European Review for Medical and Pharmacological Sciences 2019; 23: 7674-7683

Denitrosylation of nNOS induced by cerebral ischemia-reperfusion contributes to nitrosylation of CaMKII and its inhibition of autophosphorylation in hippocampal CA1

L.-M. YU, T.-Y. ZHANG, X.-H. YIN, Q. YANG, F. LU, J.-Z. YAN, C. LI

Research Center of Biochemistry and Molecular Biology and Jiangsu Key Laboratory of Brain Disease Bioinformation, Xuzhou Medical University, Xuzhou, Jiangsu, China

Li-Mei Yu and *Tai-Yu Zhang Yu* contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study is to investigate the relation between CaMKII S-nitrosylation and its activation, as well as the underlying mechanism, after global cerebral ischemia-reperfusion.

MATERIALS AND METHODS: The rat model of cerebral ischemia-reperfusion was established by four-vessel occlusion of 15 min and reperfusion of different times. nNOS inhibitor 7-nitroindazole (7-NI), exogenous nitric oxide donor GSNO (nitrosoglutathione), or N-methyl-D-aspartate receptor (NMDAR) antagonist MK-801 were administered before ischemia. The expressions of S-nitrosylation and phosphorylation of CaMKII and nNOS were detected by biotin switch assay, immunoblotting, and immunohistochemical staining after cerebral ischemia-reperfusion. The survival of hippocampal CA1 pyramidal cells after administration of the three drugs was examined by cresyl violet staining.

RESULTS: Following cerebral ischemia-reperfusion, the S-nitrosylation of CaMKII was increased, accompanied by a decrease of phosphorylation, suggesting a decrease of activity ($p<0.05$). Meanwhile, the phosphorylation and S-nitrosylation of nNOS were notably decreased at the same time point ($p<0.05$). The administration of 7-NI, GSNO, and MK-801 increased the S-nitrosylation and phosphorylation of nNOS, leading to the attenuation of increased S-nitrosylation and decreased autophosphorylation of CaMKII after cerebral ischemia-reperfusion ($p<0.05$). Administration of MK-801, GSNO, and 7-NI significantly decreased the neuronal damage in rat hippocampal CA1 caused by cerebral ischemia-reperfusion ($p<0.05$).

CONCLUSIONS: After cerebral ischemia-reperfusion, the decrease of autophosphorylation of CaMKII regulated by its S-nitrosylation may be due to the denitrosylation of nNOS and subsequent NO production. Increasing the phosphorylation of CaMKII by nNOS inhibitor, exogenous NO donor or NMDA receptor antagonist exerted neuroprotective effects against cerebral ischemia-reperfusion injury.

Key Words:
S-nitrosylation, CaMKII, nNOS, Cerebral ischemia-reperfusion.

Introduction

Ca^{2+}/calmodulin-dependent protein kinase (CaMK) family is critical to Ca^{2+} signal transduction. This family includes kinases such as CaMKI, CaMKII, and CaMKIV. CaMKII is a ubiquitous Ser/Thr specific protein kinase which is highly enriched in the central nervous system (CNS)[1]. CaMKII is a holoenzyme composed of 12 monomers, which are primary α and β subunits in neurons[2]. The α and β isoforms are especially abundant in the brain, accounting for 2% of the total protein in the hippocampus[3]. The functional domains of the kinase are attached by stalk-like appendages to a gear-shaped core, grouped into two clusters of six. Each subunit contains a catalytic, an autoregulatory, and an association domain[4].

CaMKII is known to be regulated by autophosphorylation, which increases its Ca^{2+}-independent activity[5]. Research[6,7] suggests that the S-nitrosylation of CaMKII could occur in the brain, Cys6 is a site of S-nitrosylation on CaMKII, and nitric oxide (NO) is a potent inhibitor of CaMKII through its Cys6 S-nitrosylation. NO production is reduced by phosphorylation of nNOS at

Corresponding Author: Chong Li, Ph.D; e-mail: lichong_xzhmu@sina.com

Ser847, which limits the activity of nNOS[8,9]. This nNOS phosphorylation appears to be catalyzed by $CaM{\cdot}Ca^{2+}$. Similarly, the S-nitrosylation of nNOS inhibits the activity of nNOS and decreases NO generation[10,11]. Endogenous NO derives from nNOS during ischemia-reperfusion in the brain. There are three NO synthase (NOS) isoforms in tissues of mammals. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are calcium-dependent, while inducible NOS (iNOS) is calcium-independent under most circumstances[12,13]. nNOS is regulated primarily by the influx of Ca^{2+} through receptor-dependent channels, particularly following postsynaptic stimulation of NMDA receptors by glutamate[14,15]. Small amounts of NO benefit synaptic plasticity and neuronal signaling, whereas the overproduction of NO produces specific toxic effects, such as cerebral ischemia-reperfusion injury, which is mainly through protein S-nitrosylation[16]. Protein S-nitrosylation is the interaction of reactive nitrogen oxides with cysteine sulfhydryls, i.e., a guanylate cyclase-independent and redox-dependent posttranslational modification leading to an altered protein function.

Our studies have found that the activity of CaMKII was decreased during cerebral ischemia-reperfusion, but the underlying mechanism is not clear. According to the previous reports[17], CaMKII can be S-nitrosylated, leading to the decrease of its activity.

Therefore, we suppose that during ischemia-reperfusion, CaMKII can be S-nitrosylated by nNOS-derived NO, leading to the decreased phosphorylation of CaMKII and CaMKII activity. In this work, we detected the S-nitrosylation and phosphorylation of CaMKII at different time points after cerebral ischemia-reperfusion. nNOS inhibitor 7-NI, exogenous NO donor GSNO (nitrosoglutathione), and NMDA receptors antagonist MK-801 were used to clarify the correlation between CaMKII S-nitrosylation and nNOS.

