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NEUROPROTECTION OF S-NITROSOGLUTATHIONE AGAINST ISCHEMIC INJURY BY DOWN-REGULATING FAS S-NITROSYLATION AND DOWNSTREAM SIGNALING

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Abstract—S-nitrosoglutathione (GSNO) has been reported to protect against ischemic brain injury, however, the underlying mechanisms remain to be elucidated. In the present study, we aimed to investigate the effects of GSNO pre-treatment on the S-nitrosylation of Fas and subsequent events in the Fas pathway, and reveal the correlation between Fas S-nitrosylation and nNOS activation in the rat hippocampal CA1 region after global cerebral ischemia. The results showed that GSNO pre-treatment not only facilitated the survival of hippocampal CA1 pyramidal neurons, but also abolished the activation of pro-apoptotic Caspase-8, Bid, Caspase-9 and Caspase-3. The S-nitrosylation of Fas increased sustainedly after global ischemia, and GSNO blocked such an increase. Global cerebral ischemia/reperfusion promoted the binding between neuronal nitric oxide synthase (nNOS) and postsynaptic density protein 95 that has been reported to activate nNOS, and GSNO inhibited the post-ischemic nNOS activation and NO release. A selective nNOS inhibitor 7-nitroindazole diminished the ischemia/reperfusion-induced Fas S-nitrosylation, suggesting a critical role of endogenous NO from nNOS activation in Fas S-nitrosylation. In addition, pre-administration of GSNO decreased the translocation of Fas to membrane, the formation of CD95^{hi} on the membrane, the internalization of Fas aggregates to plasma, as well as the assembly of DISC/hiDISC. These results indicate that GSNO-induced nNOS inactivation associates with the down-regulation of Fas S-nitrosylation and consequent Fas signal cascade, which is responsible for the GSNO-mediated neuronal survival after brain ischemia. The understanding of GSNO neuroprotection provides a novel strategy to find potential therapeutic targets for ischemic stroke.

Key words: cerebral ischemia, Fas, neuronal nitric oxide synthase, S-nitrosoglutathione, S-nitrosylation.

INTRODUCTION

Previous studies have shown that S-nitrosoglutathione (GSNO) attenuates neuronal loss after both global and focal cerebral ischemia. However, the underlying molecular mechanisms remain still unclear. In this study, we provide the first evidence that global cerebral ischemia promotes the S-nitrosylation of Fas (APO-1 or CD95) in the vulnerable hippocampal CA1 region, implying a role of Fas S-nitrosylation in ischemic brain injury. To elucidate the mechanism that GSNO protects neurons from ischemic injury, we will focus on the effects of GSNO on Fas S-nitrosylation and Fas signaling pathway in rat global cerebral ischemia model.

Fas belongs to the tumor necrosis factor receptor family. Activation of Fas by its physiological ligand, FasL, has been proved to definitely trigger cell apoptosis (Nagata, 1997; Krammer, 2000). FasL-Fas signaling pathway plays a critical role in immune system function, such as killing virus-infected or damaged cells and tumor suppression (Straus et al., 2001). Execution of Fas apoptosis-inducing function relies on the assembly of an intracellular death-inducing signaling complex (DISC), which involves the aspartate-specific cysteine protease, Caspase-8, its adaptor/activator, Fas-associated death domain (FADD), and its modulator, (FADD)-like interleukin 1β-converting enzyme inhibitory protein (Kischkel et al., 1995; Boldin et al., 1995; Chinnaiyan et al., 1995; Boldin et al., 1996; Muzio et al., 1996; Irmler et al., 1997). The death domains (DD), presenting in the intracellular region of both Fas and FADD, are the structural basis for the interaction of Fas-FADD (Boldin et al., 1995; Chinnaiyan et al., 1995). Similarly, the recruitment of Caspase-8 by FADD is mediated by homotypic interaction of death effector domains (DED) (Boldin et al., 1996; Muzio et al., 1996). The ligation of FasL to Fas causes rapid assembly of DISC. Subsequently, DISC induces the alteration of a series of downstream signaling proteins. First of all, the

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Abbreviations: 7-NI, 7-nitroindazole; DD, death domain; DED, death effector domain; DISC, death-inducing signaling complex; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FADD, Fas-associated death domain; GSNO, S-nitrosoglutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; I/R, ischemia and reperfusion; MMTS, methyl methanethiosulfonate; NO, nitric oxide; NOS, nitric oxide synthase; NS, normal Saline; tBid, truncated bid.

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recruitment to FADD induces the cleavage and activation of Caspase-8. Active Caspase-8 leaves the DISC and proteolytically activates its substrates, such as Caspase-3 and Bid, ultimately leading to cell apoptosis. In addition to DISC, Fas also aggregates into oligomers on the cell membrane upon the existence of FasL and consequently internalizes to facilitate the formation of cytosolic hiDISC which contains the high molecular weight aggregate, $CD95^{hi}$, resulting in cell apoptosis (Feig et al., 2007). Owing to the critical position of Fas in mediating apoptosis, mice with mutant Fas (lpr mice) sustain less ischemic damage compared with wild-type mice (Martin-Villalba et al., 1999; Rosenbaum et al., 2000). Neurons in Fas apoptotic inhibitory molecule 2 (Faim2)-deficient mice are more susceptible to combined oxygen–glucose deprivation (Reich et al., 2011). Moreover, some natural factors afford the post-ischemic neuroprotective effect through inhibiting Fas signaling pathway (Wang et al., 2007; Pan and Li, 2008). This evidence demonstrates that Fas signaling pathway plays an important role in ischemic brain injury (Rosenbaum et al., 2000; Mehmet, 2001; Ruan et al., 2008; Liu et al., 2008).

