Original Paper

Glucocalyxin B Alleviates Lipopolysaccharide-Induced Parkinson's Disease by Inhibiting TLR/NF- κ B and Activating Nrf2/HO-1 Pathway

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Key Words

Parkinson's disease • Glucocalyxin B • Toll-like receptor • Nuclear factor kappa B • Nuclear factor erythroid 2-related factor 2

Abstract

Background/Aims: Parkinson's disease (PD) is a common neurodegenerative disease in the old population, characterized by dopaminergic neuron loss, inflammation and oxidative stress injury in the substantia nigra. Glucocalyxin B (GLB), an *ent*-kauranoid diterpenoid isolated from *Rabdosia japonica*, has anti-inflammation and anti-tumor effects. However, its effects on PD remain unclear. **Methods:** PD was introduced in rats via injection of lipopolysaccharide (LPS) into cerebral corpus striatum, and GLB was given intracerebroventricularly to these rats. Their walking, climbing and sensory states were detected by Stepping, Whisker and Cylinder Tests. The expression of tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), CD11b and ionized calcium binding adaptor molecule (IBA)-1 were detected by immunohistochemical staining. The levels of a series of inflammatory factors, oxidative stress-related factors and apoptosis-related factors were measured by real-time PCR, immunoblotting and ELISA. In addition, Toll-like receptor (TLR)/nuclear factor kappa B (NF- κ B) and nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase (HO)-1 pathways were investigated to illustrate the underlying mechanism. *In vitro*, microglial cells exposed to LPS were treated with GLB. **Results:** The injection of LPS caused walking, climbing and sensory disturbances in rats, induced inflammation, oxidative stress response and apoptosis, and activated TLR/NF- κ B and Nrf2/HO-1 pathways in the cerebral tissue. GLB administration attenuated LPS-induced alterations. The TLR/NF- κ B pathway was deactivated and Nrf2/HO-1 was activated after application of GLB. *In vitro*, cytotoxic effects induced by the conditioned medium derived from microglial

cells exposed to LPS in PC12 cells were attenuated by GLB. **Conclusion:** GLB suppresses LPS-induced PD symptoms by modification of TLR/NF- κ B and Nrf2/HO-1 pathways *in vivo* and *in vitro*.

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder only after Alzheimer's disease [1]. PD is characterized by tremor, stiffness, fatigue, impairment of dexterity, a dragging of one foot, and chaotic speech [2]. In the later stage, the impairment of gait and balance results in disability in many gait-dependent activities, such as walking, dressing, bathing and housework. Moreover, PD causes other disorders, including genitourinary dysfunction, cardiovascular dysfunction, sleep disturbance, depression, dementia, olfactory and vision problems [3]. PD is an aged disease, and the median age of onset is 60 years [3].

PD is an incurable progressive disease, and its causes are not completely illustrated. However, several risk factors have been found, such as pesticides, heavy metals, occupation, traumatic brain injury and diet [3]. On the other hand, several families have been identified with parkinsonism with clear Mendelian inheritance since 1997, and multiple PD-related genes have been identified, including α -synuclein, *PTEN*-induced putative kinase 1 (*PINK1*), leucine-rich repeated kinase 2 (*LRRK2*), *DJ-1*, *Parkin*, ATPase type 13A2 (*ATP3A2*), and glucocerebrosidase (*GBA*) [1, 3]. Both environmental factors and genetic factors result in brain lesions. The primary symptoms are progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and reduction in the terminals within the dorsal striatum, leading to profound and irreversible striatal dopamine loss [4, 5]. Abnormal oxidative stress and neuronal inflammation have been reported to contribute to the loss of dopaminergic neurons. Pharmaceuticals-induced inhibition of oxidative stress and inflammation could mitigate experimental PD symptoms [6-9].

Recently, the neuroprotective effects of traditional Chinese medicine have attracted increasing attention. Some herbs and herbal formulae have been applied in PD treatment [10]. Glucocalyxin B (GLB) is an *ent*-kauranoid diterpenoid isolated from a perennial herb, *Rabdosia japonica*. GLB has been reported to suppress lipopolysaccharide (LPS)-induced inflammation in microglia [11]. GLB inhibits the proliferation of human cervical cancer cells through inducing apoptosis and autophagy, which is mediated by the PI3K/Akt signaling pathway [12]. GLB decreases growth, induces apoptosis, G2/M-phase cycle arrest, DNA damage and accumulation of reactive oxygen species (ROS) in human leukemia cells [13]. However, whether GLB can alleviate PD symptoms remains unknown. In this study, we established a rat PD model by LPS injection, and investigated whether GLB could attenuate the experimental PD symptoms and explored the underlying mechanisms.

