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ORIGINAL PAPER

Inhibition of Calcium/Calmodulin-Dependent Protein Kinase IIα Suppresses Oxidative Stress in Cerebral Ischemic Rats Through Targeting Glucose 6-Phosphate Dehydrogenase

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Abstract

Ischemic stroke is a leading cause of mortality and morbidity worldwide, and oxidative stress plays a significant role in the ischemia stage and reperfusion stage. Previous studies have indicated that both calcium/calmodulin-dependent protein kinase II (CaMKII) and glucose 6-phosphate dehydrogenase (G6PD) are involved in the oxidative stress. Thus, the aim of this study was to investigate the roles of CaMKIIα, an important isoform of CaMKII, and G6PD in a rat model of middle cerebral artery occlusion (MCAO). Intracerebroventricular injection of small interfering ribonucleic acid (siRNA) for CaMKIIα was performed at 48 h pre-MCAO surgery. Immunofluorescence Staining and western blot were performed to detect the expression of p-CaMKIIα and G6PD in the cortices. 2, 3, 5-Triphenyltetrazolium chloride (TTC) staining was performed to investigate the infarct volume. In addition, neurological deficit, reactive oxygen species (ROS), ratio of reduced-to-oxidized glutathione (GSH/GSSG) and ratio of reduced-to-oxidized oxidized nicotinamide adenine dinucleotide phosphate ($NADPH/NADP^+$) were assessed. The results indicated that both p-CaMKIIα and G6PD were widely located in the neurons and astrocytes, and their expression was gradually increased in the cortices after MCAO, which was accompanied by increased level of ROS and decreased levels of GSH/GSSG and $NADPH/NADP^+$. However, after treatment with siRNA for CaMKIIα, p-CaMKIIα expression was decreased and G6PD expression was increased. Moreover, inhibition of CaMKIIα improved the neurological deficit, reduced the infarct volume, decreased the level of ROS and increased the levels of GSH/GSSG and $NADPH/NADP^+$. The results suggested that CaMKIIα inhibition exerted neuroprotective effects through regulating G6PD expression, which provides a new target for prevention and treatment of stroke.

Keywords Ischemic stroke · Ischemia–reperfusion injury · Oxidative stress · CaMKII · G6PD

Abbreviations

I/R	Ischemia–reperfusion
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
PPP	Pentose phosphate pathway
GSSG	Oxidized glutathione
GSH	Glutathione
NADPH	Nicotinamide adenine dinucleotide phosphate
CaMKII	Calcium/calmodulin-dependent protein kinase II
G6PD	Glucose 6-phosphate dehydrogenase
MCAO	Middle cerebral artery occlusion
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
TTC	2, 3, 5-Triphenyltetrazolium chloride

Introduction

Ischemic stroke, mainly caused by sudden loss or decrease of blood supply due to thrombosis or embolism, occupies 87% of stoke cases, and it is a leading cause of mortality and morbidity worldwide [1, 2]. The cardinal therapeutic goal of ischemic stroke is timely recovery of blood supply, and reperfusion therapy using thrombolysis, such as tissue plasminogen activator (tPA) and endovascular thrombectomy, are the only FDA-approved treatments for ischemic stroke, although sudden recovery of blood supply might lead to more serious injuries, namely cerebral ischemia–reperfusion

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(I/R) injury [3, 4]. During the cerebral ischemia reperfusion phase, many pathophysiological changes occur, such as release of excitotoxic chemicals, calcium overload, free radical damage, neuronal apoptosis and neuroinflammation; wherein, free radical damage to the brain plays an important role in the process of I/R injury during reperfusion therapy [5]. Therefore, developing the novel and valid therapies against free radical damage could not only improve success rate of reperfusion therapy but also benefit to recovery from neural deficits.

