyD88, NF-κB p65, IκBα and IKKβ protein expression in the primary microglia. Data are shown as the means ± SEM. #$p < 0.05$ or ##$p < 0.01$ compared with the control group, +$p < 0.05$ or ++$p < 0.01$ compared with the LPS group.

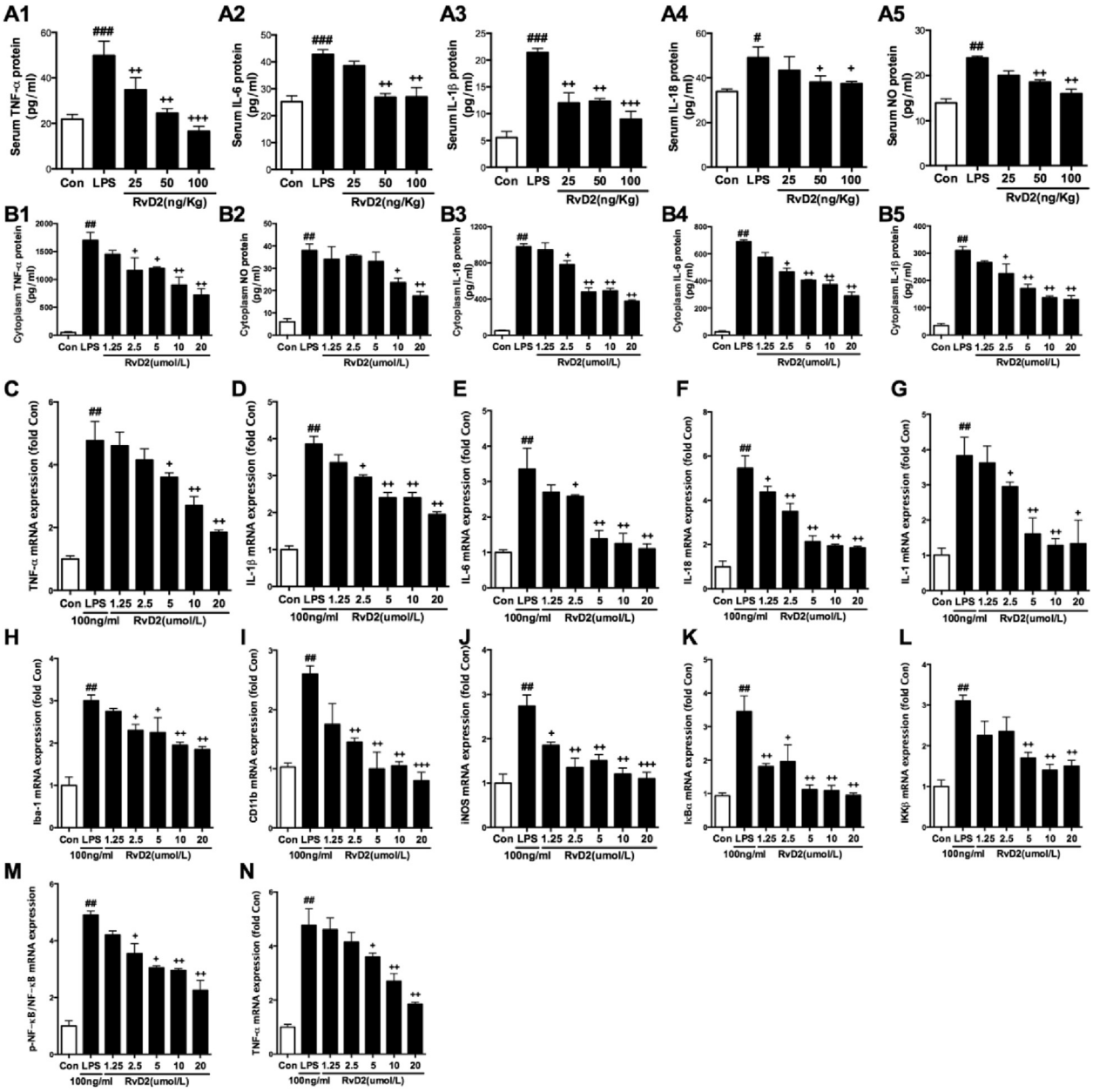

Fig. 3. Resolvin D2 suppressed LPS-induced inflammatory factors protein production in serum, cytoplasm and inflammatory gene expression. (A1)–(A5) ELISA method was used to analyze the serum inflammatory factors of NO, TNF-α, IL-6, IL-18 and IL-1β. (B1)–(B5) Microglial cells were treated with 100 ng/mL LPS for 24 h and compared with control group. ELISA also analyzed cytoplasmic inflammatory related factors as well as in serum. (C)–(N) microglial cells incubated with 100 ng/ml LPS or cultured with different concentration of RvD2. q-PCR analysis of mRNA indicated that the effect of LPS and LPS + RvD2 on the mRNA expression level. Data are shown as the means ± SEM. ##$p < 0.01$ compared with the control group, +$p < 0.05$, ++$p < 0.01$ and +++$p < 0.001$ compared with the LPS group.

