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ENDOGENOUS NITRIC OXIDE INDUCES ACTIVATION OF APOPTOSIS SIGNAL-REGULATING KINASE 1 VIA S-NITROSYLATION IN RAT HIPPOCAMPUS DURING CEREBRAL ISCHEMIA–REPERFUSION

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Abstract—Apoptosis signal-regulating kinase 1 (ASK1) is a general mediator of cell death in response to a variety of stimuli, including reactive oxygen species, tumor necrosis factor α, lipopolysaccharide, endoplasmic reticulum stress, calcium influx and ischemia. Here we reported ASK1 was activated by nitric oxide (NO) through S-nitrosylation during cerebral ischemia–reperfusion. The reagents that abrogate neuronal nitric oxide synthase (nNOS) activity such as nNOS inhibitor 7NI and *N*-methyl-D-aspartate receptor antagonist MK801 prevented ASK1 activation via decreasing ASK1 S-nitrosylation. In HEK293 cells, over-expressed ASK1 could be S-nitrosylated by both exogenous and endogenous NO and Cys869 was identified as the site of ASK1 S-nitrosylation. S-nitrosylation increased the level of ASK1 phosphorylation at Thr845, which represents ASK1 activation. Our results further confirmed that S-nitrosylation led to the increment of ASK1 dimerization. S-nitrosylation of ASK1 also activated the downstream JNK signaling and JNK-mediated nucleic pathway. The exogenous NO (SNP and GSNO) reversed the effect of endogenous NO by suppressing S-nitrosylation of ASK1 and exerted neuroprotection during ischemia–reperfusion. These results suggest that inhibiting ASK1 S-nitrosylation may be a novel approach for stroke therapy. © **2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: cerebral ischemia, apoptosis signal-regulating kinase 1, S-nitrosylation, nitric oxide.

INTRODUCTION

Nitric oxide (NO) as an easily diffusible and highly reactive molecule has been linked to numerous physiological and pathophysiological events such as smooth-muscle relaxation, cellular proliferation, apoptosis, neurotransmitter release, neurotoxicity and differentiation (Tuteja et al., 2004; Wendehenne et al., 2004; Bove and van der Vliet, 2006; Cohen and Adachi, 2006; Garcia-Brugger et al., 2006; Moncada and Bolanos, 2006; Bethke et al., 2007). It was recognized early on that NO is generated by NO synthases. There are two constitutively expressed NOS isozymes, endothelial (eNOS) and neuronal NOS (nNOS), which is Ca^{2+}-responsive and control basal NO levels, and one Ca^{2+}-insensitive inducible NOS isozyme (iNOS) which is expressed in response to specific cytokines or bacterial products in mammals (Mayer and Hemmens, 1997; Moro et al., 2004).

In addition to forming guanosine 3′,5′-cyclic monophosphate (cGMP) by binding to heme at the active site of soluble guanylyl cyclase, NO modifies a variety of proteins via S-nitrosylation (Denninger and Marletta, 1999; Jaffrey et al., 2001). The term, S-nitrosylation, refers to the coupling of a NO moiety to the thiol group of reactive cysteine residues. This direct chemical reaction with proteins modified by NO occurs through a complex chemical mechanism without the assistance of enzymes (Stamler et al., 1992; Ahern et al., 2002). A substantial body of evidence supports that S-nitrosylation regulates numerous proteins' activity in response to NO under physiological or pathophysiological conditions such as *N*-methyl-D-aspartate receptor (NMDAR), thioredoxin (Trx), Bcl-2, caspase-3, c-jun N-terminal kinases (JNKs) and eNOS (Sheehy et al., 1998; Kim et al., 1999; Park et al., 2000; Sumbayev, 2003; Mitchell and Marletta, 2005; Azad et al., 2006; Mitchell et al., 2007).

