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ORIGINAL ARTICLE

Protective Effect of Mitogen- and Stress-Activated Protein Kinase on the Rats with Focal Ischemia-Reperfusion Injury

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Abstract—Mitogen- and stress-activated protein kinase (MSK) is a recently identified nuclear cAMP-regulated enhancer B (CREB) and histone H3 kinase that responds to both mitogen- and stress-activated protein kinases. This study was designed to investigate the protective effect of MSK on the rats with focal ischemia-reperfusion injury. The rat model was established by inserting thread into the middle cerebral artery. The protein expression was measured by immunoblotting. The localization of MSK was measured by immunofluorescence assay. Highly-differentiated pheochromocytoma 12 (PC12) is used as a sympathetic neuron-like cell line and treated with glutamate to induce neurotoxicity. MSK was knocked down and overexpressed by siRNA and MSK over-expressing vector, respectively. The cell viability was measured by cell counting kit (CCK-8) assay. The coronal sections were isolated and stained with 2, 3, 5-triphenyltetrazolium chloride (TTC) to determine infarct volume. Finally, astrocytes were separated from cerebral cortexes of normal rats to analyze the effects of MSK on inflammatory response. In the rats with focal ischemia-reperfusion injury, the expression of MSK was reduced, reaching the lowest level at 3 d after ischemia-reperfusion, and then recovered gradually. MSK was found mainly localized in neurons and astrocytes. The expression levels of caspase-3, caspase-8, caspase-9, and INOS showed the opposite trend with respect to MSK. Further analysis showed that overexpression of MSK exerted a protective effect on glutamate-induced neurotoxicity through inhibiting apoptosis of PC12 cells, as well as decreased the infarct size in rat with focal ischemia-reperfusion injury. On the

Highlights

1. Expression and localization of MSK in rats with focal ischemia-reperfusion injury
2. MSK suppressed neuron apoptosis *in vitro* and reduced ischemic brain injury *in vivo*.
3. MSK can repress the inflammatory response induced by LPS.

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contrary, knockdown of MSK showed opposite results. Finally, MSK suppressed LPS-induced inflammatory response by decreasing the expression of inducible nitric oxide synthase (INOS) and increasing the expression of interleukin-10 (IL-10) in astrocytes from cerebral cortexes of normal rats. In conclusion, MSK exerted a protective effect on rat with focal ischemia-reperfusion injury through its anti-apoptotic effect on neurons and anti-inflammatory effect on astrocytes.

KEY WORDS: cerebral ischemia-reperfusion; mitogen- and stress-activated protein kinase (MSK); neuronal apoptosis; astrocytes.

INTRODUCTION

Ischemic cerebrovascular disease is one of the three major diseases that leads to human death and is a common disease in neurology, neurosurgery, cardiovasology, and cardiovascular surgery, with a high disability and death rate [23, 27]. Thus, it is urgent to understand the underlying pathogenesis of ischemic cerebrovascular disease and develop effective drugs. Ischemia can cause brain damage and restoration of blood supply, which finally leads to further injury [6]. Recent studies have shown that ischemia reperfusion is correlated with radicals, overloading Ca^{2+}, excitatory amino acid (EAA), apoptosis, inflammatory response, and so on [29].

Among them, the important effects of apoptosis and inflammatory response on cerebral ischemia-reperfusion injury have attracted increasing attention. In reperfusion after cerebral ischemia, oxidative stress and sustained depolarization lead to mitochondria damage and open mitochondrial permeability transition. Mitochondria releases CytC and apoptosis-inducing factor (AIF), initiates the apoptotic signal pathway and causes apoptosis of neurons, which is the most critical factor directly affecting the prognosis of the patients [16, 28]. Thus, exploring neuronal apoptosis-related factors may offer new treatment options for cerebral ischemia patients [32]. After cerebral ischemia, radicals and other factors activate the inflammatory factor and enzymes, which lead to release of chemokines and upregulation of adhesion molecules. The interactions between leukocytes and endothelial cells induce leukocytes adhesion to the vascular endothelial cells and obstruct microvasculars, and thus lead to microvascular occlusion named “no flow” phenomenon [1]. Aggregated cells release oxygen free radicals and proteolytic enzyme directly damage endothelial cells and lead to the damage of the blood brain barrier, secondary brain edema, cerebral hemorrhage and injury of neurons. In addition, activated leukocytes accumulate in the capillaries, release a large number of inflammatory mediators and cytokines, recruit more neutrophils and aggravate the inflammatory response. Thus, study on the molecular mechanism on the inhibition of apoptosis and inflammation may provide new approaches for the treatment of cerebral ischemia patients.

Mitogen- and stress-activated protein kinase (MSK), is a recently identified nuclear cAMP-regulated enhancer B (CREB) and histone H3 kinase that responds to both mitogen- and stress-activated protein kinases [5], which contains two protein kinase domains in a single polypeptide [2]. It was reported that phosphorylated Bad, one member of Bcl-2 family related to cell apoptosis, was significantly reduced in MSK knockout cells, indicating that MSK may affect cell apoptosis through affecting the phosphorylation of serine 112 of Bad [2]. In addition, inhibition of MSK activity significantly reduced cell death induced by lack of Mg^{2+} in MSK knockout mice. Furthermore, p38 MAPK–MSK pathway can affect Mn^{2+}-induced death of B cell lymphoma, and inhibition of MSK activity can significantly suppress the Mn^{2+}-induced activation of caspase-8 [21]. These investigations suggested the important role of MSK in inhibiting apoptosis. However, whether MSK can also inhibit apoptosis of neurons and play a role in the brain ischemia reperfusion is unclear.

