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Memantine mediates neuroprotection via regulating neurovascular unit in a mouse model of focal cerebral ischemia

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ABSTRACT

Aims: Memantine is a low-moderate affinity and uncompetitive *N*-methyl-D-aspartate receptor (NMDAR) antagonist, which is also a potential neuroprotectant in acute ischemic stroke for its particular action profiles. The present study was to reveal the mechanisms involved in the neuroprotection of memantine.
Main methods: We used a mouse model of permanent focal cerebral ischemia via middle cerebral artery occlusion to verify our hypothesis. 2,3,5-Triphenyltetrazolium chloride staining was used to compare infarct size. The amount of astrocytes and the somal volume of the microglia cell body were analyzed by immunohistochemistry and stereological estimates. Western blotting was used to determine the protein expressions.
Key findings: Memantine prevented cerebral ischemia-induced brain infarct and neuronal injury, and reduced oxygen-glucose deprivation-induced cortical neuronal apoptosis. Moreover, memantine reduced the amount of the damaged astrocytes and over activated microglia after 24 h of ischemia. In the early phase of ischemia, higher production of MMP-9 was observed, and thereby collagen IV was dramatically disrupted. Meanwhile, the post-synaptic density protein 95(PSD-95) was also severely cleavaged. Memantine decreased MMP-9 secretion, prevented the degradation of collagen IV in mouse brain. PSD-95 cleavage was also inhibited by memantine.
Significance: These results suggested that memantine exerted neuroprotection effects in acute ischemic brain damage, partially via improving the functions of neurovascular unit. Taking all these findings together, we consider that memantine might be a promising protective agent against ischemic stroke.

1. Introduction

Ischemic stroke is an acute cerebrovascular accident resulted from the blockage or plug of an artery to the brain, which results in the interruption of blood flow to the brain [20]. Due to the interruption of the supply of glucose and oxygen, over-activation of glutamate receptors, calcium overload, acidosis and oxidative stress, all of these progresses rapidly induce neuron death in the infarct core [20,25]. Now the potential therapeutic strategy for the acute ischemic stroke is neuroprotection. Therapeutic interventions, including inhibiting cytotoxic edema, excitotoxicity and production of free radical, have been demonstrated to provide neuroprotection against ischemic stroke [18]. Various drugs targeting at curtailing the early ischemic cascade have been demonstrated to exert neuroprotective effect in preclinical models. These include antagonists of *N*-methyl-D-aspartate receptors (NMDAR), Ca^{2+} channel blockers, gamma-aminobutyric acid (GABA) agonists, magnesium and free radical scavengers [2]. However, clinical trials about these drugs failed to demonstrate therapeutic efficacy in early cascade event of ischemic stroke.

Memantine is a chemical derived from amantadine, which was used in clinical practice to treat several neurological disorders including amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Alzheimer's disease (AD), stroke, epilepsy, vascular dementia and so on. Memantine can noncompetitively binds to *N*-methyl-D-aspartate receptors (NMDAR), and acts as a low-affinity open-channel inhibitor. Because of the special pharmacological properties of memantine, we considered that memantine is likely to exert favorable actions during ischemic stroke because it can block excessive NMDAR activation but not affect its physiological activity. Therefore, memantine is a moderate NMDAR antagonist and can reduce or prevent excitotoxic damage without undesired side effects, such as anesthesia, hallucination, catatonia and agitation. Although memantine mainly focuses on NMDAR, but it can also affect other targeting pathways as reported, for example it can reduce action potential firing in cultured neurons, block nicotine receptors and 5-HT_3 receptors [5,10,19,28]. However, the mechanisms about how memantine protect the brain against ischemia were still largely unknown.

In the present study, we established permanent middle cerebral artery occlusion (pMCAO)-induced stroke mouse models to explore the involved mechanisms of memantine-mediated neuroprotective roles

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[26]. Our results revealed that memantine not only protected neuronal damage induced by ischemia, but also improved glial functions and prevented degradations of extracellular matrix.

2. Materials and methods

2.1. Mouse model and experimental protocol

The study was approved by IACUC (Institutional Animal Care and Use Committee) of Nanjing Medical University. Male adult (8- to 12-week-old) C57 BL6 mice weighing between 26 and 35 g were used. By intraperitoneal (ip) injection of chloral hydrate (150 mg/kg), the mice were remained unconscious during the surgery. Continuous monitoring of body temperature was conducted and controlled at 37 ± 1 °C during the procedure. After which, permanent focal cerebral ischemia was induced by occlusion of the middle cerebral artery according to the protocol of Longa as described [21]. The mouse brains were removed at 24 h after surgery. Memantine was injected intraperitoneally (ip) once at the dose of 20 mg/kg, half an hour before middle cerebral artery occlusion (MCAO).

2.2. Infarct area measurement

2,3,5-Triphenyltetrazolium chloride (TTC) can reflect the function of mitochondria and has been shown to be a reliable marker of ischemic volume after ischemia. So TTC (Jinggong) staining was used to measure the infarct size in the ischemia-treated groups (24 h after ischemia; n = 8–10 per group). Using an image analysis system (Image-Pro Plus; Media Cybernetics) to trace the infarct area of slices which were taken at 1-mm intervals.

