Nox2 and Nox4 Participate in ROS-Induced Neuronal Apoptosis and Brain Injury During Ischemia-Reperfusion in Rats

Jinjin Wang,[$] Yin Liu,[$] Haitao Shen, Haiying Li, Zhong Wang, and Gang Chen

Abstract *Background*: Previously studies have shown that Nox2 and Nox4, as members of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase, Nox), participate in brain damage caused by ischemia-reperfusion (I/R). The aim of this study is to investigate the effects of specific chemical inhibitors of Nox2 and Nox4 on cerebral I/R--induced brain injury in rats.

Methods: At 0.5 h before MCAO surgery, the rats were pretreated with vehicle, Nox2 inhibitor (gp91ds-tat), and Nox4 inhibitor (GKT137831), respectively. After reperfusion for 24 h, the infarct sizes of brain tissues in rats in various groups are determined. The penumbra (ischemic) tissues are collected to measure ROS levels, neuronal apoptosis, and degeneration, as well as the integrity of the blood-brain barrier (BBB) in brain tissues of rats.

Results: gp91ds-tat and GKT137831 pretreatment significantly reduced the infarct sizes in brain tissues of rats, effectively suppressed I/R-induced increase in ROS levels, neuronal apoptosis and degeneration, and obviously alleviated BBB damage.

Conclusion: Under cerebral I/R conditions, Nox2 inhibitor (gp91ds-tat) and Nox4 inhibitor (GKT137831) can effectively play a protective role in the brain tissues of rats.

Keywords Cerebral ischemia-reperfusion · Nox2 · Nox4 · Middle cerebral artery occlusion · Reactive oxygen species

Introduction

Stroke and its related complications are one of the leading causes of morbidity and mortality worldwide [4, 24, 30]. Among them, the ischemic stroke patients account for 87% of stroke-related mortality [13, 21, 22, 27]. Neurons quickly die at 5 min after hypoxia; therefore, the effective time frame for treatment of the ischemic stroke is very narrow [28]. Early intravenous thrombolytic therapy is the only method of treatment in ischemic stroke patients and is currently approved [19]. Although the blood flow was restored after early intravenous thrombolytic therapy, the ischemia-reperfusion (I/R) injury caused by increased reactive oxygen species (ROS) in the reperfusion phase is particularly serious and may lead to a poor outcome in patients [11, 25].

Ischemic penumbra is a transitional area between the infarction and normal brain tissues after ischemic stroke [3]. The mechanisms underlying cerebral ischemia and potential clinical therapies have attracted more and more attention in various studies. Although there are many enzymes playing important roles in oxidative stress in various tissues and cells in many diseases, the family of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase, Nox) is considered as major source of ROS [13]. In mammals, Nox family includes Nox1, Nox2, Nox3, Nox4, dual oxidase (Duox)-1, and Duox-2 [14]. Previous studies have shown that some members of Nox family, particularly Nox2 and Nox4, are important sources of ROS in cerebral I/R [7].

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Previously, radical scavengers or natural antioxidants, such as vitamin C and vitamin E, were mostly used to inhibit oxidative stress with limited efficacy [6]. Nox family play important roles in cerebral I/R injury through oxidative stress, nitration stress, blood-brain barrier (BBB) damage, and cell apoptosis. Therefore, Nox has become a new target for the treatment of ischemic stroke [26]. Recently, it has been reported that genetic knockout and/or specific chemical inhibitor of Nox treatment can significantly reduce oxidative stress-induced brain damage ischemic stroke [23, 29]. However, the exact mechanisms of the activation of Nox are not clear, and there is lack of effective drugs that can significantly inhibit Nox in clinical practice [17]. gp91ds-tat is a specific inhibitor of Nox2 and GKT137831 is a Nox4-specific inhibitor. To our knowledge, these two Nox-specific inhibitors in the treatment of ischemic stroke have not been reported.

In this study, we hypothesized that the specific inhibitors of Nox2 and Nox4 can attenuate cerebral I/R injury in a rat model of middle cerebral artery occlusion (MCAO) through reducing ROS levels, inhibiting neuronal apoptosis and degeneration, and alleviating BBB damage.