The role of MSK in the inflammatory response has also been widely studied. MSK can activate the transcription of NF-κB and CREB, two important genes in regulating inflammatory responses [21]. Corticosteroids can translocate MSK from nucleus to cytoplasm, which abolishes the anti-inflammatory ability of MSK [3]. In macrophages, MSK also participates in the negative feedback path to prevent excessive inflammatory reaction. Lipopolysaccharide (LPS) can induce the expression of DUSP1 and IL-10 [10]. However, in cells without MSK expression, LPS does not induce expression of DUSP1 and IL-10, indicating that MSK affects the inflammatory response through affecting the expression of inflammatory factors. These results totally suggested that MSK plays an important role in the immune system through affecting

expression of inflammatory factor. However, whether MSK also plays a role in inflammatory response after cerebral ischemia-reperfusion is unclear.

To explore the role of MSK in focal ischemia-reperfusion injury, this study used Western blotting and immunofluorescence technologies to analyze the expression and location of MSK in the rat model of cerebral ischemia-reperfusion. Furthermore, we explored the effects of MSK on the apoptosis of neurons and inflammatory response of astrocytes using *in vitro* cultured well-differentiated PC12 cells and primary astrocytes, respectively. In addition, we further detected the effect of MSK on focal ischemia-reperfusion injury *in vivo*.

MATERIALS AND METHODS

Experimental Animals

Healthy adult male Sprague-Dawley (SD) rats (weighing 250–300 g, aging 3 to 4 months) were provided by the Experimental Animal Center of Nantong University School of Medicine (Jiangsu, China) and housed under diurnal lighting conditions (12-h light–dark cycle). All experimental protocols and animal handling procedures were performed in accordance with the Experimental Animal Ethics Committee of Nantong University.

Models

The cerebral ischemia reperfusion animal model (MCAO/R) was developed as described previously. Briefly, the animals were intraperitoneally anesthetized with 2% pentobarbital sodium (2 mL/100 g). Briefly, the left common carotid artery was exposed at the level of the external and internal carotid artery bifurcation. A 4–0 surgical monofilament nylon suture (Prodo Co., Ltd., Tokyo, Japan) with a blunted tip was carefully inserted into the internal carotid artery and advanced into the anterior cerebral artery to occlude the middle cerebral artery. After occluding the middle cerebral artery for 2 h, the nylon suture was removed to restore blood flow (reperfusion) for 24 h of reperfusion. Rats in the sham group underwent the surgery without suture insertion. The rats were placed in cages for recovery after incision closure, with free access to food and water. Body temperature was maintained at 37 °C using a heating pad and air conditioner during the whole procedure. After 22 h of reperfusion, neurologic deficit was evaluated. Then, three rats were randomly selected from each group, and then cortex tissues were collected and stored at − 80 °C. The whole brain tissues were collected from the other mice of each group. Neurologic score was recorded according to a method described previously [20]. Briefly, the neurologic findings were scored using a 5-point scale, which are as follows: no neurologic deficit = score 0; failure to extend forepaw fully = score 1; circling to the left = score 2; falling to the left or no spontaneous motor activity = score 3; and do not walk spontaneously and have a depressed level of consciousness = score 4. The rats with scores 0–3 were recruited into the experimental group.

Protein Extraction and Western Blotting

The right parietal lobe cortex was homogenized and lysed in a RIPA buffer (Beyotime, Shanghai, China), and then centrifuged at 12000*g* for 15 min, and the supernatant was collected for western blot analysis. The total protein content of the supernatant was determined using a BCA protein assay kit (Beyotime, Shanghai, China). Protein extracts (50 μg) were separated using SDS-PAGE and transferred to polyvinylidene fluoride membranes (Amersham, Piscataway, NJ). The membranes were immunoblotted with primary antibodies specific for MSK, caspase-3, INOS, IL-10 and GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) overnight. Secondary antibodies conjugated to horseradish peroxidase (1:1000) were then applied for 1 h. The bands were visualized using enhanced chemiluminescence (ECL) and detected using the LAS imaging software (Fuji, New York, NY).

Immunofluorescence

Tissues were fixed using 4% paraformaldehyde for 4 h. Ten-μm-thick slides were used for immunofluorescence. The slides were blocked with normal goat serum (Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. Antibody against NeuN (neural cell nuclear antigen) was from Chemicon. Antibodies against CNPase and GFAP (glial fibrillary acidic protein) were from Sigma. Antibody against CD11b was from Serotec. Secondary antibodies tagged with FITC or TRITC were from Jackson.