significantly. Of note, IL-18, IL-6, NO, TNF-α and IL-1β production were significantly inhibited by the different concentrations of RvD2 in vivo and vitro. The western blot results showed that LPS activated TLR4/MyD88 signal pathway in microglial cells, the main role of this pathway such as TLR4, NF-κB p65 and IκBα has been up-regulated by LPS. In contrast, the treatment of different concentrations of RvD2 was able to inhibit pathway.

3.4. Resolvin D2 reduced pro-inflammatory factors mRNA expression and ROS production

We further examined the effects of different concentration of RvD2 on LPS-induced primary microglia. As shown in Fig. 3C–N, the quantitative real time-PCR results showed that 100 ng/mL LPS induced cells up-regulated the expression of CD11b, Iba-1, TNF-α, NF-κB p65, iNOS, IL-1, IL-18, IL-6, IκBα, IKKβ, and IL-1β. The results suggested that RvD2 performed significant effect on inhibition of pro-inflammatory factors mRNA expression and cells activation for in vitro experiment of primary microglial cells. Moreover, As shown in Fig. 4, the results of fluorescence microscopy showed that LPS induced a significant increase in the levels of intracellular ROS. In the presence of RvD2, the level of intracellular ROS was significantly reduced; the results were consistent with the fluorescence intensity assay that measured fluorescence using a fluorescence plate reader and other results.

