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Ginkgolide K supports remyelination via induction of astrocytic IGF/PI3K/Nrf2 axis

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ABSTRACT

Although several therapies are approved, none promote re-myelination in multiple sclerosis (MS) patients, limiting their ability for sustained recovery. Thus, treatment development in MS has the opportunity to tackle the challenges, including experimental therapies targeting neuroprotection and re-myelination. Here, we provide a novel therapeutic target for Ginkgolide K (GK) that is now becoming a very critical natural compound to treat demyelination and neurodegeneration. GK improves behavioral dysfunction and demyelination in cuprizone (CPZ) model, followed by the migration and enrichment of astrocytes in the corpus callosum. Both in vitro and in vivo experiments demonstrates that GK triggers the upregulation of Nrf2/HO-1 in astrocytes and inhibition of p-NF-kB/p65, which is associated with the outcome of anti-inflammation and anti-oxidation by suppressing the production of IL-6 and TNFα as well as nitric oxide and iNOS in astrocytes. Further findings suggest that IGF/PI3K, but not BDNF, was induced in the corpus callosum after GK treatment, revealing that Nrf2 activation inhibited caspase-3 and apoptosis in O4+ oligodendrocytes possibly through IGF/PI3K signaling molecules. Since the current immunomodulatory therapies for MS have failed to prevent patients from entering the progressive phase of the disease, thus targeting Nrf2 in astrocytes with GK would be an ideal strategy for myelin protection and regeneration.

1. Introduction

Multiple sclerosis (MS), an organ-specific autoimmune disease, is an inflammatory disease of the central nervous system (CNS), characterized by the development of oligodendrocyte destruction, demyelinated plaques, and axonal degeneration. These pathological processes are paralleled by the recruitment of peripheral immune cells to the brain and the activation of microglia and astrocytes. The sequence of molecular events leading to oligodendrocyte loss and consequently demyelination is not fully understood, but different stressors are known which can induce oligodendrocyte degeneration including oxidative stress, mitochondrial dysfunction, nitric oxide, protein misfolding, or inflammatory cytokine exposure [1–4].

The current treatment of MS with immuno-modulating drugs mainly includes interferon beta (IFN-β), glatiramer acetate, natalizumab, and fingolimod [5,6]. Although there is no cure for MS patients, the primary treatment aim of these drugs and therapies is reducing the relapses and slowing the clinical progression of disability [7]. However, it is reminded that the disease may exacerbate after stopping disease-modifying drugs [8]. Based on recent progress in MS clinical and imaging technology, the demyelination and axonal degeneration are thought to be associated with the progressive loss of cognitive and motor functions [9]. Current disease-modifying therapy for the treatment of relapsing-remitting MS is immunomodulatory drugs, but this has shown limited efficacy for the treatment of progressive MS where neurodegenerative pathology is widespread [9–11]. Thus, treatment development in MS has the opportunity to tackle the challenges, including experimental therapies targeting neuroprotection and remyelination [12].

Ginkgo biloba, a slow-growing tree indigenous to Eastern Asia, is one of the oldest species of trees on the planet, with neuroprotective effects

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[13]. The neuroprotective effect has been demonstrated in several in vitro and in vivo models. In vitro, the extract of *Ginkgo biloba* protected cultured neurons against death induced by hypoxia, hydrogen peroxide, glutamate, β-amyloid, nitric oxide, and MPTP [13]). In vivo, the extract of *Ginkgo biloba* also protected neuron damage and death through different mechanisms in middle cerebral artery occlusion (MCAO), focal cerebral ischemia, hypoxia, and amyotrophic lateral sclerosis (ALS) mice or rat models [13]. Ginkgolide K (GK), a novel compound isolated from *Ginkgo biloba*, is extracted from the leaves of the *Ginkgo biloba*, attracting much attention because of its neuroprotective capacity in cerebral ischemia. In the MCAO model, the pretreatment with GK diminished the infarction volume and brain water content, and improved neurological deficit score [14]. After oxygen-glucose deprivation (OGD), the pretreatment of GK promoted astrocyte proliferation and migration via inducing protective autophagy, indicating that GK might be a potential agent for cerebral ischemia/reperfusion injury [15]. GK also exhibited neuroprotection against PC12 and A53T cells through the inhibition of ROS generation and the degradation of the unfolded protein [16,17]. Also, GK protected the heart by activating the inositol-requiring enzyme 1alpha/X box-binding protein-1 pathway [18]. In our recent research, the intervention of GK inhibited the infiltration of inflammatory cells and demyelination in the spinal cord in experimental autoimmune encephalomyelitis (EAE). Our results suggested that GK is a very promising small molecule compound for the treatment of MS [19]. However, the role of GK in the remyelination of the myelin sheath has not been reported.

Astrocytes are the most abundant glial cells in white matter and gray matter of the CNS and have a crucial role in neurophysiology. Astrocytes regulate neurotransmitters, participate in synaptogenesis, mediate the immune response, express extracellular matrix molecules and promote cell migration, differentiation, maturation of the CNS [20,21]. A previous study confirmed that astrocytes directly affect the proliferation and survival of the oligodendrocyte line [22], revealing that astrocytes are strongly associated with remyelination. Astrocytes are involved in regulating the balance between Schwann cells and oligodendrocyte remyelination, with oligodendrocyte remyelination only observed in areas where astrocytes are present. A recent study showed that testosterone promoted oligodendrocyte remyelination via astrocyte recruitment [23]. Indeed, increasing evidence shows that astrocytes directly or indirectly affect remyelination by acting on oligodendrocytes.

CPZ-induced demyelination, unlike experimental autoimmune encephalomyelitis (EAE), is independent of autoimmune attacks, and is also often used to mimic the pathology of human MS [24,25], ultimately resulting in selective loss of oligodendrocytes, extensive areas of demyelination and reactive gliosis in the corpus callosum, superior cerebellar peduncles and cerebral cortex. Remarkably, some aspects of the histological pattern induced by CPZ are similar to those found in MS. Therefore, CPZ-induced demyelination is an excellent experimental approach to study the demyelination and remyelination and is a suitable pharmacological model for developing some promising drugs of neuroprotection and remyelination. In this study, we try to explore the potential of astrocytes as a target of GK treatment for myelin protection and regeneration in CPZ-induced demyelination model.

2. Materials and methods

2.1. Animals and drug

Male C57BL/6 mice (10–12 weeks old) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). This study was approved by the Council for Laboratory and Ethics Committee of Shanxi University of Traditional Chinese medicine, Taiyuan, China. All animal protocol was performed according to the International Council for Laboratory Animal Science guidelines. All mice were maintained and housed under pathogen-free conditions in a reversed 12:12 h light/dark cycle for one week before experimental manipulation. GK was extracted and separated from ginkgo leaf, which purity was > 98% by recrystallization and high-performance liquid chromatography (HPLC) separation.

