Original Research

Sinomenine relieves oxygen and glucose deprivation-induced microglial activation via inhibition of the SP1/miRNA-183-5p/IκB-α signaling pathway

Feng Qin[1], Yang Zhao[2], Wei Shang[3], Zhi-Ming Zhao[3], Xin Qian[3], Bei-Bei Zhang[3], Hui Cai[3*]

[1] The First Clinical Medical College, Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210023, P.R. China
[2] Department of Neurology, The Third Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing , Jiangsu 210006, P.R. China
[3] Department of Integrated Traditional Chinese and Western Medicine, Jinling Hospital, Nanjing, Jiangsu 210002, P.R. China

Abstract: Studies have shown that the inflammatory activation of miroglia (MG) and nuclear factor kappa B (NF-κB) play a dominant role in inflammatory response. Previous studies have shown that sinomenine, an anti-inflammatory agent extracted from *Sinomenium acutum*, can directly protect neurons against cerebral ischemia injury. However, there are no reports on its effect on ischemia/reperfusion-induced inflammatory activation of MG. In the present study, an *in vitro* ischemia/reperfusion model was developed with mouse BV-2 microglia cells, a model of oxygen-glucose deprivation/reperfusion (OGD/R), and the inhibitory effect of sinomenine pretreatment on inflammatory activation was confirmed through measurement of inflammatory indicators. Mechanistically, sinomenine suppressed OGD/R-induced inflammatory activation through the SP1/miRNA-183-5p/IκB-α pathway. In conclusion, this study shows that sinomenine effectively inhibits OGD/R-induced inflammatory activation in MG by suppressing the activation of transcription specificity protein 1 (SP 1). This finding is of significance for the clinical use of sinomenine in treating cerebral ischemia/reperfusion injury.

Key words: Sinomenine; Microglia, IκB-α; Antiinflammation, OGD/R; miRNA-183-5p.

Introduction

During ischemic brain injury, which is characterized by high morbidity, high mortality, and high disability, the rupture or blockage of blood vessels causes hypoxic injury to cerebral cells and tissues (1-3). Although the current clinical treatment for ischemic brain injury is based on timely recovery of blood supply protects neurons from injury, the ischemia/reperfusion process inevitably aggravates the pathological injury in tissues affected by ischemia. It is therefore important to control inflammation for post-operation recovery of patients (4-6).

Inflammatory activation of MG is a marker of diseases involving brain nerve injury (7, 8). MG exist extensively in the central nervous system, accounting for 20 % of the total number of brain gliocytes, a number comparable to that of neurons. MG secrete a variety of factors under different conditions, thus playing a dual role in neural injury and recovery. Malfunction of MG is always involved in inflammatory nerve injury. It is of significance to study the association between MG and nerve injury, and to improve MG function, for improving therapy and recovery of injured nerves (9). In the normal brain, MG, with multiple branches, are at a resting status, but become activated during ischemic brain injury. It is generally understood that microglia are derived from marrow mononuclear cells or hematopoietic stem cells, which explains their immune cell-like characteristics. In fact, MG do exhibit the biological functions of mononuclear macrophages (10). During brain ischemia, MG are rapidly activated and migrate to the injured area, where they secrete multiple cytokines. Some of these cytokines are protective factors for nerve cells, reducing the number and degree of necrotic neurons, while others are pro-inflammatory factors which induce a cascade of responses and aggravate the damage to brain tissues and cells. Chronic inflammation induced by excessive activation of microglia usually leads to damage to nerve cells or brain tissues, and eventually to degenerative diseases. Therefore, the development of anti-inflammatory drugs targeted at MG is always a hotspot in research on ischemia-induced brain injury (11). Acute inflammation has been demonstrated to play a critical role in secondary brain injury induced by cerebral ischemia/reperfusion. Cell injury mediated by inflammatory cells is close to delayed neuronal death with respect to. Drugs that block every link to inflammation can be used to treat delayed damage caused by cerebral ischemia (12, 13). Studies have shown that the inflammatory activation of MG and transcription factor NF-κB play a dominant role in the inflammatory response. NF-κB is found almost in all cell types. It has been long considered to be involved in various disease pathogenesis. P65, RelB, cRel, P50, and P52 are five homologous subunits of NF-κB, which always exist as homodimers and heterodimers. In the classic NF-κB pathway, various pathogens, including reactive oxygen species, endotoxin, bacterial tumor necrosis factor alpha, etc, are considered as common stimuli for incurable inflammation (14). Heterodimers of p65 and p50 are the most abundant in cells. The IκB family includes several

proteins, one of the most important regulators of mammalian NF-κB is IκB-α. In unstimulated cells, NF-κB is sequestered in an inactive form in the cytoplasm bound to inhibitory IκB-α protein (15). Under the action of stimulatory signals, IκB-α undergoes phosphorylation, ubiquitination, and finally degradation in the cytoplasm. At the same time, NF-κB also undergoes phosphorylation, then translocates to the nucleus and activates the transcription of its target genes. Phosphorylation of NF-κB plays an important role in the dissociation of NF-κB from inhibitory proteins, nuclear translocation of NF-κB, and binding to DNA (16). Activation of NF-κB pathway is a major trigger of inflammation, in which NF-κB regulates inflammation-induced cytokines, such as TNF-α and IL-1β, through binding sites in their promoters. Thus regulation of NF-κB has been considered as a potential therapeutic for inflammatory diseases (17, 18). In NF-κB pathway, IκB-α can be activated by IκB kinase complex through ubiquitinated and degraded process, and then promoted translocation of the p65/p50 complex into the nucleus and activated the expression of downstream genes, thus the expression of IκBα was the classic negative feedback of NF-κB activation (19). SP1, a well studied transcription factor, is critical for many biological processes, including cellular growth and differentiation (20), there are few studies related to SP1 about inflammation. Interestingly, in this study, we found that SP1 was activated by OGD/R, promotes the expression of miRNA-183-5p, which, binding to the 3'-untranslated region (3'-UTR) of IκB-α mRNA, inhibits its expression, and thus activates NF-κB, resulting in inflammation. Sinomenine suppresses OGD/R induced inflammatory activation in MG through the SP1/miRNA-183-5p/IκB-α pathway.