Free radicals are divided into reactive oxygen species (ROS) and reactive nitrogen species (RNS), and the overproduction of ROS and RNS could spark oxidative stress and nitrosative stress, respectively. Thereinto, accumulating evidence indicated that oxidative stress is closely related with neuronal injuries, such as neuron loss and neuroinflammation, which contribute to neurological deficit and cognitive dysfunction following reperfusion [6–9]. In addition, reduced nicotinamide adenine dinucleotide phosphate (NADPH), the products of pentose phosphate pathway (PPP), plays an important role in transformation of oxidized glutathione (GSSG) to reduce glutathione (GSH) with the help of glutathione reductase, which is necessary to fight against oxidative stress [10]. Previous studies have found that calcium/calmodulin-dependent protein kinase II (CaMKII) is involved in oxidative stress and CaMKII inhibition relieves oxidative damage and oxidative damage-induced cell injury [11–13]. However, it remains unknown whether or not CaMKII targets at glucose 6-phosphate dehydrogenase (G6PD), a rate-limiting enzyme in PPP, to mediate production of NADPH and regulate oxidative stress. Therefore, we hypothesized that inhibition of CaMKII suppresses oxidative stress in cerebral ischemic rats through inhibiting G6PD expression.

Materials and Methods

Animals and Middle Cerebral Artery Occlusion (MCAO) Surgery

Male Sprague–Dawley rats (290–320 g) were acquired from the First Affiliated Hospital of Zhengzhou University and fed in a SPF-class housing of laboratory. All procedures were approved by the Laboratory Animal Care and Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The modified intraluminal filament method was used to induce focal cerebral ischemia according to previous studies [14, 15]. Briefly, rats underwent 90-minute occlusion of the right middle cerebral artery using a nylon filament and reperfusion by pullout of the nylon filament under anesthesia with 2% isoflurane.

Experimental Design

Experiment One

A total of 35 rats were divided into sham group (n = 5), MCAO after 3 h group (3 h; n = 5), MCAO after 6 h group (6 h; n = 5), MCAO after 12 h group (12 h; n = 5), MCAO after 24 h group (24 h; n = 10) and MCAO after 48 h group (48 h; n = 5). The rats were sacrificed at corresponding time point under anesthesia with 2% isoflurane. The brains in 24 h group (n = 5) were made frozen sections and cut into 10 μm slices to investigate the localization of CaMKIIα and G6PD by immunofluorescence staining. In addition, the cortices from each group (n = 5 per group) were collected and used to observe the expression of CaMKIIα and G6PD by western blot.

Experiment Two

To detect the effect of CaMKII on I/R injuries, we used small interfering RNA (siRNA) to inhibit the CaMKIIα expression. A total of 40 rats were divided into sham group (n = 10), MCAO group (n = 10), MCAO + Scramble siRNA group (Scr; n = 10) and + CaMKIIα siRNA group (si-CaMKIIα; n = 10). Scramble siRNA and CaMKII siRNA were purchased from Origene Technologies, Inc. (MD, USA), and intracerebroventricular injection of scramble siRNA and CaMKIIα siRNA was performed at 48 h pre-MCAO surgery according to previous study [16]. The neurological deficit of all rats were scored and the all rats were sacrificed at 24 h after MCAO surgery under anesthesia with 2% isoflurane. A part of rat brains from each group (n = 5 per group) were cut into five 2-mm slices with brain-cutting matrix for 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. A part of rats cortices from each group (n = 5 per group) were collected and used to observe the expression of protein and content of ROS, GSSG, GSH, NAPDH and oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$).

Assessment of Neurological Deficit

Assessment of neurological deficit was performed by an investigator who was blinded to the experimental groups according to a 5-score criterion described previously [17]. The concrete criterion was shown as follows: 0, normal; 2, circling to the contralateral side when mouse held by the tail on a flat surface but normal posture at rest; 3, leaning to the contralateral side at rest; 4, no spontaneous motor activity.

Assessment of Infarct Size

Infarct size was assessed by TTC staining according to the manufacturer's instructions. Briefly, the brain slices were incubated with 2% TTC (Sigma-Aldrich, St. Louis,

MO, USA) for 10 min at 37 °C and bathed in 4% formalin overnight. The TTC images were collected using a digital camera. Infarct volume was expressed as a percentage of whole-brain volume.

