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MLC901, a Traditional Chinese Medicine protects the brain against global ischemia

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ABSTRACT

Global ischemia leads to damage in the hippocampal CA1 region and is associated with behavioral deficits. NeuroAid (MLC601 and MLC901), a Traditional Chinese Medicine is used in China for patients after stroke. We have investigated here the effects of MLC901 on brain injury and deficits after global ischemia in the rat. Global ischemia induced by four-vessel occlusion resulted in degeneration of CA1 neurons. MLC901 (0.074 mg/ml) prevented both necrosis and apoptosis of neurons up to 3 h after ischemia. These positive MLC901 effects were associated with a decrease in Bax expression and in levels of the lipid peroxidation product malondialdehyde. Using the PI3-kinase inhibitor LY294002 we also demonstrated the critical role of the Akt pathway in MLC901-mediated neuroprotection. MLC901 enhanced neurogenesis. Furthermore, MLC901 improved functional recovery of rats after global ischemia as assessed by the Morris water maze. In this test MLC901 reduced the increase in escape latency and in swim distance induced by ischemia. MLC901 also improved post-ischemic grip strength. If observations made with rats can be extended to humans, then MLC901 will represent a novel therapeutic strategy after cardiac arrest with a clinically interesting time window of protection.

1. Introduction

Global cerebral ischemia is a clinical outcome occurring as a consequence of cardiac arrest, reversible severe hypotension or other situations that deprive the brain of oxygen and glucose. Each year, estimated 350,000 people undergo sudden cardiac arrest in Europe (Herlitz et al., 1999). Recovery without residual neurologic damage after global ischemia is rare. Besides the dramatic clinical aspects of the disease, subsequent neurological injuries represent a considerable financial burden in medical and rehabilitation expenses and lost productivity. Hypothermia has been described as the only therapy which can improve outcome after cardiac arrest (Nolan et al., 2003; Sterz et al., 2006; Zhao et al., 2007). The need for new therapeutic strategies is imperative (Ginsberg, 2008).

The pyramidal neurons of the CA1 region in the hippocampus are among the cells most vulnerable to loss of blood supply to the brain in humans and rodents (Pulsinelli and Brierley, 1979). Cell death occurs days after the ischemic insult, a phenomenon termed delayed neuronal death, which is associated with severe behavioral impairments. Global ischemia when it is not lethal is often followed by some types of recovery. A large part of the recovery after ischemia is associated with the capacity of the brain to induce spontaneous adult neurogenesis, which generates functional neurons in subventricular and subgranular zones of the hippocampus (Alvarez-Buylla and Lim, 2004; Eriksson et al., 1998; Sharp et al., 2002; Di Filippo et al., 2008; Zhang et al., 2008). However, brain self-repair by neuronal replacement from endogenous precursors is insufficient and functional recovery remains incomplete. Amplification of this self-repair mechanism could be a promising strategy for developing restorative therapies for global ischemia.

Interestingly, NeuroAid (MLC601) is a Traditional Chinese Medicine (TCM), which was first registered by the Sino Food and Drug Administration in 2001 after being evaluated in clinical trials in China as a drug to facilitate recovery after stroke (Chen et al., 2009). It combines 9 herbal and 5 animal components. In a previous study, we demonstrated in a rodent model of focal ischemia that MLC601 and also MLC901, which is a simplified version with only the nine herbal components, improved survival, protected the brain from ischemic injury and drastically decreased functional deficits (Heurteaux et al., 2010). MLC601/MLC901 also

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prevented neuronal death in an *in vitro* model of excitotoxicity using cultures of cortical neurons exposed to glutamate. In addition, MLC601/MLC901 treatments have been shown to induce neurogenesis, promote cell proliferation and stimulate the development of a dense axonal and dendritic network. In the present work we investigated the therapeutic effectiveness of MLC901 on brain injury and motor deficits induced by global ischemia (four-vessel occlusion model) in rats. We also deciphered some of mechanisms associated with the effects of this TCM by analyzing MLC901 actions on neuronal cell death (necrosis and apoptosis), lipid peroxidation, phosphinositide-3-kinase/Akt pathway and neurogenesis.

2. Materials and methods

2.1. Animals

All experiments were performed on male Wistar rats (250 g) from Charles River Laboratories (France) and used according to policies on the care and use of laboratory animals of European Communities Council Directive (86/609/EEC). The local Ethics Committee approved the experiments (protocol numbers NCA/2006/10-1 and NCA/2006/10-2). All efforts were made to minimize animal suffering and reduce the number of animals used. The animals were housed under controlled laboratory conditions with a 12-h dark–light cycle, a temperature of 21 ± 2 °C, and a humidity of 60–70% for at least one week prior to drug treatment or surgery. Rats had free access to standard rodent diet and tap water.

