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Maslinic acid promotes synaptogenesis and axon growth via Akt/GSK-3β activation in cerebral ischemia model

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ABSTRACT

Maslinic acid, a natural pentacyclic triterpene from *Olea europaea* plants, possesses neuroprotective effects both in vivo and in vitro. However, the mechanism of its action is not well understood. In this study, we investigated the potential effects of maslinic acid on synaptogenesis and axonal regeneration, as well as the possible signal pathway involved in a cerebral ischemia mouse model. Adult male C57BL/6J mice were subjected to 1 h of cerebral ischemia by middle cerebral artery occlusion (MCAO). Maslinic acid (0.1, 1 and 10 mg/kg) was administered intragastrically 24 h after MCAO once daily for 7 consecutive days. Axonal loss and synaptophysin expression in the ischemic boundary area was evaluated by histological assay. The Akt/GSK-3β signal pathway was determined by western blot analysis. Two Akt inhibitors, LY294002 and MK2206, were used to verify the involvement of Akt/GSK-3β pathway in maslinic acid-mediated neuroprotection. Maslinic acid significantly prevented axonal damage, promoted axonal regeneration and increased synaptophysin expression 7 days after ischemia. In addition, maslinic acid treatment was shown to enhance Akt activity and promote GSK-3β phorsphorylation in stoke mice. The increased neurite outgrowth and synaptophysin expression by maslinic acid treatment was blocked by the Akt inhibitors both in vivo and in vitro.. These findings suggested that maslinic acid promotes synaptogenesis and axonal regeneration by regulating Akt/GSK-3β signaling pathway, which may, in turn, provide neuroprotection.

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1. Introduction

Ischemic stroke is the leading cause of cerebrovascular morbidity and mortality with limited therapeutic possibilities. Axonal remodeling and dendritic plasticity is critical to nerve regeneration and brain repair in the injured area after stroke (Ueno et al., 2012), but the functional recovery after cerebral ischemia is extremely limited (Walmsley and Mir, 2007). Failure in axonal regeneration and synaptic plasticity may be partly attributed to the nonpermissive environment and reduced intrinsic growth capacity after CNS injuries, including the suppression of axonal extension via chondroitin sulfate proteoglycans, the inhibition of axonal regeneration by myelin components, and lack of neurotrophic factors and axon guidance molecules (Dill et al., 2008). Currently, strategies targeting multiple factors for axonal regeneration or enhancement of synaptic plasticity are considered as effective therapies for CNS axonal injuries, however, remain unsatisfactory (Galtrey and Fawcett, 2007; Liu et al., 2008; Walmsley and Mir, 2007).Maslinic acid (Fig. 1A), a natural pentacyclic triterpene derived from *Olea europaea*, elicits multiple bioactivities. Our previous studies have demonstrated that maslinic acid afford neuroprotection against oxygen–glucose deprivation-induced neuron damage via modulation of anti-apoptotic signaling pathways (Qian et al., 2011). Maslinic acid also exhibited protection against neuroinflammation by inhibiting NF-κB signal transducer pathway in cultured cortical astrocytes (Huang et al., 2011). Moreover, maslinic acid has been implicated in ischemic stroke in animal models. Maslinic acid administration controls hyperglycemia and prevents the exacerbation of brain lesion, along with reduced NF-κB transcriptional activity (Guan et al., 2011). Nevertheless, whether maslinic acid could prevent infarction or only delay it after cerebral ischemia, and the possible signal pathways involved in maslinic acid-mediated neuroprotection remain unclear. In the

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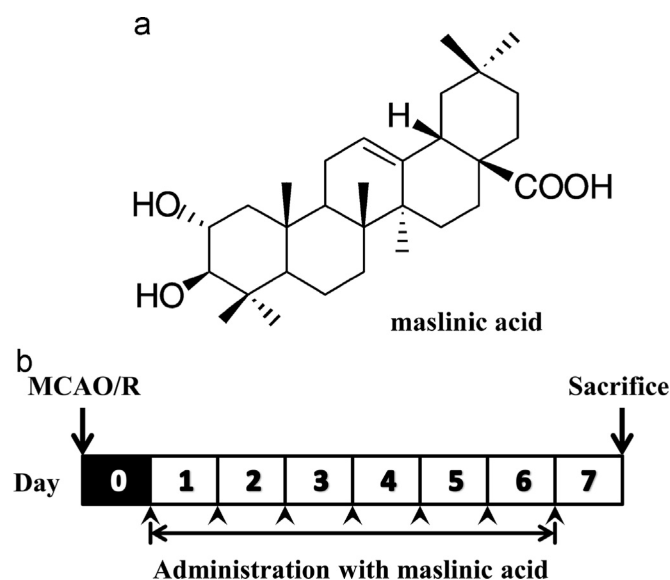

Fig. 1. (A) Chemical structure of maslinic acid. (B) Schematic diagrams showing the timeline of the experimental protocols. Mice were subjected to 1 h of MCAO followed by 24 h of reperfusion (MCAO/R). Maslinic acid was administered starting at 24 h after the reperfusion (day 0), once daily, for 7 consecutive days. At the end of day 7, the mice were killed for histology and western blot analysis.

present study, we investigated the effect of maslinic acid on axonal regeneration and synaptogenesis in a cerebral ischemia mouse model, and tried to clarify its possible mechanisms of action.