Immunofluorescence staining

The frozen sections were incubated with 4% paraformaldehyde for 30 min at room temperature. Then the sections were washed using PBS three times for 5 min and incubated with 0.5% Triton X-100 for 30 min at room temperature. After washed using PBS three times for 5 min, the sections were incubated with 10% donkey serum for 2 h at room temperature and bathed with rabbit anti-phospho (p)-CaMKIIα (T286; 1:100; Abcam, Cambridge, MA, USA), rabbit anti-G6PD (1:100; Abcam, Cambridge, MA, USA), goat anti-GFAP (1:100; Abcam) and mouse anti-NeuN (1:100; Millipore, Billerica, MA, USA) overnight at 4 °C. On the following day, sections were washed with PBS three times for 10 min and incubated with donkey anti-rabbit Alexa Fluor® 488 IgG H&L (1:100; Abcam), donkey anti-mouse Alexa Fluor® 555 IgG H&L (1:100; Abcam) and donkey anti-goat Alexa Fluor® 405 IgG H&L (1:100; Abcam) for 2 h at 37 °C in the dark. Fluorescent images were captured using a confocal scanning laser microscope (Leica, Wetzlar, Germany).

ROS Detection

ROS assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to assess the level of ROS according to the manufacturer's instructions. Briefly, cortices were cut into small pieces (about 1 mm^3) and washed with iced PBS. After pieces were treated with enzyme digestive solution at 37 °C for 20–30 min, iced PBS was used to terminate digestion and samples were filtered using nylon (300 meshes). Then, samples were centrifuged at 4 °C and 500×*g* for 10 min and the supernatant was discarded. Cells were then resuspended in the wash buffer and incubated with 10 μM DCFH-DA for 40 min at 37 °C in the dark. After incubation, cells were washed twice and analyzed by fluorescence spectrophotometer.

Assessment of ROS, GSH/GSSG and NADPH/$NADP^+$

GSH and GSSG assay kit (Beyotime, Shanghai, China) was used to assess the level of GSH/GSSG and NADPH/$NADP^+$ quantification kit (Sigma, St. Louis, MO, Cambridge, UK) was used to assess the level of NADPH/$NADP^+$ according to according to the manufacturer's instructions.

Western Blot

Total protein of the harvested cortices was extracted using RIPA buffer containing protease and phosphatase inhibitor (Beyotime, Shanghai, China) according to the manufacturer's instructions. Western blot was performed as previously described [18]. The following primary antibodies were used: rabbit anti-CaMKIIα (1:1000; Abcam), rabbit anti-p-CaMKIIα (1:1000; Abcam), rabbit anti-G6PD (1:1000; Abcam), rabbit anti-cleaved caspase-3 (1:1000, Abcam), rabbit anti-Bcl-2 (1:1000, Abcam), rabbit anti-Bax (1:1000, Abcam) and rabbit anti-GAPDH (1:4000, Abcam). Goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (1:4000, Abcam) was used as secondary antibody. The images were exhibited using enhanced chemiluminescence reagent (Beyotime). Western blot was performed five times for each protein to be able to statistically analyse the findings and that the figures were representative ones only. Protein expression was standardized to GAPDH and quantified through the Image J software.

Statistical Analysis

Data were expressed as the mean ± standard deviation and one-way analysis of variance (ANOVA) followed by a Tukey test was used to compare means of different groups. Significance level was set to 5%. There is a significant difference at $P < 0.05$.

Results

Increased Expression of p-CaMKIIα in the Cortices After MCAO

At 24 h after MCAO, the brains were made frozen sections to investigate the location of p-CaMKIIα by immunofluorescence staining. As shown in the Fig. 1a, p-CaMKIIα was located in the neurons (NeuN) and astrocytes (GFAP) in the cortices. Next, we assessed p-CaMKIIα expression using western blot at 3 h, 6 h, 12 h, 24 h and 48 h in the cortices following MCAO. The expression of p-CaMKIIα was normalized to the expression of total CaMKIIα. Surprisingly, the ratio of p-CaMKIIα/CaMKIIα was dramatically elevated as early as 3 h following MCAO, and this ratio was gradually increased at 6 h, 12 h, 24 h and 48 h after MCAO when compared with sham group ($P < 0.05$, Fig. 1b).

Increased Expression of G6PD in the Cortices After MCAO

Also, immunofluorescence staining was performed in the cortices at 24 h after MCAO to detect the location of G6PD.