Materials and Methods

Ethical Approval

All experiments are approved by the Ethics Committee of the First Affiliated Hospital of Soochow University and are in accordance with the guidelines of the National Institutes of Health on the care and use of animals. Adult male Sprague-Dawley (SD) rats (250–300 g) are purchased from Animal Center of Chinese Academy of Sciences (Shanghai, China). The rats are housed in temperature- and humidity-controlled animal quarters with a 12-h light/dark cycle.

Establishing the MCAO Model in Rats

Following the intraperitoneal anesthesia with chloral hydrate (36 mg/100 g body weight), under an operating microscope, focal cerebral ischemia in rats is achieved by right-sided endovascular MCAO. In brief, the right common, external, and internal carotid arteries (CCA, ECA, and ICA) are revealed via a midline cervical incision. Then, a piece of 4.0-monofilament nylon suture with the tip rounded by heating before coating with polylysine is inserted through the right CCA and advanced it along the ICA until the tip occluded the proximal stem of the middle cerebral artery (MCA). Rectal temperature is maintained between 36.5 and 37.5 °C with a heating pad. After 2 h of ischemia, the filament is withdrawn to allow reperfusion. At 24 h after reperfusion, the brain tissue samples of rats in various groups are obtained for further analyses.

Experimental Group and Drug Administration

The sample sizes in each group were determined by power analysis during the animal ethics dossier application. Thirty-two adult male SD rats are randomly divided into two experimental groups: sham operation group (Sham group, $n = 8$) and middle cerebral artery occlusion group (MCAO group, $n = 24$). MCAO group is then subdivided into three groups: At 0.5 h before MCAO surgery, the rats are, respectively, pretreated with vehicle ($n = 8$), gp91ds-tat ($n = 8$), and GKT137831 ($n = 8$). According to our previous study [32], the gp91ds-tat (AnaSpec., USA) is dissolved in normal saline. 20 μL gp91ds-tat solution is infused into the ventricle using a Hamilton microsyringe, and the final concentration of gp91ds-tat is 100 ng/kg. Meanwhile, the GKT137831 (MedChem Express, USA) is given by oral gavage at 60 mg/kg as the previous research [9]. At 24 h after reperfusion, eight rats in one group were randomly divided into three parts. For four rats, the total brain sections were collected for TTC staining; two rats were killed for TUNEL and FJB staining; the other two rats were used for BBB permeability and ROS assay. For TUNEL and FJB staining, representative images from at least three independent experiments using six different brain sections were shown. For Western blot analysis and ROS assay showing quantitative results, each n represents data collected from one independent experiment; combined data from six independent experiments using two different rats are shown. The "n" is always defined as number of independent experiments in every figure legend.

Assay of ROS

The levels of ROS are measured by the Reactive Oxygen Species Assay Kit (Nanjing Jiancheng Biotechnology Institute, China). Briefly, after the brain tissues are collected, the samples are homogenized and centrifuged at 12,000 g for 10 min in 4 °C. The supernatants are then collected for ROS assay. ROS concentrations are assessed using the oxidant-sensitive probe 2,7-dichlorofluorescein diacetate (DCF-DA) according to the manufacturer's proto-

col. The fluorescence intensity is tested by a fluorimetric microplate reader (FilterMax F5; Molecular Devices, USA) with excitation and emission at 485 and 530 nm, respectively. The ROS concentration is expressed as the fluorescence intensity/mg protein and then normalized to the mean value of sham group.

TTC Staining

At 24 h after reperfusion, rats are deeply anesthetized by chloral hydrate (36 mg/100 g body weight) and decapitated. The brain tissues are quickly removed and frozen in stainless steel brain matrices (−20 °C) for 10 min. The brain tissues are subsequently sectioned into 2 mm thick slices starting from the frontal pole. The cerebellum and olfactory bulb are discarded. Slices are then immersed in 10 mL of 2% TTC (Sigma, USA) for 30 min at 37 °C. After staining, the slices are washed three times with saline, fixed in 10% formalin, and then captured with a digital camera. Then, the infarct volume in brain slices of rats are calculated as described previously [21]. Briefly, the total mean infarct area of each section is calculated as the average of the area on the rostral and the caudal side. The total area is calculated by adding the average area from each section. Multiplication of the total area by 2 mm (thickness of the sections) is calculated as infarct volume. The infarct volume is expressed as a percentage of the ipsilateral hemispheric volume.

