Available online at www.sciencedirect.com
SCIENCE DIRECT
Brain Research 1037 (2005) 59–69
BRAIN RESEARCH
www.elsevier.com/locate/brainres

Research report

Calpain inhibitor MDL 28170 protects hypoxic–ischemic brain injury in neonatal rats by inhibition of both apoptosis and necrosis

Masanari Kawamura, Wako Nakajima, Akira Ishida*, Akiko Ohmura, Shinobu Miura, Goro Takada

Department of Pediatrics, Akita University School of Medicine, 1-1-1 Hondo, Akita, 010-8543, Japan

Accepted 17 December 2004

Abstract

MDL 28170 is a CNS-penetrating calpain inhibitor, and we examined the effects of MDL 28170 on hypoxic–ischemic brain injury in immature brain using the Rice-Vannucci model. Immediately after hypoxic exposure, 24 mg/kg of MDL 28170 was injected intraperitoneally as an initial dose, followed by 12 mg/kg every 4 h for a total dose of 60 mg/kg over 12 h post-HI. A vehicle control group received peanut oil injection instead. Macroscopic evaluation of brain injury revealed the neuroprotective effect of MDL 28170 after 12 h post-HI. Neuropathological quantitative analysis of cell death showed that MDL 28170 significantly decreased the number of necrotic cells in all the examined regions except for cingular cortex, and the number of apoptotic cells in caudate putamen, parietal cortex, hippocampus CA1, and laterodorsal thalamus. Western blots showed that MDL 28170 suppressed 145/150 kDa subunits of α-spectrin breakdown products (SBDP) in cortex, hippocampus, thalamus, and striatum, and also 120-kDa subunit of SBDP in all regions except for striatum. This suggests that MDL 28170 inhibited activation of calpain and caspase-3, respectively.

Our results indicate that post-hypoxic MDL 28170 injection is neuroprotective in HI newborn rat brain by decreasing both necrosis and apoptosis. SBDP expression also suggests that MDL 28170 injection inhibits both calpain and caspase-3 activation after HI insult.

Theme: Development and regeneration
Topic: Neuronal death

Keywords: Immature brain; Excitotoxicity; Calpain; Caspase-3; Neuronal cell death

1. Introduction

Calpain (EC 3.4.22.17) is a calcium-activated, neutral, cytosolic cysteine protease [32] and is proposed to participate in the turnover of cytoskeletal proteins and regulation of kinases, transcription factors, and receptors [11,54]. Over the past decade, several investigators have focused on calpain-mediated proteolysis and its contribution to necrotic neuronal death in ischemic and excitotoxic neuronal injury [63]. Many studies indicate that both apoptotic and necrotic mechanisms account for neuronal death after cerebral hypoxic ischemia (HI) in different neonatal animal models [12,24,29,41,45,62], and electron microscopy also confirms that there is a morphological continuum from apoptosis to necrosis [40]. Although calpain activation is historically assumed to result in necrotic cell death [4,51,53], contribution to apoptosis has been suggested in vivo [31,39,44,55].

Calpain activation has been demonstrated in adult [17,37,47,50] and neonatal models [7] of hypoxia and ischemia. A massive release of glutamate occurring in the brain soon after ischemia causes additional cell death [48]. Elevated glutamate triggers a large influx of calcium into neurons [14], which precipitates an intracellular cascade of events including activation of calcium-dependent proteases such as calpains [42,59]. Once activated, calpains cleave

* Corresponding author. Fax: +81 18 836 2620.
E-mail address: aishida@doc.med.akita-u.ac.jp (A. Ishida).

0006-8993/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2004.12.050

structural and regulatory proteins such as αII-spectrin, βII-spectrin, and calpastatin in the cell, leading to neuronal death.

Inhibition of calpains should be effective in neonatal brain considering the high calpain content in the rapidly growing brain [6] and upregulation of calpain activity following HI [36]. Although apoptosis is more prominent as a mode of cell death after HI in neonates compared with the adult brain [5,22,41,52], necrosis predominates in almost all regions in neonatal rat model [15,56] except for cingular cortex, retrosplenial cortex, and thalamus [30,34]. Although Blomgren et al. showed that a calpain inhibitor CX295 decreased αII-spectrin degradation in vivo neonatal brain [8], the protective effect of calpain inhibitor on HI in immature brain has not vigorously studied so far. Here we used a calpain inhibitor MDL 28170 (carbobenzylzoxy-Val-Phe-H) because of its ability of penetrating the brain rapidly [26,27]. Moreover, MDL 28170 reduced infarct volume even when administered 6 h after the initiation of ischemia in the focal cerebral ischemia model in adult rats [26]. We hypothesized that MDL 28170 would protect neonatal brain after HI and inhibit both apoptosis and necrosis. We evaluated the mode of cell death using light microscopy, TUNEL staining, and electron microscopy. Different subunits of αII-spectrin break down products, i.e., 145/150 kDa and 120 kDa proteins were also detected by Western blots to measure calpain and caspase-3 activation, respectively.