Materials and Methods

Ethical statement

Animals in this study were taken care of and used according to the Guide for the Care and Use of Laboratory Animals (8th edition-NIH). All experimental procedure was approved by Ethic Committee of China Medical University. The ethical approval number is AF-OG-23-1.1-01.

Animal model

Healthy male SD rats of 5-7 weeks (about 200 g) were randomly divided into six groups: Sham, GLB, PD, PD+GLB(L), PD+GLB(M) and PD+GLB(H). After anesthetization with 10% chloral hydrate (3.5 ml/kg), the rats in PD, PD+GLB(L), PD+GLB(M) and PD+GLB(H) groups were injected with 2 μ l LPS (5 mg/ml) (Sigma, St. Louis, MO, USA) into cerebral corpus striatum to induce experimental PD symptoms, while rats in Sham and GLB groups were injected with a same volume of sterile saline. Thereafter, the rats in GLB groups were

given intracerebroventricular injection of 5 μ l GLB (dissolved in 10% DMSO) of different concentrations (20 mg/ml in PD+GLB(L) group, 40 mg/ml in PD+GLB(M) group, 80 mg/ml in GLB and PD+GLB(H) groups). The rats in Sham and PD groups were injected with a same volume of 10% DMSO. At day 0, 7 and 14 post-injection, behavior tests were performed to evaluate the PD degree of rats in each group. After the last behavior test, all rats were sacrificed to collect their brain tissues.

There were 30 rats in each group, some were used for pathological detection, some for RNA detection, and some for protein detection. There were 6 individuals in each detection of animal experiments.

Behavior test

Stepping test was performed to assess the forelimb akinesia. The rat was set on a table, its lower body was lifted and only forepaws were touching the table. Then the rat was moved along the stable surface at a rate of approximately 1 m/min, and the number of adjusting steps in 90 cm was counted manually.

Whisker test was used to measure the sensorimotor injury. The rat's lower body was lifted, and forepaws were touching the table. Then one forepaw was restrained, and the free forepaw was still touching the table. The experimenter moved the rat to make the whisker of free side to touch the table surface. The stimulation of whisker resulted in activity of the forepaw on the same side. The forepaw activity times during ten touching processes were counted. The other side was test as the same.

Cylinder test was carried out to examine the sensorimotor function. The rat was placed into a transparent cylinder, and recorded for 5 min. The number of times that the right and left forelimbs contacted the wall was counted.

Immunohistochemical staining

The brain tissues were fixed with 10% formaldehyde overnight and washed with flow water for 4 h. Then the tissues were dehydrated with ethanol of graded concentrations (70% for 2 h, 80% overnight, 90% for 2 h, 100% for 1 h twice), permeated with xylene for 30 min, embedded in paraffin at 60°C for 2 h, and cut into sections of 5 μ m. The sections were dried at 60°C for 2 h, dewaxed with xylene for 15 min twice, rehydrated with ethanol of 100% for 5 min twice, 95% for 2 min, 85% for 2 min, 75% for 2 min, and water for 2 min. For antigen retrieval, tissue sections were soaked in boiling citrate buffer for 10 min, and blocked with 3% H₂O₂ (Sinopharm, Beijing, China) at room temperature for 15 min. After blocking with goat serum (Solarbio, Beijing, China) for 15 min, the sections were incubated with antibody against tyrosine hydroxylase (TH) (rabbit, 1:100) (Sangon, Shanghai, China), glial fibrillary acidic protein (GFAP) (mouse, 1:50) (Santa Cruz, CA, USA), CD11b (rabbit, 1:200) (Wuhanbio, Wuhan, Hubei, China) or ionized calcium binding adaptor molecule (IBA)-1 (rabbit, 1:200) (Sangon) at 4°C overnight. After rinsing with PBS, the sections were incubated with goat anti-rabbit or anti-mouse IgG labeled with biotin (1:200) (Beyotime, Haimen, Jiangsu, China) at 37°C for 30 min, and then with avidin-labeled HRP (Beyotime) at 37°C for 30 min. After reacting with DAB reagent for several seconds, the sections were stained with hematoxylin (Solarbio) for 3 min, polarized with 1% hydrochloric acid ethanol, and washed with flow water for 20 min. After dehydrating with ethanol of 75%, 85% and 95% each for 2 min, 100% for 5 min twice, and xylene for 10 min twice, the sections were mounted with gum, and photographed with a microscope (Olympus, Tokyo, Japan) at 400 $\times$ magnification. TH-positive cell, GFAP-positive cell, CD11b-positive cell and IBA-1-positive cell numbers in six sections were counted in each group.

