Int J Clin Exp Pathol 2017;10(1):258-265
www.ijcep.com /ISSN:1936-2625/IJCEP0039909

Original Article

BET protein BRD4 as a new therapeutic target in cerebral ischemic stroke

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Received September 12, 2016; Accepted November 3, 2016; Epub January 1, 2017; Published January 15, 2017

Abstract: Clinical data have shown the levels of inflammatory markers are associated with the risk of ischemic stroke. The bromodomain-containing protein 4 (BRD4), a bromodomain and extraterminal (BET) family member, is a key factor in regulating nuclear factor kappa B (NF-κB) activity. We examined the therapeutic effects of BRD4 inhibitor JQ1 in the middle cerebral artery occlusion (MCAO) model of ischemic stroke in rats. Neurological functions, infarct volume, neuronal apoptosis and neuroinflammatory response were examined 24 h after MCAO. Isolated microglial cells were studied in vitro. Results showed that BRD4 expression was significantly increased in the MCAO group. The neurological damage, infarct volume and neuronal apoptosis were all significantly inhibited in JQ1-treated rats compared to control group following MCAO. Inflammatory responses were also decreased by JQ1. In addition, JQ1 inhibited NF-κB activation following MCAO. In vitro, BRD4 silencing decreased the production of proinflammatory cytokines via inhibiting NF-κB in microglial cells after OGD/reoxygenation. These findings suggest that BRD4 could be a novel therapeutic target in ischemic stroke.

Keywords: Ischemic stroke, inflammation, BRD4, target

Introduction

Stroke is a debilitating disease that contributes greatly to behavioral and cognitive impairment involving learning, motor and memory deficits [1]. The incidence of stroke in Asia is steadily growing, and the burden of stroke is particularly high in Asian countries [2]. In the last decade, there have been substantial advances in the pathogenesis of stroke, but much remains unknown about mechanisms of inflammatory response related to ischemic and reperfusion injury [3]. Animal studies have demonstrated causal relationships of systemic inflammation with ischemic brain damage [4]. Elevated circulating inflammatory markers, such as C-reactive protein (CRP), interleukin-6 (IL-6) and serum amyloid A (SAA), are associated with an unfavorable outcome and increased mortality after stroke [5, 6]. Microglia and astrocytes contribute to the production of inflammatory mediators, such as tumor necrosis factor (TNF)-α and iNOS [7]. The role of nuclear factor-kappa B (NF-κB) in ischemic stroke has been confirmed by many studies. For ischemic stroke, anti-inflammatory agents constitute an important therapeutic strategy [8].

The bromodomain and extra-terminal domain (BET) family of adaptor proteins are comprised of BRD2, BRD3, BRD4, and BRDT, which performs diverse roles in regulating transcription by RNA polymerase II [9]. Some inhibitors of BET bromodomains, originally developed for the treatment of rare and aggressive cancers, are currently tested for their efficacy as more general anti-cancer agents in clinical trials [10]. In addition to cancer, BET proteins have attracted great interest as promising epigenetic targets for diverse human diseases, including inflammatory disease and cardiovascular disease [9, 11]. A well-studied member of the BET family is BRD4. BRD4 is capable of activating pro-inflammatory genes by interacting with

phosphorylation and acetylation of the NF-κB subunit p65 on serine 276 and lysine 310 [12]. However, the role of BET proteins in ischemic stroke remains to be explored. Thus, the objective of our study was to investigate the effects of BRD4 inhibitor JQ1 on the middle cerebral artery occlusion (MCAO) model in rats and its potential mechanisms.

Materials and methods

Middle cerebral artery occlusion

The experimental protocol was approved by the Ethics Committee for Animal Experimentation of Capital Medical University (Beijing, China). Specific-pathogen-free 8-week-old male Sprague-Dawley rats (250 to 300 g, Slack Experimental Animal Company, Shanghai, China) were housed in separate cages in the Animal Facility of Capital Medical University. The rats were acclimatized for 1 week at 23°C with a 12:12 h dark/light cycle, and food and water were provided ad libitum.

Middle cerebral artery occlusion (MCAO) was induced as previously described, with some modifications [13]. Briefly, male Sprague-Dawley rats weighting 250 to 300 g were subjected to anesthesia with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg) followed by atropine at a dose of 0.1 mg/kg. The right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were surgically exposed. The ECA was coagulated and 4-0 nylon suture with silicon was inserted into the ICA through the ECA stump to occlude the MCA. After 2 hours of MCA occlusion, the suture was carefully removed to induce reperfusion. Sham group underwent the same procedures except that the MCA was not occluded.