Materials and Methods

Antibody and Reagents

The following primary antibodies were used. Anti-CaMKIIα monoclonal was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-p-CaMKIIα (Thr286) was obtained from Promega (Madison, WI, USA). Anti-nNOS antibody (No. 4234) was obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-p-nNOS (Ser847; ab16650) was purchased from Abcam (Cambridge, MA, USA). The secondary antibody used in our experiments was goat anti-rabbit IgG, which was obtained from Sigma-Aldrich (St. Louis, MO, USA). The drugs GSNO, 7-NI, DTT, MK-801, and other chemicals used in our experiments were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animal Model

Adult male Sprague-Dawley rats weighing 250-300 g were selected for a four-vessel occlusion (4-VO) global cerebral ischemia-reperfusion, which was induced as described previously[18]. Briefly, with animals under anesthesia by inhaling isoflurane, vertebral arteries were electro cauterized, and the common carotid arteries were exposed. Rats were allowed to recover for 24 h and fasted overnight. On the following day, cerebral ischemia was induced by occluding the common carotid arteries with arterial clips for 15 min. Rats who lost their righting reflex within 30 s and whose pupils were dilated and unresponsive to light during ischemia were selected for the investigations. Rectal temperature was maintained at 36.5-37.5°C during ischemia via a temperature-regulated heating pad. Sham group rats were treated by the same surgical procedures, except for the common carotid arteries occlusion. All animal protocols were in line with the institutional guidelines, and the procedures were approved by the Animal Ethics Committee of Xuzhou Medical University.

Administration of Drugs

The rats were injected with GSNO dissolved in 0.9% NaCl at a dose of 100 ug/kg. The first injection was given 20 min before ischemia. 7-NI (25 mg/kg) dissolved in 1% dimethyl sulfoxide (DMSO) was intraperitoneally 20 min before ischemia. DTT, dissolved in 0.9% NaCl at a dose of 10 mmol/L (mM), was administered intracerebroventricularly (10 μl; bregma: 1.5 mm lateral, 0.8 mm posterior, 3.8 mm deep). MK-801 (a selective NMDA receptor antagonist)[19] was dissolved in saline at a concentration of 3 mg/kg and intraperitoneally injected 1 h before ischemia. Control rats were intraperitoneally or intracerebroventricularly given corresponding solvent, 0.9% NaCl or 1% dimethyl sulfoxide (DMSO).

Sample Preparation

Rats were decapitated immediately at different time points of reperfusion. Then, hippocampal CA1 were isolated and rapidly frozen in liquid ni-

trogen. The hippocampal tissue samples were homogenized in an ice-cold homogenization buffer containing 50 mM MOPS (pH 7.4), 100 mM KCl, 320 mM sucrose, 0.5 mM $MgCl_2$, 0.2 mM dithiothreitol (free when S-nitrosylation were tested), 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM EGTA, 1 mM Na_3VO_4, 20 mM sodium pyrophosphate, 20 mM β-phosphoglycerol, 1 mM p-nitrophenyl phosphate, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 5 μg/ml pepstatin A. The homogenates were centrifuged at 1000×g for 15 min at 4°C. Supernatants were collected, and protein concentration was determined by the method of Lowry et al[20]. Samples were stored at –80°C until used.

Determination of protein S-Nitrosylation

The measurement of S-nitrosylated nNOS or CaMKII was performed by immunoprecipitation or by the biotin switch assay with anti-nNOS or anti-CaMKIIα antibody. The biotin switch assay was performed as described previously by Jaffrey et al[21], using low-light conditions and opaque tubes. Briefly, the hippocamps were homogenized in the HEN buffer (250 mM HEPES-NaOH, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine), not containing sodium dodecyl sulfate (SDS). Free thiols were blocked by methylation with methyl methanethiosulfonate. Then, unreacted methyl methanethiosulfonate was removed by protein precipitation in 10 volumes of acetone (–20°C). Cysteine residues that had been S-nitrosylated were converted to free thiols with sodium ascorbate (1 mM). The free thiols were biotinylated with biotin-hexyl pyridyldithiopropionamide (HPDP) at 25°C for 1 h. Thus, the S-nitrosylated cysteines were switched for biotin. In some reaction mixtures, biotin-HPDP was omitted as a negative control. Proteins were precipitated by chilled acetone, while the pellet was resuspended in HENS buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 10 μM neocuproine, 1% SDS). After 2 volumes of neutralization buffer (20 mM HEPES, pH 7.7, 1 mM EDTA, 100 mM NaCl, 0.5% Triton X-100) were added, the samples were then modified with biotin in the following buffer (25 mM HEPES, pH 7.7, 0.1 mM EDTA, 1% SDS, 10 μM neocuproine, 10 mM ascorbic acid sodium salt, and 0.2 mM biotin-HPDP). After free biotin-HPDP was removed by cold acetone precipitation, biotinylated proteins were absorbed to streptavidin-agarose. Streptavidin absorbates were eluted by β-mercaptoethanol (100 mM) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting.

Western Blot

The protein was separated on polyacrylamide gels and then electrotransferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK). After being blocked for 2 h with 3% bovine serum albumin (BSA) in Tris-Buffered Saline and Tween-20 (TBST), the membranes were incubated with primary antibodies at 4°C overnight. Membranes were then incubated with secondary antibodies (l: 5000) at room temperature for 1 h, and the enhanced chemiluminescence system (ECL) was used for detection.

Histology and Immunohistochemistry

Rats were anesthetized and then underwent transcardial perfusion with 0.9% saline, followed by 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS). Brains were removed, post-fixed overnight in paraformaldehyde, gradient dehydration, and embedded in paraffin. Coronal brain sections (6 μm thick) were cut on a microtome (RM2155; Leica, Nußloch, Germany). Sections were deparaffinized in xylene and rehydrated in a gradient of ethanol and distilled water. The sections were stained with cresyl violet and examined by a light microscope.