Cell Culture and Transfection

Primary astrocytes were collected from SD rats born 1–3 days, and cultured in DMEM/F12 medium. Well-

differentiated PC12 cells were cultured in DMEM medium. Vector expressing MSK and MSK siRNA were provided by the immune microbiology laboratory, Nantong University School of Medicine. Vector or siRNA was transfected into cells using lipofectamine 2000 (Invitrogene).

Cell Morphological Changes

The morphological change of PC12 cells that transfected with MSK siRNA-3 and MSK over-expressing vector were assessed *via* staining with Hoechst 33258. The PC12 cells were seeded into 6-well plates at a final concentration of 1×10^5 cells/mL for 24 h. Then, cells were immobilized with 4% paraformaldehyde for 10 min at 4 °C and stained with Hoechst 33258 for 10 min in the dark. A fluorescence microscope (Leica, Germany) was used to observe the morphological changes of apoptotic cells.

Flow Cytometry

PC12 cells were seeded in 6-well plates (1×10^5 cells/mL) and cultured for 24 h, then transfected with MSK siRNA-3 or MSK over-expressing vector for 48 h. PC12 cells were digested with trypsin (non-EDTA), then suspended in 500 μl binding buffer and stained with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) for 15 min at room temperature in the dark. BD FACS Canto II flow cytometer was used to detect the samples.

Measurement of Infarct Volume

The rats brains were rapidly removed, and 2% 2,3, 5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) was used to stain the 2 mm coronal sections at 37 °C with 4% formalin for overnight. Infarct volume was measured by an image processing and analysis system (1.25 × objective, Q570IW, Germany) and calculated by the integration of infarct area on each brain section along the rostral-caudal axis and finally expressed as a percentage of whole-brain volume.

Statistical Analysis

All data were presented as mean ± SD. Statistical significance was analyzed using one-way analysis of variance (ANOVA) and *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Expression and Localization of MSK in Cortexes from Rats with Focal Ischemia-Reperfusion Injury

The protein level of MSK was detected in the cortexes of normal rats (N), rats in sham group and ischemia-reperfusion rats, respectively. As shown in Fig. 1A, the protein level of MSK was significantly decreased in rats with focal ischemia-reperfusion injury. However, the protein level of MSK was increased at 5-days after reperfusion, and returned to a high level that similar to that in normal rats at 14-days after reperfusion.

In order to explore the role of MSK after ischemia-reperfusion, we detected the localization of MSK in cortexes from normal rats and rats with focal ischemia-reperfusion injury by immunofluorescence at 3-days after reperfusion. NeuN, CD11b, CNPase and GFAP were used as markers for neuron, microglia, oligodendrocytes and astrocytes, respectively. As shown in Fig. 1B, MSK was mainly localized in neurons and astrocytes both in normal rats and rats with focal ischemia-reperfusion injury, indicating that MSK may be related to the biological behavior of neurons and astrocytes.

The Protein Expression Levels of Cleaved Caspase-3, -8, -9, and INOS in Rats with Focal Ischemia-Reperfusion Injury

It is well-known that neuron apoptosis occurred in the early stage of ischemia-reperfusion injury, and astrocytes were activated in the late stage. Based on the results that MSK was mainly located in neurons and astrocytes and expression level of MSK was lower in the early stage of ischemia-reperfusion, we hypothesized that MSK might be associated with neuron apoptosis. Caspase activation is a hallmark of apoptosis and induction of apoptosis involved sequential activation of caspases-8, -9, and, -3. Thus, the expression levels of cleaved caspase-3, -8, and -9 in the cortexes from rats with ischemia-reperfusion were detected by Western blotting. As shown in Fig. 2 A–D, the protein levels of caspases-8, -9, and -3 were significantly increased in rats with focal ischemia-reperfusion injury as compared with the Sham group. However, the protein levels of caspases-8, -9, and -3 were decreased at 5 days after reperfusion, and returned to a low level that similar to that in normal rats at 14 days after reperfusion.

