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A derivative of the CRMP2 binding compound lanthionine ketimine provides neuroprotection in a mouse model of cerebral ischemia

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ABSTRACT

Lanthionines are novel neurotrophic and neuroprotective small molecules that show promise for the treatment of neurodegenerative diseases. In particular, a recently developed, cell permeable lanthionine derivative known as LKE (lanthionine ketimine 5-ethyl ester) promotes neurite growth at low nanomolar concentrations. LKE also has neuroprotective, anti-apoptotic, and anti-inflammatory properties. Its therapeutic potential in cerebral ischemia and its mechanisms of neurotrophic action remain to be fully elucidated. Here, we hypothesize that the neuroprotective actions of LKE could result from induction or modulation of CRMP2. We found that treating primary cultured mouse neurons with LKE provided significant protection against *t*-butyl hydroperoxide-induced neuronal death possibly through CRMP2 upregulation. Similarly, *in vivo* studies showed that LKE pre and/or post-treatment protects mice against permanent distal middle cerebral artery occlusion (p-MCAO) as evidenced by lower stroke lesions and improved functional outcomes in terms of rotarod, grip strength and neurologic deficit scores in treated groups. Protein expression levels of CRMP2 were higher in brain cortices of LKE pretreated mice, suggesting that LKE's neuroprotective activity may be CRMP2 dependent. Lower activity of cleaved PARP-1 and higher activity of SIRT-1 was also observed in LKE treated group suggesting its anti-apoptotic properties. Our results suggest that LKE has potential as a therapeutic intervention in cerebral ischemia and that part of its protective mechanism may be attributed to CRMP2 mediated action and PARP-1/SIRT-1 modulation.

1. Introduction

Lanthionine (Lan) is a nonproteogenic amino acid formed in the central nervous system (CNS) through the transsulfuration pathway and possibly through other glutathione-dependent biochemical pathways (Hensley et al., 2010a). In the mammalian brain, Lan undergoes aminotransferase conversion to yield an unusual cyclic thioether, lanthionine ketimine (LK; 2H-1,4-thiazine-5,6-dihydro-3,5-dicarboxylic acid). LK has been observed to demonstrate neuroprotective, neurotrophic and anti-inflammatory activities (Hensley et al., 2010b). Recently, a novel cell-permeable derivative, lanthionine ketimine-ethyl ester (LKE), was shown to protect NSC-34 motor neuron-like cells from oxidative toxicity mediated by hydrogen peroxide (H_2O_2) and also to promote the growth factor-stimulated outgrowth of neurites in these cells (Hensley et al., 2010b, 2011).

One of the most significant events triggered by the loss of blood supply to the brain during ischemia is axonal injury due to the imbalance of Na^+ and/or Ca^{2+} influx into axons, which leads to the breakdown of the cytoskeleton, the final outcome of which is neuronal degeneration and death. Collapsin response mediator proteins (CRMPs) are cytoskeleton-regulating proteins expressed in dendrites, axons and growth cones. CRMPs have important roles, both developmentally and following axonal damage, as exemplified by the observation that expression of semaphorin-3A and its downstream effector CRMP2 was associated with neuronal injury in the brains of patients with epilepsy, Alzheimer's disease (AD), and cerebral ischemia (Gu et al., 2000; Kee et al., 2001).

Poly(ADP-ribose) polymerase-1 (PARP-1) activation, among several other mechanisms, has been implicated in the oxidative

Abbreviations: CRMP2, collapsin response mediator protein 2; H_2O_2, hydrogen peroxide; LKE, lanthionine ketimine-ethyl ester; MCAO, middle cerebral artery occlusion; NDS, neurologic deficit score; *t*-BuOOH, tertiary butylhydroperoxide; TFM, terpene free material; p-MCAO, permanent distal middle cerebral artery occlusion; PARP-1, poly(ADP-ribose) polymerase-1; TTC, triphenyltetrazolium chloride; SIRT-1, situin-1.

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stress-mediated cell death during ischemic injury. Release of apoptosis inducing factor (AIF) mediated by PARP-1 also plays a critical role in the cascade of neuronal loss and death (Alano et al., 2010). The histone deacetylase sir2 (silent information regulator 2) orthologue sirtuin-1 (SIRT-1), another NAD^+ dependent enzyme, is involved in regulating energy homeostasis by controlling the acetylation status and the activities of a number of enzymes and transcriptional regulators. Since both PARP-1 and SIRT-1 use NAD^+ for their activity, it has been thought that the increased activity of one molecule might interfere with the activity of the other.

In the present study, we were interested in evaluating the therapeutic potential of LKE in stroke and its implications for CRMP2 modulation as well as its ability to modulate the expression of other proteins involved in the apoptotic process following ischemia, specifically the PARP-1/SIRT-1 system. This is the first study to evaluate the role of LKE in delayed ischemic response induced by permanently occluding the distal part of middle cerebral artery (p-MCAO).