Immunohistochemistry requires antigen repair in citrate buffer (pH 6.0, 10 mM) at a 95°C high temperature for 20 min. To block endogenous peroxidase activity, sections were incubated for 30 min in 1% hydrogen peroxide (H_2O_2). To reduce nonspecific staining, sections were incubated for 2 h in a blocking solution containing 1% BSA, 2% normal goat serum, 0.3% Triton X-100. After treating with the Anti-p-CaMKIIα antibodies (1: 50) at 4°C for 1 day, these sections were incubated for 2 h in biotinylated goat anti-rabbit secondary antibody (1: 200) and then incubated with avidin-conjugated horseradish peroxidase (HRP) for 1 h at 37°C, followed by avidin-biotin-peroxidase substrate at room temperature. The sections were examined by a light microscope.

Statistical Analysis

Values were expressed as the means ± standard deviation and were obtained from at least four independent rats. Statistical analysis of the results was carried out by one-way analysis of variance

(ANOVA), followed by Duncan's new multiple range method or the Newman-Keuls test. $p<0.05$ was considered significant.

Results

S-Nitrosylation and Phosphorylation of CaMKII and nNOS after Different Time of Cerebral Ischemia and Reperfusion

To explore whether CaMKII can be S-nitrosylated during cerebral ischemia-reperfusion and whether this S-nitrosylation will change with reperfusion time, we established four-vessel occlusion model in rats to induce the global cerebral ischemia for 15 min and reperfusion for 5, 15 or 30 min. Immunoblotting showed that after cerebral ischemia-reperfusion, CaMKII could be S-nitrosylated; the S-nitrosylation of CaMKII began to increase at 5 min and reached its highest level at 30 min ($p<0.05$), while the phosphorylation of CaMKII presented an opposite trend ($p<0.05$) (Figures 1A, B). These results suggest that the activity of CaMKII decreases after cerebral ischemia-reperfusion, probably due to its S-nitrosylation.

During cerebral ischemia-reperfusion, endogenous nitric oxide (NO) mainly comes from nNOS. We speculated that the S-nitrosylation of CaMKII may be mediated by NO produced by nNOS. Results showed that after cerebral ischemia and reperfusion, the S-nitrosylation of nNOS did not change significantly at 5 min, but decreased from 15 min to the lowest at 30 min ($p<0.05$), while the phosphorylation of nNOS decreased gradually from 5 min to 30 min ($p<0.05$) (Figures 1C, D). These results suggest that after cerebral ischemia and reperfusion, the activity of nNOS increases, which may lead to an increase of S-nitrosylation of CaMKII.

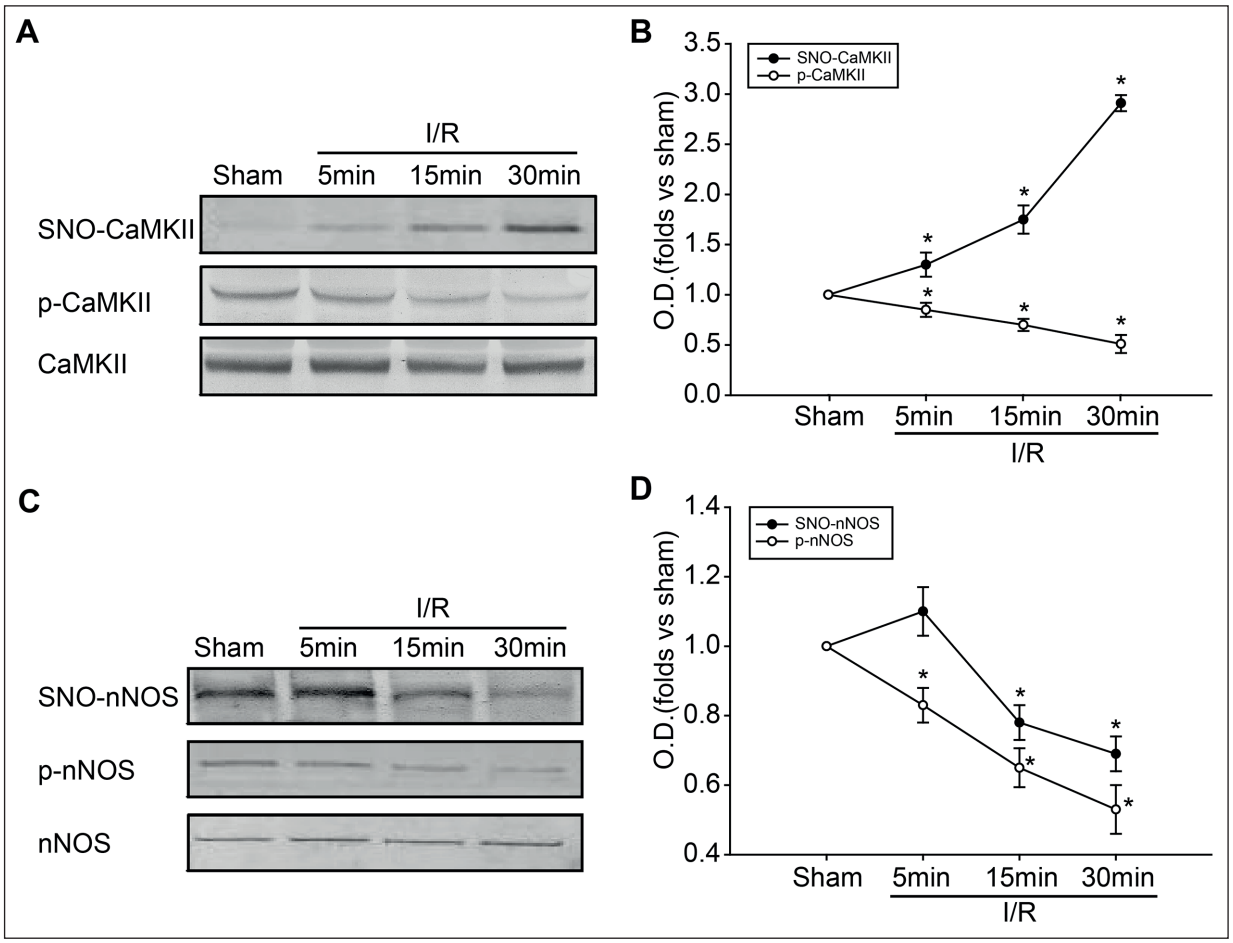

Figure 1. S-nitrosylation and phosphorylation of CaMKII and nNOS after different time of cerebral ischemia and reperfusion. ***A, B,*** S-nitrosylation and phosphorylation of CaMKII. ***C, D,*** S-nitrosylation and phosphorylation of nNOS. n=4. *$p<0.05$ *vs.* Sham.