Sinomenine, extracted from the stem and root of *Sinomenium acutum*, is soluble in ethanol, acetone, chloroform and diluted base, and slightly soluble in water, diethyl ether and benzene. According to the theory of traditional Chinese medicine, sinomenine can help to ease pain, relieve cough, lower blood pressure, and resist inflammation; it is used to treat rheumatic arthritis and neuralgia. For cerebral ischemia, sinomenine is able to inhibit nerve cell apoptosis and inflammation induced by ischemia, while promoting cell proliferation. However, no studies have been reported on its role in the inflammatory activation of MG induced by cerebral ischemia/reperfusion (21, 22). The primary objective of this study was to investigate the inhibitory action of sinomenine on OGD/R-induced inflammatory activation in BV-2 cells (a murine microglial cell line), and the regulating pathway involved. This was with a view to laying a theoretical basis for applying sinomenine in resisting inflammatory activation of MG, and in treatment of cerebral injury caused by ischemia/reperfusion.

Materials and Methods

Cell culture

BV-2 cells purchased from the Cell Bank of the Chinese Academy of Sciences (CBCAS, Shanghai, China), were maintained in DMEM (Invitrogen, CA, USA) supplemented with 10 % fetal calf serum (FCS) (Invitrogen, CA, USA) at 37 ºC in an atmosphere of 5 % CO_2. The cells were passaged at the coverage of 70 % using 0.25 % Typsin (Invitrogen, CA, USA). Cells subjected to OGD/R treatment were first cultured under normal conditions for 24 h, followed by 2 h culturing under 5 % CO_2 and 95 % N_2 in the serum and glucose free medium, and then culturing for 24 h under normal conditions. At the same time, 293 cells obtained from ATCC (MD, USA) were maintained in DMEM supplemented with 10 % FCS at 37 ºC under 5 % CO_2.

Sinomenine source and toxicity test

Analytically pure sinomenine (Shenzhen Sendi Biotech Company, ShenZhen, China) was dissolved in dimethyl sulphoxide (DMSO, Sigma, CA, USA) to prepare a 100 mM stock solution, which was diluted in culture medium to final concentrations of 25, 50, and 100 μM used for cell treatment. The BV-2 cells in logarithmic phase were seeded in 96-well plates at a density of 1×10^5 cells/well, and cultured at 37 ºC in an atmosphere of 5 % CO_2 overnight. Three groups of cells were used: control group, vehicle (DMSO) group, and sinomenine treatment group. The latter group was divided into 5 sub-groups on the basis of levels of sinomenine administered (5, 25, 50, 100, and 200 μM). After overnight culture, the medium was replaced and sinomenine was added to final concentrations of 5, 25, 50, 100, and 200 μM. The cells were cultured under normal conditions for 24 h, following by CCK-8 assay for cell proliferation. Each well was treated with 10 μl CCK-8 solution (DOJINDO, Japan), and the plates were incubated at 37 ºC under 5 % CO_2 for 4 h. Thereafter, the absorbance of each well was read at 450 nm in Multiskan GO (Thermo, MI, USA). The maximum concentration of sinomenium that produced no significant cytotoxicity was calculated and used for subsequent experiments.

OGD/R model of BV-2 cells, and assay of anti-inflammatory activity of sinomenine

BV-2 cells in logarithmic phase were seeded in 96-well plates and cultured at 37 ºC under 5 % CO_2 overnight. The cells were divided into five groups: normal group, DMSO + OGD/R group, sinomenine (25 μM) + OGD/R group, sinomenine (50 μM) + OGD/R group, and sinomenine (100 μM) + OGD/R group. After culturing overnight, the cells were pretreated with 25, 50, or 100 μM sinomenine for 2 h, and subjected to OGD/R. Following 24 h re-oxygenation, the supernatants were used for TNF-α, IL-1β and IL-6 assays. Levels of phosphorylated p65 and p50 and IκB-α were determined by western blotting.

Determination of the effect of sinomenine on mRNA and protein expressions of IκB-α

BV-2 cells in logarithmic phase were seeded in 6-well plates at a density of 3×10^5 cells/well,

and cultured overnight at 37 ºC under 5 % CO_2. The cells were divided into four groups: control group, 25 μM sinomenine group, 50 μM sinomenine group, and 100 μM sinomenine group. The cells were incubated for 24 h under normal conditions, and total RNA and protein were extracted for determination of IκB-α mRNA and protein expressions using RT-PCR and western blotting, respectively.

Determination of the binding site of mmu-miRNA-183-5p on IκB-α 3'UTR by luciferase reporter assay

TargetScan was used to predict the theoretical target (seed region) of mmu-miRNA-183-5p in the mRNA sequence of IκB-α. In this process, 293 cells were transfected with miRNA-183-5p-mimics, inhibitor, or NC, as well as pGL-wt-IκB-α and pGL-mt-IκB-α using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. The cells were harvested 48 h after transient transfection, and luciferase assays were performed. The relative luciferase activities (ratios of firefly and renilla luciferase activities) of lysates were measured using a dual luciferase reporter assay system (Promega, WI, USA).