Drug administration

JQ1 (Selleck Chemicals) from a DMSO stock was dissolved in 25% F-127 Pluronic gel (Sigma-Aldrich) as described previously [14]. Animals were subjected to MCAO for 2 h, and then BRD4 inhibitor JQ1 (50 mg/kg) or vehicle (equal volume of DMSO vehicle mixed with Pluronic gel) was intraperitoneally injected following the removal of the filament (post-reperfusion). At 24 h, all animals were killed and brains were snap frozen in isopentane for further analysis.

Tetrazolium chloride (TTC) staining

Infarct volume was measured using TTC staining as previously described [15]. At 24 h after treatment, rats were anesthetized, perfused with 200 ml of normal saline from heart into the aorta, decapitated at cervical vertebrae and then underwent craniotomy to remove the brain completely. The brain tissue was cut into five 2-mm thick sections in the coronal plane, which were stained with a 2% solution of TTC phosphate buffer for 10 min at 37°C away from light. The normal brain tissues were uniformly red, while the cerebral infarct zones were white. Images of the stained sections were taken and analyzed using Adobe Photoshop to calculate the cerebral infarct volumes.

Neurobehavioral assessments

Neurological assessments were performed by an independent researcher blinded to the treatment at 24 h after MCAO. The neurological findings were scored on a 4-point scale: 0 (no neurological deficit); 1 (Horner's syndrome); 2 (forelimb flexion); 3 (circling to right). Cumulative scores were calculated for each group.

Detection of apoptosis

For evaluation of apoptosis rate, a 4-micron paraffin slice of ischemic brain was processed. Immunofluorescence TUNEL assay was performed according to the manufacturer's instructions (Promega, Madison, WI, USA) as described previously [16]. Images were obtained by fluorescence microscope (Olympus, Tokyo, Japan) with a digital camera (Olympus).

Microglial isolation from the ischemic hemisphere

Microglial cells were isolated from brains as described previously [17]. In brief, after perfusion with ice-cold PBS, brains were dissected, weighed, and enzymatically digested using Neural Tissue Dissociation Kit (Miltenyi Biotec, Germany) for 35 min at 37°C. Further processing was performed at 4°C. Tissue debris was removed by passing the cell suspension through a 40 μm cell strainer. After myelin removal, cells were stained with PE-conjugated anti-CD11b antibodies (Miltenyi Biotec, Germany) in IMAG buffer (PBS supplemented with 0.5% BSA and 2 mM EDTA) for 10 minutes followed by

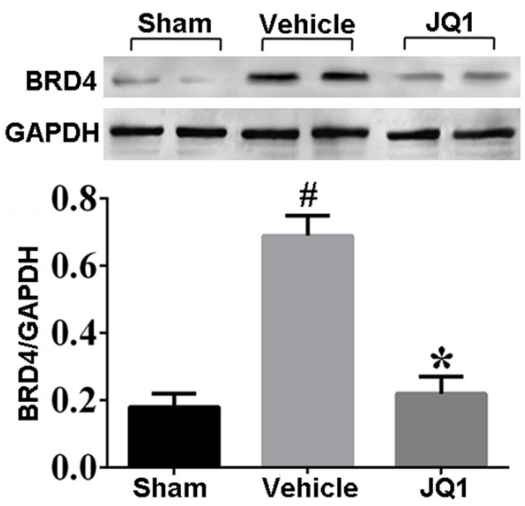

Figure 1. Effect of JQ1 on BRD4 expressions in rat cortex. Rats were given either vehicle or JQ1 (50 mg/kg, IP) immediately after MCAO. 24 h after injection of JQ1, expressions of BRD4 in rat cortex was determined by western blot. BRD4 expression was then quantitated by densitometric analysis. Bars represent mean ± SD; #P<0.01 vs sham group; *P<0.01 compared to vehicle-treated group.

incubation for 15 minutes with anti-PE magnetic beads. CD11b+ cells were separated in a magnetic field using MS columns (Miltenyi Biotec, Germany). The amounts of antibodies and magnetic beads were calculated based on the number of cells obtained after myelin removal, according to the manufacturer's guidelines. Both the CD11b+ and CD11b- (effluent) fractions were collected and used for further analysis.