Ischemic Penumbra Dissection

After indicated treatment, the rats are deeply anesthetized by chloral hydrate (36 mg/100 g body weight) and transcardially perfused with ice-cold PBS. Then, in each rat, the cerebellum and olfactory bulb are discarded, and the brain tissues are quickly removed and frozen in stainless steel brain matrices (−20 °C) for 10 min. The ischemic penumbra is determined according to the method described by Ashwal et al. [2]. Briefly, the brain tissues are subsequently sectioned into three slices beginning 3 mm from the anterior tip of the frontal lobe. The front and back slices are 3 mm in thickness. The middle slice is 4 mm in thickness, which is cut longitudinally in the ischemic hemisphere 2 mm from the midline. A transverse diagonal cut is made at the 2 o'clock position to separate the core from the penumbra. The cerebral cortical penumbra and analogous contralateral region are harvested for Western blot analysis and immunofluorescence assay.

Terminal Deoxynucleotidyl Transferase dUTP Nick End-Labeling (TUNEL) Staining and Fluoro-Jade B Staining

TUNEL staining is used to detect cell apoptosis in brain tissues and performed according to the manufacturer's protocol (Dead End Flurometric kit, Promega, USA). The sections are visualized using a fluorescence microscope (Olympus BX50/BXFLA/DP70; Olympus Co., Japan). The TUNEL-positive cells are counted by an observer who is blind to the experimental groups. To evaluate the extent of cell apoptosis, the apoptotic index is defined as the average number of TUNEL-positive cells in each section counted in six microscopic fields.

Fluoro-Jade B (FJB, Histo-Chem Inc., USA) staining is served as a marker of neuronal degeneration. Brain sections are deparaffinized and rehydrated. After incubation with deionized water for 1 min, the slides are incubated in 0.06% K permanganate (Sigma, USA) for 15 min. Slides are then rinsed in deionized water and immersed in 0.001% Fluoro-Jade working solution (0.1% acetic acid) for 30 min. Then they are washed and dried in an incubator (50–60 °C) for 10 min. Sections are cleared in xylene and coverslipped with a nonaqueous, low-fluorescence, styrene-based mounting medium (DPX, Sigma, USA). Microscopy of the stained brain sections are performed by an experienced pathologist blind to the experimental condition.

Western Blot Analysis

After brain tissues of each rat are collected, the brain samples are separately homogenized and lysed in ice-cold RIPA lysis buffer (Beyotime, China). After centrifuge at 12,000 g for 10 min at 4 °C, the supernatants are collected. The protein concentration is determined by using the bicinchoninic acid (BCA) kit (Beyotime, China). Then, protein samples (20 μg/lane) are loaded on a 15% SDS polyacrylamide gel, separated, and electrophoretically transferred to a polyvinylidene difluoride membrane (PVDF, Millipore, USA). The membrane is blocked with PBST (PBS + 0.1% Tween-20) containing 5% skim milk for 1 h at 37 °C. Subsequently, the membrane is incubated with the primary antibodies against albumin and GAPDH (abcam, USA) overnight at 4 °C. Next, the membrane is incubated with the horseradish peroxidase (HRP)-linked secondary antibody (abcam, USA) for 2 h at 37 °C and then washed with PBST for three times. Then, the membrane is revealed with use of an enhanced chemiluminescence detection kit. The relative quantity of proteins is

analyzed by using Image J program (NIH, USA), and the data are normalized to that of loading controls.

Statistical Analysis

All data are expressed as mean ± SEM and GraphPad Prism 6.0 (GraphPad, USA) is adopted for all statistical analyses. Data sets are tested for normality of distribution with Kolmogorov-Smirnov test. Data groups (two groups) with normal distribution are compared using two-sided unpaired Student's t-test, and the Mann-Whitney U test is used for nonparametric data. $P < 0.05$ is considered as statistically significant difference.