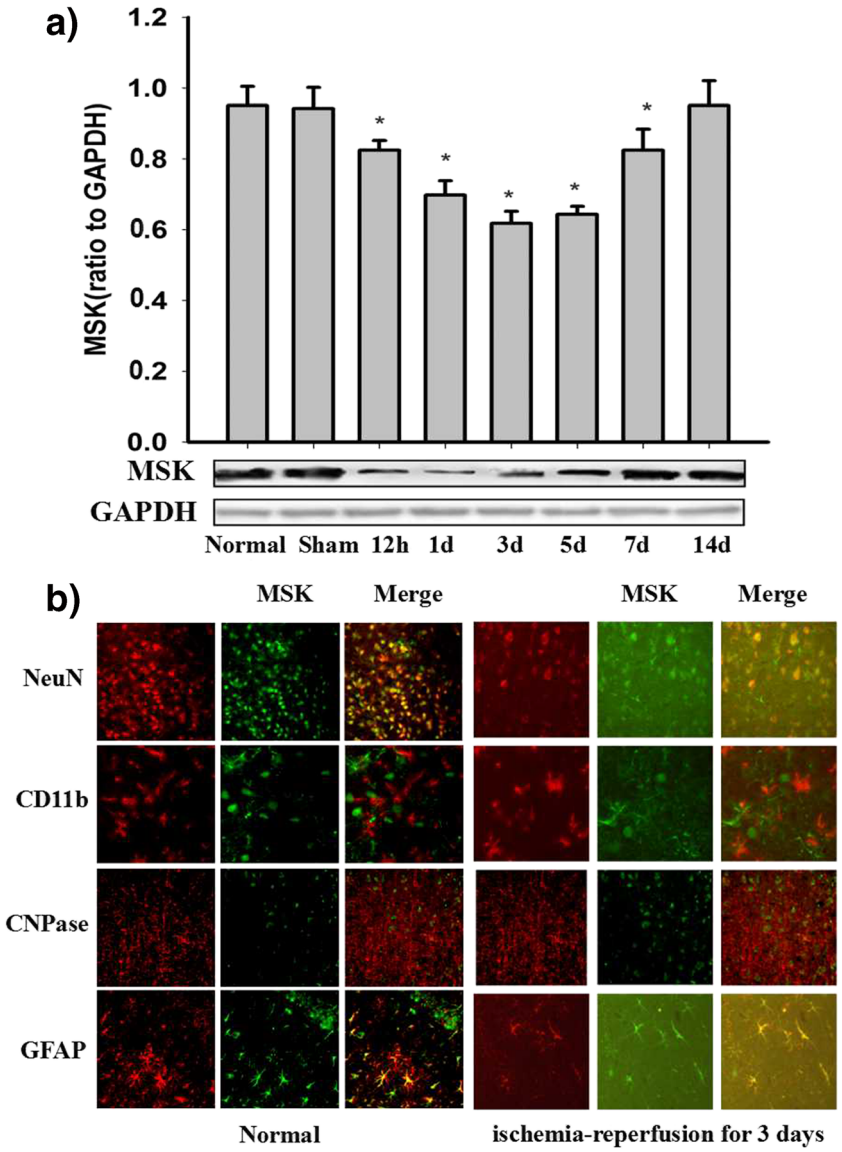

Fig. 1. Expression and localization of MSK in cortexes from rats with focal ischemia-reperfusion injury. **a** Western blotting was used to detect the protein expression of MSK in the cortex tissues from normal rats, sham rats, and rats with focal ischemia-reperfusion injury 12 h, 1 day, 3 days, 5 days, 7 days, and 14 days after ischemia-reperfusion. Data were presented as mean ± SD ($n = 6$). *$P < 0.05$ *versus* normal group. **b** Immunofluorescence technique was used to determine the location of MSK in cortex of normal rat (left) and rat after ischemia-reperfusion for 3 days (right).

Moreover, since the expression level of MSK was increased in the late stage of ischemia-reperfusion, we also hypothesized that MSK might be associated with the activation of astrocytes. Brain inflammatory response can be triggered by the activation of microglial cells and astrocytes in response to various injury of the central nervous system [25]. INOS is a key regulator in the inflammatory response and activated by a variety of pro-inflammatory cytokines [12]. Thus, we detected the expression level of INOS in the cortexes of rats after ischemia-reperfusion. As shown in Fig. 2E, the protein level of INOS was significantly increased in rats with focal ischemia-reperfusion injury as compared with the normal group. However, the protein level of INOS was decreased at 5 days after reperfusion, and returned to a