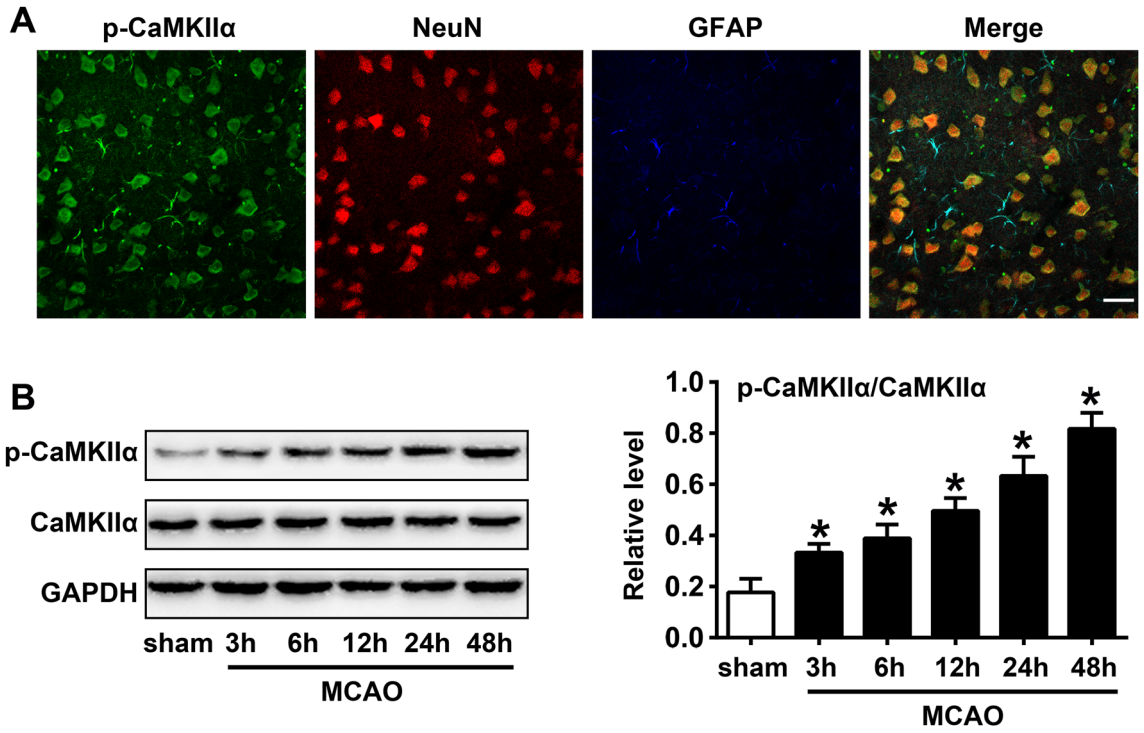

Fig. 1 Increased expression of p-CaMKIIα in the cortices after MCAO. **a** Representative immunofluorescence staining for p-CaMKIIα (green), NeuN (red) and GFAP (blue) in the cortices. Scale bar = 50 μm. **b** Representative images and statistical charts of western blot for p-CaMKIIα in the cortices (n = 5 per group). The levels of p-CaMKII were normalized to the levels of CaMKIIα. *P < 0.05 versus sham group. *CaMKIIα* calcium/calmodulin-dependent protein kinase IIα (Color figure online)

As shown in the Fig. 2a, G6PD was expressed in the neurons (NeuN) and astrocytes (GFAP) in the cortices. Then, western blot was conducted to observe G6PD expression in the cortices at 3 h, 6 h, 12 h, 24 h and 48 h following MCAO. Similarly, G6PD expression was gradually increased in the cortices at 3 h, 6 h, 12 h, 24 h and 48 h after MCAO when compared with sham group (P < 0.05, Fig. 2b).

CaMKIIα siRNA Improved Neurological Deficit and Infarct Volume

High level of p-CaMKIIα expression was detected in the cortices of MCAO rats. Therefore, we tried to use CaMKIIα siRNA to inhibit CaMKIIα expression, which could decrease phosphorylated level of CaMKIIα. Scramble siRNA and CaMKIIα siRNA were injected through lateral ventricles at 48 h before MACO surgery, and western blot was conducted to detect the expression of p-CaMKIIα. As shown in the Fig. 3a, the ratio of p-CaMKIIα/CaMKIIα was observably decreased in the si-CaMKIIα group when compared with Scr group (P < 0.05). At 24 h after MCAO, neurological deficit was assessed according to Longa's scoring [18]. We found obvious neurological deficit in the MCAO rats when compared with sham rats (P < 0.05, Fig. 3b). Whereas, significant ameliorative neurological deficit was investigated in the si-CaMKIIα group when compared with Scr group (P < 0.05, Fig. 3b), which suggested that inhibition of CaMKIIα could improve neurological deficit. In addition, TTC staining was conducted to assess the infarct volume. As shown in the Fig. 3c, we found bigger infarct volume in the MCAO rats when compared with sham rats (P < 0.05). However, infarct volume was significant reduced in the si-CaMKIIα group when compared with Scr group (P < 0.05), which suggested that inhibition of CaMKIIα could decrease infarct volume.