Cell culture

Microglial cells were cultured in medium (DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1 × antibiotic/antimycotic; Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator (10% CO_2, 90% air).

Oxygen-glucose deprivation

To simulate ischemic conditions, cells were subjected to oxygen glucose deprivation (OGD). Before induction of OGD, serum-containing media were removed from the cell cultures before adding low-glucose medium (1.0 g/L, supplemented DMEM). The cultures containing low-glucose medium were placed in a hypoxia chamber (Billups-Rothenburg, Del Mar, CA, USA), which was flushed with a mixture of 95% N_2 and 5% CO_2 for 1 hour, and then closed for the duration of the experiment. O_2 levels decreased to 0.1% to 0.4% at 4 hours, and were maintained throughout the experiment (18 hours in total).

Transfection of small-interfering RNA targeting BRD4

Scrambled siRNA (Ambion) or BRD4 siRNA (Santa Cruz) was transfected with HiPerFect Transfection Reagent (Qiagen, Shanghai, China). The ability of the siRNA to inhibit BRD4 expression was assessed 48 h post-transfection by western blot.

Enzyme-linked immunosorbent assay

Ischemic brain tissues were collected and frozen at -80°C immediately until analysis of cytokine concentrations with commercial kits for tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-17 (RayBiotech, Inc.) according to the manufacturer's instructions.

Western blot

Total proteins were extracted from cortex. Nuclear-cytoplasmic fractionation was conducted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's protocol. Protein concentration was measured by BCA kit (Thermo Scientific). Equal amounts of protein lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane and then immunoblotted with antibodies against BRD4 (1:1000 dilution; Cell Signaling Technology); NF-κB p65 (1:1000 dilution; Cell Signaling Technology), IκBα (1:1000 dilution; Cell Signaling Technology), Lamin B (1:1000 dilution; Cell Signaling Technology) and GAPDH (1:1000 dilution; Cell Signaling Technology). Then, the membranes were incubated with HRP-conjugated secondary antibody. The signals were detected with a chemiluminescence system (ECL Plus, Amersham Pharmacia).

Statistical analysis

Quantitative data are presented as mean ± SD. Comparisons of parameters among multiple