2.2. Cuprizone-induced demyelination model

To be induced demyelination, mice were fed with 0.2% (w/w) cuprizone (Sigma-Aldrich, USA) in chow diet ad libitum for a total of 6 weeks. After 4 weeks of cuprizone feeding, mice were randomly divided into three groups (n = 8 per group) as follows: (i) normal group fed a normal diet (normal); (ii) cuprizone group, fed cuprizone and injected with NS as control (CPZ) and (iii) GK-treated group, which were intraperitoneally injected with GK (20 mg/kg), starting from four weeks until sixth weeks for consecutive 14 days (CPZ + GK). For the control of cuprizone-containing diet intake, both body weights and food consumption were closely monitored twice daily.

2.3. Behavioral tests

The elevated plus maze (EPM) is a measurement method for analyzing anxiety reaction. The design includes a circular platform, two relatively closed quadrants, and two relatively open quadrants, eliminating the time required for Plus in the maze of the elevated cross and allowing continuous measurement. The mice moved freely for 5 min at the start of each experiment. The distance in the open area was recorded to measure anxiety.

The forced swimming test (FST) was conducted by previously report [26]. The mice were placed individually to swim in a plastic cylinder (height: 30 cm, diameter: 10 cm) filled with 20 cm of 25 ± 1 °C water. The mean swimming distance was analyzed by blind individuals via SNART V3.0 software (RWD Life Science Co. USA).

2.4. Primary astrocyte culture and treatment

Primary cortical astrocytes were prepared from newborn mice at postnatal 24 h as previously described with minor modifications (Ding et al., 2009). Briefly, the small cubes (< 1 mm^3) of meninges-free cortices were digested with 0.25% trypsin at 37 °C for 15 min. The suspension of tissue fragments was passed through a 70 um cell strainer and allowed pre-adherence for 1 h to remove contamination from fibroblast. The supernatant containing unattached cells was transferred to 75-cm^2 flasks (Corning, USA) and cultured in an incubator with 5% CO_2 at 37 °C. The culture medium was changed every 3–4 days. When the cultures reached 80–90% confluence, cells were sub-cultured for another 7 days before being used. The purity of astrocytes was determined by staining glial fibrillary acidic protein (GFAP, astrocytic marker). > 95% of cells showed GFAP immunoreactive in the cultures.

For oxygen-glucose deprivation (OGD), cells were exposed to 95% nitrogen and 5% CO_2 at 37 °C (Forma Anaerobic System, Thermo, USA). Oxygen tension was kept at 1–2% during the duration of the experiment. The media used in these OGD experiments consisted of sugar-free culture medium containing normal salts, 10% fetal bovine serum, 100 U/ml penicillin-streptomycin. Control groups were grown under normal culture conditions (5% CO2in atmosphere). Astrocytes were exposed to OGD for 3 h. After OGD, cells were re-oxygenated for 24 h. Before re-oxygenation, astrocytes were exposed to GK (30 μg/ml, dissolved in DMSO, the ratio of GK: medium = 1:1000). The same volume of DMSO was added as a control.

2.5. Tissue preparation

Half of the mice in each group were deeply anesthetized with 10% chloral hydrate and perfused intracardially with saline, followed by 4% paraformaldehyde in PBS (0.01 M, pH = 7.4). Brains were removed and cryo-protected by immersion in 15%, 25%, and 30% sucrose solutions

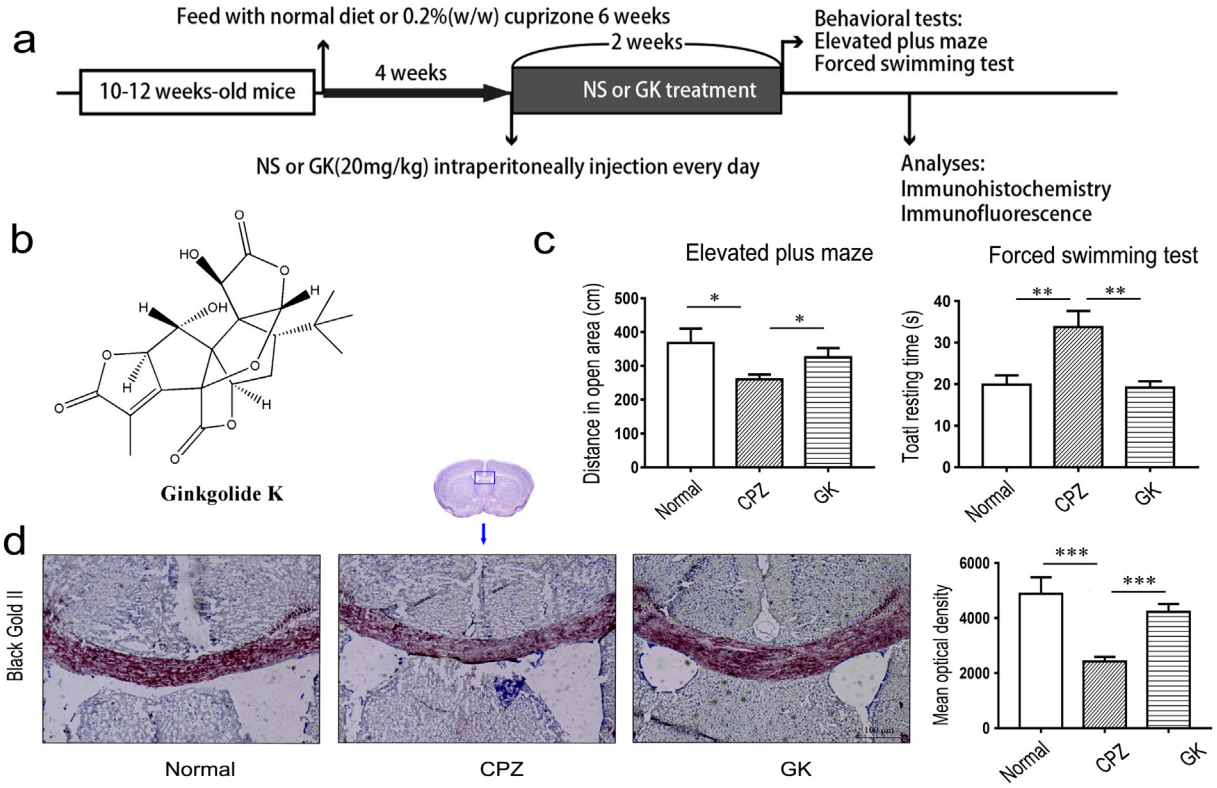

Fig. 1. GK improved behavioral abnormality and protected demyelination. Mice were intraperitoneally injected with GK, starting from fourth weeks until sixth weeks for consecutive 14 days, without CPZ withdrawal. (a) the number of entering close arms in EPM and mean swimming distance of FST ($n = 8$ for both group). (b) the histopathology of myelin sheath by Black Gold II staining ($n = 4$ for both group). The results were quantified by Image-Pro Plus 6.0 software. All data represents the means ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. CPZ group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

one day for each concentration. Brain coronal sections (10 μm) were cut using a cryostat microtome (Leica CM1850, USA) and stored at 4 °C for immunofluorescence staining. Another half of the mice were deeply anesthetized with 10% chloral hydrate and perfused intracardially with saline, without 4% paraformaldehyde. Brains were removed and quickly stored at −80 °C for ELISA and western tests.

