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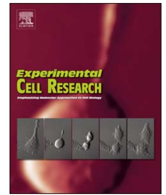

Research article

Memantine attenuates cell apoptosis by suppressing the calpain-caspase-3 pathway in an experimental model of ischemic stroke

Bin Chen[a,b], Guoxiang Wang[b], Weiwei Li[c], Weilin Liu[a], Ruhui Lin[a], Jing Tao[a], Min Jiang[b], Lidian Chen[a,*], Yun Wang[b,*]

[a] *Fujian Rehabilitation Tech Co-innovation Center (2011 Project), Fujian Rehabilitation Engineering Research Center & Fujian Key Lab of Motor Function Rehabilitation, Academy of Integrative Medicine Biomedical Research Center, Fujian University of Traditional Chinese Medicine, Fuzhou 350122, China*

[b] *Institutes of Brain Science and State Key Laboratory for Medical Neurobiology, Collaborative Innovation Center for Brain Science, Fudan University, Shanghai 200032, China*

[c] *Department of Anesthesiology, Shanghai Changzheng Hospital, Shanghai 200003, China*

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ABSTRACT

Ischemic stroke, the second leading cause of death worldwide, leads to excessive glutamate release, over-activation of N-methyl-D-aspartate receptor (NMDAR), and massive influx of calcium (Ca^{2+}), which may activate calpain and caspase-3, resulting in cellular damage and death. Memantine is an uncompetitive NMDAR antagonist with low-affinity/fast off-rate. We investigated the potential mechanisms through which memantine protects against ischemic stroke *in vitro* and *in vivo*. Middle cerebral artery occlusion-reperfusion (MCAO) was performed to establish an experimental model of ischemic stroke. The neuroprotective effects of memantine on ischemic rats were evaluated by neurological deficit scores and infarct volumes. The activities of calpain and caspase-3, and expression levels of microtubule-associated protein-2 (MAP2) and postsynaptic density-95 (PSD95) were determined by Western blotting. Additionally, Nissl staining and immunostaining were performed to examine brain damage, cell apoptosis, and neuronal loss induced by ischemia. Our results show that memantine could significantly prevent ischemic stroke-induced neurological deficits and brain infarct, and reduce ATP depletion-induced neuronal death. Moreover, memantine markedly suppressed the activation of the calpain-caspase-3 pathway and cell apoptosis, and consequently, attenuated brain damage and neuronal loss in MCAO rats. These results provide a molecular basis for the role of memantine in reducing neuronal apoptosis and preventing neuronal damage, suggesting that memantine may be a promising therapy for stroke patients.

1. Introduction

Ischemic stroke is one of the most common causes of mortality and morbidity in both pediatric and adult populations [1,2]. The reduction of cerebral blood flow that occurs during acute ischemic stroke leads to oxygen and glucose deprivation and to subsequent hyperactivation of postsynaptic glutamate/glycine-gated ion channels, specifically, the N-methyl-D-aspartate receptor (NMDAR) [1,3]. Overstimulation of NMDAR induces an excessive influx of Ca^{2+}, which may activate calpain and caspase-3, eventually resulting in neuronal dysfunction and cell death by necrosis or apoptosis [3–7]. Therefore, NMDARs have been considered drug targets in treating stroke.

Many previous NMDA receptor antagonists tested in clinical trials have been unsuccessful because of their unacceptable neurotoxic side effects, which are partially caused by their blockade of physiological synaptic transmission [8–10]. However, memantine is an uncompetitive NMDA receptor antagonist with a low-affinity and a fast off-rate, which preferentially blocks pathological NMDA receptor activity, particularly at the extrasynaptic position, without disrupting physiological synaptic transmission [10–13]. Previous studies have revealed that memantine exerts a neuroprotective in different *in vitro* and *in vivo* models of excitotoxicity [14–16]. Moreover, memantine reduces ischemic brain injury and enhances recovery in rats and mice when administered in the acute or chronic stroke phase [17–21]. Additionally, clinical trials of memantine for the treatment of ischemic stroke are currently under way (Clinical Trials.gov identifier:

Abbreviation: NMDAR, N-methyl-D-aspartate receptor; LDH, lactate dehydrogenase; MCAO, middle cerebral artery occlusion-reperfusion; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; ATP-D, ATP depletion; MEM, memantine; CBF, cerebral blood flow; MAP-2, microtubule-associated protein-2; PSD95, postsynaptic density-95; TTC, 2,3,5-triphenyl tetra-zolium chloride; TUNEL, TdT-mediated dUTP Nick-End Labeling

* Corresponding authors.
E-mail addresses: fjtcm1958@sina.com (L. Chen), yunwang@fudan.edu.cn (Y. Wang).

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NCT02144584). These findings suggest that memantine may be a viable pharmacological agent for treating stroke patients.

Memantine has received considerable attention and has been examined as a potential therapeutic drug for several neurological disorders, including Alzheimer's disease, vascular dementia, neuropathic pain, and stroke. However, the molecular mechanisms of memantine in treating ischemic stroke remain largely unknown. In the present study, we investigated whether memantine could exert neuroprotective effects against ischemic stroke and attenuate neuronal apoptosis by suppressing calpain-caspase-3 pathways in the penumbra of MCAO rats.