Mouse IκB-α's 3'-UTR (130 bp) was amplified from cDNA obtained through the reverse transcription of the total RNA of mouse brain tissues, using the primers 5'-GCTCTAGATGACACCTAAGAAATTTACTG-TGAAAAAAGC-3' and 5'-GCTCTAGATAGTGTG-GTAATTTTTAATATTAAAAGTAA-3'. The amplification procedures were 32 cycles of denaturation at 95 °C for 10 sec, annealing at 58 °C for 30 sec, and extension at 72 °C for 30 sec. The product was then digested with XbaI and inserted into the pGL3-promotor vector (Promega, WI, USA). The seed region was subjected to point mutation from 5'-AGTGCCA-3' to 5'-TGACG-CA-3'. The resulting vectors were named pGL3-wt-IκB-α and pGL3-mt-IκB-α. Chemically synthesized miRNA-183-5p-mimics (5'-GUCACUUAAGAUG-GUCACGGC Att-3'), inhibitor (5'-UGCCGUGAC-CAUCUUAAGUGACtt-3') and NC (5'-CCAAUG-GUUAAGCGUGUCAGCAtt-3') were obtained from Invitrogen (Invitrogen, Shanghai, China).

Determination of TFBS of SP1 in miRNA-183-5p promoter by luciferase assay

A 2500-bp sequence upstream of the transcriptional start site of miRNA-183-5p precursor sequence was obtained from NCBI. The promoter sequence was predicted by Promoter 2.0, and the binding sites on the promoter were predicted using http://jaspar.genereg.net. The results indicated that SP1 had a theoretical binding site on the miRNA-183-5p promoter. SP1's transcription factor binding site (TFBS) 5'-GGCGGG-3' was found. The CDS of mouse SP1 was amplified using the primers 5'-GCTCTAGAGCCACCATGAGCGACCAAGAT-CACTCC-3' and 5'-CGGGATCCTTAGAAACCAT-TGC CACTGA-3', which contain an XbaI restriction site (5'-TCTAGA-3'), a Kozak sequence (5'-GCCACC-3') and a BamHI restriction site (5'-GGATCC-3'), respectively. The RNA isolated from BV-2 cells were reverse-transcribed to cDNA, and the PCR product was digested and cloned into the pcDH1-CMV expression vector (Systermbio, CA, USA); the recombinant vector was named pcDH1-SP1. About 500 bp of the miRNA-183-5p precursor promoter sequence was cloned from mouse genomic DNA and inserted into a luciferase expression vector, pGL3-Enkancer, to construct a wild-type TFBS reporter gene expression vector, pGL3-wp-183. The TFBS was mutated from 5'-GGCGGG-3' and 5'-GCGGGG-3', to construct a mutant TFBS reporter gene expression vector pGL3-wp-183. To eliminate interference from endogenous miRNA-183-5p promoter on SP1, luciferase reporter assays were also carried out. Cells (293) were transfected with pcDH1-SP1 and pGL3-wp-183 or pGL3-mp-183, and harvested 48 h later for luciferase activity assay. The transfection experiment was carried out in 24-well plates, following the instructions for Lipofectamine 2000 (Invitrogen, CA, USA). Each well was transfected with PGL-TK (100 ng) (Promega, WI, USA) as an internal reference for the luciferase assay.

Determination of the specificity of the SP1/miRNA-183-5p/IκB-α pathway

Cells were divided into seven group, control, model (OGD/R), Sinomenine + model, NC transfection + Sinomenine + model, Lv-SP1 + Sinomenine + model, Lv-miR-183-5p + Sinomenine + model, and Lv-shR-NA- IκB-α + Sinomenine + model. Twenty-four hours after transfection, BV-2 cells were seeded into 6-well plates and cultured under normal conditions overnight, and then incubated in medium containing 100 μM of sinomenine 2h under normal conditions prior to OGR/R. The cells were collected 24 h after re-oxygenation for measuring relevant genes and proteins.

Measurement of TNF-α, IL-1β and IL-6 by ELISA

Total protein was extracted and quantified using the bicinchoninic acid (BCA) method, and intracellular TNF-α, IL-1β and IL-6 were measured using appropriate mouse ELISA kits (Invitrogen, CA, USA) according to the manufacturer's instructions. The levels of TNF-α, IL-1β and IL-6 were calculated according to the A450.

Determination of mature miRNA-183-5p

Total RNA (2 μg) was reverse-transcribed into cDNA with the specific primers for mouse U6 snRNA: Oligo dT, miRNA-183-5p: 5'-GTCGTATCCAGTGCG-TGTCGTGGAGTCGGCAA TTGCACTGGATACGA-CAGTGA-3'. Then, 2 μl of the product was used as the template for fluorogenic quantitative PCR to measure miRNA-183-5p. The results were analyzed by $2^{-\Delta\Delta Ct}$ with U6 as the internal reference. The PCR primers were: U6-forward: 5'-TAGAGCTTGTGGACAAATGT-3'; U6-reverse: 5'-TTCTGTGACATCTTCCAACACC-3'; miRNA-183-5p forward: 5'-TATGGCACTGGTA-GAATTCACT-3'; miRNA-183-5p reverse: 5'-GTCG-TATCCAGTGCGTGTCGTG-3'.

The PCR reaction system contained 10 μl of SYBR Premix Ex Tap (Takara, Dalian, Shanghai), forward and reverse primers (20 μM, 0.2 μl each); cDNA (2 μl,) and dH$_2$O added to bring the reaction volume to 20 μl. The reaction process entailed 40 cycles of denaturation at 95°C for 10 sec, annealing at 58 °C for 10 sec, and elongation at 72 °C for 10 sec.

Determination of IκB-α mRNA contents

Total RNA was reverse- transcribed into cDNA with Oligo dT18, and $2^{-\Delta\Delta Ct}$ method was used to analyzed the results of PCR with beta actin as the internal reference. The PCR primers were: β actin-forward: 5'-AACCCTAAGGCCAACCGTGAAAAG-3', β actin-reverse: 5'-TGGCGTGAGGGGAGAGCATAGC-3'; IκB-α-forward: 5'-CCGTCCTGCAGGCCACCAA-CTACA-3', IκB-α-reverse: 5'-CAAGAGCGAAA-CCAGGTCAGGATT-3'. The PCR reaction system

contained SYBR Premix Ex Tap (10 μl), forward and reverse primers (20 μM, 0.2 μl each), cDNA (2 μl), and dH$_2$O added to bring u the volume to 20 μl. The reaction consisted of 40 cycles of denaturation at 95°C for 10 sec, annealing at 56°C for 10 sec, and elongation at 72 °C for 10 sec.