2.6. Myelin staining and analysis

The black gold staining is a widely used approach for visualizing myelin morphology with excellent resolution of individual myelin fibers [27]. Pre-warmed Black Gold II solution (0.3% in NS) was added onto 4% PFA-fixed sections (slides) and incubated at 60 °C for 25 min. The slides were rinsed with Milli-Q water twice. Pre-warmed 1% sodium thiosulfate (1% in Milli-Q water) was added to the slides and incubated at 60 °C for 3 min. After rinsing with Milli-Q water thrice, the slides were stained with cresyl violet at room temperature (RT) for 3 min. Slides were rinsed again and dehydrated using a series of graduated alcohols and finally in a xylene substitute for 2 min and coverslipped with mounting media. The area of demyelination in the corpus callosum was manually measured using Image-Pro Plus 6.0 software and represented as percent demyelination.

2.7. Preparation of splenic mononuclear cells

At the end of the experiment, mice were sacrificed and spleens were removed under aseptic conditions. The weight and volume of spleens were observed and recorded. Mononuclear cells (MNCs) from spleens were prepared by grinding the organ through a 70 μm nylon mesh in medium, and erythrocytes were osmotically lysed. Cells were then washed 3 times and re-suspended in the complete medium and adjusted to 5 × 106/ml. Cells were incubated in the presence or absence of MOG (10 μg/ml) for 48 h.

2.8. Immunofluorescent

At the end of the experiment, mice were perfused with saline, and 4% buffered paraformaldehyde and brains were removed. Brains were sliced (10 μm), and immunofluorescent were detected. Briefly, the slides of brain (10 μm) and cells (astrocytes) were blocked with 1% BSA (Sigma, USA) at room temperature (RT) for 30 min for blocking non-specific binding, followed by the incubation with anti-GFAP (Abcam, USA), anti-Nrf2 (Abcam, USA), anti-HO-1 (Abcam, USA), anti-Ki67 (Abcam, USA), anti-p-NF-kB/p65 (Cell Signaling Technology, USA), anti-IGF (Abcam, USA), anti-BDNF (Abcam, USA), anti-O4 (R & D, system, USA), anti-PI3K (Abcam, USA) and anti-caspase3 (Cell Signaling Technology) at 4 °C overnight and with corresponding secondary antibodies at RT for 2 h. Meanwhile, additional sections were treated similarly, but the primary antibodies were omitted as a negative control. Results were visualized under the fluorescent microscope (BX60, Olympus Imaging America Inc., USA) by Image-Pro Plus 6.0 software in a blinded fashion. Quantification was performed on three sections per mouse.

2.9. Western blot analysis

At the end of the experiment, mice were perfused with saline, and 4% buffered paraformaldehyde and brains were removed. Brains were homogenized with a glass homogenizer using RIPA Lysis Buffer (Thermo Fisher Scientific Inc., USA) supplemented with the protease inhibitor and phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc., USA). The homogenates were centrifuged at 12,000 ×g for 30 min

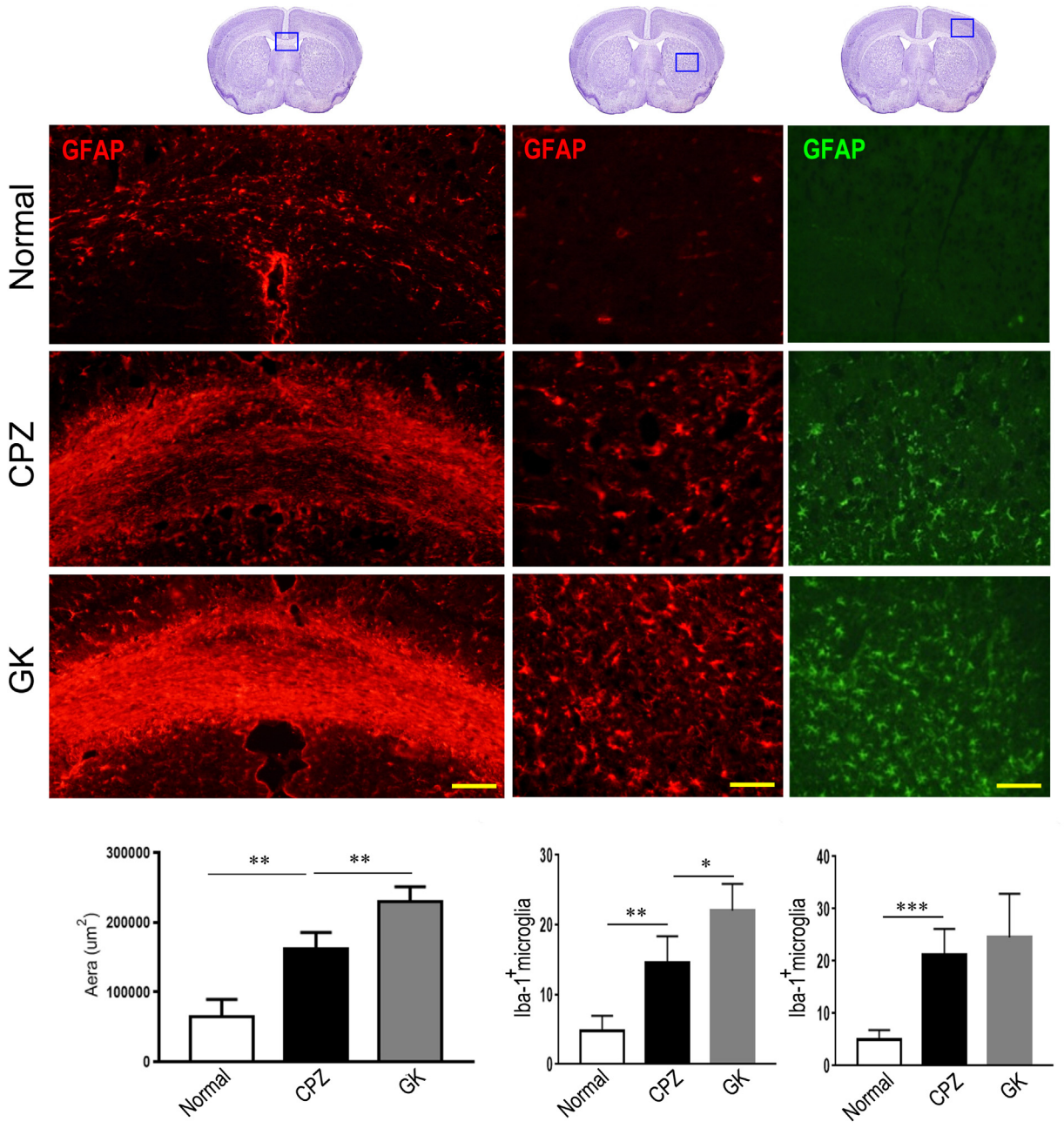

Fig. 2. The migration and enrichment of GFAP+ astrocytes in the myelin regions. At the end of the experiment, immunohistochemistry staining of GFAP was performed in the corpus callosum, striatum, and cortex of the brain. Representative microphotograph images were obtained from 4 mouse brains/each group with similar results. The results of immunohistochemistry were quantified by Image-Pro Plus 6.0 software. Data represents the means ± SEM (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, vs. CPZ group).

at 4 °C, and the supernatants were collected. Protein concentration was measured by BCA kit (Thermo Fisher Scientific Inc., USA). Equal amounts of protein (30 μg) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Protran 0.2 NC; GE Healthcare Life Sciences, USA). After blocking with 5% milk at RT for 1 h, the membranes were incubated at 4 °C overnight with primary antibodies against Nrf2 (Abcam, USA), HO-1 (Abcam, USA), p-NF-kB/p65 (Cell Signaling Technology, USA), GAPDH (Abcam, USA). In the following day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, USA)) at RT for 1 h. Bands were visualized by chemiluminescence (ECL) kit (EMD Millipore, Billerica, MA, USA) under a ChemiDoc XRS+ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH was used as the optical density of internal reference. Analysis and quantification were performed by Image Lab 5.2 software (Bio-Rad, USA).