Results

General Observation

No significant differences in mortality rate, body weight, mean arterial blood pressure, heart rate, temperature, or blood gas data are detected in any experimental groups (data not shown).

Nox2 Inhibitor (gp91ds-tat) and Nox4 Inhibitor (GKT137831) Treatments Reduce ROS Levels in Brain Tissues of Rats After MCAO

Compared to the sham group, the level of ROS is significantly increased in penumbra of brain tissues at 24 h after reperfusion in rats in the MCAO group ($P < 0.01$, Fig. 1). However, treatment with Nox2 inhibitor (gp91ds-tat) or Nox4 inhibitor (GKT137831) significantly decreased the ROS levels in penumbra of brain tissues of rats compared with that in the MCAO group (both $P < 0.01$, Fig. 1).

gp91ds-tat and GKT137831 Treatments Reduce Cerebral Infarction Area at 24 h After Reperfusion in MCAO Rats

As shown in Fig. 2, there is a significant infarct area in the brain tissues of rats in the MCAO group compared to the sham group ($P < 0.01$, Fig. 2). However, the cerebral infarct area is significantly reduced after treatment with gp91ds-tat or GKT137831 when compared with that in the MCAO group (both $P < 0.01$, Fig. 2).

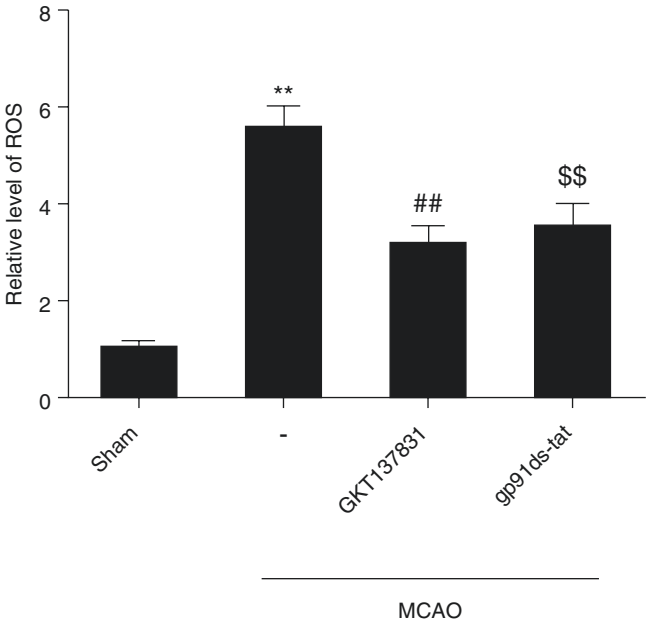

Fig. 1 ROS levels in the penumbra are significantly higher in the MCAO group than that in the sham group; Nox2 inhibitor (gp91ds-tat) or Nox4 inhibitor (GKT137831) treatment significantly reduced the ROS levels in brain tissues compared with the MCAO group. All data are expressed as mean ± SD, $^{**}P < 0.01$, vs. Sham group; $^{\#\#}P < 0.01$, vs. MCAO group; $^{\$\$}P < 0.01$, vs. MCAO group; $n = 6$

gp91ds-tat and GKT137831 Treatments Decrease the Rate of Neuronal Apoptosis in Penumbra of Brain Tissues in Rats at 24 h After Reperfusion After MCAO

Compared with the sham group, the TUNEL-positive neurons are obviously increased in the penumbra of brain tissues at 24 h after reperfusion in rats after MCAO ($P < 0.01$, Fig. 3). However, the gp91ds-tat or GKT137831 treatment significantly reduced the numbers of TUNEL-positive cells in brain tissues of rats after MCAO compared with that in the MCAO group, (both $P < 0.01$, Fig. 3).

gp91ds-tat and GKT137831 Treatments Reduce Neurodegeneration in Penumbra of Brain Tissues in Rats After MCAO