Assay of protein expression and phosphorylation by western blotting

Total protein was extracted from the cells using M-PER mammalian protein extraction reagent (Pierce, IL, USA). Equal amounts of protein (20 μg per lane) estimated with BCA protein assay kit (Pierce), were loaded onto 11% SDS-PAGE gels and transferred onto nitrocellulose membranes. The blots were probed with a monoclonal antibody against mouse anti-p65 (1:300 dilution, sc-8008), anti-P-p65 (1:500 dilution, sc-136548), anti-p50 (1:600 dilution, sc-166588), anti-P-p50 (1:1500 dilution, sc-271908), anti-IκB-α (1:600 dilution,sc-1643) and anti β actin (1:2000 dilution, sc-81178) (Santa Cruz, CA, USA). This was followed by application of the secondary HRP-conjugated anti-mouse antibody ((1:300 dilution, sc-516102, Santa Cruz, CA, USA). After washing, the bands were detected by chemiluminescence and imaged with X-ray films. β-Actin was used as an endogenous reference for normalization.

Statistical analysis

All data are expressed as mean ± SD, and analyzed by one-way ANOVA. Least Significant Difference (LSD) was used for multiple comparisons between any two means. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS 13.0 software.

Results

Toxicity and anti-inflammatory activity of sinomenine in BV-2 cell model of OGD/R

Results from CCK-8 assay showed that 200 μM sinomenine significantly inhibited cell proliferation ($p < 0.01$), but there was no statistically significant difference between 100 μM sinomenine and control ($p > 0.05$) (Fig. 1A). BV-2 cells pretreated with sinomenine for 2h were subjected to OGD/R. Following 24 h reoxygenation, the inflammatory factors were assayed to confirm that the model was established, and to determine the effect of sinomenine on the model. ELISA assay showed that TNF-α, IL-1β and IL-6 levels in the supernatant from the OGD/R model were significantly increased ($p < 0.01$), when compared with the control group, indicating inflammatory activation of BV-2 cells. Pretreatment with 100 μM sinomenine significantly and dose-dependently decreased the levels of TNF-α, IL-1β and IL-6 in the culture supernatant, relative to the model group ($p < 0.01$, Fig. 1B). It is generally understood the inflammatory activation is ascribed to inactivated regulation of NF-κB. Therefore, phosphorylated p65 and p50 and IκB-α were assayed for. The data showed that OGD/R treatment stimulated the phosphorylation of p65 and p50, and reduced IκB-α ($p < 0.01$). These effects were reversed in a significant and dose-dependent manner by sinomenine pretreatment, with reduction in phosphorylated p65 and p50 levels, and enhancement in
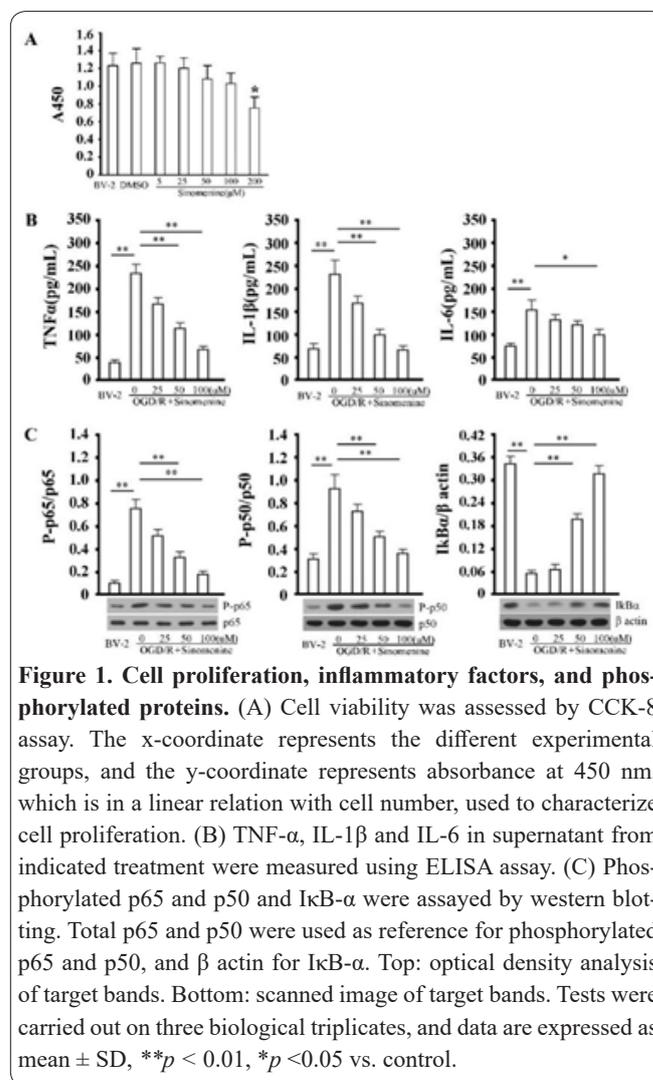

Figure 1. Cell proliferation, inflammatory factors, and phosphorylated proteins. (A) Cell viability was assessed by CCK-8 assay. The x-coordinate represents the different experimental groups, and the y-coordinate represents absorbance at 450 nm, which is in a linear relation with cell number, used to characterize cell proliferation. (B) TNF-α, IL-1β and IL-6 in supernatant from indicated treatment were measured using ELISA assay. (C) Phosphorylated p65 and p50 and IκB-α were assayed by western blotting. Total p65 and p50 were used as reference for phosphorylated p65 and p50, and β actin for IκB-α. Top: optical density analysis of target bands. Bottom: scanned image of target bands. Tests were carried out on three biological triplicates, and data are expressed as mean ± SD, **$p < 0.01$, *$p < 0.05$ vs. control.

