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Spatio-temporal expression of Hexokinase-3 in the injured female rat spinal cords

Yu-Hong Lin [a, b, c, 1], Yan Wu [a, b, c, 1], Ying Wang [a, b, 1], Zong-Feng Yao [a, b], Jie Tang [b, c], Rui Wang [b], Lin Shen [b], Shu-Qin Ding [a], Jian-Guo Hu [a, b, **], He-Zuo Lü [a, b, c, *]

[a] Clinical Laboratory, The First Affiliated Hospital of Bengbu Medical College, Anhui 233004, PR China
[b] Anhui Key Laboratory of Tissue Transplantation, The First Affiliated Hospital of Bengbu Medical College, Anhui 233004, PR China
[c] Department of Immunology, Bengbu Medical College, Anhui Key Laboratory of Infection and Immunity at Bengbu Medical College, Anhui 233030, PR China

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ABSTRACT

Hexokinase-3 (HK3) is a member of hexokinase family, which can catalyze the first step of glucose metabolism. It can increase ATP levels, reduce the production of reactive oxygen species, increase mitochondrial biogenesis, protect mitochondrial membrane potential and play an antioxidant role. However, the change of its expression in spinal cord after injury is still unknown. In this study, we investigated the spatio-temporal expression of HK3 in the spinal cords by using a spinal cord injury (SCI) model in adult female Sprague-Dawley rats. Quantitative reverse transcription-PCR and western blot analysis revealed that HK3 could be detected in sham-opened spinal cords. After SCI, it gradually increased, reached a peak at 7 days post-injury (dpi), and then gradually decreased with the prolonging of injury time, but still maintained at a higher level for up to 28 dpi (the longest time evaluated in this study). Immunofluorescence staining showed that HK3 was found in GFAP$^+$, β-tubulin III$^+$ and IBA-1$^+$ cells in sham-opened spinal cords. After SCI, in addition to the above-mentioned cells, it could also be found in CD45$^+$ and CD68$^+$ cells. These results demonstrate that HK3 is mainly expressed in astrocytes, neurons and microglia in normal spinal cords, and could rapidly increase in infiltrated leukocytes, activated microglia/macrophages and astrocytes after SCI. These data suggest that HK3 may be involved in the pathologic process of SCI by promoting glucose metabolism.

1. Introduction

Hexokinase (HK) is an enzyme that catalyses the transfer of phosphate from ATP to glucose in the first step of glucose metabolism (Wilson, 1995, 2003). In mammalian, HK has four important isozymes which are designated as HK1, HK2, HK3 and HK4 (Wilson, 1995, 2003). HK1 is considered to be “brain HK”, it is the predominant HK form in the brain. However, it is ubiquitously expressed in all mammalian tissues, and most physiological, hormonal and metabolic factors have no effect on its expression (Calmettes et al., 2015; Purich and Fromm, 1971). HK2 is also called “muscle HK”, it is primarily expressed in insulin-sensitive tissues, such as skeletal muscle, heart and adipose tissues (Calmettes et al., 2015; Mandarino et al., 1995; Nederlof et al., 2014). HK3 is ubiquitously expressed in most tissues at relatively low level; however, its higher abundance levels have been reported in lung, liver and kidney (Lowes et al., 1998; Okatsu et al., 2012; Wyatt et al., 2010). In myeloid cells, particularly granulocytes, HK3 is the predominant hexokinase. (Federzoni et al., 2014). HK4, is also known as glucokinase, mainly exists in the liver and pancreas. In terms of kinetics and function, it is different from the other three HKs. HK4 has a lower affinity for glucose than the other three HKs (Cardenas et al., 1998).

The function and tissue specific distribution of the HK isoforms are affected by the organism's pathophysiological state. For example, the expression and activity of some isoforms are increased in tumor tissue, this makes the tumor tissue can still get enough energy under hypoxia, and many intermediates of glycolysis can be used by tumor cells to synthesize proteins, nucleic acids

* Corresponding author. Anhui Key Laboratory of Tissue Transplantation, The First Affiliated Hospital of Bengbu Medical College, 287 Chang Huai Road, Bengbu 233004, PR China.
** Corresponding author. Clinical Laboratory, The First Affiliated Hospital of Bengbu Medical College, Anhui 233004, PR China.
E-mail addresses: jghu9200@163.com (J.-G. Hu), lhz233003@163.com (H.-Z. Lü).
[1] These authors contributed equally to this work.