Real-time PCR

Total RNA was extracted from tissues or cells by a total RNA Rapid Extraction Kit (BioTeke, Beijing, China) according to the manufacturer's protocols. After the concentration measurement, RNA was reversely transcribed into cDNA via Super M-MLV reverse transcriptase (BioTeke), in presence of Oligo(dT) and random primers. All instruments in this section were pre-treated with Surface RNase Erase (TIANDZ, Beijing, China), and all reagents were RNase-free.

The cDNA sample was used for real-time PCR by using 2 $\times$ Power Taq PCR MasterMix (BioTeke) and SYBR Green (Solarbio) to detect the mRNA levels of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6, with the homologous β -actin as the internal control. The real-time PCR primers used in this study were purchased from Songon, and the sequence information was shown in Table 1.

Western blot

The total protein was extracted from tissues or cells with RIPA lysis buffer (Beyotime), and the nuclear or cytoplasmic protein was extracted by a Nucleus and Cytoplasm Protein Extraction Kit (Beyotime). After denaturation, the protein was separated by SDS-PAGE and transferred onto the PVDF membrane (Millipore, Boston, MA, USA). After blocking with 5% skim milk (YILI, Hohhot, Inner Mongolia, China), the PVDF membrane was incubated with one of the following antibodies at 4°C overnight: rabbit anti-inducible nitric oxide synthase (iNOS) (Boster, Wuhan, Hubei) (1:500), rabbit anti-cleaved caspase-3 (Abcam, Cambridge, UK) (1:400), rabbit anti-Bax (Boster) (1:400), rabbit anti-Bcl-2 (Boster) (1:400), rabbit anti-nuclear factor kappa B (NF-κB) (Boster) (1:400), rabbit anti-nuclear factor erythroid 2-related factor 2 (Nrf2) (Bioss) (1:1000), rabbit anti-heme oxygenase (HO)-1 (Santa Cruz, CA, USA) (1:500), rabbit anti-Toll-like receptor (TLR)2 (Bioss) (1:500), rabbit anti-TLR4 (Bioss) (1:500), rabbit anti-glutathione S-transferase (GSTA) 1 (Bioss) (1:500), rabbit anti-NAD(P)H:quinine oxidoreductase (NQO) 1 (Bioss) (1:500), mouse anti-β-actin (Santa Cruz) (1:1000) or rabbit anti-Histone H3 (Santa Cruz) (1:500). After washing with TBST, the PVDF membrane was incubated with the goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP (Beyotime) (1:5000) at 37 °C for 45 min, and exposed with ECL reagent (7 sea, Shanghai, China). β-actin was used as the cellular internal control, and Histone H3 as nuclear control.

Cell culture

Mouse microglial cell line BV2 was purchased from CHI (Jiangyin, Jiangsu, China) and cultured with RPMI-1640 (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) at 37°C in 5% CO₂. Rat adrenal pheochromocytoma cell line PC12 was purchased from Zhongqiaoxinzhong (Shanghai, China) and cultured with DMEM (Gibco) supplemented with 10% FBS at 37°C in 5% CO₂.

Measurement of nitric oxide (NO) content

The tissues were mixed with a 9-fold volume of PBS, freeze-thawed thrice, smashed by homogenizer, and centrifuged at 10005 g for 10 min. The supernatant was collected. Cells were cultured for 48 h, and the culture supernatant was collected. The protein concentration was determined with a BCA Protein Quantification Kit (Beyotime). The NO content in tissue and cell supernatant was measured with the NO Assay Kit (Jiancheng, Nanjing, Jiangsu, China).

Measurement of reactive oxygen species (ROS)

The tissue was mixed with a 20-fold volume of PBS, freeze-thawed thrice, smashed by homogenizer, centrifuged at 10005 g for 10 min, and the supernatant was collected. The ROS content in the supernatant was measured with dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay using a ROS Assay Kit (Jiancheng) according to the manufacturer's protocols. The ROS content was presented as the relative fluorescent density with the emission light at 525 nm and excitation light at 485 nm. The ROS content in the cells was directly detected with the ROS Assay Kit according to the protocols.