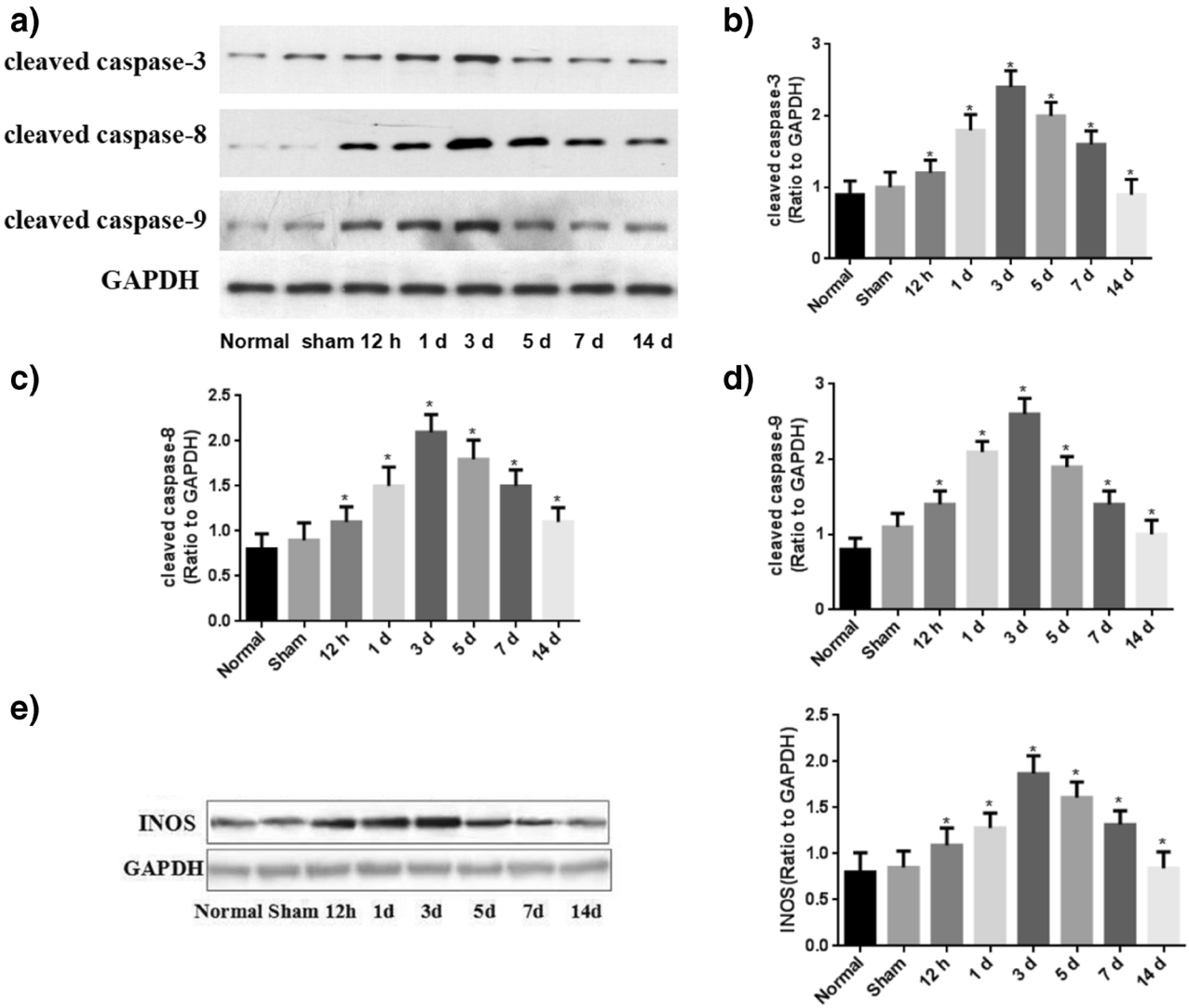

Fig. 2. The protein expression levels of cleaved caspase-3, -8, -9, and INOS in rats with focal ischemia-reperfusion injury. **a** Western blotting was used to detect the protein expression levels of cleaved caspase-3, -8, and -9 in the cortex tissues from normal rats, sham rats, and rats with focal ischemia-reperfusion injury at 12 h, 1 day, 3 days, 5 days, 7 days, and 14 days after ischemia-reperfusion. **b** Quantification on the protein expression of cleaved caspase-3. **c** Quantification on the protein expression of cleaved caspase-8. **d** Quantification on the protein expression of cleaved caspase-9. **e** Western blotting was used to detect the protein expression of INOS in the cortex tissues from normal rats, sham rats, and rats with focal ischemia-reperfusion injury 12 h, 1 day, 3 days, 5 days, 7 days, and 14 days after ischemia-reperfusion. Data were presented as mean ± SD ($n = 6$). *P < 0.05 *versus* normal group.

low level that similar to that in normal rats at 14 days after reperfusion. Interestingly, the trends on the expression levels of caspases-8, -9, -3, and INOS were opposite to Fig. 1A.

Taken together, these data suggest that MSK might be correlated with the apoptosis of neurons in the early stage of ischemia-reperfusion, and correlated with the inflammatory response of astrocytes in the middle-late stage of ischemia-reperfusion.

MSK Suppressed Neuron Apoptosis *In Vitro* and Reduced Ischemic Brain Injury *In Vivo*

Highly-differentiated PC12 is a sympathetic neuron-like cell line [14], which was used here to explore the role of MSK in the apoptosis of neurons *in vitro*. PC12 cells were incubated with different concentrations of glutamate, and the cell viability was detected by CCK-8 kit. As shown in Fig. 3A, the viability of PC12 cells was significantly