We also assessed neuronal degeneration in the penumbra of brain tissues at 24 h after reperfusion by FJB staining. In the

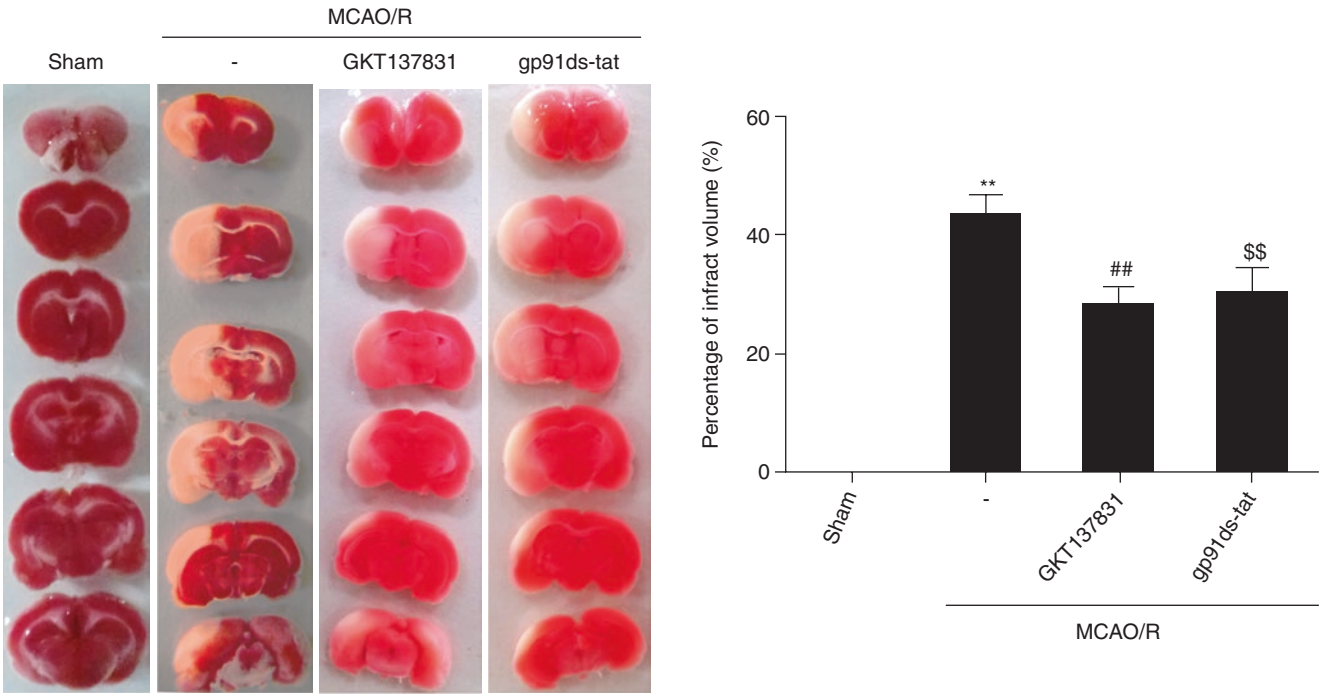

Fig. 2 It is shown that there is a significant infarct area in the brain tissues of rats in the MCAO group compared with the sham group, gp91ds-tat or GKT137831 treatment significantly reduced the percentages of the infarct volume compared with the MCAO group. All data are expressed as mean ± SD, $^{**}P < 0.01$, vs. Sham group; $^{\#\#}P < 0.01$, vs. MCAO group; $^{\$\$}P < 0.01$, vs. MCAO group; $n = 4$

