Neurochemistry International 61 (2012) 649–658

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Glycine attenuates cerebral ischemia/reperfusion injury by inhibiting neuronal apoptosis in mice

Yan Lu [a,b], Jing Zhang [a,b], Bingqing Ma [a], Kexue Li [a,b], Xiaoyu Li [a], Hui Bai [b], Qing Yang [b], Xudong Zhu [a,b], Jingjing Ben [a,b], Qi Chen [a,b,*]

[a] Atherosclerosis Research Centre, Laboratory of Molecular Intervention with Cardiovascular Diseases, Nanjing Medical University, Nanjing 210029, PR China
[b] State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 210029, PR China

ARTICLE INFO

Article history:
Received 5 March 2012
Received in revised form 18 June 2012
Accepted 3 July 2012
Available online 10 July 2012

Keywords:
Glycine
Cerebral ischemia/reperfusion
Apoptosis
SH-SY5Y cell
siRNA
Neurons
Glycine receptor

ABSTRACT

Glycine is a cytoprotector to protect cells against ischemic damage by counteracting neuronal depolarization. However, whether it can directly inhibit neuronal apoptosis is unknown. In this study, we demonstrated that glycine could attenuate ischemia/reperfusion (I/R) induced cerebral infarction and improved neurological outcomes in mice. The protective effect of glycine was associated with reduction of terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) positive cells, deactivation of phosphor-JNK, inhibition of caspase-3 cleavage, down-regulation of FasL/Fas, and up-regulation of bcl-2 and bcl-2/bax in the mouse I/R penumbra. The beneficial effect of glycine against oxygen and glucose deprivation (OGD) induced injury was also confirmed in SH-SY5Y cells as well as in primary cultured neurons, which was significantly dampened by knockdown of glycine receptor α1 (GlyR α1) with siRNA transfection or by preventing glycine binding with glycine receptor using a specific antibody against glycine receptor. These results suggest that glycine antagonize cerebral I/R induced injury by inhibiting apoptosis in mice. Glycine could block both extrinsic and intrinsic apoptotic pathways for which GlyR may be required.

1. Introduction

Stroke is a leading cause of death and adult disability worldwide. Of all strokes, ischemic stroke accounts for approximately 80% (Feigin et al., 2003; Liu et al., 2010). Thrombolysis and revascularization of the obstructed blood vessels are effective and safe therapies to restore the cerebral blood flow. However, the efficacy of these interventions is satisfactory in a selected few types of stroke and is limited by a narrow time window (usually in the very early period) (Hankey and Warlow, 1999; Kwiatkowski et al., 1999). Thus, finding more effective therapeutic neuroprotective agents has become a priority in the field.

Inhibitory amino acid taurine and L-serine can protect the brain from ischemic injury. It is supposed to be mediated by activating glycine receptors (GlyRs) (Wang et al., 2007, 2010). GlyRs, as well as glycine-containing fibers and cell bodies, are widely distributed in brain rather than restricted to the spinal cord and brain stem (Legendre, 2001). As a major inhibitory neurotransmitter, glycine can activate GlyR to reach chloride influx induced postsynaptic hyperpolarization and reduces neuronal excitability. This anti-excitotoxic property is by counteracting neuronal depolarization and subsequent cascade of biochemical events that would result in cell death (Wang et al., 2010).