2. Materials and methods

2.1. Unilateral cerebral hypoxic–ischemia model

All animals were treated in a manner that complied with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences in the Physiological Society of Japan and all experiments were performed in accordance with approved institutional animal care guidelines of Akita University School of Medicine. Wistar rats were purchased from Japan SLC (Shizuoka, Japan). Unilateral (right) cerebral hypoxic ischemia in the postnatal day (PND) 7 was based on the Rice-Vannucci model as previously reported [46,61]. In brief, PND7 rat pups were placed in a temperature-controlled incubator set to an ambient temperature 35 °C. Under anesthesia with inhaled diethyl ether, the right common carotid artery was isolated, double-ligated, and cut between the ligatures. After the surgical procedure, the animals recovered for 90 min in the temperature-controlled incubator and then were exposed to an 8% oxygen hypoxia. If an animal died before the 90 min of hypoxic period, hypoxic exposure was stopped at that time.

2.2. In vivo calpain inhibition

Preliminary experiments were done to decide the drug administration protocol. To know the toxic total amount of MDL 28170, each 80 mg/kg, 160 mg/kg, and 240 mg/kg in total was administered, referring that the optimal and lethal dose for adult rabbit when given by intravenous injection was 30 mg/kg and 60 mg/kg, respectively [33]. The total dose was divided and given intraperitoneally every 4 h until 24 h post-HI according to the half-life of MDL 28170 [26]. The pups given both 160 and 240 mg/kg MDL 28170 seized 12 h after the first injection. Next, we decided to use peanut oil as a vehicle because it was safely used for newborn rat pups [19]. Either a mixed solution of polyethylene glycol (PEG) and ethanol [33] or dimethyl sulfoxide [20] that was previously used as the vehicle prevented dams from feeding pups probably due to their specific smells. To decide the drug administration protocol, we prepared 5 groups; i.e., vehicle control group, each 60 mg/kg of MDL 28170 and 80 mg/kg up to 12 h, and each 120 mg/kg and 160 mg/kg to 24 h. The macroscopic brain injury in the right hemisphere was scored at 48 h post-HI using our rating scale (see Section 2.5) and each score was 2.8 ± 0.5, 1.4 ± 0.4, 2.3 ± 1.3, 1.9 ± 1.2, and 2.1 ± 0.6 (n = 5 in each group), therefore we adopted 60 mg/kg in total amount up to 12 h. We also confirmed that 24 mg/kg of MDL 28170 injection (the loading dose when we administered 60 mg/kg in total amount to 12 h) did not bring non-specific drug-induced hypothermia for 2 h after injection (MDL 28170 group, 36.7 ± 0.2 °C vs. vehicle control group, 36.6 ± 0.2 °C, n = 5 in each group).

From these results, we decided the protocol as follows: immediately after the end of hypoxic exposure, the first dose, 24 mg/kg of MDL 28170, was injected intraperitoneally. Pups subsequently received 12 mg/kg of MDL 28170 every 4 h up to 12 h post-HI. MDL 28170 was diluted with peanut oil, so the injection volume was 0.05 ml per animal. Equal volume of peanut oil was injected in vehicle control group.

2.3. Tissue preparation

Pups were sacrificed at 0, 6, 12, 24, and 48 h after hypoxic exposure for Western blotting (n = 6 per time point) and histological analysis (n = 6 per time point). For Western blotting sample, we separated a hemisphere to cortex, striatum, thalamus, and hippocampus. The tissue samples were homogenized in lysis buffer and centrifuged at 23,500 × g for 60 min at 4 °C. The supernatant was decanted and stored at −70 °C until analysis. For histological study, pups were exsanguinated with PBS, then perfused and fixed with paraformaldehyde. Brains were embedded in paraffin, sectioned at a thickness of 2.5 μm, and processed for H and E, Nissl stain, and TUNEL as described previously [61]. To confirm the finding of light microscopy, additional 4 brains were processed for electron microscopy. Twenty-four and 48 h after HI, pups (n = 2 at each time point) in the vehicle control group were anesthetized with ether and perfused with 70 ml of PBS followed by 150 ml of 1% paraformaldehyde and 1.25% glutaraldehyde (GA) in 0.1 M PBS (pH 7.4). After perfusion, brains were removed and