2.3. Determination of neurological symptoms

The severity of neurological symptoms of the experimental animals was graded on a scale of 0′–5′ in a blinded manner: 0-no neurological deficit; 1′-retracts left forepaw when lifted by the tail; 2′-circles to the left; 3′-falls while walking; 4′-does not walk spontaneously; 5′-dead. Neurological symptoms were evaluated 24 h after MCAO.

2.4. Evans blue staining

The integrity of Blood-Brain-Barrier (BBB) was studied with Evans blue Staining. Briefly, 2% Evans blue (4 ml/kg, in 0.9% normal saline) was intravenously administered at 24 h post-MCAO and was allowed to circulate for 20 min. Then mice were transcardially perfused with saline to remove the intravascular dye. After that, animals were sacrificed, the entire brains were removed. The brain hemisphere was weighed rapidly, then homogenized in 50% trichloroacetic acid, and then centrifuged (12,000 × *g* for 20 min). The amount of Evans blue in the collected supernatant was quantified at 620 nm by spectrofluorophotometry.

2.5. Brain fixation

Using chloral hydrate (150 mg/kg, i.p) to euthanize the animals. Exposing the heart to make an incision in the right ventricle, and a hypodermic syringe was carefully placed in the deep of the ventricle to perfuse with phosphate-buffered saline (PBS) and then 20 ml of 4% paraformaldehyde (PFA) in PBS. After perfusion the whole brain was removed and then soaked in 4% PFA for 24 h at 4 °C. Immunohistochemistry evaluation and conventional Nissl staining were conducted with sections of 40-μm. The total number of Nissl-positive cells was calculated with the optical disector following a semiautomated system and fractionator rules (StereoInvestigator software, Microbrightfield, VT, USA) [16,33].

2.6. Immunohistochemistry

Immunohistochemistry was performed as described previously [12]. First, sections were restored to room temperature and then washed in PBS. Using 3% H_2O_2 in PBS to quench endogenous peroxidase for immuno-peroxidase localization. After the quench, the sections were soaked in 5% bovine serum albumin (BSA) in PBS to block the non-specific binding sites for an hour, and then incubated overnight with different primary antibody (monoclonal mouse anti-Mac-1, 1:1000 Serotec; polyclonal rabbit anti-GFAP, 1:5000, Abcam; polyclonal rabbit anti-collagen IV, 1:1000, Abcam) at 4 °C. The sections were brought to room temperature and then rinsed with PBS. After that the sections were incubated with corresponding secondary antibody for 1 h at 37 °C. Different from immunohistochemistry, for immunofluorescence, special secondary antibodies conjugated to FITC were used to detect the primary antibody (mouse anti-PSD-95, Abcam, 1:800). It was incubated for 1 h at 37 °C in the dark.

2.7. Quantification of immunostaining

An individual who was unaware of treatment conditions of each animal performed the data collection. After choosing a starting point randomly, each sixth section along the dorsoventral extent of the hippocampus was chosen, each animal yield a mean of 8 sections for analysis. Firstly, they demarcate the boundaries of the stereological analysis with a low power magnification lens (6.3×, 0.16 NA). The total number of GFAP-positive cells was calculated with the optical disector following a semiautomated system and fractionator rules (StereoInvestigator software, Microbrightfield, VT, USA) [16,33].

The nucleator probe was used to measure the somal volume of the microglia cell body in each fifth counted microglial cell.

2.8. Primary cortical neurons cultures

Cultured primary cortical neurons were prepared from the cortex of C57BL6 mouse embryos (E14/15) as described previously but with some modifications [8]. Firstly, we use trypsinization [0.02% EDTA and 0.25% (w/v) trypsin in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution] to disaggregate cells at 37 °C for 5–6 min, and then gently triturate tissue in plating medium (h-DMEM containing 10% fetal bovine serum(FBS) and 10% horse serum; Sijiqing). Afterward cells were diluted to a density of 2.5×10^5 cells/cm^2 and plated on poly-L-lysine (Sigma)-coated 24-well plates and 25-cm^2 T-flask. Cells were maintained at 37 °C in 5% CO_2 atmosphere. After cell adherence, we replaced the stale medium by Neurobasal medium which containing 2% B-27 (Gibco-BRL) and 0.5 mM L-glutamine (Sigma) to replace and treated the cells with 1 M cytosine arabinoside (Sigma) for one day to avoid glial cell proliferation. Every 3.5 days, half of the medium was replaced with fresh medium. After 7 days the neurons could be used.

2.9. Oxygen–glucose deprivation (OGD) and drug treatment

We took advantage of a hypoxia chamber (Thermo) to perform a procedure of OGD. At the 7th day in vitro, cortical neurons were subjected to an OGD as described previously [27]. Briefly, before the experiment, the medium was replaced by OGD medium and treated with 10 μM memantine. After that, the cells were incubated in the hypoxia chamber programmed at 1% O_2, 5% CO_2, and 37 °C for 30 min. After OGD, cells were removed from the hypoxic chamber and incubated under normal conditions for 24 h for later experiments. In the normal conditions, neurons were switched to the normal feeding medium, and memantine (10 μM) was again added during reoxygenation. Control cell cultures were incubated under normal conditions throughout the procedure. We used medium as a vehicle.