Measurement of superoxide dismutase (SOD) activity

The tissues were mixed with a 9-fold volume of PBS, freeze-thawed thrice, smashed by homogenizer, centrifuged at 10005 g for 10 min, and the supernatant was collected. The cells were lysed with the RIPA

Table 1. Sequences of real-time PCR primers used in this study

Name	Sequence (3'-5')
Rat TNF-α F	3'- TGGCGTGTTCATCCGTTCT'5'
Rat TNF-α R	3'- CCACTACTTCAGCGCTCGT'5'
Rat IL-1 β F	3'- TCCAGTCAGGCTTCCTTG'5'
Rat IL-1 β R	3'- CGAGATGCTGCTGTGAGATT'5'
Rat IL-6 F	3'- GTTGCTTCTTGGGACTGATGT'5'
Rat IL-6 R	3'- ATACTGGTCTGTGTGGGTGGT'5'
Rat β-actin F	3'- GGAGATTACTGCCCTGGCTCCTAGC'5'
Rat β-actin R	3'- GGCCGGACTCATCGTACTCTGCTT'5'
Mouse TNF-α F	3'- GAGTCCGGGAGGCTACTT'5'
Mouse TNF-α R	3'- CCAGGTCACTGTCCAGCATC'5'
Mouse IL-1 β F	3'- TGTGACATCAGCACCTCACA'5'
Mouse IL-1 β R	3'- GAAGGCATTAGAAACAGTCC'5'
Mouse IL-6 F	3'- ACTTCCATCCAGTTGCCTTCTT'5'
Mouse IL-6 R	3'- TCATTTCACGATTTCACAGA'5'
Mouse β-actin F	3'- CTGTGCCCATCTACGAGGCTAT'5'
Mouse β-actin R	3'- TTTGATGTACGCACGATTTC'5'

lysis buffer, centrifuged at 10005 g for 10 min, and the supernatant was collected. The SOD activity in tissue or cell supernatant was measured with an SOD Assay Kit (WST-1 method) (Jiangcheng) according to the manufacturer's protocols.

Measurement of myeloperoxidase (MPO) activity

The tissues were smashed in 10-fold volume of saline, and the cells were lysed with lysis buffer. The MPO activity in tissues or cells was detected with an MPO Assay Kit (Jiangcheng) according to the manufacturer's protocols.

MTT assay

BV2 cells were seeded into 96-well plates at a density of 3×10^3 per well. After adhesion, fresh culture media containing LPS at different concentrations (0, 0.1, 1, 10, 100, 1000 ng/ml) was added into each well. Twenty-four hours later, cell viability was determined by MTT assay. In short, MTT was added into medium with the final concentration of 5 mg/ml to incubate the cells for 4 h. Then, cell supernatant was removed, 200 μ l DMSO was added to dissolve the crystal, and the optical density of the solution was detected with a microplate reader.

The BV2 cells were incubated with GLB at different concentrations (0, 150, 300, 600, 1200, 12500 nM) for 24 h, and then the cell viability was detected with the MTT assay as well.

On the other hand, the BV2 cells were seeded into 6-well plates: Control, LPS, LPS+GLB. After adhering to the plates, BV2 cells in LPS+GLB group were pre-treated with GLB (the concentration based on the previous MTT results) for 30 min, and then treated with LPS (the concentration based on the previous MTT result) for 24 h. The culture supernatant was collected as Control culture supernatant (Control CS), LPS CS and LPS+GLB CS. PC12 cells were seeded in 96-well plates at a density of 3×10^3 per well. After adhesion, PC12 cells were cultured in culture supernatants from BV2 cells for 24 h, and the viability was also detected with the MTT assay.

Flow cytometry

PC12 cells were seeded in 6-well plates, and cultured in culture supernatant from BV2 cells for 48 h as previously described. Subsequently, the PC12 cells were collected, treated with Annexin V-FITC Apoptosis Detection Kit (KeyGEN, Nanjing, Jiangsu, China), and detected with a flow cytometer (BD, Franklin Lakes, NJ, USA).