Effects of nNOS Inhibitor and Exogenous NO Donor on S-nitrosylation and Phosphorylation of CaMKII

Based on the above experimental results, we chose 30 min of reperfusion as the time observation point to further explore the correlation between the S-nitrosylation of CaMKII and the activity of nNOS. Rats were administrated with exogenous NO donor GSNO to increase the S-nitrosylation of nNOS or with selective nNOS inhibitor (7-NI) to inhibit the activity of nNOS before ischemia. As shown in Figure 2, after cerebral ischemia-reperfusion, GSNO and 7-NI increased the S-nitrosylation and phosphorylation of nNOS ($p<0.05$), and decreased the S-nitrosylation of CaMKII which led to the increase of phosphorylation of CaMKII ($p<0.05$). Moreover, DTT can counteract the effect of GSNO ($p<0.05$).

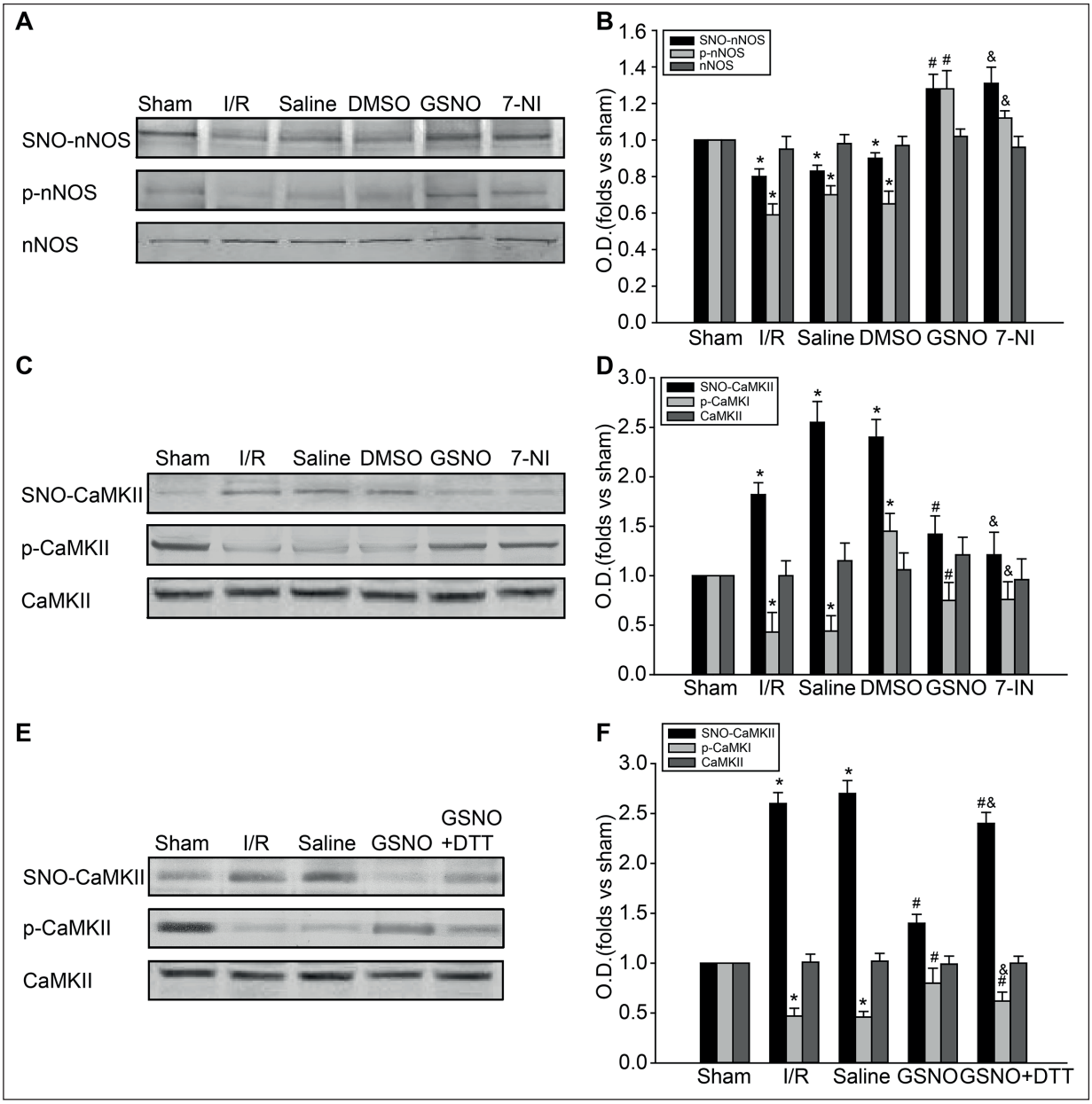

Figure 2. Effects of nNOS inhibitor and exogenous NO donor on S-nitrosylation and phosphorylation of CaMKII. ***A, B,*** S-nitrosylation and phosphorylation of nNOS. ***C, D, E, F,*** S-nitrosylation and phosphorylation of CaMKII. n=4. $^{*}p<0.05$ *vs.* Sham, $^{\#}p<0.05$ *vs.* Saline, $^{\&}p<0.05$ *vs.* DMSO (B, D), $^{\&}p<0.05$ *vs.* GSNO (F).