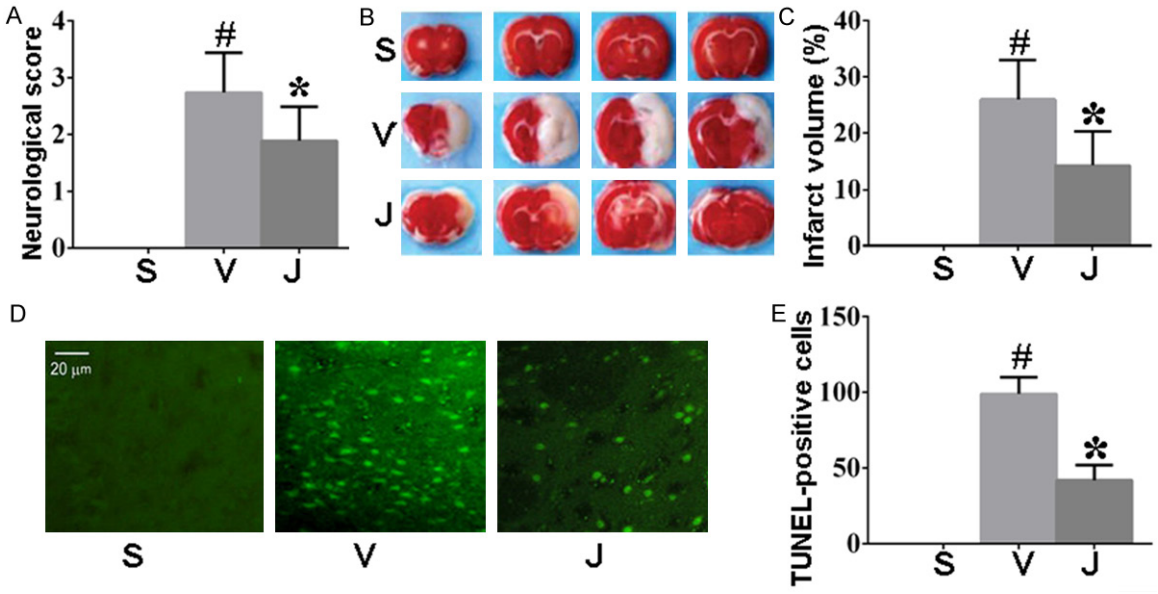

Figure 2. Effect of JQ1 on neurological deficits and infarct volume after MCAO. A. Elevated neurological deficit scores after MCAO were significantly attenuated by JQ1, and neurologic deficits were significantly ameliorated in the JQ1 group compared with the control group at 24. B. Representative TTC stained brain sections of different groups were shown. Bars represent mean ± SD (n = 8). C. Quantification of infarct volume at 24 h. The ratio of corrected infarct volume to the nonischemic hemisphere volume was calculated for the cerebral infarct size. Infarct volume was decreased at 24 h with JQ1 treatment. D. Representative images and quantitative analysis of apoptosis using fluorescent TUNEL assay. E. TUNEL-positive cells (green) were considered as apoptotic cells. Bars represent mean ± SD; #P<0.01 vs sham group; *P<0.01 vs vehicle-treated group, n = 8 per group. Sham (S), Vehicle (V), JQ1 (J).

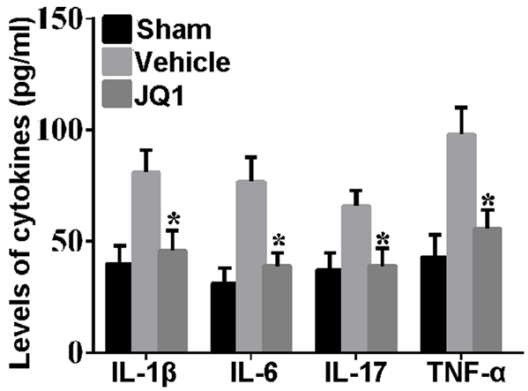

Figure 3. Effect of JQ1 on inflammatory response in the ischemic brain. JQ1 treatment down-regulated the level of IL-1β, IL-6, IL-17 and TNF-α at 24 after ischemia. Bars represent mean ± SD; #P<0.01 vs sham group; *P<0.01 vs vehicle-treated group, n = 8 per group.

groups were made by one-way analysis of variance, and comparisons of different parameters between each group were made by Bonferroni's post-hoc test. A *p* value <0.05 was considered statistically significant.

Results

Effect of JQ1 on BRD4 expression in the rat ischemic cortex

First of all, we detected the BRD4 expression in the rat ischemic cortex. As shown in **Figure 1**, BRD4 expression was significantly increased in the MCAO group, compared with sham group. JQ1 has been reported to inhibit BRD4 activity [18]. As expected, JQ1 treatment markedly decreased BRD4 expression in ischemic cortex.

Effect of JQ1 on neurological functions, infarct volume and neuronal apoptosis

Neurologic deficits, at 24 h after cerebral ischemia, were significantly reduced in JQ1 group compared with vehicle group (P<0.01, **Figure 2A**).

Infarct volume was measured by TTC at 24 h after cerebral ischemia. Infarct volumes in the JQ1 group were significantly reduced compared with the vehicle group (P<0.01, **Figure 2B**, **2C**).

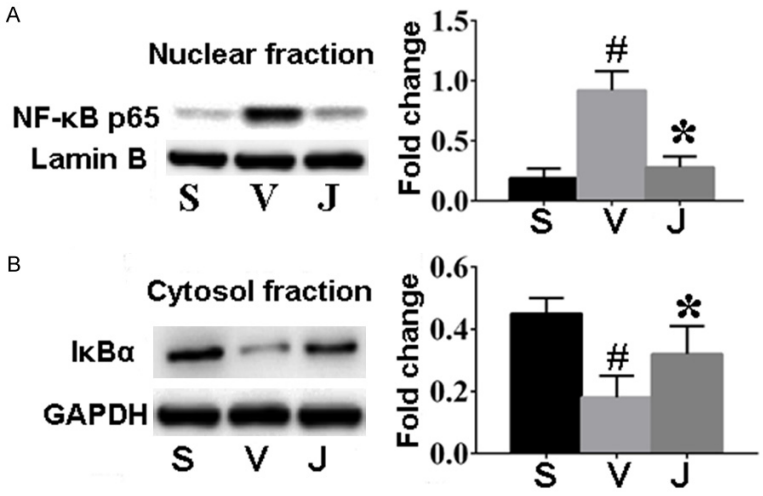

Figure 4. JQ1 treatment inhibits NF-κB signaling pathway at 24 hours after MCAO. A. Expression of NF-κB p65 in nuclear fractions in Sham (S) and infarcted brain tissue of vehicle (V) and JQ1(J)-treated rats was determined by immunoblotting. B. Expression of IκBα in cytosol fractions in Sham (S) and infarcted brain tissue of vehicle (V) and JQ1(J)-treated rats was determined by immunoblotting. #P<0.01 vs sham group; *P<0.01 vs vehicle-treated group, n = 8 per group.