IκB-α level ($p < 0.01$, Fig. 1C).

Effect of sinomenine on mRNA and protein expressions of IκB-α

Sinomenine suppressed inflammatory activation in BV-2 cells by inhibiting NF-κB activation, and the process was directly associated with increased IκB-α expression (Fig. 1). In order to determine whether the increased IκB-α expression was regulated at the transcriptional or post-transcription level, cells were exposed to sinomenine at different concentrations for 24 h, and IκB-α mRNA and protein were assayed. The results showed that there was no apparent difference in IκB-α mRNA level between cells treated with sinomenine and untreated cells ($p > 0.05$), but IκB-α protein was higher in the group treated with sinomenine ($p < 0.01$, Figures 2A and 2B). These results suggest that IκB-α may mediate the inhibition of NF-κB by sinomenine, and the increase in IκB-α by sinomenine was through a post-transcription regulation. To elucidate how sinomenine regulates the expression of IκB-α, attention was focused on post-transcription by miRNA. Thus, miRNAs with the potential to bind to the 3' UTR of mouse IκB-α were searched for using TargetScan Human 7.1, and changes in the miRNAs were determined through RT-PCR. It was found that miRNA-183-5p was negatively correlated with IκB-α protein levels, that is, sinomenine pretreatment increased miRNA-183-5p ($p < 0.01$, Fig. 2C).
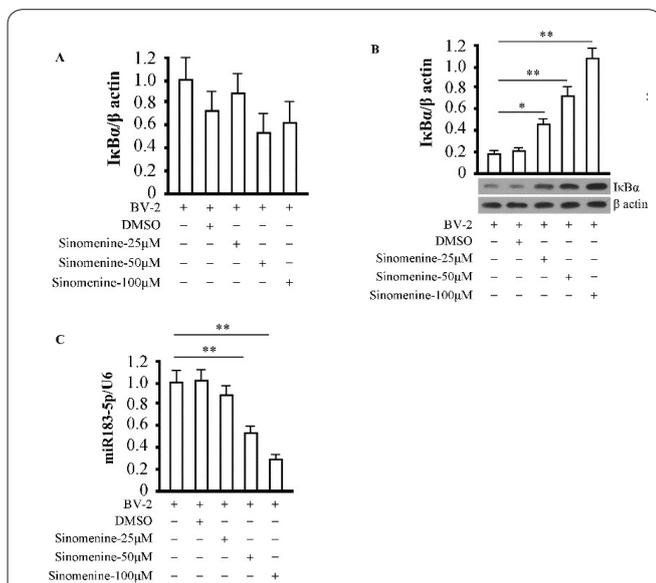
Figure 2. mRNA and protein of IκB-α and relative expression of miRNA-183-5p. (A) IκB-α mRNA was assayed in BV-2 cells treated with sinomenine at indicated concentrations for 24 h, with β-actin as reference. The relative content of target was analyzed using the $2^{-\Delta\Delta Ct}$ method. (B) IκB-α protein was determined in BV-2 cells treated with sinomenine at indicated concentrations for 48 h. Bottom: image of scanned target bands; top: optical density analysis for IκB-α protein. (C) miRNA-185-5p relative content was studied using RT-PCR, with β-actin as reference. The relative content of miRNA-183-5p was analyzed using the $2^{-\Delta\Delta Ct}$ method. Tests were carried out on three biological triplicates, and data are expressed as mean ± SD, **$p < 0.01$, *$p < 0.05$ vs. BV-2 or DMSO group.

Figure 3. Determination of interaction between miRNA-183-5p and SP1. (A) Predicted binding site of miRNA-183-5p in the 3'UTR of SP1; (B) 293 cells were transfected with pGL3-WT or pGL3-MT in the presence or absence of miRNA-183-5p-mimic or miRNA-183-5p-inhibitor. The histogram indicates relative firefly luciferase activities in the different transfected groups. Error bars represent standard deviation and were obtained from at least three independent experiments. * $p < 0.05$; ** $p < 0.01$.

Binding site of mmu-miRNA-183-5p on IκB-α 3'UTR

Based on the existence of a 7-base mmu-miRNA-183-5p seed sequence in the 3'-UTR of IκB-α mRNA as predicted by bioinformatics (Fig. 3A), a luciferase reporter vector was constructed and used to verify the binding site. The reporter vectors containing the wild-type IκB-α 3'UTR or the 3'UTR with a mutated miRNA-183-5p target site expressed luciferase at a high level (Fig. 3B), and miRNA-183-5p-mimic significantly inhibited the activity of luciferase in cells transfected with the reporter vectors carrying the wild type 3'UTR, from 26.83 ± 6.43 to 1.12 ± 7.54 ($p < 0.05$). In addition, miRNA-183-5p-inhibitor increased luciferase activity from 26.83 ± 6.43 to 46.32 ± 5.82 ($p < 0.05$). For the reporter vector containing the mutated miRNA-183-5p target site, neither miRNA-183-5p mimics nor miRNA-183-5p-inhibitor had observable effects on luciferase activity ($p > 0.05$). The co-transfection of miRNA-183-5p-NC had no obvious effects on the luciferase expressed by both vectors ($p > 0.05$). These results indicate that there is a target site of mmu-miRNA-183-5p in IκB-α mRNA through which mmu-miRNA-183-5p down-regulates IκB-α.