Statistical analysis

Data in this study were presented as mean $\pm$ standard deviation (SD) in six (*in vivo*) or three (*in vitro*) replicates, and analyzed by one-way or two-way ANOVA test followed by post hoc Bonferroni's multiple comparisons. A p value was considered as statistical significant when less than 0.05. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: no significance)

Results

Establishment of rat PD model by injection of LPS

As inflammation and nerve injury are the significant symptom of PD, the rats were injected with LPS in their brain corpus striatum to induce PD. At day 0, 7 and 14 post-injection, Stepping, Whisker and Cylinder tests were performed to evaluate whether the rat PD model was established successfully. As shown in figure 1, the Stepping, Whisker and Cylinder test grades of rats in PD group were increased by 88%, 76% and 64% on day 7, and increased by 80%, 68% and 48% on day 14, respectively (Fig. 1A-C), compared to sham-operated rats, suggesting that the injection of LPS could cause representative PD symptoms in rats. The intracerebroventricular injection of GLB increased the Stepping test grade by 2.5 folds (Fig. 1A), Whisker test grade by 2.06 folds (Fig. 1B), and Cylinder test grade by 1.43 fold on day 14 (Fig. 1C). Moreover, the injection of GLB hardly changed these test grades in sham-operated rats (Fig. 1A-C), indicating that GLB did not affect the brain function and behaviors of rats under physiological condition. Next, the expression levels of TH (a key enzyme of dopamine),

Fig. 1. Establishment of rat Parkinson's disease (PD) model by injection of lipopolysaccharide (LPS). (A-C) Stepping test (A), Whisker test (B) and Cylinder test (C) were performed to measure PD degree of rats treated with PD or/and Glucocalyxin B (GLB). (D) Immunohistochemical staining of tyrosine hydroxylase (TH), glial fibrillary acidic protein (GFAP), CD11b and ionized calcium binding adaptor molecule (IBA)-1 was carried out to determine the cerebral injury of rats with different treatments. (E-H) The numbers of TH-positive (E), GFAP-positive (F), CD11b-positive (G) and IBA-1-positive cells (H) in immunohistochemical staining were quantitatively analyzed. (n=6, *P<0.05, **P<0.01, ***P<0.001, ns: no significance, the scale bar in the images represented 50 μ m).

GFAP (a marker of astrocytes), CD11b (a microglia marker) and IBA-1 (a marker of activated microglia) in rat brain tissues were detected by immunohistochemical staining. The results showed that the number of TH-positive cells was decreased by 70% after injection of LPS, and was increased 1.14-fold, 1.58-fold and 2.19-fold after GLB treatment (Fig. 1D-E). The amounts of GFAP-positive, CD11b-positive and IBA-1-positive cells were increased 2.22-fold, 3.32-fold and 6.83-fold post LPS injection, and were decreased by 38%, 44% and 60%, upon GLB administration (Fig. 1D, F-H). In addition, the magnified immunohistochemistry

Fig. 2. GLB alleviated inflammation, oxidative stress response and cell apoptosis induced by LPS. (A-C) The mRNA levels of tumor necrosis factor (TNF)-α (A), interleukin (IL)-1β (B) and IL-6 (C) in cerebral tissues of rats treated with PD or/and GLB were detected by real-time PCR. (D) The nitric oxide (NO) content in rat cerebral tissue samples. (E) The expression level of inducible nitric oxide synthase (iNOS) in rat cerebral tissue samples was detected by western blot. (F) The reactive oxygen species (ROS) content in each group. (G) The superoxide dismutase (SOD) activity in each group. (H) The myeloperoxidase (MPO) activity in each group. (I-K) The expression levels of cleaved caspase-3 (I), Bax (J) and Bcl-2 (K) in rat cerebral tissue sample were detected by western blot. (n=6, *P<0.05, **P<0.01, ***P<0.001, ns: significance).

images showed a deep staining of GFAP and CD11b in LPS-injected cerebral tissues (Fig. 1D). The above results suggested that LPS decreased dopamine production, and augmented the activation of astrocytes and microglia, and that these alterations were partly restored by GLB *in vivo*.

Fig. 3. LPS activated Toll-like receptor (TLR)/nuclear factor kappa B (NF- κ B) and nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase (HO)-1 pathway in vivo. (A-B) The expression levels of TLR2 (A) and TLR4 (B) in rat cerebral tissue samples were detected by western blot. (C) The cytoplasmic level of NF- κ B (p65) in rat cerebral tissue samples. (D) The level of NF- κ B (p65) in the nucleus fraction in cerebral tissue samples. (E) The nuclear level of Nrf2 in rat cerebral tissue samples. (F) The cellular level of HO-1 in rat cerebral tissue samples. (G-H) The expression levels of glutathione S-transferase (GSTA) 1 (G) and NAD(P)H:quinine oxidoreductase (NQO) 1 (H) in rat cerebral tissue samples were detected by western blot. (n=6, *P<0.05, **P<0.01, ***P<0.001, ns: significance).