Glycine also exhibits a directive protection against ischemia induced injury to organs and tissues (Yin et al., 2002; Zhang et al., 2003; Omasa et al., 2003; Tang et al., 2006). We showed that cytoprotection against ATP depletion by glycine is mediated by GlyR (Pan et al., 2005). The extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 mitogen-activated protein kinase and AKT pathways constitute GlyR-coupled signaling pathways (Jiang et al., 2010), suggesting a potential anti-apoptotic property should be involved in cytoprotection of glycine. Apoptosis occurs in the delayed neuronal death in the ischemic lesions (Kametsu et al., 2003; Waldmeier, 2003). In order to clarify if glycine antagonizes ischemic injury by an anti-apoptotic mechanism in the brain, we used an experimental transient middle cerebral artery occlusion

Abbreviations: CNS, central nervous system; ERK1/2, extracellular signal-regulated kinase 1 and 2; GlyRs, glycine receptors; GlyR α1, glycine receptor α1; IACUC, institutional animal care and use committee; I/R, ischemia/reperfusion; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MCAO, middle cerebral artery occlusion; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate; OGD, oxygen and glucose deprivation; TTC, 2,3,5-triphenyl tetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

* Corresponding author. Address: Atherosclerosis Research Center, Key Laboratory of Cardiovascular Disease and Molecular Intervention, Nanjing Medical University, 140 Hanzhong Road, Nanjing, Jiangsu 210029, PR China. Tel.: +86 25 86862610; fax: +86 25 86508960.
E-mail address: qichen@njmu.edu.cn (Q. Chen).

0197-0186/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuint.2012.07.005

(MCAO) model in mice. Our results demonstrated that glycine may attenuate mouse cerebral ischemia/reperfusion (I/R) injury by inhibiting cell apoptosis.

2. Materials and methods

2.1. Animals

Male ICR mice were supplied by the Experimental Animal Center of Nanjing Medical University, Nanjing, China. The experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University. All experiments were performed in accordance with international standards on the ethical treatment of animals. Mice were housed under climate-controlled conditions with a 12-h light/dark cycle and provided standard food and water ad libitum. Glycine (Sigma, St. Louis, MO, USA) was dissolved in physiological saline and was intraperitoneally injected into mice 30 min before MCAO for the pretreatment study. All MCAO mice were divided into receiving vehicle, low-dose glycine (250 mg/kg), and high-dose glycine (1000 mg/kg) groups. For the posttreatment experiments, drug treatments were given via intraperitoneal injection 10 min after reperfusion.

2.2. MCAO model

Transient focal cerebral ischemia was induced by right MCAO with a modified intralumenal filament technique (Hata et al., 1998). Briefly, mice were anesthetized by 3.5% chloral hydrate (1 mg/kg). A 5-0 monofilament surgical nylon suture with a heat-blunted tip was introduced into the left internal carotid artery through the stump of the external carotid to the base of the right to stop blood flow. After 90 min, the filament was withdrawn to allow blood reperfusion. Meanwhile the ipsilateral common carotid artery remained ligated, the neck skin was closed, and the skin was sutured. Rectal temperature, monitored with a digital thermometer inserted 2 cm into the anus, was maintained at 37–38 °C throughout the operation using a feed back-regulated heating blanket. All animals recovered from anesthesia within 30 min of wound closure. Sham-operated animals underwent the same procedure except for MCAO.

2.3. Measurement of the area of early ischemic brain injury

Mice were examined for neurological deficit using a four-tiered grading system before giving anesthesia, 24 h, and 72 h after MCAO and reperfusion (Connolly et al., 1996a,b). A score of 1 was given if the animal was demonstrated normal spontaneous movements; 2 was given if the animal was noted to be turning to the right (i.e., clockwise circles) when viewed from above (i.e., toward the contralateral side); 3 was given if the animal was observed to spin longitudinally (clockwise when viewed from the tail); and 4 was given if the animal was crouched on all fours, unresponsive to noxious stimuli. This scoring system is based upon similar scoring systems used in rats (Bederson et al., 1986; Menzies et al., 1992) which are based upon the contralateral movement of animals with stroke. After cerebral infarction, the contralateral side is "weak" and so the animal tends to turn toward the weakened side. Mice were killed by decapitation 24 h or 72 h after MCAO. Brains were removed and chilled in ice-cold saline for 5 min. Four 2-mm consecutive coronal slices were made by using a brain slicer, beginning from the anterior pole. Slices were incubated in saline solution containing 2% 2,3,5-triphenyl tetrazolium chloride (TTC) (Sigma, St. Louis, MO, USA) at 37 °C for 30 min. They were then fixed by 10% formalin neutral buffer solution (pH 7.4) for 1 h. Infarct area in each slice was evaluated by scanned digital images with an image analyzer. Infarct volume of each slice was obtained by multiplying the infarct area by 2-mm thickness. Total infarct volume was determined by summing up infarct volume of five consecutive slices. All brain slices were analyzed for their infarct volume using the Image-J analysis software. Percentage infarct volume was calculated as follows: $[(VC - VL)/VC] \times 100$, where VC is the volume of control hemisphere (Right side), VL is the volume of non-infarcted tissue in the lesioned hemisphere (Right side).