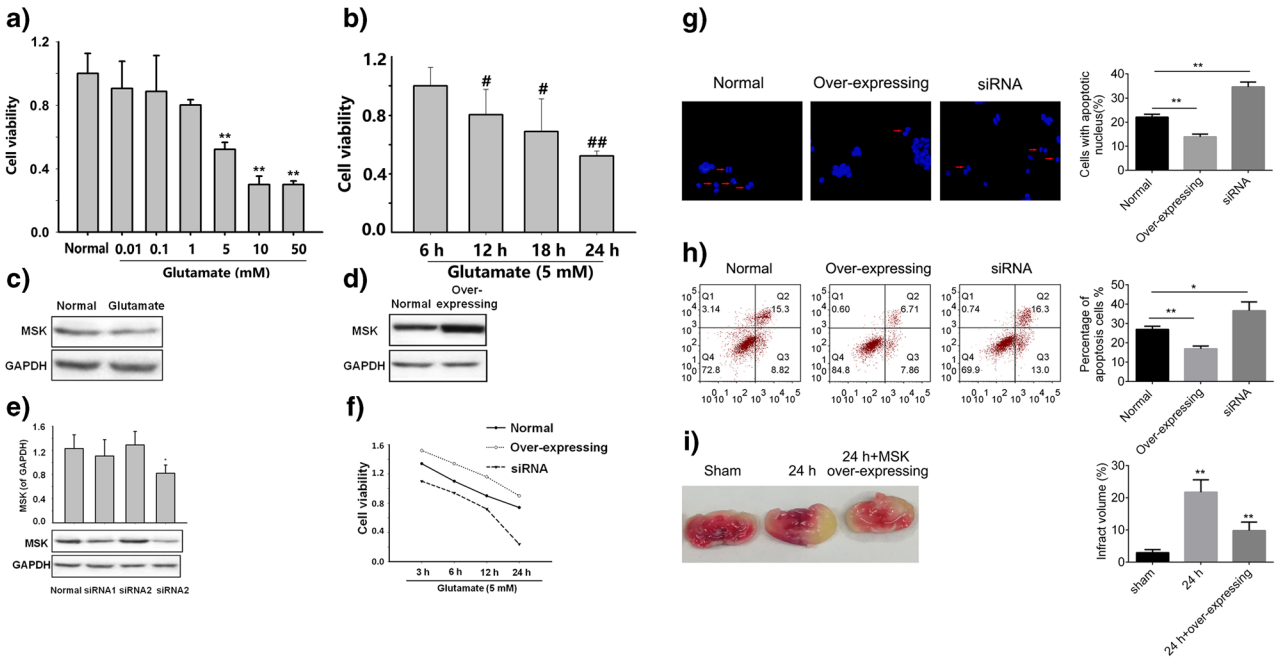

Fig. 3. MSK suppressed neuron apoptosis *in vitro* and reduced ischemic brain injury *in vivo*. **a** The viability of PC12 cells that were treated with different concentrations of glutamates. Cell viability was detected by CCK-8 kit. **b** The viability of PC12 cells that were treated with 5 mM glutamate for 6 h, 12 h, 18 h, and 24 h. Cell viability was detected by CCK-8 kit. **c** Western blotting was used to detect the protein expression of MSK in PC12 cells without (normal) or with glutamate treatment. **d** Western blotting was used to detect the protein expression of MSK in PC12 cells that were transfected with MSK overexpressing vector. **e** Western blotting was used to detect the protein expression of MSK in PC12 cells that were transfected with siRNAs againt MSK. **f** The viability of PC12 cells that were transfected with MSK overexpressing vector or siRNA against MSK. **g** The morphological changes in PC12 cells that were transfected with MSK overexpressing vector or siRNA against MSK, which was detected by fluorescence microscopy. Hoechst 33258 was used to stain the nuclear. **h** PC12 cells were transfected with MSK overexpressing vector or siRNA against MSK for 48 h, the percentage of apoptotic cells was determined using Annexin V-FITC and PI. **i** Representative images of TTC staining in the coronal sections from rats with focal ischemia-reperfusion injury 24 h after ischemia-reperfusion. Data were presented as mean ± SD ($n = 6$). $^{*}P < 0.05$ *versus* normal group, $^{\#}P < 0.05$ *versus* 6 h group.

decreased after glutamate treatment in a concentration-dependent manner. Then, we treated PC12 cells with 5 mM of glutamate for 6, 12, 18, and 24 h, and the viability of cells were respectively detected. As shown in Fig. 3B, the viability of PC12 cells was significantly decreased after glutamate treatment in a time-dependent manner. To explore whether MSK expression level was changed in PC12 cells after glutamate treatment, we detected the expression level of MSK by Western blotting and found that the protein level of MSK was significantly decreased after glutamate stimulation (Fig. 3C).

MSK was knocked down and overexpressed by siRNA and MSK over-expressing vector to investigate the effect of MSK on the viability of PC12 cells after glutamate treatment. After three generation of laboratory culture, MSK over-expressing vector EGFP-N_2-MSK and MSK siRNA were transfected into PC12 cells, respectively. The expression level of MSK was significantly higher in the cells that transfected with MSK over-expressing vector than normal cells (Fig. 3D). On the contrary, the expression level of MSK was significantly decreased in cells transfected with MSK siRNA-3 than normal cells, which was used in the following experiments (Fig. 3E).

Cells were then incubated with 5 mM of glutamate and the viability of cells was detected at 3, 6, 12, and 24 h after glutamate treatment. As shown in Fig. 3F, over-expression of MSK partially reversed the glutamate-induced reduction in viability of PC12 cells, but MSK siRNA aggravated glutamate-induced reduction in viability of PC12 cells.

The fluorescence microscopy was observed at 48 h after PC12 cells were transfected with MSK siRNA-3 or MSK over-expressing vector. The results showed that MSK over-expression decreased the number of apoptotic cells, and knockdown of MSK increased the apoptosis of PC12 cells, accompanied with nuclear chromatin

condensation (Fig. 3G). The flow cytometry assay showed that the apoptotic rate of MSK over-expressing group was less than that of normal group, while the MSK siRNA group exhibited significant increased rate of apoptotic cells as compared to that of normal group (Fig. 3H). These results were consistent with the CCK-8 assay and Hoechst staining, demonstrating that MSK exerted a protective effect on glutamate-induced neurotoxicity through mechanisms related to anti-apoptosis ofPC12 cells.