Effects of NMDA Receptor Antagonist on S-Nitrosylation and Phosphorylation of CaMKII

Previous studies have shown that after cerebral ischemia-reperfusion, glutamate mediates the complex ternary assembling of NMDA, postsynaptic density protein 95 (PSD95) and nNOS, leading to the translocation of nNOS from the plasma membrane to the cytoplasm, the dephosphorylation of nNOS and nitric oxide (NO) production. We speculate that inhibiting the assembly of this module will also affect the production of endogenous NO, as well as the S-nitrosylation of CaMKII. As shown in Figure 3, the NMDA receptor antagonist (MK-801) increased the S-nitrosylation and phosphorylation of nNOS ($p<0.05$), decreased the S-nitrosylation, and increased the phosphorylation of CaMKII ($p<0.05$). The results of immunohistochemistry further confirmed GSNO, 7-NI, and MK-801 could increase the phosphorylation of CaMKII (Figure 4).

Inhibiting the S-Nitrosylation of CaMKII has Neuroprotective Effect on Cerebral Ischemia-Reperfusion Injury

To study the effect of inhibiting the decrease of CaMKII activity on the cerebral ischemia-reperfusion injury, cresyl violet staining was used to detect the survival of pyramidal cells in the hippocampal CAl region. Round and light-stained cells were counted as normal cells, while those with shrinkage and nuclear condensation were counted as dead cells. As shown in Figure 5, 15 min of ischemia and 5 days of reperfusion resulted in severe neuronal death in hippocampal CAl in rats. Compared with vehicle group of saline or DMSO, MK-801, GSNO, and 7-NI could significantly reduce neuronal death ($p<0.05$).

Discussion

According to the present results, CaMKII can be S-nitrosylated during cerebral ischemia-reper-

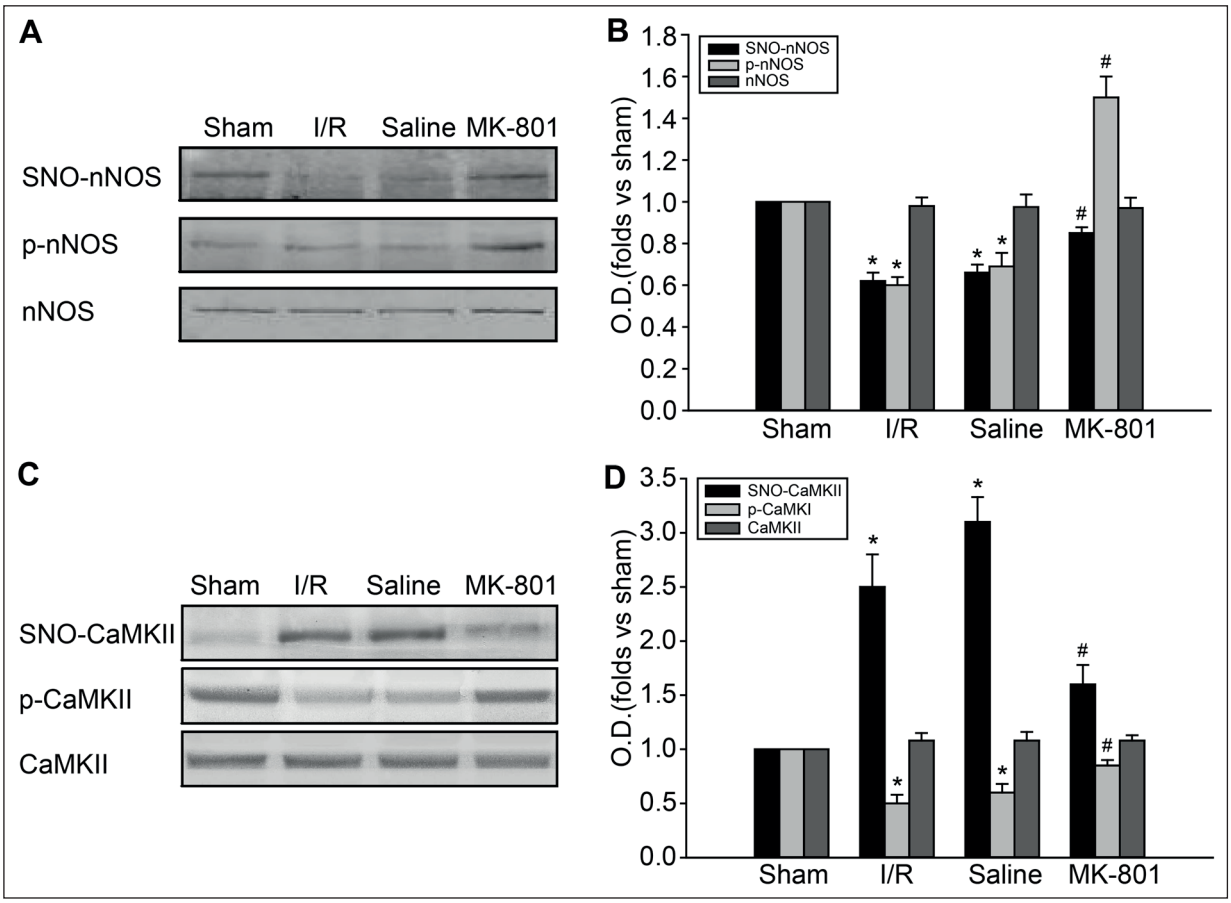

Figure 3. Effects of NMDA receptor antagonist on S-nitrosylation and phosphorylation of CaMKII. ***A, B,*** S-nitrosylation and phosphorylation of nNOS. ***C, D,*** S-nitrosylation and phosphorylation of CaMKII. n=4. *$p<0.05$ *vs.* Sham, $^{\#}p<0.05$ *vs.* Saline.