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase (MAPK) family that activates both the MKK4/MKK7-JNK and MKK3/MKK6-p38 signaling cascades (Ichijo et al., 1997; Chang et al., 1998; Liu et al., 2000). ASK1 is a general

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Abbreviations: 7-NI, 7-nitroindazole; AMT, 2-amion-5,6-dihydro-6-methyl-4H-1,3-thiazine; ASK1, apoptosis signal-regulating kinase 1; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid disodium; eNOS, endothelial nitric oxide synthase; FasL, Fas ligand; GSNO, S-nitrosoglutathione; HPDP, biotin-hexyl pyridyldithiopropionamide; iNOS, inducible nitric oxide synthase; JNK, c-jun N-terminal kinase; MKK, mitogen-activated protein kinase kinase; MMTS, methyl methanethiosulfonate; NEM, *N*-ethylmaleimide; NMDAR, *N*-methyl-d-aspartate receptor; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PBST, phosphate-buffered saline with 0.1% Tween 20; PSD-95, postsynaptic density protein 95; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNP, sodium nitroprusside; Trx, thioredoxin.

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mediator of cell death in response to a variety of stimuli, including reactive oxygen species (ROS), tumor necrosis factor-α (TNFα), lipopolysaccharide (LPS), endoplasmic reticulum (ER) stress, calcium influx and ischemia (Tobiume et al., 2001; Nishitoh et al., 2002; Yamaguchi et al., 2003; Saadatzadeh et al., 2004; Matsuzawa et al., 2005; Wu et al., 2006). Oxidative stress induces phosphorylation of Thr845 in the activation loop of ASK1, which is correlated with ASK1 activity and ASK1-dependent apoptosis (Tobiume et al., 2002). On the other hand, Akt1 and ASK1 are physically associated. Phosphorylation at Ser83 triggered by Akt1 is related to a decrease in stimulated ASK1 kinase activity and promotes cell survival (Kim et al., 2001; Yano et al., 2001). ASK1 is regulated by physical interaction with proteins such as 14-3-3, Trx, glutathione S-transferase Mu, and p21 (Saitoh et al., 1998; Asada et al., 1999; Zhang et al., 1999; Cho et al., 2001; Ko et al., 2001). Park's study suggested that endogenous NO mediates the IFN-γ-induced suppression of ASK1 activation in macrophage cells by means of a thiol–redox mechanism (Park et al., 2004).

In the present study, we aimed to investigate whether NO could affect the activation of ASK1 via S-nitrosylation during cerebral ischemia–reperfusion and the possible mechanism of alteration of ASK1 activity triggered by S-nitrosylation. We also examined the effect of ASK1 S-nitrosylation in ischemic brain injury, which suggested a novel potential therapy for cerebral ischemia.

EXPERIMENTAL PROCEDURE

Antibodies and reagents

The following primary antibodies were used: anti-ASK1 (#3762), anti-P-ASK1 (Thr845, #3765), anti-p-ASK1 (Ser83, #3761), anti-p-MKK4 (#9156), anti-p-MKK7 (#4171), anti-MKK7 (#4172), anti-c-jun (#9162), anti-FasL (#4273) were acquired from Cell Signaling Biotechnology (Boston, MA, USA). Anti-p-JNKs (sc-6254), anti-p-c-jun (sc-16312), anti-Fas (sc-716) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-JNK3 antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). The secondary anti-mouse IgG (A 1682) or anti-rabbit IgG (T 6778) used in our experiments was bought from Sigma (St. Louis, MO, USA). BCIP and NBT were from Promega (Shanghai, China). nNOS AS (Wang et al., 2005): 5′-ACGTGTTCTCTTCCAT-3′, nNOS MS: 5′-TAAAGGGAGAA-CACGT-3′, were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). The drugs sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO), (*N*-ethylmaleimide) NEM, dithiothreitol (DTT), 7-nitroindazole (7-NI), 2-amion-5,6-dihydro-6-methyl-4H-1, 3-thiazine (AMT) and other chemicals used in our experiments were all acquired from Sigma unless indicated otherwise. Old GSNO solution was obtained by leaving the freshly prepared GSNO solution under a lamp (100 W) for 2 days at room temperature.