2.10. Cytokine ELISA assay

At the end of the experiment, splenic MNCs (5×10^6/ml) were incubated for 48 h at 37 °C. Supernatants were harvested and measured for cytokine concentrations of IL-1β, IL-6 and TNFα (R&D System, USA) by a sandwich ELISA kits following the manufacturer's instructions. All data were performed in triplicate in 3 independent experiments.

2.11. TUNEL assay

The presence of apoptotic cells in brain sections was determined

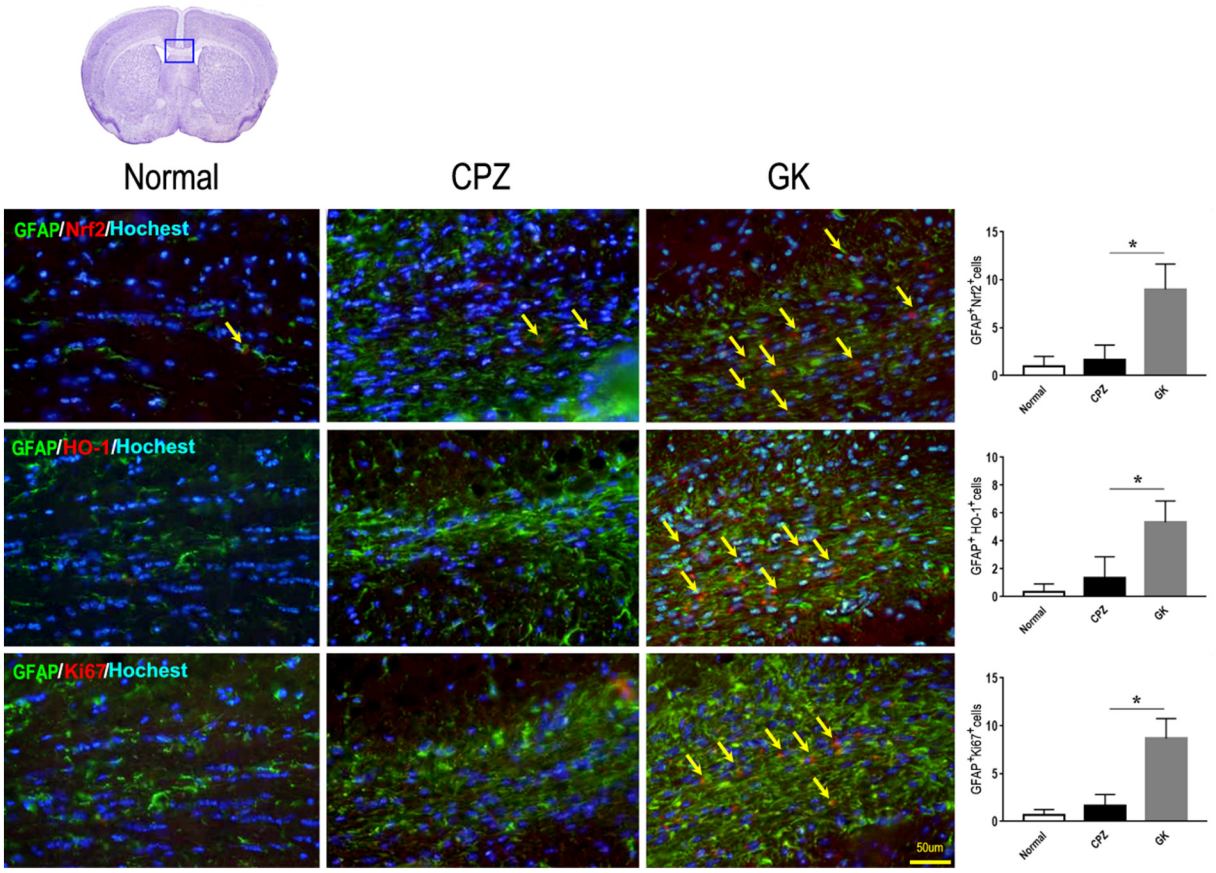

Fig. 3. The expression of Nrf2 and HO-1 in the corpus callosum. At the end of the experiment, immunohistochemistry staining of Nrf2, HO-1, and ki67 was performed in the corpus callosum of the brain. Representative microphotograph images were obtained from 4 mouse brains/each group with similar results. The results of immunohistochemistry were quantified by Image-Pro Plus 6.0 software. Data represent the means ± SEM (*p < 0.05, vs. CPZ group).

using a terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL) assay kit (Beyotime Biotechnology, Shanghai, China). Briefly, after fixing in 4% paraformaldehyde/PBS at RT for 60 min, brain sections were incubated with a cocktail containing terminal deoxynucleotidyl transferase enzyme and fluorescence-labeled nucleotides and examined under fluorescence microscopy (BX60, Olympus Imaging America Inc., USA) in a blinded fashion. Analysis and quantification were performed on three sections per mouse by Image-Pro Plus software.

2.12. Statistical analysis

Data were expressed as the mean ± SEM (Standard Error of Mean). For parametric data, comparison of different groups was performed by one-way analysis of variance, followed by Tukey's post hoc test for multiple comparisons. The Statistic analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. GK improves behavioral dysfunction and demyelination

To define the effect of GK on myelin protection/regeneration in CPZ-induced demyelinating model, GK was intraperitoneally injected from fourth weeks until sixth weeks during CPZ feeding for consecutive 14 days. The behavioral O maze test showed that the distance in the open area was decreased, and the mean swimming distance of FST was prolonged in CPZ-fed mice compared to normal mice (Fig. 1a, $p < 0.05$ and $p < 0.01$ respectively), which was extended and reduced by GK treatment (Fig. 1a, $p < 0.05$ and $p < 0.01$ respectively).

Myelin content of brain in the corpus callosum was detected using standard Black Gold II staining. CPZ feeding induced profound demyelination in the corpus callosum as evidenced by a marked decrease in the intensity of Black Gold II (Fig. 1b, $p < 0.001$). GK treatment enhanced the intensity of Black Gold II staining in the corpus callosum (Fig. 1b, $p < 0.001$). The results indicate that GK protects myelin and promotes regeneration in CPZ-induced demyelinating model.