2. Materials and methods

2.1. Materials

Maslinic acid (96.5% purity, No. 0060324) was supplied by Center for Drug Discovery, China Pharmaceutical University. The compound was suspended in 0.5% sodium carboxymethyl cellulose (W/V in normal saline, for animal administration) or dissolved in Dimethyl sulfoxide (DMSO, 0.5%, final concentration, for cell culture treatment). The MAP-2 antibody was purchased from Cell Signaling Technology (Danvers, MA); the synaptophysin, Akt and phospho-Akt antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the GSK-3β and phospho-GSK-3β (ser9) antibodies were from Cell Signaling Technology, Inc., (Cell Signaling, Danvers, MA), and the glyceraldehyde phosphate dehydrogenase (GAPDH) antibody was obtained from Kangchen Bio-Tech (Shanghai, China). MK2206 was obtained from Biovision (Milpitas, CA). Lithium chloride (LiCl) and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animal treatment and middle cerebral artery occlusion

All experiment protocols were approved by the ethics committee of China Pharmaceutical University. Animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male C57/BL6J mice (24–28 g) were supplied by the Experimental Animal Center of China Pharmaceutical University. Mice were housed in a temperature-controlled environment (18–22 °C) with a 12 h light-dark cycle and allowed free access to food and water before the experiment. $N=8$ group sizes were used. All efforts were made to minimize animal suffering and reduce the number of animals used.

Mice were randomly divided into six groups: sham-operation group (Sham), middle cerebral artery occlusion (MCAO) group treated with vehicle (Vehicle), MCAO group treated with lithium chloride (LiCl, as the positive control) (Chuang et al., 2011; Xie et al., 2014), and MCAO groups treated with 0.1 mg/kg, 1 mg/kg, and 10 mg/kg of maslinic acid. Stroke was induced by MCAO as previously described, with some modifications (Belayev et al., 1999). Mice were anesthetized with chloral hydrate (400 mg/kg intraperitoneally) (Gauillard et al., 2002; Ozden and Isenmann, 2004). Under an operating microscope, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a ventral midline incision. A 6-0 monofilament nylon suture, coated with silicon hardener mixture, was introduced into the ECA lumen and gently advanced 10 mm from the bifurcation of the CCA to occlude the MCA. Relative cerebral blood flow (rCBF) was measured using a laser Doppler flowmetry (Moor Instruments Ltd, UK). Blood flow was observed to drop to $<30\%$ of the baseline and remained at that level throughout the occlusion period. Mice whose rCBF remained $\geq 30\%$ of baseline were excluded from the experiment. After 1 h of occlusion, MCA blood flow was restored by withdrawal of the suture. The rectal temperature was monitored and kept at 37.0 ± 0.5 °C with a thermostatically controlled heating pad. The left femoral artery was catheterized for continuous arterial blood gas monitoring to ensure the physiological conditions were the same in the vehicle- and drug-treated animals at baseline. Sham mice underwent a similar procedure, with the omission of MCAO. Lithium chloride (5 mmol/kg), maslinic acid (0.1, 1 and 10 mg/kg) or vehicle was administered intragastrically starting at 24 h after reperfusion, once daily, for 7 consecutive days. At the end of day 7 (24 h after drug administration), the mice were killed for histological analysis and western blot assay (Fig. 1B).

2.3. Intracerebroventricular administration of LY294002

Mice were randomly divided into four groups: sham-operation group (Sham), MCAO group treated with vehicle (Vehicle), MCAO group treated with maslinic acid (MA), and MCAO group treated with maslinic acid and LY294002 (MA+LY). Intracerebroventricular administration of LY294002 into the left lateral ventricle was performed after 24 h of ischemia, as previously described (Shimamura et al., 2013). Mice were placed in the stereotaxic apparatus under anesthesia and a burr-hole was drilled in the skull (anteroposterior 1.5 mm, lateral 1.0 mm from bregma, depth 2.5 mm). LY294002 (2 μL of 25 μM in 25% DMSO in PBS) or vehicle was intracerebroventricularly injected into the left lateral ventricle via the hole. Maslinic acid (10 mg/kg) or vehicle was administered intragastrically starting at 24 h after reperfusion once daily. The histological analysis and western blot assay were evaluated at 72 h after ischemia/reperfusion injury.

2.4. Primary neuron cultures

Cerebral cortical neurons were prepared as previously described (Xiong et al., 2004), with some modifications. In brief, cortices were dissected from embryonic day 18 (E18) Sprague-Dawley rat embryos and incubated with 0.05% EDTA-trypsin then passed through a nylon sieve (80 μm pore size) into Minimum Essential Medium (MEM) supplemented with glucose (0.6% wt/vol), penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% (vol/vol) horse serum. The cells were adjusted to approximately 1×10^9/L and planted into 6-well plates, which were pre-coated with 10 mg/L poly-L-lysine, at 37 °C in an atmosphere of 5% CO_2 and 95% O_2. After 4 h, the medium was removed, and neurons were maintained in Neurobasal medium supplemented with 0.02% B-27 and L-glutamine (0.5 mM). Under these conditions, the cultures typically contained more than 95% neurons as assessed by MAP2-specific immunocytochemical detection.

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