TUNEL assay showed that JQ1 treatment decreased the number of apoptotic cells in infarcted area (**Figure 2D**, **2E**).

JQ1 modulated pro-inflammatory and anti-inflammatory cytokines in the ischemic brains

To confirm whether JQ1 was immunomodulatory in experimental stroke, we tested the levels of several pro-inflammatory cytokines, including IL-1β, IL-6, IL-17 and TNF-α by ELISA at 24 h after cerebral ischemia. In the ischemic brain, JQ1 treatment reduced the level of IL-1β, IL-6, IL-17 and TNF-α (**Figure 3**).

JQ1 treatment inhibited NF-κB after MCAO

NF-κB pathway is the major mediator regulating the production of inflammatory cytokines, and we further studied the effects of JQ1 treatment on NF-κB pathway in MCAO model. The nuclear NF-κB p65 (**Figure 4A**) was higher, whereas the cytosolic IκB expression was lower in infarcted brain tissues compared with sham tissue (**Figure 4B**), indicating that NF-κB was activated in experimental stroke. Treatment of JQ1 significantly attenuated the nuclear NF-κB p65 and upregulated cytosolic IκB level (**Figure 4A**, **4B**). These results indicate that JQ1 suppresses NF-κB activation during ischemic stroke.

BRD4 siRNA suppressed the production of proinflammatory cytokines in microglial cells via inhibiting NF-κB

As NF-κB is essentially involved in regulating production of proinflammatory cytokines, we investigated whether BRD4 siRNA inhibits expression of proinflammatory cytokines via inhibition of NF-κB activity. BRD4 siRNA inhibited the level of IL-1β, IL-6, IL-17 and TNF-α (**Figure 5A**). And, treatment of microglial cells with OGD markedly increased the NF-κB activity, which was significantly inhibited by treatment with BRD4 siRNA (**Figure 5B**).

Discussion

BET proteins are a group of epigenetic readers of histone acetylation involved in chromatin remodeling and transcriptional regulation [11]. Multiple small-molecule inhibitors were developed to target BET members in cancers and inflammatory diseases [19]. For instance, BET inhibitor I-BET-762 can cause long-lasting suppression of the proinflammatory functions of Th1 cells [20]. JQ1 is the best characterized BET inhibitor for its emerging preclinical and clinical evidence in haematological malignancies [21]. Recent discoveries extend applications for BET inhibitors [22]. Interestingly, results in this present study show that BRD4 expression was significantly increased in the MCAO group. The neurological deficit, infarct volume and neuronal apoptosis were all significantly reduced in JQ1-treated rats. JQ1 reduced inflammatory responses both in vitro and in vivo. Thu, BRD4 inhibition was protective in the rat cerebral ischemic stroke.

Increased production of cytokines contributes greatly to inflammatory response during cerebral ischemia. Proinflammatory cytokines modulate tissue injury in experimental stroke and are therefore considered as potential targets in stroke [23]. In mice, exogenous TNF-α exacerbates focal ischemic injury and blocking endogenous TNF-α is neuroprotective [24]. Studies