Site-directed mutagenesis of ASK1

Mutant ASK1 plasmid (pCDNA3-ASK1C869A) was constructed by using the pCDNA3-ASK1 plasmid as a template. The following sets of forward and reverse primers were used to introduce mutation at Cys869: 5′-CTGGTCTCTGGGCGGTACA-ATCATTGAAATGG-3′ (forward) and 5′-CCATTTCAATGATTG-TACCGCCCAGAGACCAG-3′ (reverse). The mutant plasmid was constructed by using the QuikChange II kit (Stratagene, La Jolla, CA, USA). Mutagenesis was confirmed by automated nucleotide sequencing (ABI 3730XL sequencer, Life Technologies, Carlsbad, CA, USA).

Cell culture and transfection

Hippocampal neurons from the fetal SD rat (18 days gestation) hippocampus were cultured. Briefly, fetal rats were anesthetized with ether and hippocampi were meticulously isolated in ice-cold high-glucose DMEM (h-DMEM, GibcorBRL, Grand Island, NY, USA). Hippocampal cells were dissociated by trypsinisation [0.25% (w/v) trypsin and 0.05% ethylenediamine tetraacetic acid disodium (EDTA) in Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution] at 37 °C for 15 min, followed by gentle triturating in plating medium (h-DMEM supplemented with 10% fetal bovine serum). Cells were seeded onto poly-L-lysine (Sigma, St. Louis, MO, USA)-coated 24-well plates at a density of 1×10^5 cells per cm^2 and incubated at 37 °C in 5% CO_2 atmosphere. Each well contains 0.3-ml medium. After 18–24 h, cells were incubated in Neurobasal Medium supplemented with 2% B-27 (GibcorBRL) and 2 mM glutamine and then half-replaced twice a week.

Human embryonic kidney (HEK) 293 cells, obtained from SGST.CN. CellBank, were maintained under an atmosphere of 5% CO_2 at 37 °C in h-DMEM supplemented with 10% fetal bovine serum. For transfection, HEK293 cells were detached from the surface by trypsinisation, resuspended in 4-ml fresh medium, and seeded at 3×10^5 cells/ml onto 25 cm^2 flasks precoated with poly-D-lysine. The plasmids expressing ASK1, ASK1C869A and nNOS were transfected into HEK293 cells using lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The CalPhos Mammalian Transfection Kit (Clontech Mountain View, CA, USA) was also utilized to transfect HEK293 cells for the examining of phosphorylation of ASK1.

The human SH-SY5Y neuroblastoma cell line was cultured in DMEM supplemented with 10% fetal bovine serum in a 5% CO_2 humidified incubator at 37 °C. Medium was changed every 2–3 days. The plasmids for eukaryotic expression were transfected into SH-SY5Y cells using the CalPhos Mammalian Transfection Kit according to the manufacturer's protocol and the method of Jiang (Jiang et al., 2004).

Ischemic animal model and drug treatment

Adult male Sprague–Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of Science) weighing 200–250 g were used. Transient brain ischemia (15 min) was induced by the four-vessel occlusion method (4-VO) as described previously (Pulsinelli and Brierley, 1979). Briefly, rats were anesthetized with chloral hydrate (300 mg/kg, intraperitoneally) before both vertebral arteries were occluded permanently by electrocautery and both carotid arteries were isolated. Then rats were allowed to recover for 24 h, and both carotid arteries were occluded with aneurysm clips to induce cerebral ischemia without the administration of chloral hydrate. After occlusion for 15 min, the aneurysm clips were removed for reperfusion. Rats that lost their righting reflex within 30 s and those whose pupils were dilated and unresponsive to light were selected for the experiments. Rectal temperature was maintained at 36.5–37.5 °C during ischemia (15 min) and the 2-h reperfusion. Sham control rats were treated using the same surgical procedures except that the carotid arteries were not occluded. The rats were injected intraperitoneally with SNP (5 mg/kg) dissolved in saline with an interval of 1.5 h three times, the first SNP administration was 30 min before ischemia. 7-NI (25 mg/kg) was administered to rats by intraperitoneal injection 30 min before ischemia. MK-801 (3 mg/kg) dissolved in saline was administered to rats by intraperitoneal injection 100 min before ischemia. 10 nmol