GLB reduced LPS-induced inflammation, oxidative stress response and cell apoptosis in vivo

The levels or activities of several inflammatory cytokines and oxygen stress response related molecules were detected on day 14. As shown in figure 2, the levels of TNF- α , IL-1 β and IL-6 in cerebral tissues of the PD rats were increased 4.26-fold, 4.68-fold and 3.3-fold as compared to that of the sham-operated rats. The injection of GLB decreased their levels by 50%, 61% and 52%, respectively (Fig. 2A-C). In addition, the level of NO, a messenger molecule of the inflammatory response, was increased 2.84-fold post LPS injection. GLB treatment decreased its level by 17%, 36% and 47% in a dose-dependent manner (Fig. 2D). Like NO, LPS-stimulation-induced elevation of iNOS (a crucial synthase of NO) and ROS level was also decreased by GLB (Fig. 2E-F). Further, LPS injection decreased the activity of SOD (a pivotal anti-oxidative enzyme) by 51%, while increased that of MPO (a vital pro-oxidative enzyme) by 3.69 folds (Fig. 2G-H). GLB application enhanced SOD activity slightly, and suppressed MPO activity dramatically (Fig. 2G-H). In addition, LPS injection increased the levels of cleaved caspase-3 (the apoptosis executor) and Bax (a pro-apoptotic protein) 3.1-fold and 3.16-fold, whereas decreased that of Bcl-2 (an anti-apoptotic protein) by 69%. These LPS-induced alterations were attenuated by GLB (Fig. 2I-K).

Fig. 4. The condition optimization of LPS and GLB treatments in BV2 cells. (A) The viability of BV2 cells treated with LPS of indicated concentrations was tested by MTT assay. (B) The NO content in BV2 cells treated with LPS of indicated concentrations was detected. (C) The ROS content in BV2 cells. (D) The viability of BV2 cells treated with GLB of graded concentrations was measured by MTT assay. (The data in each group controlled to which in 0 ng/ml or 0 nM group. $n=3$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns: significance).

Fig. 5. GLB alleviated LPS-induced inflammation, oxidative stress response and apoptosis in BV2 cells. (A) The mRNA levels of TNF- α , IL-1 β and IL-6 in BV2 cells treated with LPS or/and GLB were detected by real-time PCR. (B) The NO content in culture supernatant of BV2 cells with different treatments was detected. (C) The ROS content in BV2 cells with different treatments was detected with dichlorodihydro-fluorescein diacetate (DCFH-DA) assay. (D-E) The activity of SOD (D) and MPO (E) in BV2 cells was detected. ($n=3$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns: no significance).

Fig. 6. GLB inhibited TLR/NF- κ B activation and activated Nrf2/HO-1 signaling in BV2 cells (A-B). The expression levels of TLR2 (A) and TLR4 (B) in BV2 cells with different treatments were detected by western blot. (C-D) The cytoplasmic (C) and nuclear levels of NF- κ B (p65) (D) in BV2 cells were detected. (E) The nuclear level of Nrf2 in BV2 cells was detected by western blot. (F) The cellular level of HO-1 in BV2 cells. (G-H) The expression levels of GSTA1 (G) and NQO1 (H) in BV2 cells. (n=3, *P<0.05, **P<0.01, ***P<0.001, ns: no significance).

LPS activated the TLR/ NF- κ B and Nrf2/ HO-1 pathway

We further investigated whether GLB affected the activation of TLR/NF- κ B and Nrf2/HO-1 pathways, two essential signaling transduction pathways involved in inflammation and anti-oxidative response. The western blot results showed that the expression levels of TLR2 and TLR4 were increased 3.41-fold and 4.81-fold post LPS injection (Fig. 3A-B). The nucleus accumulation of NF- κ B (p65) was augmented by LPS (Fig. 3C-D). Administration of GLB suppressed LPS-induced changes in TLR/NF- κ B pathway related molecules (Fig. 3A-D). Furthermore, the level of nuclear Nrf2 increased 2.36-fold after LPS injection, and it further increased 1.39-fold, 1.88-fold and 2.38-fold after GLB treatment (Fig. 3E). The cellular HO-1 expression level showed similar changes to nuclear Nrf2 (Fig. 3F). In addition, the expression levels of GSTA1 and NQO1, two anti-oxidant enzymes regulated by Nrf2/HO-1 pathway, increased 2.2-fold and 1.59-fold in response to LPS stimulation, and further increased 2.3-fold and 3-fold after GLB treatment (Fig. 3G and H).