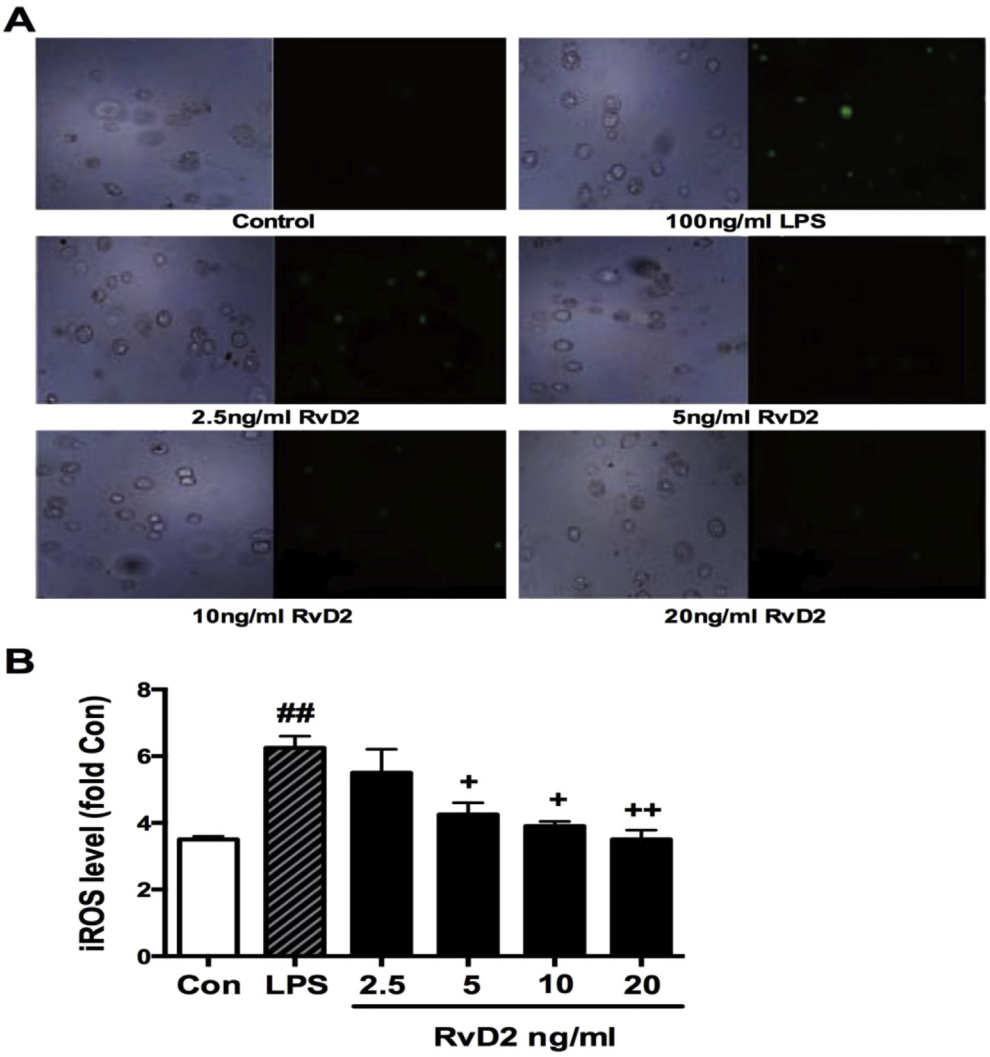

Fig. 4. Resolvin D2 inhibited LPS-induced ROS production. (A) The cells were incubated in 96-well plates and pre-treated with RvD2 for 2 h before 100 ng/mL LPS or normal medium was added. After 2 h, the cells were exposed to serum-free DMEM/F12 containing the fluorescent probe DCFH-DA (10 nM), for 30 min at 37 °C. (B) The results of fluorescence microscopy showed that LPS induced a significant increase in the levels of intracellular ROS. In the presence of RvD2 (especially for treatment of 20 ng/mL), the level of intracellular ROS was significantly reduced. Fluorescence intensity was measured using a fluorescence plate reader. Data are shown as the means ± SEM. ##$p < 0.01$ compared with the control group, +$p < 0.05$ and ++$p < 0.01$ compared with the LPS group.

4. Discussion

It is fact that a variety of cell types have been proved, including peripheral immune cells, neurons, microglia and astrocytes infiltration into the brain tissue, which are source of cytokines in the central nervous system (CNS), studies still show that microglial cells are primary sources of pro-inflammatory and immune-regulatory cytokines. In various types of neurons in central nervous system, some pro-inflammatory stimuli, such as lipopolysaccharides on Gram-negative bacteria cell wall, can activate microglia to induce selective loss of neurons [7,20]. Both in vivo and in vitro experiments showed that lipopolysaccharides could activate microglia and cause the death of neurons and release of cytokines. Resolvin D2 as a novel family of lipid mediators including RvD1 and RvE1, show remarkable potency in treating disease conditions associated with inflammation. In this study, we first discussed the relationship between RvD2 and resolution of microglial cells inflammation in LPS induced inflammatory rat model, and whether RvD2 attenuates inflammatory response of microglia in PD. we also examined whether RvD2 can suppress microglial cells activation following nerve injury induced by LPS in rat model, and further indicated the possible mechanisms involved in cultured microglia activated by LPS in vitro.

