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Temporal–spatial expression of ENOLASE after acute spinal cord injury in adult rats

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ABSTRACT

ENOLASE enzymes are abundantly expressed, cytosolic carbon–oxygen lyases known for their role in glucose metabolism. Recent accumulation of evidence revealed that, in addition to its glycolytic function, enolase is also associated with ischemia, hypoxia and to be a neurotrophic factor. To analysis the certain expression and biological function in central nervous system, we performed an acute spinal cord contusion injury model in adult rats. Western blot analysis indicated a marked upregulation of ENOLASE after spinal cord injury (SCI). Immunohistochemistry revealed wide distribution of enolase in spinal cord, including neurons and glial cells. Double immunofluorescent staining for proliferating cell nuclear antigen and phenotype-specific markers showed increases of enolase expression in proliferating microglia and astrocytes. Our data suggest that enolase may be implicated in the proliferation of microglia and astrocytes after SCI.

1. Introduction

Traumatic SCI is the most common form of acquired spinal cord injury. It is generally accepted that the acute SCI is a two-step process involving primary and secondary injury mechanisms (Zhao et al., 2011). Primary injury is due to irreversible tissue detrition and necrosis which resulted from external mechanical forces (Shen et al., 2008). The pathophysiologic changes of secondary injury involve multiple cellular and molecular events such as ischemia, edema, excitotoxicity, inflammatory injury, electrolyte imbalance, free radical damage, and apoptosis (Kwon et al., 2004; McDonald and Sadowsky, 2002). These events impact on the prognosis of spinal cord severely by aggravating the injury degree and expanding the range of lesion. Consequently, primary injury leads to edema and ischemia, thus triggering a secondary injury cascade that consists of inflammation and the release of free radicals and cytotoxic levels of excitatory amino acids. This secondary injury cascade causes a further damage to axons and contributes to the death of numerous cell types.

ENOLASE was discovered in 1934 by Lohman and Mayerhof. It is one of the most abundantly expressed cytosolic proteins in many organisms. It is a key glycolytic enzyme that catalyzes the dehydratation of 2-phosphoglycerate to phosphoenolpyruvate, in the last steps of the catabolic glycolytic pathway (Pancholi, 2001). It is a metalloenzyme that requires the metal ion magnesium (Mg^{2+}) to be catalytically active. Enolase is found from archaebacteria to mammals, and its sequence is highly conserved (Piast et al., 2005). Recently, glycolytic enzyme has also been reported to have diverse biological functions in different organisms (Aaronson et al., 1995; Mathur et al., 1992; Merkulova et al., 1997; Johnstone et al., 1992). And, ENOLASE, which has been well characterized for their catalytic function in glucose metabolism, is no longer considered to be the house-keeping enzymes only. In particular, ENOLASE has been reported to be a neurotrophic factor, 14-3-2 (Hattori et al., 1994, 1995), a hypoxic stress protein (Aaronson et al., 1995), c-Myc binding protein and transcription factor (Ray and Miller, 1991), and a strong plasminogen (PGn) binding protein (Nakajima et al., 1994; Pancholi and Fischetti, 1998). Furthermore, many of these non-glycolytic functions have been implicated in hypoxia and ischemia (Sousa et al., 2005).

Although the glycolytic functions of enolase have been proved in the brain (Pancholi, 2001), its certain expression and other biological function in central nervous system (CNS) have been poorly researched. The finding which demonstrate its functions in hypoxia and ischemia attract our attention to examine whether ENOLASE have any other functions after SCI. So, we have attempted to investigate the expression and distribution of ENOLASE in the spinal cord

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after injury. Our work is conducted to achieve greater insight into the physiologic function of ENOLASE and its association with the molecular mechanisms underlying CNS lesion and repair.

2. Materials and methods

2.1. Animals and surgery

Male Sprague Dawley rats ($n = 44$) with an average body weight of 250 g (220–275 g) were used in this study. The rats were deeply anesthetized with chloral hydrate (10% solution, 0.3 ml/100 g i.p.) and surgery was performed under aseptic conditions. Dorsal laminectomies were carried out at the level of the ninth thoracic vertebra (T9). Contusion injuries ($n = 34$) were performed using the NYU impactor (Gruner, 1992). The exposed spinal cord was contused by dropping a rod 2.0 mm in diameter and 10 g in weight from a height of 10 cm. After contusion, the overlying muscles and skin were respectively closed in layers with 4-0 silk sutures and staples, and the animals were allowed to recover on a 30 °C heating pad. Postoperative treatments included saline (2.0 cm^3, s.c.) for rehydration and Baytril (0.3 cm^3, 22.7 mg/ml, s.c., twice daily) to prevent urinary tract infection. Bladders were manually expressed twice daily until reflex bladder emptying returned. Animals were sacrificed at 6 h and 12 h and 1, 3, 5, 7, and 14 days after injury. Sham-operated animals ($n = 8$) were served as non-injured controls. They were deeply anesthetized with chloral hydrate (10% solution, 0.3 ml/100 g i.p.) and surgery was performed under aseptic conditions. Dorsal laminectomies were carried out at T9. However, contusion injuries were not performed. Then the overlying muscles and skin were, respectively, closed in layers with 4-0 silk sutures and staples, and the sham-operated animals were allowed to recover on a 30 °C heating pad. All surgical interventions and postoperative animal care were carried out in accordance with the Guide for the Care and Use of Laboratory Anima and were approved by the Chinese National Committee to the Use of Experimental Animals for Medical Purposes, Jiang Branch. All efforts were made to minimize the number of animals used and their suffering.