Rats were euthanized at 24 h after focal ischemia-reperfusion injury, and brain tissues were isolated immediately and the coronal sections were stained with TTC to determine infarct volume. As shown in Fig. 3I, the infarct volume of rats in the MSK over-expressing group was significantly decreased as compared with Sham rats. Infarct volume is one of the common indexes for evaluating the extent of ischemic brain injury after focal cerebral ischemia [19]. These results suggest that MSK may play an important role in reducing ischemic brain injury in rats with focal ischemia-reperfusion injury.

MSK Suppressed LPS-Induced Inflammatory Response of Astrocytes from Cerebral Cortexes of Normal Rats

Astrocytes were separated from cerebral cortexes of normal rats, cultured, and treated with different concentrations of LPS (0.001, 0.01, 0.1, 1 and 10 μg/mL). Then the expression levels of INOS and IL-10 were detected by Western blotting. As shown in Fig. 4 A and B, the expression levels of INOS and IL-10 were induced by LPS in a dose-dependent manner. MSK siRNA and MSK overexpressing vector were transfected into astrocytes, respectively. Astrocytes were treated with LPS (1 μg/mL) for 24 h and the expression levels of INOS and IL-10 were detected. As shown in Fig. 4 C and D, overexpression of MSK significantly decreased the expression level of INOS and increased the expression level of IL-10, suggesting that MSK repressed the inflammatory response induced by LPS.

DISCUSSION

Ischemic brain injury is the most common cerebral vascular disease [7]. Many studies have shown that the apoptosis of neurons and inflammatory response are two main factors for cerebral ischemic injury [33]. To illustrate the protective role of MSK in the ischemic injury, the expression level and localization of MSK was firstly detected in rat model of focal ischemia-reperfusion injury. And the effects of MSK on the apoptosis of neurons and inflammatory responses of astrocytes were analyzed.

MSK, a recently identified nuclear cAMP response element-binding protein and histone H3 kinase [5], was mainly localized in lung, placenta, and brain tissues [24], but its role in these tissues is still unclear. Our results showed that the protein level of MSK was significantly gradually decreased in rats with focal ischemia-reperfusion injury. However, its level increased 5 days after reperfusion, and returned to a high level that similar to that in normal rats 14 days after reperfusion. The results suggested that MSK may play a potential role during ischemia-reperfusion. Previous study showed that the phosphorylation of MSK1 increased after ischemia in isolated adult cardiac myocytes [5]. Another study showed that endothelin-1, used to induce model of transient cerebral ischemia, increased the expression of phospho-MSK1 in the subgranular zone of the dentate gyrus [22].

Further analysis showed that MSK was mainly located in neurons and astrocytes, indicating MSK may exert functions in these two types of cells. In consistent with our results, previous study showed that MSK1 is expressed at highest levels in striatal and olfactory tubercle neurons [11]. Another study suggested that MSK1 mRNA and protein levels gradually declined, reaching the lowest point at 3 days, and increased thereafter in a subarachnoid hemorrhage model [26] and an acute spinal cord injury model [18]. Besides, the expression of active caspase-3 was negatively correlated with MSK1 level and they were colocalizated in neurons and astrocytes [26]. Similarly, our study also detected the protein levels of cleaved caspase-3, -8, and -9, and found that their protein expressions were increased after reperfusion for 12 h, reached a peak at 3-h time point, and then decreased. Furthermore, it seems that the expression levels of cleaved caspase-3, -8, and -9 were negatively correlated with MSK level. In addition, we also found that the expression of INOS level was also negatively correlated with that of MSK.

Focal ischemia-reperfusion involves two-phase injury, including an initial acute ischemic insult and subsequent inflammatory reperfusion injury [8, 17]. Astrocytes exposed to certain inflammatory stimulants and activated astrocytes are detrimental to neighboring neurons in neuroinflammatory processes [8]. Combined our investigations with these conclusions, we thus hypothesized that MSK has anti-apoptotic effects on neurons and anti-inflammatory role in astrocytes. Well-differentiated PC12 have characteristics similar to neurons, which were used in this study. Glutamate successfully induced the