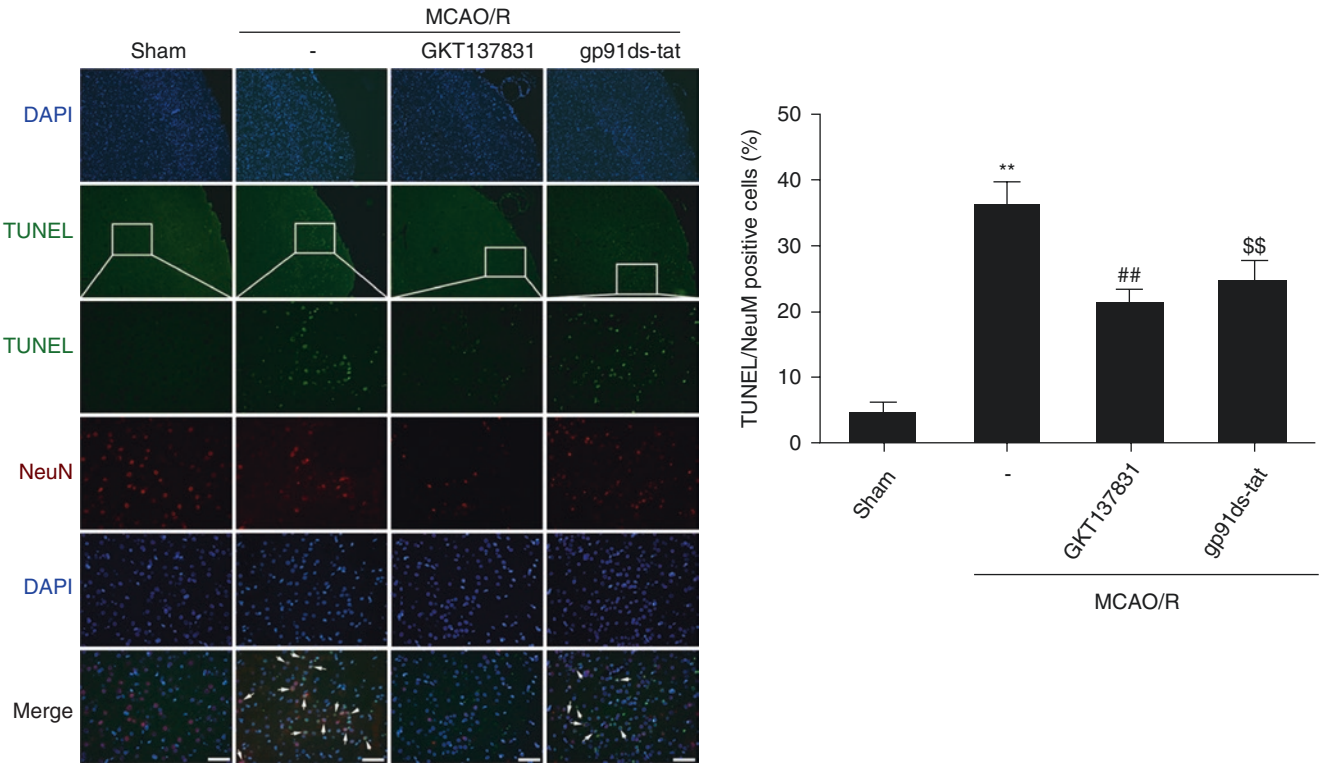

Fig. 3 The TUNEL-positive neurons are significantly increased in brain tissues of rats in the MCAO group relative to the sham group; while gp91ds-tat or GKT137831 treatment significantly reduced the numbers of apoptosis neurons compared with that in the MCAO group. All data are expressed as mean ± SD, $^{**}P < 0.01$, vs. Sham group; $^{\#\#}P < 0.01$, vs. MCAO group; $^{\$\$}P < 0.01$, vs. MCAO group; scale bar = 100 μm; $n = 6$