Genetic intervention through a lentiviral approach

For BV-2 cells, conventional transfection methods can hardly achieve a high gene delivery efficiency: lipofection transfection can hardly apply to G0 cells, and eletro-transfection damages gliocytes. Thus, a lentiviral system was selected to solve overcome this problem so as to achieve efficient and stable intervention. As mentioned earlier, miR-183-5p promoter sequence was get,
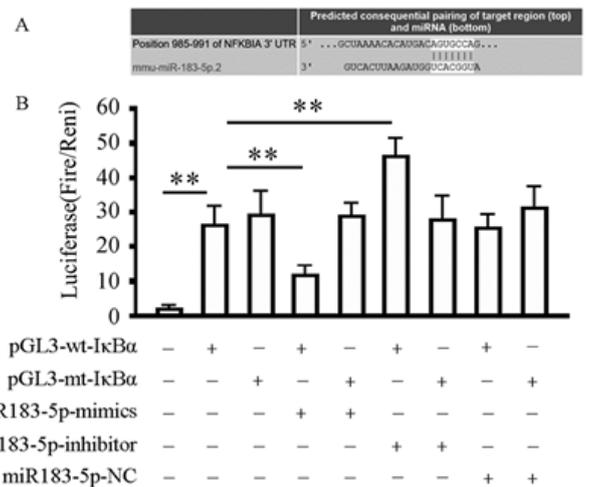
software "JASPAR" was used to predict the transcription factors that may have binding sites on the miRNA-183-5p promoter. The results indicated that SP1 had a theoretical binding site on the miRNA183-5p promoter. To knock down and overexpress target genes in validating the specificity of the pathway, the recombinant viruses Lv-SP1, Lv-miRNA-183-5p and Lv-shRNA-IκB-α were constructed. Green fluorescent protein was observed in most cells 72 h after BV-2 cells were infected with Lv-SP1, Lv-miRNA-183-5p or Lv-shRNA-IκB-α, and the gene delivery efficiency was evaluated by calculating the percentage of the cells expressing GFP relative to the total cells, and found to be close to 90 % in the three groups (Figures 4A, 4B and 4C). Moreover, Lv-SP1 infection but not Lv-miRNA-183-5p and Lv-shRNA-IκB-α significantly increased SP1 mRNA ($p < 0.01$, Fig. 4A right). In addition, Lv-SP1 and Lv-miRNA-183-5p significantly increased Lv-miRNA-183-5p ($p < 0.01$), and Lv-shRNA-IκB-α did not significantly change Lv-miRNA-183-5p ($p > 0.05$, Fig. 4B, right). Lv-SP1, Lv-miRNA-183-5p and Lv-shRNA-IκB-α all decreased SP1 mRNA levels ($p < 0.01$, Fig. 4C, right): there were no apparent differences between them.

TFBS of SP1 in miRNA-183-5p promoter

The promoter sequence of miRNA-183-5p gene was cloned from mouse genomic DNA. To verify whether the promoter was active, we cloned this promoter sequence upstream of the plasmid carrying the fluorescent marker green fluorescent protein (GFP), followed by transfection of 293 cells. 48 hours after transfection, a large amount of GFP was expressed in the cells under fluorescence microscopy, indicating that this promoter is fully capable of directing transcription of downstream genes (Fig. 5A).

Bioinformatics analysis showed the theoretical binding site of SP1 on the miR-183-5p promoter (Fig. 5B). Luciferase activity assay showed that overexpression of SP1 increased the activity of luciferase in cells transfec-
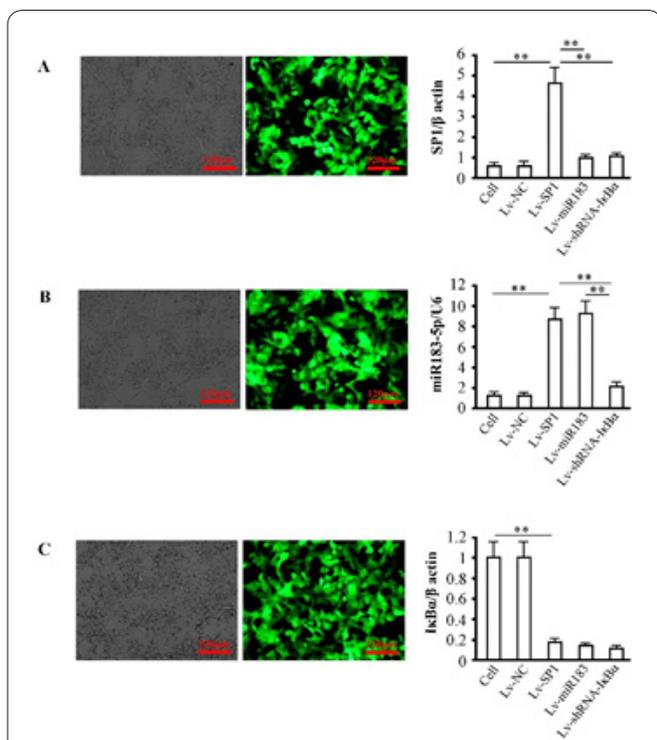
Figure 4. Infection of BV-2 cells and quantification of miR-NA-183-5p and mRNA of SP1 and IκB-α 72 h after infection. (A - C) Left: Seventy-two h after BV-2 cells were infection with recombinant virus, GFP expression showed a gene delivery efficiency close to 100 % (left = dark field, right = corresponding fluorescence field). Gene delivery efficiency was estimated by dividing the number of cells expressing GFP by the total cell number. MOI = 20 (x120). (A - C) Right: quantitative measurement of SP1, miR-NA-183-5p and IκB-α in cells infected with indicated recombinant virus. Data are expressed as mean ± SD, and tests were carried out on three biological replicates, ** *p* < 0.01, * *p* < 0.05.
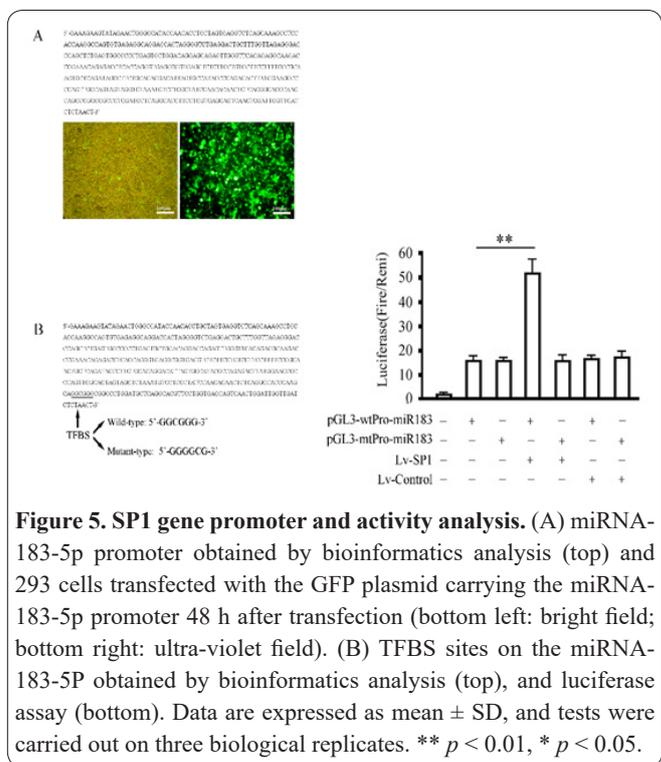