2. Materials and methods

2.1. Animals

All animal studies were conducted in accordance to the protocol approved by The University of Toledo Health Science Campus Institutional Animal Care and Use Committee and the guidelines prescribed by the National Institutes of Health. C57BL/6 male (5–10 weeks old; 25–30 g) and female (timed 17 days pregnant) mice were procured from Charles River Laboratories, Wilmington, MA and were housed at 22 ± 1 °C with a 12 h:12 h light/dark cycle with water and food available *ad libitum*.

2.2. Permanent middle cerebral artery occlusion (p-MCAO)

We used our previously optimized method to occlude the distal portion of the MCA (Shah et al., 2011). Briefly, mice were anesthetized with isoflurane (Baxter Healthcare, Deerfield, IL), initially with 3–5% in the induction chamber and then maintained at 1% throughout the surgical procedure via the nasal cone. With the aid of a surgical microscope, a 10 mm vertical skin incision was made between the right eye and ear, and the underlying temporal bone was exposed by moving the temporal muscle aside. Accordingly, a 2.0-mm burr hole was drilled and the distal part of MCA was occluded directly using a bipolar coagulator, and complete cessation of blood flow at the occlusion site was confirmed by laser Doppler flowmetry. Mice having complete cessation of the blood flow were selected for the study. Rectal temperature was monitored continuously with a rectal probe and maintained at 37.0 ± 0.5 °C during the surgery with a heating blanket. After the surgery, animals were left to recover in a temperature-regulated recovery chamber before shifting to home cages. No mortality was observed in this model and all the mice survived.

2.3. Drug treatment

Mice were randomized into different treatment groups, and personnel working on the study were blinded from the experimental design. LKE was synthesized as described previously (Hensley et al., 2010a) and solubilized to 25 mg/mL in physiological saline by careful titration to neutrality with 2 N NaOH. In the pre-treatment paradigm, LKE (100 mg/kg p.o.) or vehicle (physiological saline) was administered for 7 days prior to p-MCAO. In the post-treatment paradigm, LKE (100 mg/kg) was administered via an intraperitoneal (i.p.) injection 4 h after the p-MCAO and then daily for 7 days. This dose was chosen based on the limits of LKE solubility in saline and reported dose efficacy in a mouse model of ALS (Hensley et al., 2010b).

2.4. Locomotor activity

Locomotor activity was evaluated by means of a rotarod task by a person blinded to the treatment groups. Mice were placed on a horizontal rod (Columbus Instruments, OH) that was made to rotate at 1 rpm with an acceleration rate of 1 rpm every 10 s until the animal fell from the rod. Each animal was tested three times per trial. All the animals were trained on the rotarod assembly prior to surgery. The duration for which each animal was able to stay on the accelerating rod was recorded as the latency to fall and registered manually. Locomotor activity was monitored 4 h before and 2, 5 and 7 days after p-MCAO surgery.

2.5. Grip strength

Grip strength was evaluated by holding the mice by tails and placing their forelimbs on a specially designed pull bar assemblies (Grip strength meter, Columbus Instruments, OH). Peak amount of force animals exert was displayed on the digital display and noted. Each animal was tested three times per trial at 4 h before, 2 and 7 days after p-MCAO surgery.

2.6. Neurological deficit scores (NDS)

NDS were evaluated by a 28-point score pattern optimized by our group (Shah et al., 2011). A person blinded to the treatment evaluated NDS 7 days after p-MCAO; the evaluation included both sensory and motor deficits, such as body symmetry, gait, climbing, circling behavior, front limb symmetry, compulsory circling, and whisker response. Each of the seven tests included in the 28-point NDS was graded from 0 to 4, with higher scores indicating severe deficits.

2.7. Infarct volume analyses

Animals from all the groups were euthanized 7 days after p-MCAO. Brains were dissected out and sliced into five 2-mm-thick coronal sections before incubating in 1% triphenyltetrazolium chloride (TTC) (Sigma Co., MI). The infarct area was estimated from five slices of each brain, measuring rostral and caudal sides of each individual slice in conjunction with the thickness and expressed as a percentage of the volume of the contralateral hemisphere. A person blinded to the treatment groups measured the infarct volume with the help of ImageJ software provided by NIH.

2.8. Western blots

Brain cortices of ischemic and non-ischemic mice were dissected out, weighed, and homogenized as described previously (Shah et al., 2011). Protein samples were analyzed by loading equivalent amounts of protein (25 μg) onto 10% SDS–polyacrylamide gels and incubated with different primary antibodies; rabbit anti-actin, 1:3000 (Sigma Co., MI); rabbit anti-CRMP2, 1:50,000 (Millipore, Billerica, MA), rabbit anti PARP-1, 1:1000 (cell signaling, Danvers, MA); rabbit anti SIRT-1, 1:1000 (Cell Signaling, Danvers, MA) and/or rabbit anti histone H3, 1:3000 (ThermoScientific, Waltham, MA). Images were analyzed using ImageJ software provided by the NIH. The densitometric values were normalized with respect to β-actin/histone H3.