2. Experimental procedures

2.1. Animals

Sprague-Dawley (SD) rats were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). They were individually housed in plastic cages at a controlled temperature (22 ± 1 °C), relative humidity (55 ± 10%), and photoperiod (light/dark conditions 12/12 h lights on 7:00 a.m.). All experiments were performed strictly in accordance with the International Ethical Guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Primary hippocampal neuronal culture

Primary hippocampal neurons were prepared and cultured similarly to our previous reports [22]. Briefly, hippocampi were dissected out from 18-day rat embryos of either sex in cold HBSS. Then, the tissue was digested with 0.05% trypsin–EDTA for about 20 min at 37 °C, followed by trituration with pipettes in the plating media (DMEM with 10% FBS, 10% F12 and 25 u/ml penicillin/streptomycin). After rinsing twice, cells were counted and plated onto glass coverslips precoated with 0.1 mg/ml poly-D-lysine (Sigma). After culturing for 1 day, half of the media were changed into neuronal culture media (neurobasal media (GIBCO) containing 2 mM GlutaMAX™-I Supplement, 2% B27 and 25 u/ml penicillin/streptomycin). 2 μM Ara-C (Sigma) was added 6–8 day after plating during the culture medium change, and cells were fed twice weekly thereafter. All cells were grown at 37 °C and in 5% CO_2. Unless stated otherwise, all tissue culture reagents were obtained from Invitrogen (San Diego, CA).

2.3. ATP depletion and assessment of neuronal death

Primary cultured neurons (11–12 DIV) were washed twice with PBS and then were incubated at 37 °C for 20 min in either normal growth medium (control cells) or glucose-free DMEM containing 3 mM 2-deoxyglucose and 5 mM NaN3 to yield ATP-depleted cells. Following ATP depletion, hippocampal neurons were exposed with or without memantine (1, 10 and 50 μM). Neuronal death was determined either by visualizing survival neurons stained with NeuN or measuring lactate dehydrogenase (LDH) activity released from damaged cells into culture medium at 24 h after memantine treatment. Measurement of LDH release was performed by using a Cytotox 96 Kit (Promega) with the instructions of the manufacturer. Data are presented as the difference in LDH levels as a percentage of control.

2.4. Transient focal cerebral ischemia

Transient focal ischemia was induced by intraluminal suturing of the middle cerebral artery occlusion and reperfusion (MCAO), which was originally described by Longa et al. [23]. Briefly, SD rats were anesthetized with isoflurane (3% initial, 2% maintenance) and nitrous oxide (60%) in oxygen with spontaneous respiration via a mask. A Laser Doppler Flowmetry device (LDF100C, Biopac Systems, Goleta, CA), attached to the skull (5 mm lateral and 1 mm posterior to bregma) with dental cement, was used for measuring ipsilateral cerebral blood flow. Under the operating microscope, proximal portions of the left common carotid artery (CCA) and external carotid artery (ECA) were ligated, and a 4–0 silicon-coated nylon suture was introduced into the CCA and advanced about 18 mm beyond the carotid bifurcation for transient occlusion of the middle carotid artery (MCA). Ischemic rats that showed a stable drop of 80% in Blood Perfusion Units compared with baseline levels (before MCAO) were used for further experimentation. Reperfusion was achieved by removing the intraluminal occlusive embolus 1 h after MCAO. Animals subjected to the sham operation were treated similarly, except without ligations and occlusions.

2.5. Experimental design and drug treatment

Experimental animals were divided into sham, saline, and memantine (MEM) groups. Immediately after MCAO, drugs were injected intraperitoneally. (1) The MEM group received memantine at a dose of 20 mg/kg (Sigma, 5 mg/ml in 0.9% sterile saline), followed by a maintenance dose of 1 mg/kg at 12 h intervals to sustain levels of approximately 1–10 μM in brain tissue because the half-life of the drug is approximately 12 h [24]. (2) The saline group animals were subjected to MCAO and received an equivalent volume of vehicle. (3) The sham group served as sham-operated control and received 0.9% sterile saline.

2.6. Neurological assessment

The neurological deficit score of each rat was measured 24 h after MCAO induction in a blinded fashion according to a well established five-point neurological scale [23]: score 0=no apparent deficits; score 1=failure to fully extend the right forepaw; score 2=circling to the right; score 3=falling or leaning over to the right; score 4=no spontaneous walking and a depressed level of consciousness; score 5=dead.

2.7. Measurement of cerebral infarct volume

For determination of the infarct volume, rats were deeply anesthetized with 3.5% isoflurane and sacrificed by decapitation. Brains were quickly removed, and six 2-mm-thick coronal sections were prepared with a brain matrix device (ASI Instruments, Inc.). Brain sections were stained with 2% TTC (w/v) at 37 °C for 15 min and fixed in 4% paraformaldehyde (PFA). Images were analyzed using Image J software (NIH, Bethesda, MD, USA). The infarct volume data was expressed as a percentage of the contralateral hemisphere and calculated as described previously [25].

2.8. Western blotting

At 24 h after MCAO, rats (n=4 per group) were deeply anesthetized, and the ischemic penumbras were microdissected according to established protocols in rodent models of unilateral proximal MCAO [26]. Briefly, a 4-mm thick coronal brain slice was cut, beginning 5 mm from the anterior tip of the frontal lobe. Then a longitudinal cut (from top to bottom) was made approximately 2 mm from midline through the ischemic hemisphere to remove medial portions. Finally, a transverse diagonal cut was made at approximately the “2 o'clock” position to separate the wedge-shaped penumbra.

The harvested brain tissues were homogenized in RIPA lysis buffer containing protease inhibitor cocktails (Roche Diagnostics) and PMSF. After centrifugation, the protein concentration was quantified by the BCA assay (Pierce Biotechnology). Brain homogenates (30 μg) were separated by SDS-PAGE and transferred onto PVDF membranes, which were blocked for 2 h with 5% non-fat dry milk at room temperature. The blots were incubated overnight at 4 °C with the

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