2.2. Experimental design

The study was carried out on rats divided into three groups. (i) Sham group, (ii) Ischemic group + vehicle and (iii) Ischemic group + MLC901. MLC901 was provided by Moleac (Singapore). The composition of MLC901/capsule was the following: 0.57 g Radix astragali, 0.114 g Radix salvia miltiorrhizae, 0.114 g Radix paeoniae rubra, 0.114 g Rhizoma chuanxiong, 0.114 g Radix angelicae sinensis, 0.114 g *Carthamus tinctorius*, 0.114 g *Prunus persica*, 0.114 g Radix polygalae, 0.114 g Rhizoma acori tatarinowii. The TCM was diluted in saline (as vehicle) at the concentration of 74 mg/ml (Stock solution) and incubated under agitation for 1 h at 37 °C. The solution was then filtered with 0.22 μm filter. Rats were intraperitonally injected with a single dose of MLC901 (500 μl/rat) 30 min, 1, 2, 3 or 6 h after ischemia followed by one injection per day for three or seven days after reperfusion. The MLC901 intraperitoneal dose used has been selected based on the concentrations used in humans (oral administration: 4 capsules three times a day (Chen et al., 2009)) and reported to the rat according to the formula for dose translation based on BSA (Body Surface Area) from animal to human (Reagan-Shaw et al., 2008). In the neurogenesis and behavioral experiments where the reperfusion time exceeded one week MLC901 was administered in drinking water at the concentration of 10 mg/ml. Researchers, who carried out the ischemic surgery and measures of brain injury were blinded in regard to the treatment code.

2.3. Induction of the transient global ischemia

Forebrain ischemia was performed by four-vessel occlusion (Pulsinelli and Brierley, 1979). In brief, rats were anesthetized by inhalation of 2% halothane mixed with 30% oxygen and 70% nitrous oxide. Core temperature was monitored with a rectal probe. Core temperature was registered from the onset of anesthesia to 1 h reperfusion. Body temperature was maintained at 37 °C with a heating blanket during and in the hours following surgery. Heart rate, blood pressure and percutaneous arterial oxygen saturation were monitored. No difference in physiological parameters was seen between the different groups (data not shown). Animals were placed in a Kopf stereotaxic frame and the vertebral arteries were irreversibly occluded by electrocoagulation. The common carotid arteries were then exposed and a polyester non absorbable suture (Ethicon) was looped around the carotid arteries. On the following day, the animals were ventilated with 1% halothane and miniature aneurysm clips were attached to occlude both carotid arteries. Anesthesia was then disconnected and carotid arteries were clamped for 20 min. Rats lost their righting reflex during ischemia. Following ischemia, reperfusion was achieved by declamping the arteries. Negative controls were sham-operated rats. Each animal was anesthetized, the carotid arteries were isolated, but they were not clamped.

2.4. Tissue preparation and analysis of neuronal density (Cresyl violet and Fluoro-Jade B stainings)

One, 3 or 7 days following ischemia, animals were anesthetized, brains quickly extracted and fresh frozen in isopentane at −40 °C. Coronal sections (10 μm) were cut on a cryostat (Leica) and post-fixed by immersion in 4% paraformaldehyde/10^{-2} M phosphate-buffered saline (PBS) for 30 min. Slides were then dehydrated in ethanol baths (50, 70 and 100%), air-dried and stored at −20 °C until use. Cresyl Violet, a dye that stains the Nissl bodies in the stellate somas of viable neurons was used to analyze the cell survival in the different groups. For each brain studied (n = 6 per time point and treatment), two sections were placed on 3-aminopropylethoxysilane-coated slides, and 10 slides (randomly chosen) per rat were used in each stage of analysis. Slides were dipped into a solution of 1% Cresyl Violet in 0.25% acetic acid for 3 min, rinsed, dehydrated and mounted with Entellan. A neuropathologist, blind to experimental conditions, performed the histological assessment using light microscopy. The neuronal density of the hippocampal CA1 subfield was determined 7 days post-ischemia on cresyl-violet stained sections of the dorsal hippocampus corresponding to brain sections located between 3.14 and 4.16 mm posterior to bregma (Pulsinelli and Brierley, 1979). The total linear length of CA1 sector was measured by means of a digitizer. The number of living neurons in the stratum pyramidale within the CA1 subfield was counted using a Leica Aristoplan photomicroscope at a magnification of 400X. Neurons that had shrunken cell bodies with surrounding empty spaces were excluded. The neuronal density of CA1 sector, i.e. the number of intact pyramidal cells per 1 mm linear length of the CA1 stratum pyramidale observed in each 10-μm section was quantified. Thus, a mean value for each hippocampal CA1 substructure was obtained from 10 bilateral measurements on two sections per slide and 10 slides per rat, for the six animals in each of the experimental groups. The neuronal density for a given animal represents the average of both right and left hippocampal neuronal cell densities. Neuronal density values are expressed as mean ± S.D. Data analysis was performed by two-factor (experimental condition and brain region) analysis of variance followed by Tukey's w test for multiple comparisons.