Figure 5. SP1 gene promoter and activity analysis. (A) miRNA-183-5p promoter obtained by bioinformatics analysis (top) and 293 cells transfected with the GFP plasmid carrying the miRNA-183-5p promoter 48 h after transfection (bottom left: bright field; bottom right: ultra-violet field). (B) TFBS sites on the miRNA-183-5P obtained by bioinformatics analysis (top), and luciferase assay (bottom). Data are expressed as mean ± SD, and tests were carried out on three biological replicates. ** *p* < 0.01, * *p* < 0.05.
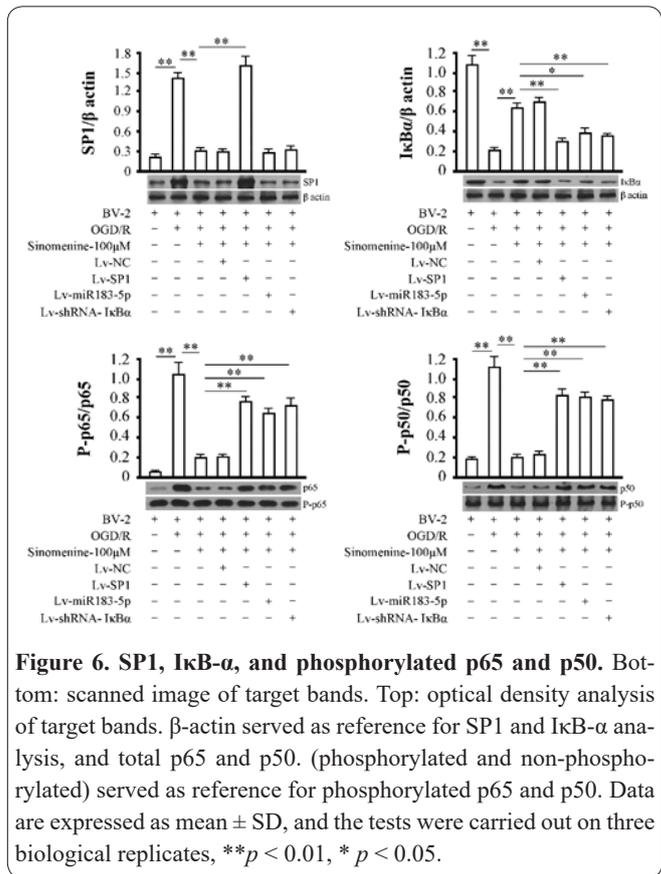

Figure 6. SP1, IκB-α, and phosphorylated p65 and p50. Bottom: scanned image of target bands. Top: optical density analysis of target bands. β-actin served as reference for SP1 and IκB-α analysis, and total p65 and p50. (phosphorylated and non-phosphorylated) served as reference for phosphorylated p65 and p50. Data are expressed as mean ± SD, and the tests were carried out on three biological replicates, ** *p* < 0.01, * *p* < 0.05.

ted with the wild-type reporter gene expression vector (*p* < 0.01), but had no apparent effect on the reporter vector carrying a mutant promotor (*p* > 0.05, Fig. 5B), indicating that SP1 did bind to the miRNA-183-5p through the *TFBS* and did positively regulate its transcription.

Specificity of the SP1/miRNA-183-5p/IκB-α pathway

Based on data obtained so far, it was posited that there might be a SP1/miRNA-183-5p/IκB-α pathway in the inhibition of OGD/R-induced inflammatory activation of BV-2 cell by sinomenine. If the pathway did exist, it was important to confirm whether it played a dominant role in the inhibition. So recombinant viruses Lv-SP1, Lv-miR183-5p and Lv-shRNA-IκBα were constructed, respectively, and transfected BV-2cells to overexpress SP1, miR-183-5p or silence IκBα, then the resultant changes in SP1, IκB-α and phosphorylation of p65 and p50 were studied to investigate the role of the pathway in sinomenine protection against ischemia reperfusion. The results showed that OGD/R significantly increased SP1 protein, reduced IκB-α, and promoted the phosphorylation of p65 and p50 (*p* < 0.01). Pretreatment with 100 μM of sinomenine inhibited the increases in SP1 protein, as well as increases in phosphorylated p65 and p50, and the decreases in IκB-α (*p* < 0.01). Overexpression SPI increased SP1 protein content (*p* < 0.01), but miRNA-183-5p overexpression or IκB-α silencing had no observable effect on SP1 (*p* > 0.05). The expressions of SP1 and miRNA-183-5p and silencing of IκB-α both significantly decreased IκB-α expression (*p* < 0.01), and increased the levels of phosphorylated p65 and p50 (*p* < 0.01, Fig. 6).