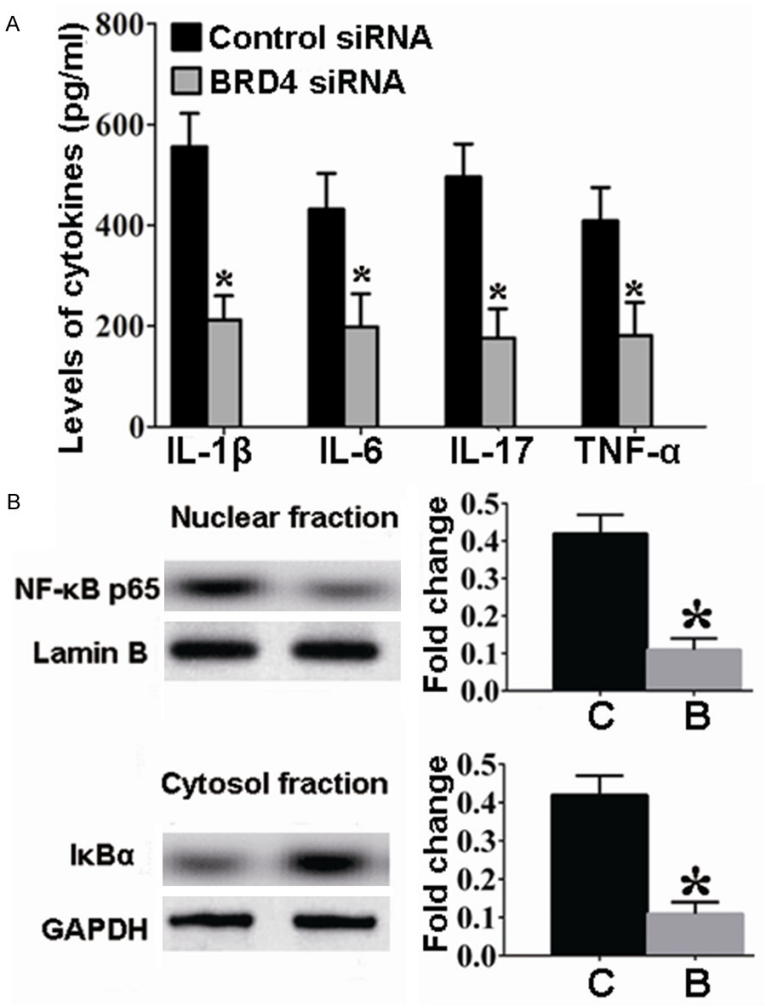

Figure 5. BRD4 siRNA inhibited NF-κB dependent proinflammatory cytokines in microglial cells after oxygen-glucose deprivation/reoxygenation (OGD). A. Microglial cells were treated with control siRNA or BRD4 siRNA (10 nM) for 30 min. ELISA was then conducted. B. Microglial cells were pretreated with control siRNA, BRD4 siRNA (10 nM) for 30 min before OGD. The effects of BRD4 siRNA on the NF-κB were detected. C: Control siRNA, B: BRD4 siRNA. *P<0.01 vs control siRNA-treated group.

have also shown that localized striatal injection of IL-1β led to the exacerbation of ischemic brain damage and recombinant human IL-1 receptor antagonist markedly protects against focal cerebral ischemia in the rat [25, 26]. Elevated levels of IL-6 are associated with an unfavorable functional outcome and increased mortality after stroke and inhibition of IL-6 is a potential therapeutic strategy in stroke [27]. IL-17, another powerful pro-inflammatory cytokine, may promote brain I/R injury through increasing calpain-mediated TRPC6 proteolysis, outlining a novel neuroprotective strategy with increased effectiveness for the inhibition of excess brain IL-17A in cerebral I/R injury [28, 29]. Also, inhibiting the microglial activation in the ischemic regions has been shown to protect brain against focal cerebral ischemia [30]. Our results show that JQ1 treatment markedly suppressed the elevation of IL-1β, IL-6, IL-17 and TNF -α in both ischemic brain and the microglia isolated from the ischemic hemisphere. Therefore, the neuroprotective mechanisms of JQ1 was at least in part attributed to the decrease of IL-1β, IL-6, IL-17 and TNF-α.

NF-κB is activated by a huge array of stimuli, including proinflammatory cytokines such as TNF and IL-1β [31]. NF-κB is activated in neurons during human stroke, and activation of NF-κB in the brain may contribute to infarction in pMCAO [32]. NF-κB activation involves nuclear translocation of the subunits p50 and increased DNA binding of p50 homodimers. MCAO in the rat is followed over hours by NF-κB activation, as demonstrated by increased p65 and p50 expression in ischemic region neurons, and gel shifts of homogenized lesional tissue [33]. NF-κB inhibition caused a reduction in stroke size, neurodeficit as well as apoptosis [34, 35]. It was also shown that over-expression of NF-κB p65 in astrocytes significantly increased the co-cultured neuronal apoptosis under OGD followed by oxygen-glucose regeneration condition [36]. In addition, suppression of NF-κB in microglia attenuated the severity of experimental stroke [37]. In this study, JQ1 inhibited NF-κB activation following MCAO in vivo, and BRD4 silencing decreased the production of proinflammatory cytokines via inhibition of NF-κB pathway in microglial cells after OGD/reoxygenation. Therefore, the

neuroprotective mechanisms of JQ1 was associated with the inhibition of NF-κB activation.

Disclosure of conflict of interest

None.

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