GLB alleviated LPS-induced inflammation, oxidative stress response by inhibiting TLR/NF- κ B and activating Nrf2/HO-1 pathway in vitro

In vitro study using BV2 cells as the major model was further performed to confirm the *in vivo* neuroprotective effects of GLB. BV2 cells were treated with LPS from 0.1 to 1000 ng/ml, and their viability, NO content and ROS content were measured. As shown in Fig. 4, 100 ng/ml of LPS hardly influenced the cell viability, but significantly promoted the production of NO and ROS (Fig. 4A-C). This concentration was thus selected to stimulate inflammation and oxidative stress response in BV2 cells. Meanwhile, the viability of BV2 cells exposed to graded concentrations of GLB was also determined. The results showed that GLB of ≤ 1200 nM hardly impacted cell viability (Fig. 4D). Therefore, the GLB of 1200 nM was selected to incubate cells in the following study.

We found that LPS increased the levels of TNF- α , IL-1 β , IL-6, NO and ROS 3.68-fold, 3.36 folds, 5.06-fold, 3.4-fold and 1.93-fold, respectively (Fig. 5A-C). The activity of MPO was

increased 4.36-fold, while that of SOD was decreased by 73% upon LPS stimulation (Fig. 5D-E). GLB treatment reduced the levels of TNF- α by 39%, IL-1 β by 41%, IL-6 by 51%, NO content by 31%, ROS content by 39%, MPO activity by 37%, and enhanced SOD activity by 2.24-fold (Fig. 5A-E). Similar to the results from animal experiments, the TLR2 and TLR4 expression levels were increased 3.32-fold and 4.84-fold, the cytoplasmic NF- κ B (p65) level was decreased by 57% and nuclear NF- κ B (p65) level was increased 2.16-fold after LPS treatment (Fig. 6A-D). GLB treatment attenuated these changes (Fig. 6D). Thereafter, BV2 cells were treated with GLB for 24 h, 48 h and 72 h. We found that the nuclear level of Nrf2 and expression levels of HO-1, GSTA1 and NQO1 increased in a time-dependent manner in presence of LPS (Fig. 6E-H). These results demonstrated that GLB relieved LPS-induced inflammation and oxidative stress response by inhibiting TLR/NF- κ B and activating Nrf2/HO-1 pathway.

GLB decreased microglia-mediated cytotoxic effect in vitro

It is well-known that microglia act as macrophages in the center nervous system, and play a crucial role in inflammation and oxidative stress response of neurodegenerative diseases. The previous results demonstrated that GLB reduced LPS-induced inflammation and oxidative stress response in microglia, next we explored whether GLB mitigated the activated microglia-induced cytotoxic effects on neurons. PC12 cells were incubated in culture supernatant (CS) derived from BV2 cells treated with LPS, LPS+GLB or nothing. The MTT assay results showed that the 48-h treatment of LPS-stimulated CS decreased PC12 cell viability by 18%, and GLB restored the cell viability (Fig. 7A). The flow cytometry results revealed that GLB suppressed the apoptosis in PC12 cells induced by LPS-stimulated CS (Fig. 7B-C). Collectively, the LPS-activated BV2 cells displayed a cytotoxic effect on neuronal cells, which were suppressed by GLB.

Discussion

PD is a common neurodegenerative disease, characterized by the loss of dopaminergic neurons, inflammation and oxidative stress injury in the substantia nigra. In this study, we established a rat PD model via injection of LPS in the corpus striatum. The LPS-induced rats showed walking, climbing and sensory disturbances. The number of TH-positive cells was decreased in the brain tissue after LPS exposure, while that of GFAP-positive cells,

Fig. 7. GLB inhibited microglia-mediated cytotoxic effect on PC12 cells. (A) MTT assay was performed to detect the viability of PC12 cells cultured with BV2 cells culture supernatant (CS). (B) Cell apoptosis rate of PC12 cells was detected with flow cytometry. (C) Flow cytometry assay images of PC12 cells in each group. (n=3, *P<0.05, **P<0.01, ***P<0.001, ns: no significance).

