Neuroscience Letters 545 (2013) 64–68

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

A selective inhibitor of Drp1, mdivi-1, protects against cell death of hippocampal neurons in pilocarpine-induced seizures in rats

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HIGHLIGHTS

- Mdivi-1 attenuates seizure-induced neuronal death in the hippocampus.
- The neuroprotection effect of mdivi-1 was dose-dependent.
- The mechanisms involve CytC release, AIF translocation and caspase-3 activation.

ARTICLE INFO

Article history:
Received 1 February 2013
Received in revised form 29 March 2013
Accepted 6 April 2013

Keywords:
Mitochondrial fission
Mdivi-1
Epilepsy
Apoptosis-inducing factor
Cytochrome c

ABSTRACT

Mdivi-1 is a selective inhibitor of a mitochondrial fission protein Drp1. Recent studies demonstrated that inhibition of Drp1 provides neuroprotection in vitro and in vivo. In this study, we examined the role of mdivi-1 in hippocampal neuron death after seizures induced by pilocarpine. Our data showed that pretreatment with mdivi-1 (1.25 mg/kg) significantly attenuated the neuronal death in hippocampus induced by seizures. This neuroprotective effect was dose-dependent. In addition, the seizures resulted in up-regulation of Drp1 expression and mdivi-1 treatment had no effect on the expression. Moreover, we also found that mdivi-1 (1.25 mg/kg) treatment reversed the release of cytochrome c (CytC), translocation of apoptosis-inducing factor (AIF) induced by seizures while inhibiting the activated caspase-3. Altogether, our data suggested that mdivi-1 exerts neuroprotective effects against cell death of hippocampal neurons induced by seizures, and the underlying mechanism may be through inhibiting CytC release, AIF translocation and suppression of the mitochondrial apoptosis pathway.

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1. Introduction

Mitochondrial dysfunction has been shown to be associated with epileptic seizures in the central nervous system [1,6,13,14]. Mitochondria play an important role in cell death by mediating both intrinsic and extrinsic apoptosis signaling pathways. A new development in the understanding of mitochondrial regulation in apoptosis is the discovery of drastic morphological changes in the organelles. Mitochondria change their morphology by undergoing either fusion or fission [7]. Mitochondrial fission plays important roles in the regulation of apoptosis [21]. Mitochondrial fission involves the constriction and cleavage of mitochondria by fission proteins, such as dynamin-related protein (Drp1) and Fission 1 (Fis1) [17,20]. Drp1 has recently been demonstrated to be an intrinsic component of multiple mitochondria-dependent apoptosis pathways [8].

Mitochondrial division inhibitor (mdivi-1) is a highly efficient small molecule that selectively inhibits mitochondrial fission Drp1. It inhibits Drp1 GTPase activity by blocking the self-assembly of Drp1 in vitro and causes the rapid reversible and dose-dependent formation of netlike mitochondria in wild-type cells [5,18]. Recent study has shown that pretreatment with mdivi-1 could provide neuroprotection against glutamate toxicity and oxygen-glucose deprivation (OGD) in vitro and ischemic brain damage in vivo [9].

To our knowledge, the role of mdivi-1 has not yet been studied in any animal model of epilepsy. Thus, in this study we investigated whether mdivi-1 might attenuate hippocampal neuron damage after seizures induced by pilocarpine. In addition, we also examined the potential protective mechanisms initiated by mdivi-1, including cytochrome c (CytC) and apoptosis-inducing factor (AIF)-dependent mitochondrial apoptosis pathway.

2. Materials and methods

2.1. Animals and model of seizures

Adult male Wistar rats (Experimental Animal Center of Zhengzhou University) weighing 250–300 g were used. All rats

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http://dx.doi.org/10.1016/j.neulet.2013.04.026

were maintained at room temperature (20 ± 2 °C), with controlled illumination (12 h light and dark cycle) and free access to water and food. The experimental procedures were approved by the Commission of Zhengzhou University for ethics of experiments on animals in accordance with international standards.

The experimental rats were given lithium chloride intraperitoneally (3 mEq/kg, i.p.). Twenty hours after lithium chloride treatment, experimental rats received pilocarpine (30 mg/kg, i.p. Sigma, USA) and control rats received the same volume of 0.9% saline instead of pilocarpine. Scopolamine methylnitrate (1 mg/kg) was injected subcutaneously 30 min before the administration of pilocarpine to prevent peripheral cholinergic effects. Rats showing stage 4 or 5 convulsive seizures [16] were included in the experimental group. Seizures were allowed to continue for 60 min and then terminated by an injection of diazepam (10 mg/kg, i.p. Sigma, USA). Rats were killed by decapitation at 2, 8, 24, and 72 h after seizures.

2.2. Drug administration and experimental groups

Rats were randomly divided into 5 treatment groups: (1) control, as described above; (2) pilocarpine, lithium chloride-pilocarpine administered, as described above; (3) pilocarpine + DMSO, an intravenous bolus of DMSO (0.1 ml of 0.1% DMSO) was given before pilocarpine injection. (4) Mdivi-1 dose 1 group, an intravenous bolus of mdivi-1 (1.25 mg/kg) was given 15 min before pilocarpine injection; (5) Mdivi-1 dose 2 group, an intravenous bolus of mdivi-1 (0.25 mg/kg) was given 15 min before pilocarpine injection.