2.2. Western blot analysis

To obtain samples for Western blot, the sham-operated or injured spinal cords were excised and snap frozen at −70 °C until used. The portion of spinal cord extending 5 mm rostral and 5 mm caudal to the injury epicenter was immediately removed. To prepare lysates, frozen spinal cord samples were minced with eye scissors in ice. The samples were then homogenized in lysis buffer (1% NP-40, 5.0 mmol/l Tris, pH 7.5, 5 mmol/l EDTA, 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1% Triton X-100, 1 mmol/l PMSF, 10 mg/ml aprotinin, and 1 mg/ml leupeptin) and clarified by centrifuging for 20 min in a microcentrifuge at 4 °C. After determination of its protein concentration with the Bradford assay (Bio-Rad), the resulting supernatant (50 μg of protein) was subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore) by a transfer apparatus at 350 mA for 2.5 h. The membrane was then blocked with 5% nonfat milk and incubated with primary antibody against ENOLASE (antirabbit, 1:500; Santa Cruz), proliferating cell nuclear antigen (PCNA, anti-mouse, 1:1000; Santa Cruz), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, anti-rabbit, 1:1000; Sigma). After incubating with the fluorescently labeled secondary antibodies (Odyssey, 1:10,000), the membranes were detected on the Odyssey instrument.

2.3. Immunohistochemistry

After defined survival times, sham-operated and injured rats were terminally anesthetized and perfused through the ascending aorta with saline, followed by 4% paraformaldehyde. After perfusion, the sham-operated and injured spinal cords were removed and post-fixed in the same fixative for 3 h and then cryoprotected with 20% sucrose for 2–3 days, following 30% sucrose for 2–3 days. After treatment with sucrose solutions, the tissues were embedded in O.T.C compound. Then, 8-μm frozen cross sections at two spinal cord levels (2 mm rostral and caudal to the injury epicenter) were prepared and examined. All of the sections were blocked with 10% goat serum with 0.3% Triton X-100 and 1% (w/v) bovine serum albumin (BSA) for 2 h at room temperature (RT) and incubated overnight at 4 °C with anti-ENOLASE anti-body (anti-rabbit, 1:200; Santa Cruz), followed by incubation in biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). Staining was visualized with diaminobenzidine (Vector Laboratories). The primary antibodies were deleted from the procedure in the negative control. In addition, to verify the ENOLASE-positive cells in the results were just neurons, astrocytes or microglia, we only specified NeuN (neuron marker, 1:600; Chemicon), GFAP (astrocyte marker, 1:200; Sigma) or CD11b (microglia marker, 1:50; Serotec) in both sham and injured sections to make sure the certain types of the neural cells. Cells with strong or moderate brown staining were counted as positive, cells with no staining were counted as negative, while cells with weak staining were scored separately.

2.4. Double immunofluorescent staining

Slide-mounted sections were removed from the freezer and kept in an oven at 37 °C for 30 min, sections were blocked with 10% normal serum blocking solution species, the same as the secondary antibody, containing 3% BSA and 0.1% Triton X-100 and 0.05% Tween 20, 2 h at RT in order to avoid unspecific staining. Then, the sections were incubated with both rabbit polyclonal primary

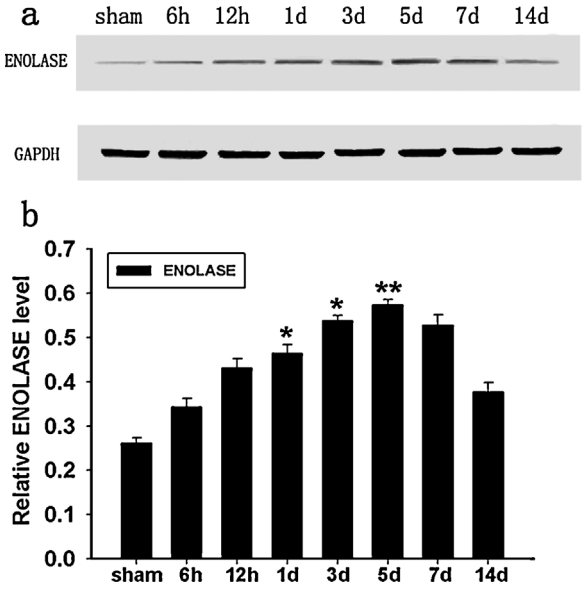

Fig. 1. The expression changes of ENOLASE in adult rat spinal cord following contusion injury. (a) Spinal cord tissues from rats at various survival times after SCI were homogenized and subjected to immunoblot analysis. Sample immunoblots probed for ENOLASE and GAPDH are shown above. (b) The bar chart below demonstrates the ratio of ENOLASE protein to GAPDH for each time point by densitometry. The data are means ± SEM ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$, significantly different from the sham group).

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