CaMKIIα siRNA Increased G6PD Expression in the Cortices After MCAO

In order to test whether CaMKIIα expression could affect G6PD expression or not, western blot was used to detect G6PD expression after MCAO rats were treated with scramble siRNA and CaMKIIα siRNA. As shown in the Fig. 4, G6PD expression was increased in the MCAO group when compared with the sham group (P < 0.05). Moreover, G6PD expression was increased in the si-CaMKIIα group when compared with Scr group (P < 0.05). These data indicated that CaMKIIα inhibition could up-regulate G6PD expression.

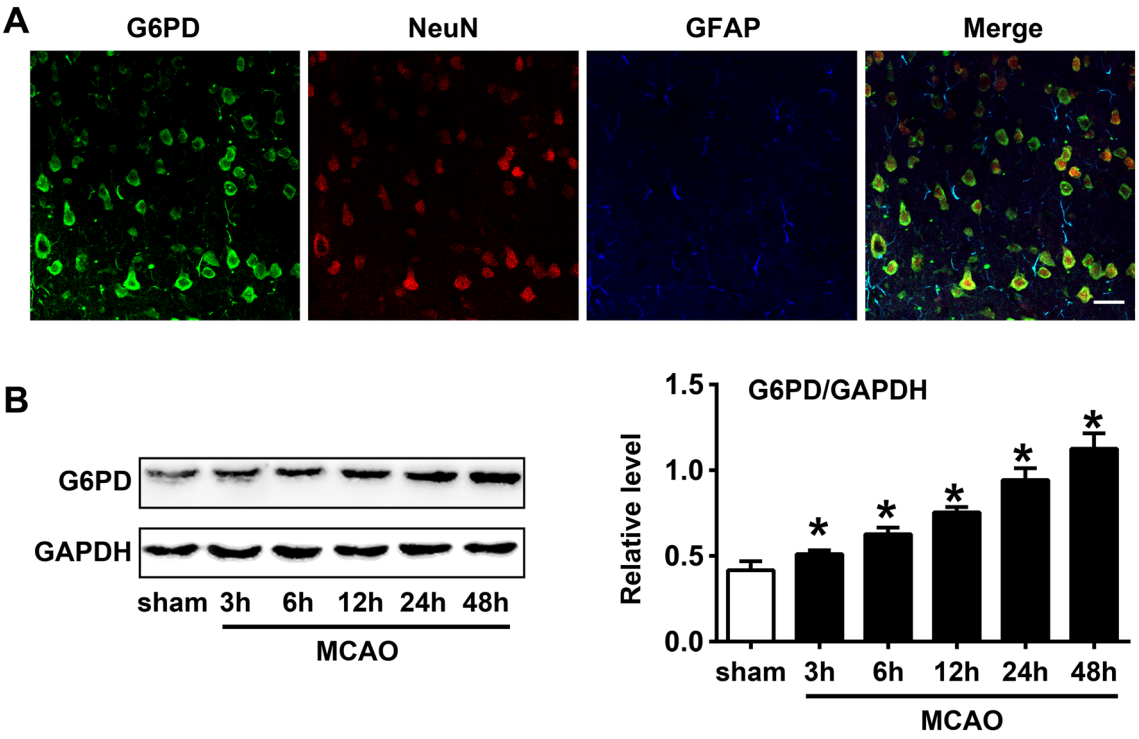

Fig. 2 Increased expression of G6PD in the cortices after MCAO. **b** Representative immunofluorescence staining for G6PD (green), NeuN (red) and GFAP (blue) in the cortices. Scale bar = 50 μm. **b** Representative images and statistical charts of western blot for G6PD in the cortices (n = 5 per group). The levels of G6PD were normalized to the levels of GAPDH. *P < 0.05 versus sham group. *G6PD* glucose 6-phosphate dehydrogenase, *MCAO* middle cerebral artery occlusion (Color figure online)