2.3. Histology

Rats under anesthesia were intracardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline and the brains were removed. The paraffin-fixed brains were cut into coronal sections 10 μm thickness. Nissl staining with toluidine blue was then performed. For every tenth section (six sections per animal), we counted in a blinded manner the number of surviving hippocampal CA1 and CA3 pyramidal cells per 1-mm length of the bilateral hemispheres by use of a microscope with high magnification (400×). The detailed procedures were carried out as described previously [11].

2.4. Cell fraction preparation

To obtain the whole-cell fraction, the hippocampi were homogenized in 10 volumes of the ice-cold homogenization buffer and then centrifuged at 12,000 × *g* for 10 min at 4 °C. Supernatants were collected, and protein concentrations were determined by using BCA protein assay kit (Beyotime, Jiangsu, China). To obtain mitochondrial, cytosolic and nuclear protein fractions, hippocampi were homogenized in a buffer. The fractions were isolated by centrifugation, as described previously [12]. Protein contents were determined before storage at −80 °C.

2.5. Western blot

Western blot was performed as described previously [19]. Briefly, thirty micrograms of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking in 5% fat-free milk in Tris-buffered saline with Tween buffer for 2 h, the membranes were incubated with primary antibodies such as anti-Drp1 (1:2000; Santa Cruz, USA), anti-CytC (1:3000; Santa Cruz, USA), anti-AIF (1:300; Abcam, UK), anti-caspase-3 (1:300; Abcam, UK) at 4 °C overnight. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000; Jingmei, Beijing, China) for 1 h at 37 °C. Immunoreactivity was intensified by chemiluminescence kit and exposed to film. Band intensity was quantified by densitometric analysis using a densitometer.

Table 1
Effects of mdivi-1 on neuron loss in hippocampus after seizures.

Group	Neuron number (mean ± S.D.)	
	CA1	CA3
Control	110.5 ± 7.8	126.8 ± 8.6
Pilo	52.6 ± 10.8*	66.3 ± 12.6*
Mdivi-1 dose 1	105.2 ± 8.7#	110.6 ± 11.8
Mdivi-1 dose 2	56.2 ± 9.8**	70.2 ± 9.6**

The numbers of surviving pyramidal cells per 0.5 mm length of the CA1 and CA3 subfields of the hippocampus were counted under light microscopy. Data are mean ± S.D. (n = 6/group). pilo, pilocarpine.
* $P < 0.05$ vs. control.
** $P > 0.05$ vs. pilo.
# $P < 0.05$ vs. pilo.

2.6. Statistical analysis

Data was expressed as mean ± S.D. The results were statistical analyzed using one-way ANOVA and the Newman–Keuls test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of mdivi-1 against neuron loss in hippocampus after seizures

To examine the extent of neuron loss in hippocampus after pilocarpine-induced seizures, toluidine blue was used for staining. Our results showed that seizures led to severe cell death at 72 h after seizures. The surviving neuron numbers were sharply decreased in pilocarpine group compared with control ($P < 0.05$) but no significant difference in neuron loss between the pilocarpine and pilocarpine + DMSO groups was found (data not shown). Moreover, mdivi-1 (1.25 mg/kg) treatment significantly attenuated the neuron loss induced by seizures ($P < 0.05$). However, there was no difference in the surviving neuron numbers between pilocarpine and mdivi-1 dose 2 groups ($P > 0.05$) (Fig. 1; Table 1).

3.2. Expression of Drp1 in hippocampus after seizures

Our data showed that Drp1 protein levels started to increase at 2 h ($P < 0.05$) after seizures, reached a maximum level at 8 h and remained significantly elevated until 72 h ($P < 0.05$) (Fig. 2A). Mdivi-1 treatment has no effect on the expression of Drp1 ($P > 0.05$) (Fig. 2B).

3.3. Effect of mdivi-1 on CytC release, AIF translocation and caspase-3 activation

AIF translocated from mitochondria to the nucleus in hippocampus beginning at 2 h and peaking at 24 h after seizures ($P < 0.05$) (Fig. 3A and C). Consistent with nuclear translocation, AIF level decreased in the mitochondrial fraction ($P < 0.05$) (Fig. 3A and C). As well, CytC level increased in the cytosolic fraction and decreased in the mitochondrial fraction after seizures ($P < 0.05$) (Fig. 3A and B). Moreover, the active cleavage product of caspase-3 appeared at 24 h after seizures and increased until 72 h ($P < 0.05$) (Fig. 4A). Compared with pilocarpine group, pretreatment with mdivi-1 (1.25 mg/kg) significantly suppressed the translocation of AIF ($P < 0.05$) (Fig. 3D and F), release of CytC ($P < 0.05$) (Fig. 3D and E) and caspase-3 activation ($P < 0.05$) (Fig. 4B) at 24 h after seizures

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