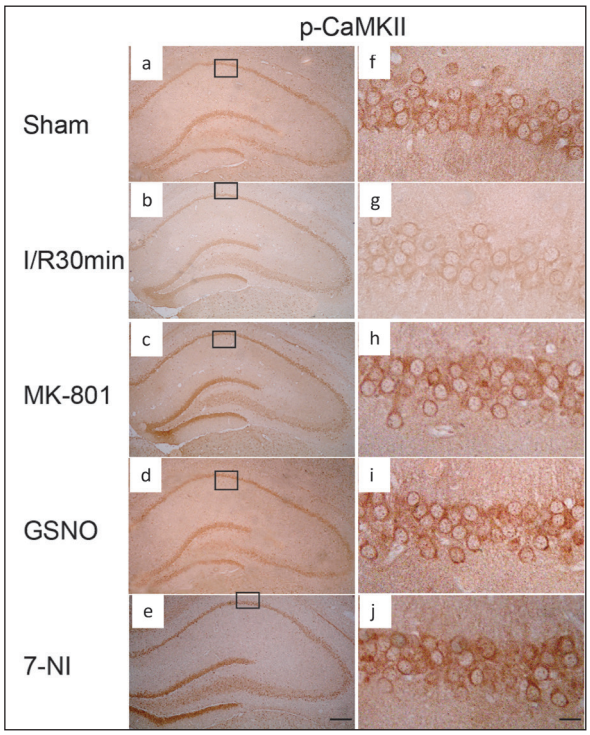

Figure 4. Immunohistochemical staining of phosphorylation of CaMKII in coronal sections of hippocampus and histological analysis of rats subjected to 15 min of ischemia followed by 5 days of reperfusion. Low magnification (40×; a, b, c, d, e): Scale bar =200 μm. High magnification (400×; f, g, h, i, j): Scale bar =20 μm. n=4.

fusion. The possible molecular mechanism is shown in Figure 6, which illustrates that after reperfusion, the increased level of Ca^{2+} leads to nNOS denitrosylation and activation, producing an excessive amount of NO. NO facilitates CaMKII S-nitrosylation and decreases CaMKII phosphorylation, thus inhibiting CaMKII activity. This is possibly mediated by the NMDAR●PSD95●nNOS signaling module. During cerebral ischemia-reperfusion, NMDA receptor can promote Ca^{2+} influx and nNOS activation, leading to the S-nitrosylation of CaMKII. Exogenous NO donor GSNO, nNOS inhibitor 7-NI, and the NMDA receptor antagonist MK-801 can reduce the S-nitrosylation of CaMKII. As a sulfhydry-reducing regent, DTT can reduce the disulfide bonds to free thiol groups. We demonstrated that DTT reversed the effect of GSNO on the S-nitrosylation and phosphorylation of CaMKII during ischemia-reperfusion. The reduced S-nitrosylation of CaMKII finally played a neuroprotective role in hippocampal CA1 neuron injury induced by cerebral ischemia-reperfusion.

Since endogenous NO derives from NOS during ischemia-reperfusion, in our work, we mainly focused on the Ca^{2+}-dependent nNOS. Researches[8,13,22] showed that the decreased phosphorylation of nNOS at Ser847 resulted in a reduction of its enzyme activity. Also, the S-nitrosylation of nNOS inhibits its activity[9]. In this study, we examined the time course of nNOS phosphorylation, as well as S-nitrosylation during cerebral ischemia-reperfusion. Results implied the increased nNOS activation and increased the production of NO at 30 min after reperfusion. Our results also showed that the S-nitrosylated CaMKII changed at different time points, which reached a peak at 30 min after reperfusion. These results implied that the S-nitrosylation of CaMKII was related to the activation of nNOS. Therefore, we selected 30 min after reperfusion to analyse the correlation between nNOS and CaMKII.

The exogenous NO donor GSNO enhanced the S-nitrosylation and phosphorylation of nNOS during ischemia-reperfusion, which inferred a decreased activation of nNOS and a reduction of endogenous NO production. Thus, did the nNOS inhibitor 7-NI and the NMDA receptor antagonist MK-801. These results suggest that NO donor, nNOS inhibitor, and NMDA receptor antagonist might lead to the reduction of the S-nitrosylation of CaMKII by increasing the S-nitrosylation and phosphorylation of nNOS, inhibiting nNOS activity and reducing the production of NO.

The previous reports[23-26] found that after global ischemia in rat hippocampus, the phosphorylation of nNOS at Ser847 was catalyzed by CaMKII, which was dependent on Ca^{2+} and CaM. The phosphorylation of nNOS at Ser847 in the nervous system indicated a down-regulation of nNOS activity[7,8], which inferred that CaMKII could phosphorylate nNOS and inhibit its activation. However, our results showed the opposite situation. During cerebral ischemia-reperfusion, the nNOS activity was not inhibited but rather activated, quickly leading to CaMKII S-nitrosylation by nNOS derived NO. This phenomenon may be involved in the following mechanism: both nNOS and CaMKII are dependent on Ca^{2+} and CaM. However, due to the space location, the NMDAR●PSD95●nNOS signaling module makes nNOS closer to receptor channel than CaMKII, which may lead to a spatio-temporal difference between nNOS and CaMKII. Therefore, during cerebral ischemia-reperfusion, nNOS denitrosylation may