Discussion

Ischemic brain injury is one of the common diseases in the aged: it causes almost 200 deaths in China every hour. During ischemic brain injury, the rupture or blockage of blood vessels causes obstructed circulation and hypoxia injury in cerebral cells and tissues (2). The

main therapeutic approach is focused on the recovery of blood supply combined with multiple cytokines, which, if applied in time, will effectively improve the patient's symptom. However, clinical statistics have revealed some adverse effects of this regimen. Reperfusion, which is inevitable in recovery of circulation, often leads to inflammation in MG. Activated MG release a lot of inflammatory factors and mediators, thereby exposing the brain neurons to inflammation and apoptosis (4, 11). The control of ischemia/reperfusion-induced inflammatory activation of MG is important for reducing the loss of brain nerve function caused by ischemia, and for improving prognosis.

Microglia account for about one-fifth of total gliocytes in brain. They have some characteristics of immunocytes, and are very biologically active: they secrete a variety of factors under different conditions, thus playing dual roles in neural injury and recovery. Since MG and neurons are interwoven in the brain, inflammatory nerve injury is closely related to MG malfunction. During ischemia, activated MG move to the damaged areas and secrete multiple nutritional factors so as to maintain the basic functions of neurons. However, reperfusion leads to excessively activation of MG and secretion of more inflammatory factors, resulting in a cascade inflammatory response, which may aggravate neuronal malfunction and exacerbate neuronal injury (23, 24). So far, brain injury caused by cerebral ischemia/reperfusion can only be relieved: there are no effective treatments. The development of efficient drugs for treatment cerebral ischemia/reperfusion-induced injury is always a subject of immense interest in cerebral ischemia studies.

Studies have shown that, in general, inflammatory response of MG in mice occurs 3 h after ischemia/reperfusion, and peaks at 48 h. According to a long clinical monitoring study, MG activation starts at 72 h after cerebral ischemia/reperfusion, and lasts for over one month. Therefore, the essence of ischemia/reperfusion treatment is to effectively inhibit inflammatory activation of MG (25). The development of anti-inflammation drugs targeted at MG remains a major objective of research on ischemia-induced brain injury. Acute inflammation plays a critical role in secondary brain injury induced by cerebral ischemia/reperfusion, and cell injury mediated by inflammatory cells resembles delayed neuronal death in terms of timing. Drugs that block all inflammation links could be used to treat delayed damage caused by cerebral ischemia (26, 27). So far, the drugs that resist inflammatory activation of MG after cerebral ischemia are pro-inflammatory factor inhibitors: a non-selective adenosine receptor agonist 2-Cado can effectively suppress the expression of TNF-α in ischemia-injured tissue and cells, and brazilein has been shown to effectively protect brain tissue by inhibiting TNF-α and IL-6 expressions (28, 29). Mechanistic studies have shown that NF-κB, a classical regulator of inflammatory response, plays a dominant role in ischemia-reperfusion-induced MG activation. Therefore, the suppression of excessive activation of NF-κB is an important step in the treatment of ischemia-reperfusion injury (30).

An important way for developing new drugs is to search for lead compounds, improve their activities, and reduce their toxicities through structural modifications. Sinomenine is an isoquinoline alkaloid with anti-inflammatory, immune-strengthening, pain-relieving, and blood pressure-lowering properties. Resistance to inflammation is an important property of sinomenine which has been used in the clinical treatment of rheumatic arthritis. Indeed, studies have shown that sinomenine downregulates IL-1, TNF-α, and IL-6 expressions in arthritic mice (31). In addition, mechanistic studies have shown that sinomenine activity may be associated with the inhibition NF-κB and expression of IκB-α (32, 33). Therefore, it is worthwhile to investigate the effect of sinomenine in resistance to inflammation. Although a few studies have been reported on its role in cerebral ischemia-reperfusion, none has focused on the inflammatory activation of MG.

In the present study, an *in vitro* OGD/R model was created using BV-2 cells, and used to study the effect of sinomenine on inflammatory activation of MG. Based on assessments of NF-κB activation and IκB-α expressions, the anti-inflammatory activity of sinomenine relies on the suppression of NF-κB activation by upregulation of IκB-α. Results from quantitative PCR and western blotting revealed that sinomenine upregulated IκB-α in a post-transcription manner. From bioinformatical analysis and validation experiments, it can be concluded that sinomenine resists inflammatory activation of BV-2 cells via the SP1/miRNA-183-5p/IκB-α, that is, it inhibits the transcription of miRNA-183-5p, and thus increases the expression of IκB-α. This, in turn limits the phosphorylation of p65 and p50, and the activation of NF-κB, thereby suppressing OGD/R-induced inflammation. Further investigations on the specificity of the pathway showed that it dominates anti-inflammatory activity of sinomenine. This is considered of significance in the use of sinomenine for treatment of ischemia-reperfusion injury and development of sinomenine derivatives targeting SP1. However, the mechanism involved in the sinomenine-induced suppression of SP1 expression needs further studies. Moreover, it is not clear whether SP1 is a direct target of sinomenine. Based on current data and bioinformatical analysis, a preliminary hypothesis on how sinomenine suppresses SP1 expression can be proposed: sinomenine binds to SP1, exposes its ubiquitination site, and thus induces its ubiquitination. However, this hypothesis needs to be verified.

In conclusion, the present study has elucidated the role and mechanism of sinomenine in resisting OGD/R-induced inflammatory activation of MG. These findings are significant because they can help develop new sinomenine-based regimens for treating ischemia-reperfusion, and they are of benefit for prospective studies on sinomenine and gene combination therapy. Moreover, the findings can help in the modification of sinomenine to derivatives with potent drug properties. Thus, these findings provide a solid theoretical base for the role of sinomenine in resisting inflammation and improving nerve injury in ischemic brain injury.

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