3.2. GK promotes the enrichment of astrocytes in the corpus callosum

After 6 weeks of CPZ feeding, the number and enrichment of GFAP + astrocytes increased markedly in the corpus callosum of CPZ mice, as compared with normal mice (Fig. 2, $p < 0.01$). In the striatum and cortex of normal mice, GFAP+ astrocytes were rarely or hardly detectable, but CPZ feeding increased GFAP+ astrocytes (Fig. 2, $p < 0.01$ and $p < 0.001$ respectively). GK treatment further enhanced the migration and accumulation of GFAP+ astrocytes in the corpus callosum (Fig. 2, $P < 0.01$) and increased the number of GFAP+ astrocytes in the striatum (Fig. 2, $p < 0.05$). There was no difference in astrocytes in the cortex between CPZ and CPZ + GK (Fig. 2). Based on the role of protection or regeneration of GK in the myelin sheath, GK-derived enrichment of GFAP+ astrocytes in the corpus callosum should be beneficial to resist myelin damage mediated by CPZ feeding.

3.3. GK upregulates the expression of Nrf2/HO-1 in astrocytes

It remains to be determined the association between myelin protection/regeneration and astrocyte enrichment in the corpus callosum. Further evidence for the importance of astrocytic Nrf2 expression

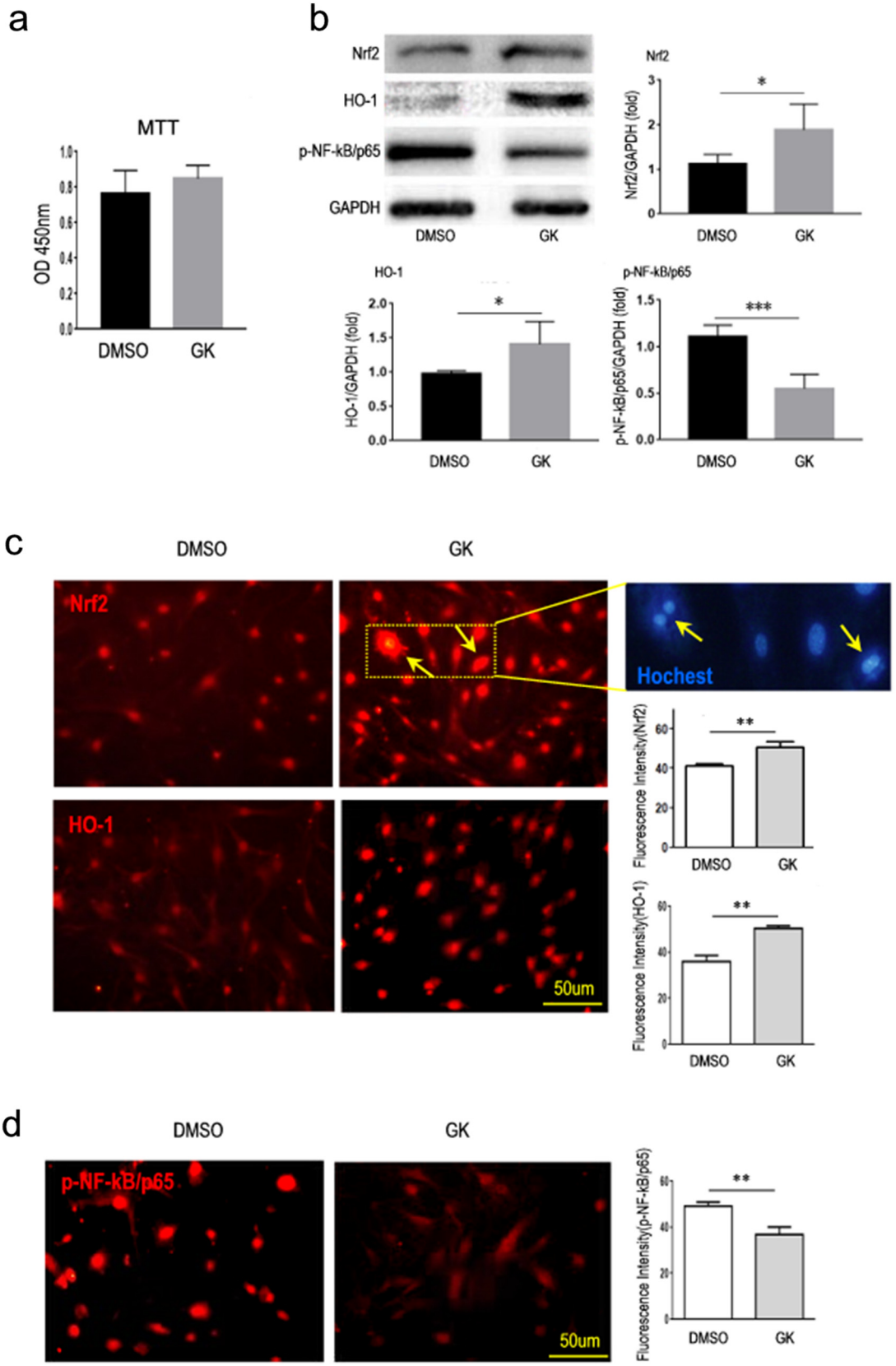

Fig. 4. The expression of Nrf2, HO-1, and p-NF-kB/p65 in primary astrocytes. a) Immunofluorescent staining of GFAP in primary astrocytes. b) cell viability in primary astrocyte treated GK by MTT assay. c) the expression of Nrf2, HO-1, and p-NF-kB/p65 protein in the extract of primary astrocytes by Western blot. d) the expression of Nrf2, HO-1, and p-NF-kB/p65 protein in primary astrocytes by Immunofluorescent. A graph inserted on the left side is the number of primary astrocytes, and a graph inserted on the right side is Hochest staining. Representative microphotograph images were obtained from 2 independent experiments with similar results. The results were quantified by Image Lab 5.2 software and Image-Pro Plus 6.0 software. Data represent the means ± SEM (*p < 0.05, vs. CPZ group).