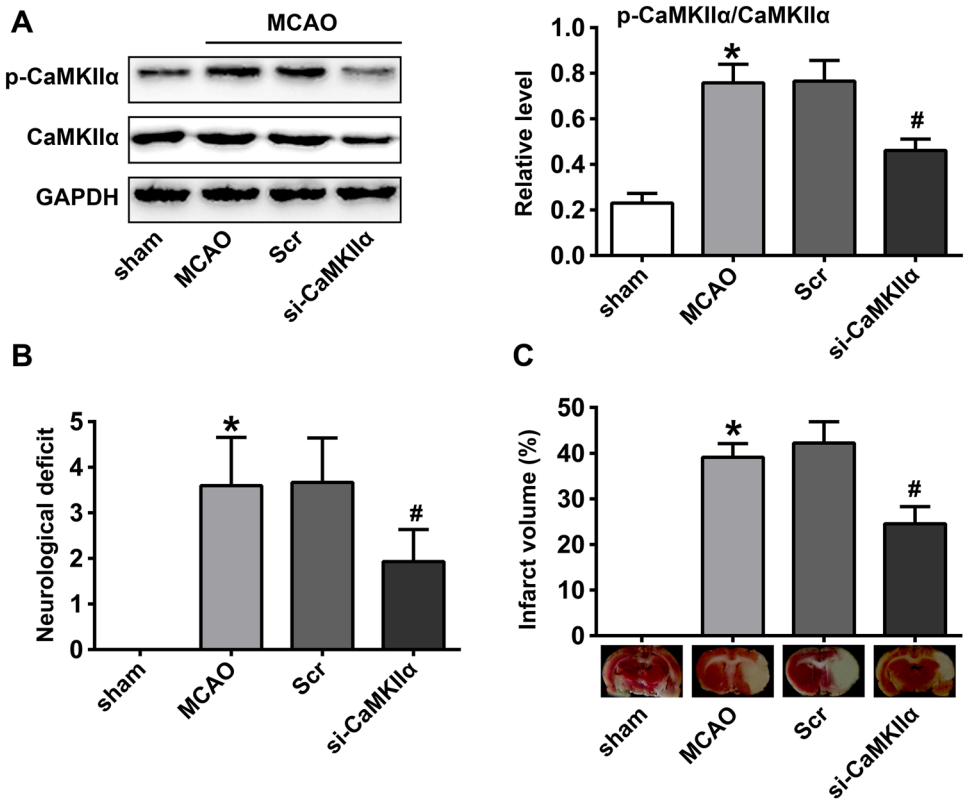

Fig. 3 CaMKIIα siRNA improved neurological deficit and infarct volume. **a** Representative images and statistical charts of western blot for p-CaMKIIα in the cortices (n = 5 per group). **b** The statistical charts for neurological deficit (n = 10 per group). **c** Representative images and statistical charts of TTC staining (n = 5 per group). *P < 0.05 versus sham group; #P < 0.05 versus Scr group. *CaMKIIα* calcium/calmodulin-dependent protein kinase IIα, *TTC* 2, 3, 5-triphenyltetrazolium chloride