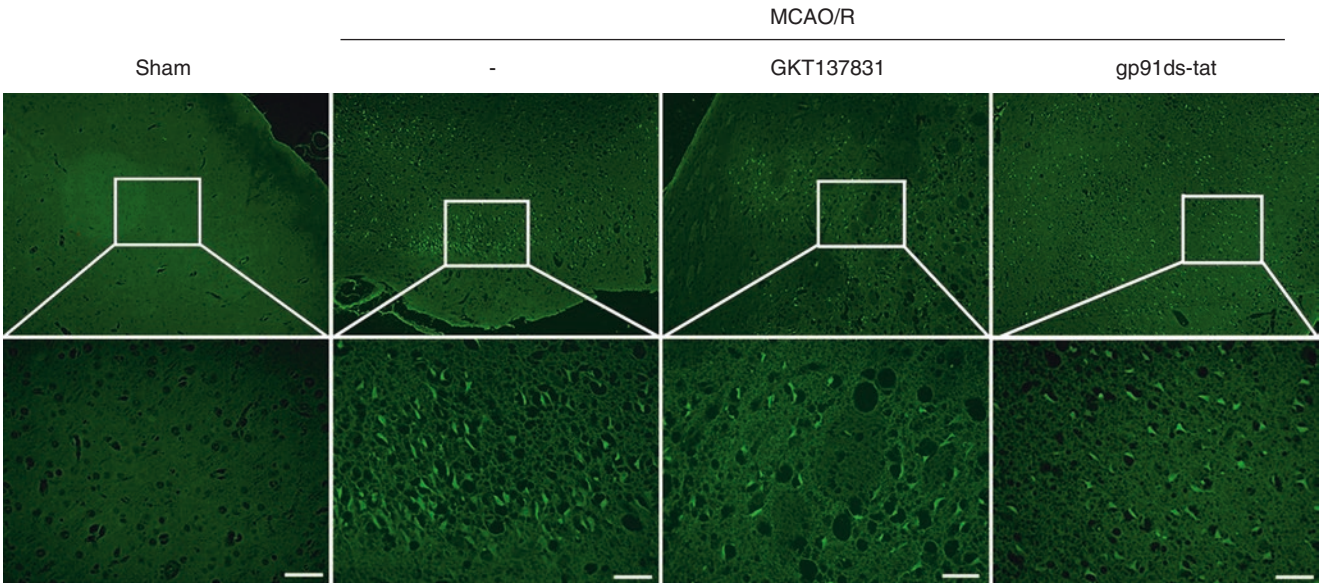

Fig. 4 The FJB-positive cells are significantly increased in brain tissues of rats in the MCAO group relative to the sham group; while gp91ds-tat or GKT137831 administration significantly reduced the numbers of neurodegenerative cells compared with that in the MCAO group; scale bar = 100 μm

MCAO group, FJB-positive cells are significantly increased compared with that in the sham group (Fig. 4). However, treatments with gp91ds-tat or GKT137831 significantly reduce the numbers of FJB-positive cells in brain tissues of rats compared with that in the MCAO group (Fig. 4).

gp91ds-tat and GKT137831 Treatments Protect the BBB Integrity at 24 h After Reperfusion in Rats After MCAO

In order to determine the integrity of BBB in rats, we assess the levels of albumin in brain tissues by Western blot analysis. Compared with the sham group, the level of albumin is significantly increased in brain tissues of the rats in the MCAO group ($P < 0.01$, Fig. 5), which suggests that there is albumin leakage in brain tissues of rats after MCAO, resulting in BBB injury. However, compared with the MCAO group, gp91ds-tat or GKT137831 treatment significantly reduced the levels of albumin in brain tissues in rats after MCAO (both $P < 0.01$, Fig. 5).