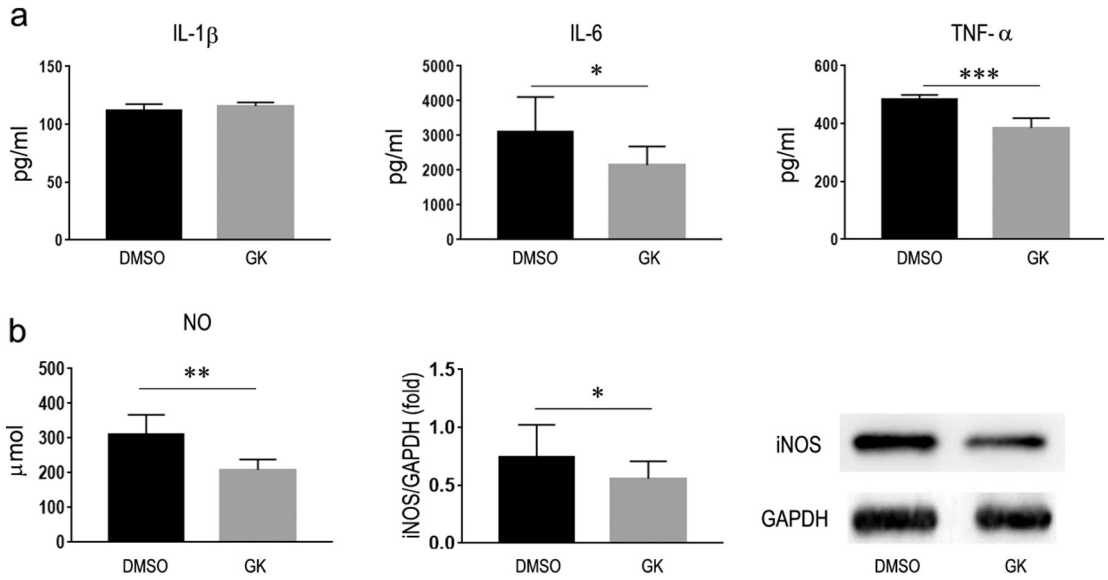

Fig. 5. The expression of inflammatory cytokines and oxidative species in primary astrocytes. a) the concentration of IL-1b, IL-6, and TNFα in the supernatant of primary astrocytes by ELISA. b) the levels of NO and the expression of iNOS in the supernatant and extract of primary astrocytes by Griess assay or Western blot. The results were obtained from 2 independent experiments with similar results and quantified by Image Lab 5.2 software. Data represents the means ± SEM (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, vs. CPZ group).

comes from the resistance to MPTP toxicity in PD model and protection against the myelin sheath in the cuprizone model. By double immunohistochemistry staining, the expression of Nrf2 and HO-1 on GFAP + astrocytes was upregulated in GK-treated mice compared to CPZ mice (Fig. 3, $p < 0.05$ respectively). Also, GFAP + Ki67 + astrocytes in the corpus callosum in GK-treated mice increased (Fig. 3, $p < 0.05$). Although GFAP + astrocytes increased in the corpus callosum, no difference was observed between normal and CPZ mice in the expression of Nrf2, HO-1, and Ki67 on GFAP + astrocytes (Fig. 3).

In vitro experiment of cultured astrocytes (Fig. 4a), we further confirm the expression of Nrf2 and HO-1 in astrocytes and explore the effects and molecules in the upstream and downstream of Nrf2 and HO-1 pathway. First, there was no difference in cell viability when experimental dose GK was used (Fig. 4b). The results show that GK treatment upregulated the expression of Nrf2 and HO-1 in astrocytes (Fig. 4c, $p < 0.05$, respectively). Simultaneously, the expression of p-NF-kB/p65 was decreased in astrocytes treated with GK (Fig. 4c; $p < 0.001$). Consistent with the results by Western blot, Immunofluorescent staining confirmed that GK treatment induced the expression of Nrf2 and HO-1 (Fig. 4d, $p < 0.01$ respectively) and inhibited the expression of p-NF-kB/p65 in astrocytes (Fig. 4d, $p < 0.01$). Careful observation found that that Nrf2 + astrocytes were proliferating. Thus the number of astrocytes increased after GK treatment (Fig. 4d, insert with Hochest staining, $p < 0.05$).

Nrf2/HO-1 expression and p-NF-kB/p65 activation have been shown to be crucial for anti-oxidation and anti-inflammation in astrocytes. Therefore, we examined the effect of GK on inflammatory cytokines and antioxidant capacity in astrocytes. The treatment of GK led to inhibition of inflammatory cytokine IL-6 and TNFα in astrocytes (Fig. 5a, $p < 0.05$ and $p < 0.001$ respectively), while IL-1β had no significant difference (Fig. 5a). The treatment of GK inhibited the production of astrocyte-derived NO (Fig. 5b, $p < 0.001$) and the expression of iNOS protein in astrocytes (Fig. 5b, $p < 0.05$). These results indicate that GK exhibits anti-inflammatory and antioxidant capacity in astrocytes by activating Nrf2/HO-1 and inhibiting NF-kB pathways.

To further investigate whether Nrf2/HO-1 activation can prevent cell apoptosis in CPZ-fed mice, we detected the caspases-3 expression and TUNEL staining in O4 + oligodendrocytes. As shown in Fig. 6a, immunolabeling of caspase-3 and TUNEL cells was increased in CPZ-fed mice ($p < 0.05$ and $p < 0.01$ respectively), which was inhibited by GK treatment ($p < 0.05$ and $p < 0.01$ respectively).

3.4. GK induces IGF/PI3K signaling pathways in astrocytes

Phosphatidylinositol 3-kinase (PI3K) pathway has been proved to be one of the potential upstream signaling regulators of Nrf2 [28]. Next, we detected the expression of PI3K in the corpus callosum, where GFAP + astrocytes enriched. CPZ feeding resulted in the enrichment of GFAP + astrocytes in the corpus callosum but did not cause the upregulation of PI3K. However, as expected, the treatment of GK induced the expression of PI3K compared with CPZ mice (Fig. 6a).

The PI3K signaling pathway is regulated by insulin-like growth factor (IGF) and brain-derived neurotrophic factor (BDNF) membrane receptors [29,30]. The results show that CPZ feeding resulted in the upregulation of IGF, but did not affect BDNF expression in the corpus callosum, cortex, and striatum (Fig. 7). Treatment of GK activated astrocytes to further upregulate the expression of IGF in the corpus callosum, cortex, and striatum, especially in the corpus callosum (Fig. 7a). However, Treatment of GK did not change the BDNF expression as compared with both normal and CPZ-fed mice (Fig. 7).

4. Discussion

In our recent results, GK inhibited the infiltration of inflammatory cells and the expression of the inflammation-related molecules TLR4, NF-κB, and COX2 in the spinal cord, indicating that GK intervention can inhibit the inflammatory microenvironment of the spinal cord in EAE mice. Also, GK modified the imbalance between regulatory T cells (Treg) and T helper 17 cells (Th17). Additionally, GK shifted macrophage/microglia polarization from M1 to M2 cell type [19]. But, the role of GK in the remyelination of the myelin sheath has not been reported.

The role of protection or regeneration of the myelin sheath of GK on CPZ model has been observed in this study. From our findings, GK exhibits antioxidant and anti-inflammatory efficacies by activating IGF/PI3K/Nrf2 and inhibiting NF-kB signaling pathways on astrocytes, which may contribute to the beneficial effect of GK in the treatment of MS or myelin injury-related diseases.