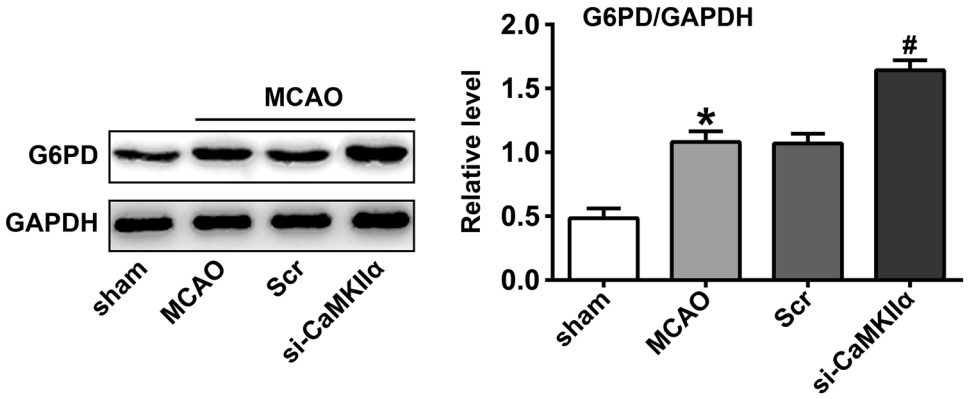

Fig. 4 CaMKIIα siRNA increased G6PD expression in the cortices after MCAO Representative images and statistical charts of western blot for G6PD in the cortices (n = 5 per group). *P < 0.05 versus sham group; #P < 0.05 versus Scr group. *CaMKIIα* calcium/calmodulin-dependent protein kinase IIα, *G6PD* glucose 6-phosphate dehydrogenase, *MCAO* middle cerebral artery occlusion

CaMKIIα siRNA Decreased Content of ROS and Increased Levels of GSH/GSSG and NADPH/NADP$^+$ in the Cortices After MCAO

Finally, we assessed the levels of ROS, GSH/GSSG and NADPH/NADP$^+$ in the cortices after MCAO. The results indicated that increased level of ROS ($P < 0.05$, Fig. 5a) and decreased levels of GSH/GSSG ($P < 0.05$, Fig. 5b) and NADPH/NADP$^+$ ($P < 0.05$, Fig. 5c) were detected in the MCAO group when compared with sham group. However, treatment with si-CaMKIIα could decrease the levels of ROS ($P < 0.05$, Fig. 5a) and increase levels of GSH/GSSG ($P < 0.05$, Fig. 5b) and NADPH/NADP$^+$ ($P < 0.05$, Fig. 5c). These data suggested that inhibition of CaMKIIα could suppress oxidative stress through increasing the levels GAPDH and GSH.

Discussion

In the present study, we investigated the effects of CaMKIIα on I/R injuries. We found both p-CaMKIIα and G6PD were widely expressed in the neurons and astrocytes, and gradually increased expression of p-CaMKIIα and G6PD was observed in the rats cortices following MCAO. However, after MCAO rats received treatment with si-CaMKIIα, neurological deficit and infarct volume were distinctly improved, which was paralleled by increase of G6PD expression and improvement of oxidative stress. The results indicated that CaMKIIα might be involved in the oxidative stress through regulating G6PD expression, and inhibition of CaMKIIα shows neuroprotective effects in the I/R injuries.

It is well known that oxidative stress is closely related with various pathological changes in the central nervous system, such as apoptosis, neuroinflammation, synaptic plasticity, etc. [19–22]. Under normal conditions, endogenous enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), and nonenzymatic antioxidative molecules, including GSH, Vitamins

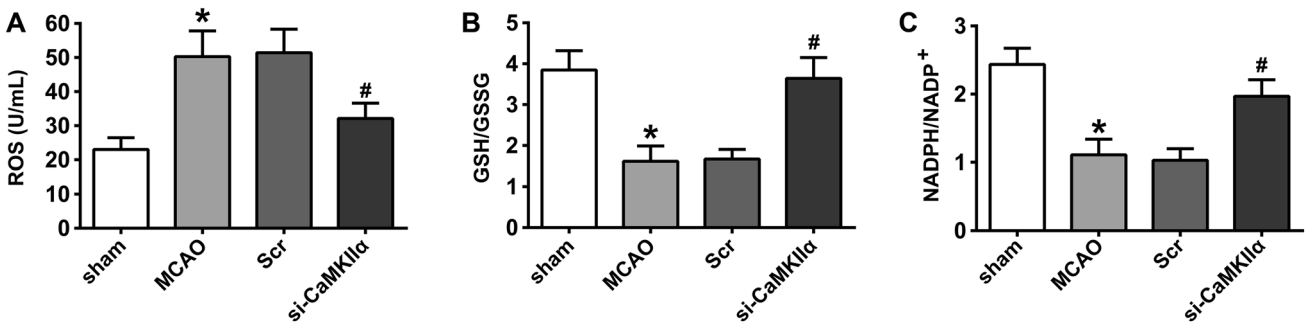

Fig. 5 CaMKIIα siRNA decreased content of ROS and increased levels of GSH/GSSG and NADPH/NADP$^+$ in the cortices after MCAO. The statistical charts for ROS (**a**), GSH/GSSG (**b**) NADPH/NADP$^+$ (**c**) in the cortices (n = 5 per group). *P < 0.05 versus sham group; #P < 0.05 versus Scr group. *CaMKIIα* calcium/calmodulin-dependent protein kinase IIα, *ROS* reactive oxygen species, *GSH* glutathione, *GSSG* oxidized glutathione, *NADPH* nicotinamide adenine dinucleotide phosphate, *NADP*$^+$ oxidized nicotinamide adenine dinucleotide phosphate, *MCAO* middle cerebral artery occlusion