CD11b-positive cells and IBA-1-positive cells were increased. Quiescent microglia monitor surrounding environment through filopodia expansion and retraction protrude. When activated, the microglia act as phagocytes, and the filopodia become shorter and thicker [14, 15]. The activated astrocytes show thick and bushy processes [16]. The morphological changes of microglia and astrocytes presented in our study also revealed the activation of microglia and astrocytes after LPS treatment. The increase of CD11b-positive cells in LPS-stimulated brain may be due to the migration of activated microglia.

In this study, we found that LPS injection caused serious cerebral inflammation, oxidative stress response and apoptosis, and induced activation of TLR/NF- κ B and Nrf/HO-1 signaling pathways. NF- κ B is a family of transcription factors that modulate the inflammatory response, innate and adaptive immunity [17–20]. The NF- κ B family contains five member protein monomers in mammals: RelA/p65, RelB, cRel, p50 and p52. These members form homodimers or heterodimers to bind to DNA. In resting cells, NF- κ B exists in the cytoplasm as a complex with the p65/p50/inhibitor (I κ B). When receiving external stimuli, the inhibitor of NF- κ B kinase (IKK) is activated to phosphorylate I κ B, and then promotes the ubiquitination and degradation of I κ B. Subsequently, p65/p50 heterodimers are released to translocate into the nucleus, and regulate the transcription of the downstream genes [21, 22]. Postmortem demonstrated that the proportion of NF- κ B (p65)-immunoreactive dopaminergic neurons in idiopathic PD patients was 70-fold higher than that in control subjects [23]. In 6-hydroxydopamine-induced rat PD model, NF- κ B is activated, and the inhibition of NF- κ B nuclear translocation could protect dopaminergic neurons [24]. It has been known that NF- κ B is regulated by TLRs [25]. TLRs are a class of pattern recognition receptors (PRRs) that interact with components of pathogens and mediate intracellular inflammatory response [26]. In brain, TLRs are primarily expressed in immune cells such as microglia and astrocyte [27]. TLR4 is increased in 1-methyl-4-phenylpyridinium ion (MPP⁺)-induced PD model *in vitro* [28], and TLR2 is upregulated in the brain tissues of PD patients [29]. TLRs can be activated by LPS and inflammatory cytokines [30, 31], and they can further activate NF- κ B via MyD88 and TNF receptor-associated factor 6-dependent pathway [32, 33]. In this study, we found that LPS increased the expression levels of TLR2 and TLR4, and accelerated NF- κ B nuclear translocation in rat brain and BV2 cells. These changes were restored by application of GLB. We speculate that GLB ameliorates LPS-induced PD via TLR/NF- κ B signaling. This speculation is more credible with the performance of reverse experiments, which will be investigated in our next study.

Similar to NF- κ B, Nrf2 is sequestered in cytoplasm by Kelch-like ECH-associated protein 1 (Keap 1) that promotes the ubiquitination and degradation of Nrf2. Under oxidative stress conditions, Nrf2 is released from the oxidized Keap 1 and translocates to the nucleus to activate the transcription of cytoprotective genes [34–36]. HO-1 is an enzyme controlled by Nrf2 and it catalyzes the rate-limiting step in heme degradation, leading to the generation of iron ions, biliverdin and carbonic oxide (CO) [37]. Nrf2 predominantly localizes in the cytoplasm of nigral dopaminergic neurons taken from normal control subjects, whereas expresses in the cell nucleus in PD patients' brains. This nuclear accumulation of Nrf2 suggests a compensation effect [38]. The serum level of HO-1 is higher in PD patients than that in the healthy controls [39]. The activated Nrf2 signaling exerts neuroprotective function in PD animal models and toxic cells [40]. Our data illustrated that GLB could further augment the LPS-induced activation of Nrf2 *in vivo* and *in vitro*, suggesting that the GLB may exert its neuroprotective effect at least by modulating Nrf2 pathway. In addition, we examined the cytotoxic effect of activated microglia on neuronal cells. The microglia cells play essential roles in the inflammatory response of nervous system. We found that the LPS-activated BV2 cells suppressed the viability and caused apoptosis in PC12 cells. This cytotoxic effect was restrained by GLB.

Together, we demonstrated that GLB alleviated LPS-induced inflammation and oxidative stress response *in vivo* and *in vitro*, and attenuated the dyskinesia and sensory disturbances of PD rats by inhibiting TLR/NF- κ B and activating Nrf2/HO-1 pathway. These findings may be of great significance for clinical therapy of PD.

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Disclosure Statement

The authors declare no Disclosure Statement.

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