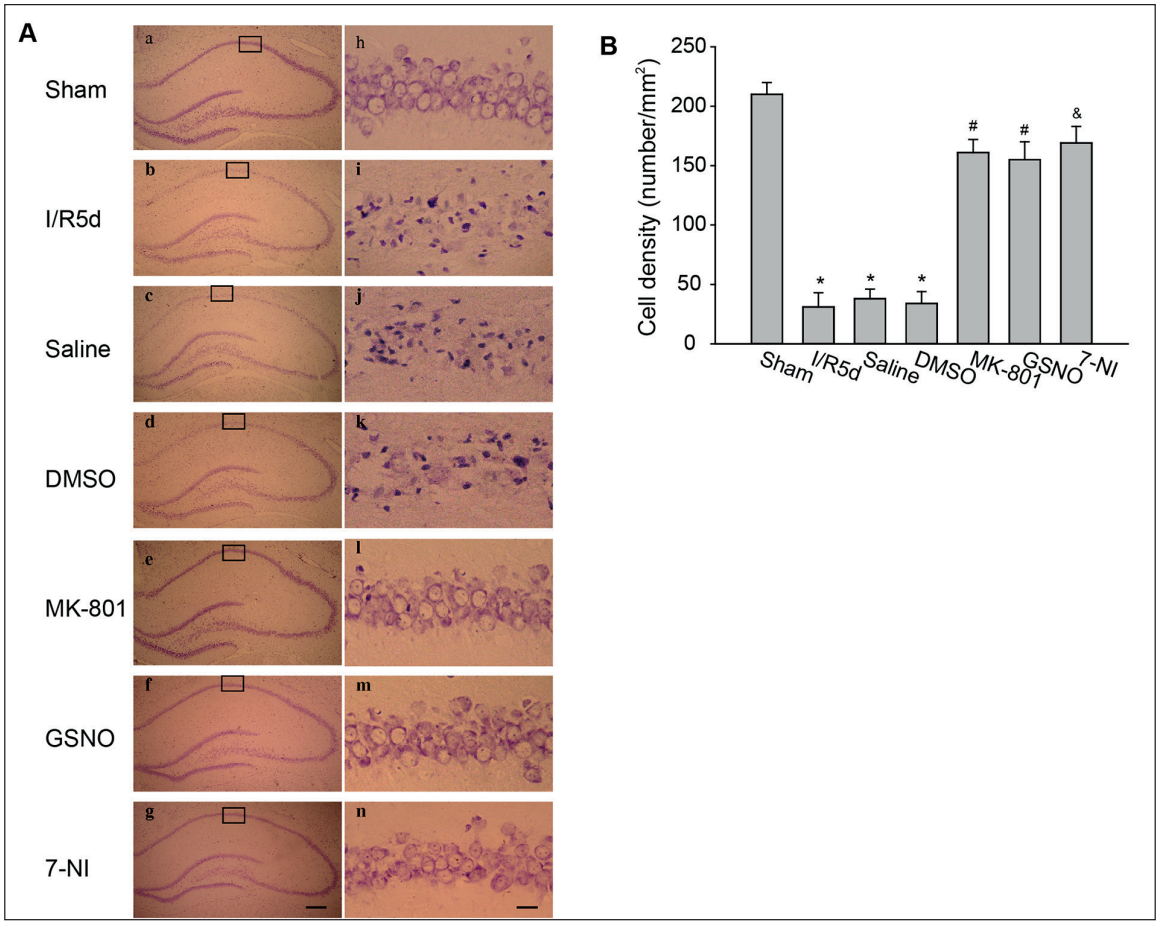

Figure 5. Cresyl violet staining of the survival of pyramidal cells in hippocampal CAl region after 15 min of ischemia and 5 days of reperfusion. ***A,*** Low magnification (40×; a, b, c, d, e, f, g): Scale bar =200 μm. High magnification (400×; h, i, j, k, l, m, n): Scale bar =20 μm. ***B,*** Cell density is expressed as the number of cells per 1 mm length of the CA1 pyramidal cells counted under a light microscope. n=4. *p<0.05 *vs.* Sham, $^{\#}p$<0.05 *vs.* Saline, $^{\&}p$<0.05 *vs.* DMSO.

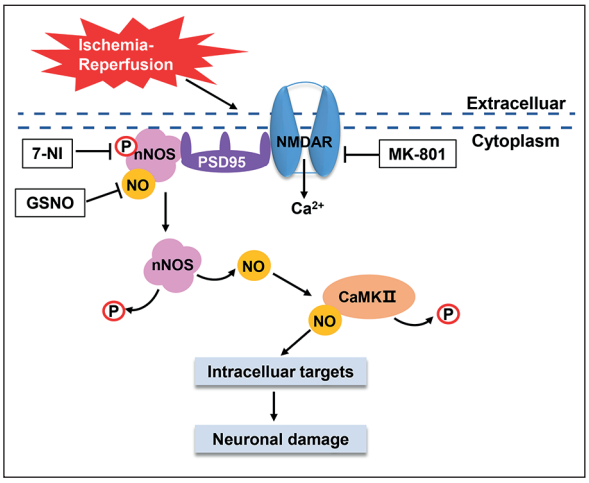

Figure 6. Schematic diagram providing a possible mechanism for the relationship between nNOS and CaMKII signaling pathway via S-nitrosylation induced during cerebral ischemia-reperfusion.

precede the phosphorylation of nNOS by CaMKII. Therefore, Ca^{2+} influx did not inhibit but increased the activity of nNOS. Once nNOS was activated, a large amount of NO was released, leading to an increase of S-nitrosylation of CaMKII and a decrease of CaMKII activity.

Finally, we found that NMDA receptor antagonist, exogenous NO donor, and nNOS inhibitor have neuroprotective effects in cerebral ischemia-reperfusion. This discovery may provide new targets and ideas for the treatment of ischemic stroke.

Conclusions

After cerebral ischemia-reperfusion, the decrease of autophosphorylation of CaMKII regulated by its S-nitrosylation might be due to the

denitrosylation of nNOS and subsequent NO production. Increasing the phosphorylation of CaMKII by nNOS inhibitor, exogenous NO donor or NMDA receptor antagonist exerted neuroprotective effects against the cerebral ischemia-reperfusion injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

This work was supported by grants from National Natural Science Foundation Youth Fund (81601070) and Blue Engineering Science and Technology Innovation Team and Major Natural Science Projects of the Jiangsu Ministry of Education (15KJA180009).