In the present study, we demonstrated that the treatment of different concentration of RvD2 for neuronal damage in the nigrostriatal dopaminergic system. Given the fact that the degree of apomorphine-elicited rotational behavior is related to LPS-induced inflammatory reactions and RvD2 improved apomorphine induced rotational behavior and inhibited the TH-positive neurons loss of the SNpc in LPS-treated rats, suggesting that RvD2 improved the behavioral deficit as a consequence of the protection of dopaminergic neurons. The activated microglial cells can release a large number of pro-inflammatory cytokine, nitric oxide, reactive oxygen metabolites and other substances, which perform toxic effects on neurons. IL-1β and TNF-α are two major pro-inflammatory cytokine produced by microglia in the central nervous system inflammation [14–16]. LPS can significantly induce an increasing production of IL-1β and TNF-α in microglia [6–8,13]. Cultured microglia exhibits neurotoxicity effects, including damage to dopaminergic cells. In sporadic and familial

PD, there are numerous reactive microglial cells. The presence of activated microglia in mesencephalic substantia nigra and the death of monkeys exposed to MPTP for several years have proved long-standing inflammation in PD process. In such view of the situation, we used CD11b to investigate microglial activation and morphological changes during neurodegenerative inflammation; the results showed that in LPS-injected rats, activated microglia were easily identifiable throughout the SNpc by their thicker processes and more rounded cell bodies. Treatment with RvD2 significantly inhibited the activation of microglia, and the OD value of CD11b were significantly decreased by about 19%, 25% and 43%.

In various nervous system diseases, the injured and died neurons can activate microglial cells through a variety of cytokines [19–21]. For the in vitro experiment, we study the possible mechanisms involved in cultured microglia activated by LPS. TNF-α, iNOS, IL-1, IL-18, IL-6 and IL-1β as the main cytokines were used to evaluate the level of activated microglial cells. The cells were incubated in RvD2 and then treated with LPS (100 ng/mL) in order to test whether RvD2 could attenuate cytokines' mRNA expression. The qPCR results showed that cells with LPS treatment significantly increased the expression of cytokines and glial activation just like we mentioned above. In contrast, the group with RvD2 treatment performed the down-regulation level in expression of pro-inflammatory factors and activation of NF-κB related signal pathway. These suggest that RvD2 exhibits effect on inhibition of NF-κB, TNF-α, IL-1, IL-18, IL-6, IL-1β and iNOS mRNA expression in vitro experiment of primary microglial cells.

Nuclear factor-kappa B, an important factor of inflammatory process and autoimmune diseases, has recently been proved to be involved in neuropathic pain [5–7,21]. In this study, we investigate the possible mechanism of NF-κB p65 impact on inflammation of microglial cells. The qPCR and western blot results showed that cells with treatment of 100 ng/mL LPS significantly increased the expression of TLR4/NF-κB pathway mRNA and p65 protein. Also, the mRNA and protein expression could be inhibited by different concentration of RvD2 and with concentration increasing more inhibition performed in LPS-induced microglial cells. This phenomenon suggested that in an LPS-induced acute inflammation rat model, RvD2 down-regulated NF-κB p65 mRNA expression by reducing the nuclear translocation of NF-κB p65 in microglial cells. Additionally, we also use ELISA to analyze the main pro-inflammatory factors protein expression in serum and glial cytoplasm. Our present results indicate that the level of intracellular and serum inflammatory factors can be inhibited by RvD2, and the function of suppression increases with the concentration of RvD2.

Summary, in this regard, we study the effect of RvD2 on LPS-induced microglial cells in vitro and dopaminergic neurons in vivo experiment. The results showed that RvD2 could reduce microglial cells inflammation and perform the possible mechanisms by inhibiting microglial activation, downstream signaling of TNF-α, IL-1β and iNOS, up-regulated NF-κB p65 related pathway mRNA expression, NO release and ROS production.

Conflict of interest

The authors declare that there are no conflicts of interest related to this manuscript.

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