Under normal conditions, astrocytes modulate the synaptic function and provide nutrients and support that are needed for neuronal

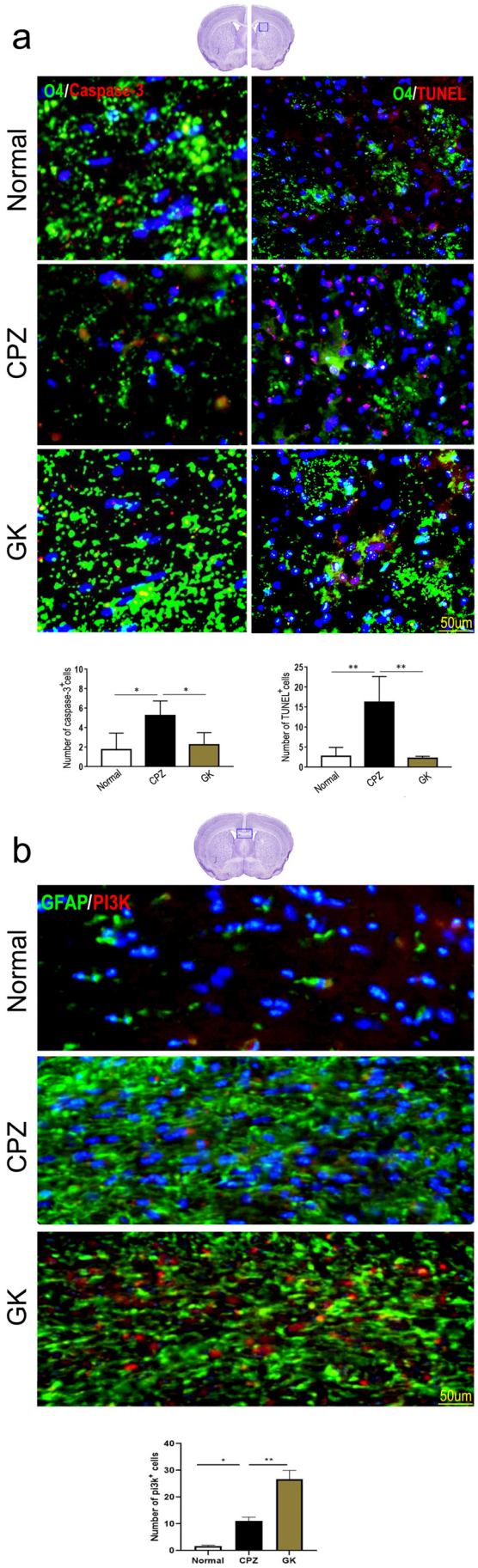

Fig. 6. The expression of PI3K, caspase-3, and TUNEL in the corpus callosum and striatum. At the end of the experiment, Immunofluorescent staining of PI3K (a), caspase-3, and TUNEL (b) were performed in the corpus callosum and striatum of the brain. Representative microphotograph images were obtained from 4 mouse brains/each group with similar results. The results of immunohistochemistry were quantified by Image-Pro Plus 6.0 software. Data represents the means ± SEM (*p < 0.05, **p < 0.01, vs. CPZ group).

function and survival. In the CPZ-induced model, microglia can be activated directly by apoptotic oligodendrocytes and damaged myelin, or indirectly by cytokines or chemokines [31,32]. Astrocytes in MS are considered to possess a dual role since beneficial as well as detrimental effects of astrocytes on de- and remyelination have been observed [33,34]. Thus, astrocytes may be critical players in complex interactions with oligodendrocytes and microglia supporting remyelination at least in the CPZ-induced model [35].

In this study, we found that CPZ feeding caused demyelination, accompanied by astrocyte migration and enrichment to the corpus callosum. However, GK treatment further promoted astrocyte enrichment, but demyelination improved significantly. The results seem to reveal that the enrichment of astrocytes in the corpus callosum may be related to myelin protection and regeneration. In recent years, endogenous cellular anti-oxidative responses focus on nuclear factor Nrf2. Nrf2 activation can inhibit or diminish cellular damage in different tissues and organs [36]. The activation of Nrf2 has been shown to be a critical step in the cellular response against several common diseases, including neuroinflammation, aging, diabetes, cardiovascular disease and neurodegenerative diseases, and cancer [37–39]. However, the endogenous Nrf2 response to disease is insufficient to prevent neuronal damage and myelin lesion. Thus, targeting the Nrf2/HO-1 pathway with natural GK could be a potential strategy for the prevention or treatment of demyelination. Here, the ability of GK to activate astrocytic Nrf2 underpins myelin protection and generation in CPZ mice, which is not yet targeted by other MS therapies. In EAE, some pharmacological inducers of Nrf2 showed protective effects [40,41], suggesting Nrf2 activation may be involved in its therapeutic action.

Regarding elucidating which cells are most important for Nrf2 activation, astrocytes may be the predominant cell type for activation of Nrf2 under pathogenetic conditions [42]. Overexpression of Nrf2 in astrocytes delayed motor pathology and alpha-synuclein aggregation in the alpha-synuclein mutant (A53T) mouse model [43]. Transplantation of astrocytes overexpressing Nrf2 can protect against 6-OHDA-induced damage in both in vitro and in vivo [44]. In addition, GFAP-Nrf2 protected against MPTP toxicity in GFAP-Nrf2(+)/Nrf2−/− mice, suggesting that astrocytic Nrf2 is a promising target for therapeutics aimed at reducing or preventing neuronal death in PD [45]. Besides, it was also reported that astrocyte-specific activation of Nrf2 ameliorated the demyelinating lesions in the CPZ-induced model, and prevented oligodendrocyte loss and axonal damage [46], demonstrating that astrocyte-specific Nrf2 activation is sufficient to prevent pathology in the cuprizone model. In this study, GK promoted the migration and enrichment of astrocytes in the corpus callosum, which also indicates that GK improved the demyelination possible by activating astrocytic Nrf2.

Nrf2 regulates oxidative stress and also represses inflammatory response [47]. In human clinical studies, Nrf2 inducer Tecfidera (dimethyl fumarate) has been approved for the treatment of MS [48], in part based on its anti-inflammatory function. It is now clear that robust NF-κB and Nrf2 activity is essential for maintaining coordinated cellular responses to resolve the inflammatory status of the cell/tissue. The imbalance between Nrf2 and NF-κB pathways is associated with a significant number of diseases ranging from neurodegeneration and autoimmune disorders [49,50]. Compared with the mechanism of Nrf2 antioxidation, the mechanism underlying Nrf2-mediated anti-inflammation has not been clarified in detail. The previous opinion considers that Nrf2 function was limited to oxidative stress control and that

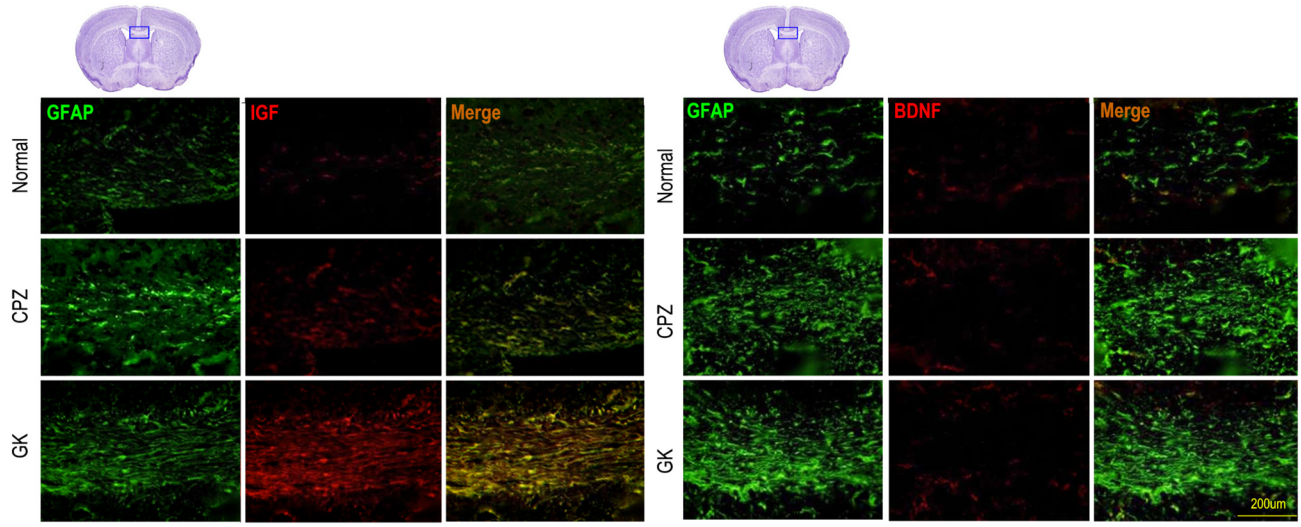

Fig. 7. The expression of IGF and BDNF in the corpus callosum, cortex, and striatum. At the end of the experiment, Immunofluorescent staining of GFAP, IGF, and BDNF were performed in the corpus callosum, cortex, and striatum of the brain. Representative microphotograph images were obtained from 4 mouse brains/each group with similar results.