E and C exert crucial roles in suppressing overproduction of ROS [23]. Previous study indicated that GSH in mitochondria is the only defense available against hydrogen peroxide to moderate oxidative stress. Moreover, the antioxidant capacity of GSH is usually assessed by the ratio of reduced-to-oxidized glutathione (GSH/GSSG) and the level of GSH/GSSG ratio is negatively correlated with ROS production [24]. In the stroke, a large number of studies have observed an increase in the ROS level and a decrease in GSH/GSSG ratio in the experimental model, and many researchers suggested that increasing GSH/GSSG ratio might be a valid strategy against oxidative stress [25–27]. Wang et al., for instance, have indicated that lavender oil could inhibit the increase of ROS level and the decrease of GSH/GSSG ratio, which ameliorated neurological deficit and infarct volume [27]. Similarly, in the present study, we also found that ROS level was markedly increased and GSH/GSSG ratio was significantly decreased, which was accompanied by more serious neurological deficit and infarct volume in the cortices of MCAO rats when compared with sham rats. However, treatment with si-CaMKIIα abolished those change and showed neuroprotective activity, which suggested that inhibition of CaMKIIα could suppress ROS production in the cerebral ischemic rats through mediating GSH/GSSG ratio.

CaMKII comprises a family of closely related kinases with four isoforms (α, β, γ, and δ), and CaMKIIα is almost exclusively expressed in brain, making up nearly 1% of the total brain protein [28–30]. Hence, we measured the location of p-CaMKIIα by the immunofluorescence staining, and the results indicated that p-CaMKIIα was widely expressed in the neurons and astrocytes in the cortices, which is consistent with the previous study [16]. In addition, we also found that the p-CaMKIIα expression was gradually up-regulated following MCAO. Previous studies indicated that CaMKIIα is activated by autophosphorylation of at T286 and persists kinase activity after removal of Ca^{2+} and CaM when Ca^{2+} binds to calmodulin (CaM), which sustains the activation of CaMKII and regulates the downstream molecules [16, 31, 32].

As is known to all, G6PD is an important rate-limiting enzyme of PPP, which supplies NADPH for transformation of GSSG to GSH, contributing to the elimination of excess ROS [10, 33]. Overproduction of ROS due to I/R could trigger the compensatory increase in G6PD expression to maintain more supplies of NAPDH to fight against ROS-induced oxidative stress [25, 34, 35]. Additionally, over-expression of G6PD increases the levels of NADPH and GSH, and suppresses ROS-induced oxidative stress, which is accompanied by the amelioration of neurological deficit and the decrease of infarct volume [25]. Similarly, we not only found wide location of G6PD in the neurons and astrocytes and but also observed gradually increased expression of G6PD after I/R. In addition, inhibition of CaMKIIα also decreased the ratio of p-CaMKIIα/CaMKIIα and up-regulated the expression of G6PD in cerebral ischemic rats. Therefore, we concluded that inhibition of CaMKIIα might increase G6PD expression and exerted obvious anti-oxidant effects following I/R.

In summary, this study showed that CaMKIIα inhibition can alleviate neurological deficit and decrease infarct volume through sparking G6PD-induced anti-oxidant effects. The findings provide further insight into the mechanism by which CaMKIIα inhibition exerts its neuroprotection and suggest that exploitation of CaMKIIα inhibitors might be implicated for the final aim of treating patients after stroke to prevent ROS and subsequent damage. There are some limitations in the present study. Firstly, CaMKIIα-involved oxidative stress is quite complicated and G6PD is not the only downstream target of CaMKIIα. Whether other molecules are implicated into this process remains to be studied further. Meanwhile, how CaMKIIα affects the G6PD expression and whether other molecules may exist in the CaMKIIα-mediated expression of the G6PD also need further studies. Even so, this study provides a new target involved in oxidative stress, which can be further broaden to study the prevention and treatment of stroke.

Compliance with Ethical Standards

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

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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