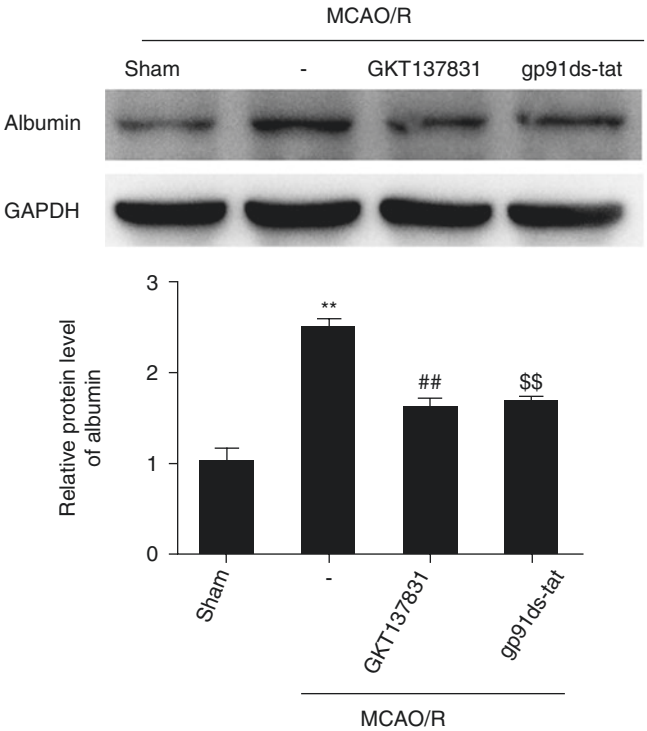

Fig. 5 The protein levels of albumin are significantly increased in brain tissues of rats in the MCAO group relative to the sham group; however, gp91ds-tat or GKT137831 administration significantly reduced the protein levels of Albumin in brain tissues of rats compared with that in the MCAO group. All data are expressed as mean ± SD, $^{**}P < 0.01$, vs. Sham group; $^{\#\#}P < 0.01$, vs. MCAO group; $^{\$\$}P < 0.01$, vs. MCAO group; $n = 6$

Discussion

Previous studies have shown that the increasing expression and activation of Nox induced the massive production of ROS in cerebral I/R injury [16]. Excessive ROS may cause the peroxidation of lipid, protein, and nucleic acids [1, 8], destruction of the blood-cerebrospinal fluid structure and further increase 3 cysteine-aspartic acid proteases, decrease Bcl-2/Bax ratios, and finally result in cell apoptosis [12]. In addition, oxidative stress and inflammation can influence and interact with each other in a synergistic manner in cerebral I/R-induced brain injury [5]. Another previous research also found that Nox acti-

vation and ROS production may lead to activation of microglia and production of inflammatory mediators, such as IL-1β and TNF-α in a mice model with focal cerebral I/R injury [15].

As our previous report, Nox2 and Nox4 may play greater roles than other members of Nox family in cerebral I/R--induced brain injury [22]. It is reported that Nox2 increases from 24 to 72 h after reperfusion in endothelial cells and microglia of the penumbra in a mice model of MCAO. Nox4 is also been conformed that it was increased in the brain tissues after ischemic stroke. In a mice model of MCAO, Nox4 mRNA levels in neurons increase within the 24 h, peak between days 7 and 15, and slowly decline until day 30 [10, 31]. In analysis of whole brain tissues, the mRNA and protein levels of Nox2 and $p22^{phox}$ increase in the ischemic hemisphere in a rat model of MCAO [20], and Nox4 increases in the ischemic cortex and basal ganglia after ischemic stroke in mice [18]. In this study, our results show that Nox2-specific inhibitor (gp91ds-tat) and Nox4-specific inhibitor (GKT137831) have significant protective effects in brain injury induced by cerebral I/R in a rat model of MCAO. It is expected that Nox2 and Nox4 specific inhibitors may have superior effects than omni-spectrum inhibitors of the Nox family.

The present study also has the following limitations. Firstly, we use healthy adult rats in this study, which do not maximally mimic human high-risk populations, such as the elderly, women, and patients with cardiovascular diseases. Additionally, the mechanisms underlying the effects of treatment with Nox2 and Nox4 inhibitors on cerebral I/R induced brain injury are also not fully investigated. Of course, these limitations involved in Nox2-specific inhibitor (gp91ds-tat) and Nox4-specific inhibitor (GKT137831)-induced neuroprotective effects showed in this study would be explored in our future work.

In conclusion, this study suggests that Nox2 inhibitor (gp91ds-tat) and Nox4 inhibitor (GKT137831) treatment could, respectively, inhibit I/R-induced excessive increasing of ROS in the penumbra of brain tissues, reduce cerebral infarction area, mitigate neuronal apoptosis and degeneration, and alleviate BBB damage. Meanwhile, our study provides a theoretical basis for the clinical use of Nox2-specific inhibitor (gp91ds-tat) and Nox4-specific inhibitor (GKT137831) for stroke therapy.

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Conflict of Interest: **The authors declare that they have no conflict of interest.**

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