2.4. TUNEL staining

Samples from sham-operated, vehicle, and glycine-high groups were used for experiments. Mice were deeply anesthetized with chloral hydrate 72 h after reperfusion. After transcardiac perfusion with 50 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), brains were removed and stored in the same paraformaldehyde solution overnight. Multiple, paraffin-embedded, coronal sections (4 μm thick) were taken from brain and stained by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay. The apoptotic neurons were counted using an inverted microscope (Olympus BX51; Japan) in the peri-infarct cortex (three fields were counted in each case at ×400 magnification) in a blinded manner.

2.5. Western blot analysis

The right ischemic cortex in the middle cerebral artery region (middle cerebral artery cortex) was obtained from preoperation (healthy control) and 24 h after MCAO. Protein concentration was determined using BCA protein assay kit (PIERCE, Rockford, IL, USA). Individual samples (60 μg each) were separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, USA). Immunoblotting was performed by incubation of the membrane in 5% dry milk for 1 h and in the specific antibody overnight at 4 °C. Rabbit antibodies against P-JNK, caspase-3, bcl-2 and bax were obtained from Cell Signaling Techonology (Beverly, MA, USA). Rabbit anti-FasL antibody was obtained from Abcam (Cambridge, UK). Rabbit anti-GlyRα1/2 antibody was obtained from Abd serotec (Oxford, UK). After washing twice with Tris-buffered saline-Tween 20, each membrane was incubated with a secondary antibody (1:5000) for 2 h. Cellular β-actin or GAPDH was used as a loading control to prove that all of the lanes were loaded with the same amount of protein. The membranes were then developed using an enhanced chemiluminescence system. The figures were analyzed with Image J.

2.6. RNA extraction and real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen, San Diego, CA, USA) in accordance with the manufacturer's protocol. After extraction, 0.5 μg of total RNA was used as template to synthesize cDNA using a first strand synthesis kit (Invitrogen, San Diego, CA, USA). The cDNA from this synthesis was then used in quantitative real-time PCR analysis (ABI-Prism 7700 sequence detection system; Applied Biosystems, Foster City, CA, USA) using SYBR Green dye. The following primer pairs for murine were used: Fasl, 5′-TGAATTACCCATGTCCCCAG-3′ (forward) and 5′-AAACTGACCCTGGAGGAGCC-3′ (reverse); Fas, 5′-TGGCAGAGGAGCCTAGTTGT-3′ (forward) and 5′-CACACCCAGGAACAGTCCTT-3′ (reverse); bcl-2, 5′-ATGATAACCGGGAGATCGTG-3′ (forward) and 5′-GTTCAGGTACTCAGTCACC-3′ (reverse); bax, 5′-ACCAGCTCTGAACAGATCATG-3′ (forward) and 5′-TGGTCTGGATCCAGACAAG-3′ (reverse); actin, 5′-TTCGTTGCCGGTCCACA-3′ (forward) and 5′-ACCAGCGCAGCGATATCG-3′ (reverse) (Pinkoski et al., 2002; Guo et al., 2003; Symonds et al., 2005; Yoshimatsu et al., 2009).