References

1) Braun AP, Schulman H. The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. Annu Rev Physiol 1995; 57: 417-445.
2) Soderling TR, Chang B, Brickey D. Cellular signaling through multifunctional Ca2+/calmodulin-dependent protein kinase II. J Biol Chem 2001; 276: 3719-3722.
3) Erondu NE, Kennedy MB. Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. J Neurosci 1985; 5: 3270-3277.
4) Hudmon A. Neuronal Ca2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. Annu Rev Biochem 2002; 71: 473-510.
5) Shamloo M, Kamme F, Wieloch T. Subcellular distribution and autophosphorylation of calcium/calmodulin-dependent protein kinase II-alpha in rat hippocampus in a model of ischemic tolerance. Neuroscience 2000; 96: 665-674.
6) Song T, Hatano N, Kambe T, Miyamoto Y, Ihara H, Yamamoto H, Sugimoto K, Kume K, Yamaguchi F, Tokuda M, Watanabe Y. Nitric oxide-mediated modulation of calcium/calmodulin-dependent protein kinase II. Biochem J 2008; 412: 223-231.
7) Song T, Hatano N, Sugimoto K, Horii M, Yamaguchi F, Tokuda M, Miyamoto Y, Kambe T, Watanabe Y. Nitric oxide prevents phosphorylation of neuronal nitric oxide synthase at serine1412 by inhibiting the Akt/PKB and CaM-K II signaling pathways. Int J Mol Med 2012; 30: 15-20.
8) Hayashi Y, Nishio M, Naito Y, Yokokura H, Nimura Y, Hidaka H, Watanabe Y. Regulation of neuronal nitric-oxide synthase by calmodulin kinases. J Biol Chem 1999; 274: 20597-20602.
9) Komeima K, Hayashi Y, Naito Y, Watanabe Y. Inhibition of neuronal nitric-oxide synthase by calcium/ calmodulin-dependent protein kinase IIalpha through Ser847 phosphorylation in NG108-15 neuronal cells. J Biol Chem 2000; 275: 28139-28143.
10) Abu-Soud HM, Wang J, Rousseau DL, Fukuto JM, Ignarro LJ, Stuehr DJ. Neuronal nitric oxide synthase self-inactivates by forming a ferrous-nitrosyl complex during aerobic catalysis. J Biol Chem 1995; 270: 22997-23006.
11) Gorren AC, Schrammel A, Riethmüller C, Schmidt K, Koesling D, Werner ER, Mayer B. Nitric oxide-induced autoinhibition of neuronal nitric oxide synthase in the presence of the autoxidation-resistant pteridine 5-methyltetrahydrobiopterin. Biochem J 2000; 347: 475-484.
12) Bredt DS, Snyder SH. Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem 1994; 63: 175-195.
13) Marletta MA. Nitric oxide synthase: aspects concerning structure and catalysis. Cell 1994; 78: 927-930.
14) Garthwaite J, Garthwaite G, Palmer RM, Moncada S. NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. Eur J Pharmacol 1989; 172: 413-416.
15) Rameau GA, Chiu LY, Ziff EB. Bidirectional regulation of neuronal nitric-oxide synthase phosphorylation at serine 847 by the N-methyl-D-aspartate receptor. J Biol Chem 2004; 279: 14307-14314.
16) Hu SQ, Ye JS, Zong YY, Sun CC, Liu DH, Wu YP, Song T, Zhang GY. S-nitrosylation of mixed lineage kinase 3 contributes to its activation after cerebral ischemia. J Biol Chem 2012; 287: 2364-2377
17) Song T, Hatano N, Kambe T, Miyamoto Y, Ihara H, Yamamoto H, Sugimoto K, Kume K, Yamaguchi F, Tokuda M, Watanabe Y. Nitric oxide-mediated modulation of calcium/calmodulin-dependent protein kinase II. Biochem J 2008; 412: 223-231.
18) Pulsinelli WA, Brierley JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 1979; 10: 267-272.
19) Yanamoto H, Miyamoto S, Tohnai N, Nagata I, Xue JH, Nakano Y, Nakajo Y, Kikuchi H. Induced spreading depression activates persistent neurogenesis in the subventricular zone, generating cells with markers for divided and early committed neurons in the caudate putamen and cortex. Stroke 2005; 36: 1544-1550.
20) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.
21) Jaffrey SR, Erdjument-Bromage H, Ferris CD, Tempst P, Snyder SH. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nat Cell Biol 2001; 3: 193-197.
22) Rameau GA, Tukey DS, Garcin-Hosfield ED, Titcombe RF, Misra C, Khatri L, Getzoff ED, Ziff EB. Biphasic coupling of neuronal nitric oxide synthase phosphorylation to the NMDA receptor regulates AM-

PA receptor trafficking and neuronal cell death. J Neurosci 2007; 27: 3445-3455.

23) Osuka K, Watanabe Y, Usuda N, Nakazawa A, Fukunaga K, Miyamoto E, Takayasu M, Tokuda M, Yoshida J. Phosphorylation of neuronal nitric oxide synthase at Ser847 by CaM-KII in the hippocampus of rat brain after transient forebrain ischemia. J Cereb Blood Flow Metab 2002; 22: 1098-1106.

24) Watanabe Y, Song T, Sugimoto K, Horii M, Araki N, Tokumitsu H, Tezuka T, Yamamoto T, Tokuda M. Post-synaptic density-95 promotes calcium/calmodulin-dependent protein kinase II-mediated Ser847 phosphorylation of neuronal nitric oxide synthase. Biochem J 2003; 372: 465-471.

25) Yan XB, Meng FJ, Song B, Zhang GY. Brain ischemia induces serine phosphorylation of neuronal nitric oxide synthase by Ca(2+)/calmodulin-dependent protein kinase II in rat hippocampus. Acta Pharmacol Sin 2004; 25: 617-622.

26) Song T, Hatano N, Horii M, Tokumitsu H, Yamaguchi F, Tokuda M, Watanabe Y. Calcium/calmodulin-dependent protein kinase I inhibits neuronal nitric-oxide synthase activity through serine 741 phosphorylation. FEBS Lett 2004; 570: 133-137.