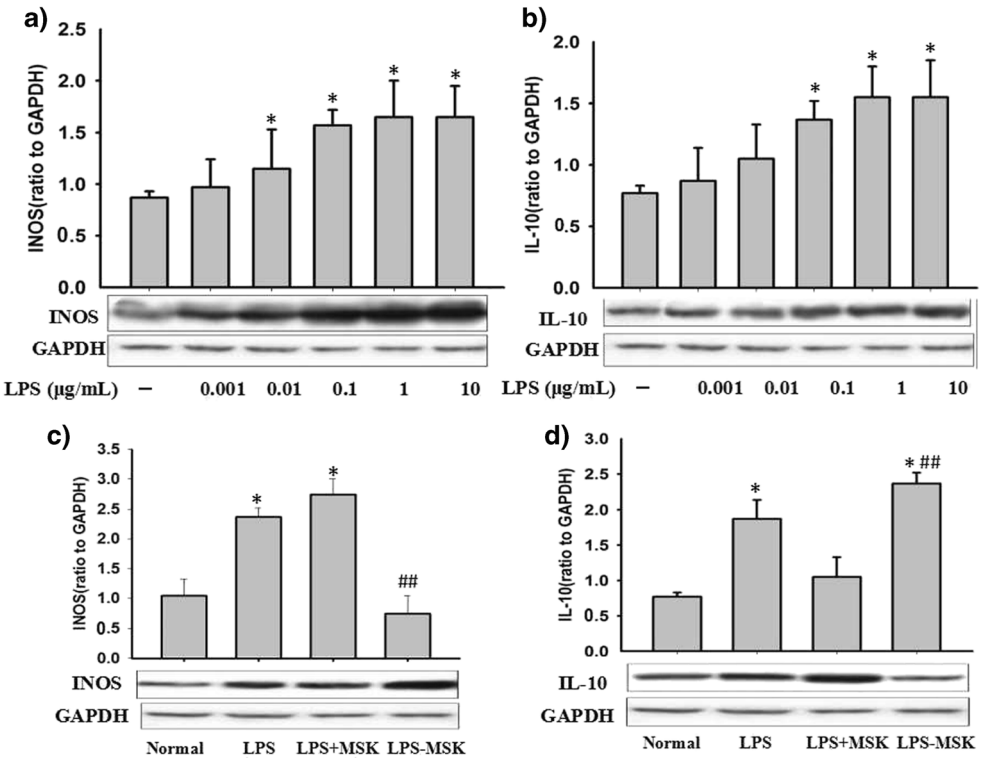

Fig. 4. MSK and suppressed LPS-induced inflammatory response of astrocytes from cerebral cortexes of normal rats. **a**, **b** Western blotting was used to detect the protein expressions of INOS and IL-10 in primary astrocytes that were treated with different concentrations of LPS. **c**, **d** Western blotting was used to detect the protein expressions of INOS and IL-10 in LPS treated primary astrocytes that were transfected with MSK overexpression vector. Data were presented as mean ± SD ($n = 6$). $^{*}P < 0.05$ *versus* normal group. $^{\#\#}P < 0.01$ *versus* LPS+MSK group.

neurotoxicity on PC12 cells. However, MSK exerted a protective effect on glutamate-induced neurotoxicity through mechanisms related to anti-apoptosis of PC12 cells. We also proved that overexpression of MSK decreased the infarct volume of rats with focal ischemia-reperfusion injury *in vivo*. These results indicated that MSK might protect brain from injury through inhibiting the apoptosis of neurons and reducing ischemic brain injury after ischemia-reperfusion.

In order to explore the role of MSK in astrocytes, we treated primary astrocytes with LPS and detected the protein levels of inflammatory factor INOS and anti-inflammatory factor IL-10. The results showed that the levels of INOS and IL-10 were positively induced by LPS, suggesting the inflammatory response to LPS in astrocytes. siRNA of MSK further enhanced the expression level of INOS induced by LPS and reduced the expression of IL-10 induced by LPS, while overexpression of MSK showed contrary results, indicating that MSK may exert an anti-inflammatory effect through reducing the expression of inflammatory cytokines and enhancing anti-inflammatory cytokines.

Taken together, these results verified our primary hypothesis. Bo *et al.* [26] indicated that MSK1 is related to astrocytic and neuronal apoptosis following subarachnoid hemorrhage in rats. Karelina *et al.* [13] found that MSK1/2 paly an important role in regulating ischemia-induced progenitor cell proliferation and neurogenesis. Previous study indicated that MSK1induced NF-κB by the chemokine CX3CL1 in microglial cells, which are essential mediators of neuroinflammatory processes [9]. Vermeulen *et al.* also proved that MSK plays an important role in the inflammatory process. MSK regulates the inflammatory response mainly through two inflammation related pathways of NF-κB and CREB, and plays an anti-inflammatory role through phosphorylation of Histone H3 [30]. *In vitro* studies have shown that MSK is mainly activated by MAPK2/ERK2 and/or SAPK2/p38 signaling

pathways [4]. MSK can also be activated by ultraviolet, oxidation, and chemical stress in addition to growth factors and phorbol ester [15]. Besides, MSK1/2 can tightly regulate the transactivational potential of numerous transcription factors [5, 31]. It seems that MSK is involved in complex signaling pathway and participates in cell growth, differentiation, apoptosis, inflammation and autophagy and so on. Therefore, more deep investigations should be performed to explore the various and complex roles of MSK in focal ischemia-reperfusion injury.

In conclusion, this study confirmed that MSK could inhibit the apoptosis of neurons and suppress the inflammatory response of astrocytes and thus had protective effects on the brain after ischemia-reperfusion. A clearer understanding of the role of MSK in mediating recovery of ischemia-reperfusion may ultimately help guide the development of novel therapeutic options.

AUTHOR CONTRIBUTION STATEMENT

Yanfeng Zhou: conception and design, collection, and assembly of results. Guangzhong Gao: analysis of the data and drafting the paper. Xuedong Li and Lin Jiang: revising the article critically for important intellectual content.

COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of Interest. The authors declare that they have no conflict of interest.

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