For assessment of neuronal degeneration, we used staining with Fluoro-Jade B, a polyanionic fluorescein derivative which sensitively and specifically binds to degenerating neurons (both apoptotic and necrotic). Due to its high affinity it is an excellent marker for detecting degenerating neurons. Fluoro-Jade B staining was performed 7 days following ischemia according to the protocol described by Schmued et al (Schmued et al., 1997).

2.5. TUNEL staining

Terminal deoxynucleotidyl transferase (TdT)-mediated 2′-deoxyuridine 5′-triphosphate-biotin nick-end labeling (TUNEL) staining was performed 3 days following ischemia by using the *In Situ* Cell Death Detection Kit (Roche, Basel, Switzerland), which allows to detect the DNA fragmentation of apoptotic cells in ischemic brain tissue. Coronal 10 μm frozen sections were rehydrated in ethanol (95, 70 and 50%) followed by PBS and bathed in 0.3% hydrogen peroxide/methanol to inactivate endogenous peroxidase. Sections were then permeabilized in 0.3% Tween/PBS and washed twice in PBS. Labeling of 3-OH terminal DNA fragments was then performed at 37 °C for 2 h by using the TUNEL reaction mixture according to the manufacturer's protocol. Positive control was obtained by pre-incubation of a section with DNAse I (20 μg/ml) for 15 min at 37 °C before incubation with biotinylated dUTP. Quantitative analyses *i.e* counting of the number of TUNEL-positive cells/0.5 mm^2 were performed in the hippocampal CA1 subfield. TUNEL-positive cells displayed a brown staining within the nucleus in the apoptotic cells.

2.6. Immunohistochemistry

Frozen brain sections (25 μm thick) were immersed in 0.3% H_2O_2/methanol for 10 min, permeabilized in 0.1% Triton/PBS for 10 min and blocked with 3% goat serum/PBS for 2 h at room temperature. After a PBS rinse sections were incubated with the primary antibody overnight. The antibodies used were the rabbit polyclonal Bax antibody (Santa Cruz, SC493, diluted 1/300) or the rabbit polyclonal Akt (1:1000, Cell Signaling Technology, USA), phospho-Ser-473-Akt (p-Akt) (1:1000, Cell Signaling Technology, USA). After the primary incubation and three rinses in PBS, sections were then incubated in biotinylated horse anti-rabbit IgG (Jackson ImmunoResearch, diluted 1/15,000) for 2 h at room temperature. Bax expression was visualized by 3,3-diaminobenzidine (DAB) staining using VectaStain ABC kit (Biovalley). All sections were washed and mounted with Entellan. In the experiments with the PI3-kinase inhibitor LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1benzopyran-4-one), we intracerebroventricularly injected this drug (5 μl bregma, dorso-ventral, −3.6 mm below the cortical surface, medio-lateral, 1.4 mm from bregma, antero-posterior,−0.8 mm from bregma) at the dose of 50 nmol in 25% dimethyl sulfoxide in PBS 1 h before ischemia.

2.7. Western blotting

Whole-cell protein extraction was performed using standard procedures. Samples were obtained from ischemic hippocampi (vehicle- and MLC901-treated) and from sham-operated controls. (n = 3 per group). Fresh brain tissue was cut into pieces after 24 h of reperfusion and homogenized in four volumes of cold lysis buffer (20 nmol/l Tris pH: 7.5, 137 mmol/l NaCl, 2 mmol/l EDTA, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail) on ice. The homogenates were centrifuged at 12,000 g for 30 min at 4 °C. The supernatant was stored at −70 °C until further use. Protein concentrations were measured using conventional Bradford's method. Fifty microgram proteins from each experimental group were applied to 10% SDS