S-nitrosylation is the covalent attachment of a nitric oxide group (–NO) to a cysteine thiol side chain. The –NO group either comes from NO directly or is transferred from other S-nitrosylated proteins. In mammalian cells, the endogenous NO is mainly generated by L-Arg-dependent nitric oxide syntheses (NOS) – neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3) (Foster et al., 2009). S-nitrosylation of cysteine is an important regulation mode universally occurring in most proteins. Brain ischemia induces the activation of nNOS (Eliasson et al., 1999). Several receptors and signaling proteins are found to be S-nitrosylated after ischemic stroke (Di et al., 2012; Hu et al., 2012), which may mediate neuron injury.

Therefore, to investigate the effects of GSNO on Fas S-nitrosylation and Fas signaling pathway will be helpful to reveal the molecular mechanism of GSNO neuroprotection against ischemia. A better understanding of the beneficial role of GSNO in neuronal survival might direct us to find potential therapeutic methods in treating ischemic stroke.

EXPERIMENTAL PROCEDURES

Materials

Anti-FasL (#sc-6237), anti-Fas (#sc-716), anti-Bid (#sc-11423) and anti-Caspase-9 (#sc-70506) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Caspase-8 (#9746), anti-pro-Caspase-3 (#9662S), anti-cleaved-Caspase-3 (#9661S), anti-nNOS (#4234) and anti-β-Actin (#4970L) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Anti-FADD (AAM-212) antibody was from Enzolifescience (New York, NY, USA). Anti-tBid (AB10002) antibody was from Millipore Technology (Temecula, CA, USA). Anti-PSD-95 (P246) antibody, GSNO and 7-nitroindazole (7-NI) were obtained from Sigma–Aldrich (St Louis, MO, USA). Kits for nNOS activity or NO concentration assay were from the Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Animal model of ischemia and drug administration

Adult male Sprague–Dawley rats (250–300 g) were used. Anesthesia was induced with 20% choral hydrate [(350 mg/kg, intraperitoneal (i.p.)], then vertebral arteries were electrocauteriezed and common carotid arteries were dissected free. Rats were allowed to recover for 24 h while food was withheld overnight. To achieve global ischemia, the common arteries were occluded for 15 min with aneurysm clips. The rats which lost the right reflex within 15s with pupils dilated were selected for experiments. Rectal temperature was maintained between 36.5 °C and 37.5 °C throughout the procedure. The clips were released to restore carotid artery blood flow (Pulsinelli and Brierley, 1979). Sham controls were obtained using the same surgical procedures mentioned above, excepting those used to obtain occlusion. All the rats were kept in a room maintained at a constant temperature of 23–25 °C. GSNO (25 μg:10 μl) in normal saline (NS) was administered to rats intracerebroventricularly (i.c.v., bregma: 1.5 mm lateral, 0.8 mm posterior, 3.5 mm deep) 40 min prior to ischemia. GSNO control rats were treated with GSNO without ischemia. The reductant dithiothreitol (DTT), which can reduce the S-nitosylated cysteine, was administered 20 min prior to ischemia (15 μg:10 μl, i.c.v.) to prove that the method adopted to examine S-nitrosylation of Fas and nNOS are credible. A selective nNOS inhibitor 7-NI (25 mg/kg, i.p.) was administered 20 min before ischemia.

Sample preparation

Rats were decapitated and hippocampi were dissected. The CA1 fraction samples were homogenized in 800 μl ice-cold homogenization buffer consisting of 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS, PH 7.4), 50 mM NaF, 20 mM NaPPi, 20 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA and protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml pepstatin) with a Teflon-glass homogenizer. This was followed by centrifugation at 4 °C, 1000 g for 15 min. Supernatants were then centrifuged at 4 °C, 100 000 g for 30 min. Supernatants deemed as cytosol fractions were collected. The pellets deemed as membrane fraction were resuspended with 400 μl homogenization buffer containing 1% Triton X-100 and exposed to ultrasound.

S-nitrosylation assay

S-nitrosylation was detected using the biotin-switch method (Jaffrey et al., 2001). Homogenizer was diluted in buffer (250 mM HEPES PH 7.7, 1 mM EDTA, 0.1 mM neocuproine, 1% Nonidet P-40, 150 mM NaCl and protease inhibitor mixture). Homogenizer was then mixed with an equal volume of methyl methanethiosulfonate (MMTS) buffer [25 mM HEPES PH 7.7, 0.1 mM EDTA, 10 μM neocuproine, 5% Sodium