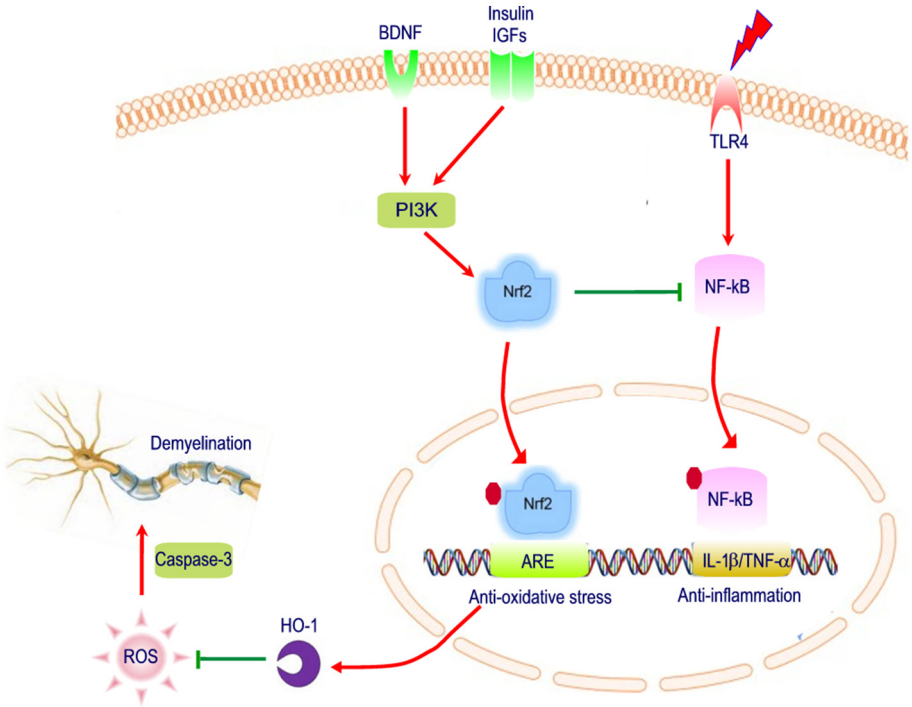

Fig. 8. Pattern diagram of GK action for myelin protection or regeneration in CPZ-induced demyelinating model. GK promotes the enrichment of astrocytes in the corpus callosum, where astrocyte-derived Nrf2 and HO-1 are induced, and p-NF-kB/p65 is inhibited. Therefore, GK inhibits the microenvironment of inflammation and oxidative stress in the brain, which can cause caspase-3-mediated apoptosis. Further observation shows that IGF, but not BDNF, might be involved in PI3K/Nrf2/HO-1 signaling pathway.

anti-inflammation was merely a consequence of ROS elimination [47].

In contrast to this dogma, recent findings support the notion that the Nrf2 regulated negatively the target genes that encode inflammatory cytokines [47]. This notion coincides with our finding that GK inhibits the NF-κB-mediated transcription of proinflammatory cytokine genes possibly by activating the Nrf2/HO-1 pathway. Direct evidence for the involvement of Nrf2 in NF-κB-mediated transcription of proinflammatory cytokine demonstrates that the $Nrf2^{-/-}$ macrophages do not exhibit this anti-inflammatory capacity [51]. Nrf2 protects against oxidative damage triggered by injury and neuroinflammation. In our results, caspase-3 activation, which has been shown to be upregulated in TUNEL-positive cells, revealed that GK could inhibit caspase-3 activation and oligodendrocyte apoptosis possibly through inducing Nrf2/HO-1 pathway.

It was reported that Nrf2 activation was regulated through two signaling pathway: 1) IGF/PI3K/Nrf2 pathway and 2) BDNF/PI3K/Nrf2 pathway. A study indicates that IGF-1 protected SH-SY5Y cells against $A\beta_{25\text{-}35}$-induced cell injury via the PI3K/Akt-Nrf2 signaling pathway [52]. Another study demonstrates that BDNF activated Nrf2 in a $TrkB.T1\text{-}p75^{NTR}$-dependent manner [53]. In this study, we try to confirm the upstream signaling molecules for Nrf2 activation mediated with GK in astrocytes. Our results indicate that IGF-1, but not BDNF, should be involved in GK-mediated Nrf2 activation in astrocytes. Previous studies found that IGF-1 deficient mice showed a decreased expression of Nrf2 and increased oxidative stress and apoptosis [54]. IGF-1 also protected neurons against Aβ-induced cell death through activation of the Nrf2/ARE system [52]. Moreover, a specific PI3K inhibitor, LY294002, was found to completely abolish the effect of IGF-1 on Nrf2/HO-1 expression, suggesting that IGF-1 protects against Aβ25-35-induced neurotoxicity via activation of PI3K/Nrf2 signaling

pathway in SH-SY5Y cells [52].

In conclusion, we provide a novel therapeutic target for GK that is becoming a very critical natural compound to treat demyelination and neurodegeneration. GK promotes the migration and enrichment of astrocytes in the corpus callosum. Both in vitro and in vivo experiments demonstrates that GK triggers the upregulation of Nrf2/HO-1 in astrocytes, which is associated with the outcome of anti-inflammation and anti-oxidation. Further findings suggest that IGF/PI3K, but not BDNF, was induced after GK treatment, revealing that Nrf2 activation may be regulated with IGF/PI3K signaling (Fig. 8). Since the current immunomodulatory therapies for MS have failed to prevent patients from entering the progressive phase of the disease, thus targeting Nrf2 in astrocytes with GK would be an ideal strategy for myelin protection and regeneration.

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Authors' contributions

The study was designed and supervised by BGX, WX, and WBY. QYL performed all aspects of the cell culture and animal experiments and analyzed these data. The Behavioral tests were performed by QM and RXS. The manuscript was written by WBY and BGX with input from all the authors, who approved the final version of the manuscript.

Ethics approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was approved by the Council for Laboratory and Ethics Committee of Shanxi University of Traditional Chinese medicine, Taiyuan, China. All animal protocol was performed according to the International Council for Laboratory Animal Science guidelines.

Declaration of competing interest

None of the authors has any potential financial and non-financial conflict of interest related to this manuscript.

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