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and lipids, thus providing the necessary material basis for the growth and proliferation of tumor cells (Cho et al., 2015; Gao and Chen, 2015). Some isoforms are also been reported relating to injury, inflammation, apoptosis and cell survival (Clemons and Toledo-Pereyra, 2015; Wyatt et al., 2010). Spinal cord injury (SCI) is the injury of spinal cord resulting in devastating loss of motor and sensory functions (Gwak et al., 2016). SCI also includes inflammation, apoptosis and other pathophysiological processes (Cox et al., 2015). However, the changes in expression and function of these isoforms have not been reported in the injured spinal cords so far. Recently, using RNA-Sequencing, we found that HK3 was significantly upregulated at mRNA level in the injured spinal cords at subacute stage (Shi et al., 2017). This is an interesting phenomenon. HK3 lacks hydrophobic N-terminal sequences which are known being used to bind mitochondria by HK1 and HK2 (Wyatt et al., 2010). Using Novikoff rat hepatoma cell line and HK3-overexpressed HEK293 cells, Wyatt et al. reported that HK3 could exert cytoprotective effects against oxidative stress by increasing ATP levels, reducing oxidant-induced ROS, preserving mitochondrial membrane potential and increasing mitochondrial biogenesis (Wyatt et al., 2010). However, its expression, cellular localization and function in the lesion microenvironment after SCI are still unclear.

In this study, a SCI model in adult female Sprague-Dawley rats was performed and the dynamic changes of HK3 expression in the spinal cords were investigated. Quantitative reverse transcription-PCR, immunohistochemistry and western blot analysis were used to reveal its spatio-temporal expression and cellular localization in the lesion microenvironment after SCI.

2. Materials and methods

2.1. Animals

A total of 78 eight-week-old female Sprague-Dawley rats (220–250 g, RRID: RGD_5508397, Vital River Laboratory Animal Technology Co. Ltd. Beijing, China) were used in this study. The animal care in the surgical procedures and after operation were in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China; revised in June 2004) and the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent Survival Surgery approved by the Animal Care and Ethics Committees of Bengbu Medical College.

2.2. Contusive SCI

A New York University Impactor was used to perform contusive SCI as previously described (Lu et al., 2008). Briefly, the rats were anesthetized with 50 mg/kg pentobarbital intraperitoneally. This dose could produce satisfactory anesthetic effect in most animals. Occasionally it was insufficient for complete surgical anesthesia, we supplemented the anesthetic, but it should not exceed the 1/5 of total dose. The depth of anesthesia was judged by the breathing, corneal reflex, tension of the muscles, and the reaction of the skin to pinching. When the respiration became deeper and slower, the sensitivity of the corneal reflex decreased or disappeared, the limbs and abdominal muscles relaxation and the skin showed no obvious pain response to pinching, it showed that no pain was perceived by the animals. After being anesthetized, the rats received a laminectomy at the T9 vertebra. There are three main reasons for choosing T9 as the spinal cord segment for contusive SCI. First, thoracic SCI models are apparently reliable and easy to reproduce. Second, T9 is easy to locate, the inclination of its spinous process is biggest. Last but not least, choosing T9 does not affect the cardiorespiratory function, could reduce mortality, and can produce typical lower limb dysfunction. To stabilize the spine, the T7 and T11 vertebrae were clamped. For making contusive injury, the cord's exposed dorsal surface received a 25 mm weight drop by using a 10 g rod (2.5 mm diameter). Sham-operated (sham) rats only received a laminectomy without contusive injury. After closing the muscles and skin in layers, the rats were placed in a temperature- and humidity-controlled chamber and received manual bladder emptying three times daily until be able to urinate autonomously. For analgesia, the animals received buprenorphine (0.05 mg/kg, SQ; Reckitt Benckise, Hull, England) every 12 h for 3 consecutive days. To prevent infections, chloramphenicol (50–75 mg/kg) was used via drinking water for 10 consecutive days.

2.3. RNA extraction and real-time reverse transcription PCR

A separate series of animals was used for RNA extraction ($n = 6$ in every group). Before removing the spinal cords, the animals were perfused with phosphate buffer saline (PBS) under deep anesthesia (80 mg/kg pentobarbital, intraperitoneal). The 1 cm spinal cord segments of sham, and the spinal cords (1 cm spinal cord segments containing the injury epicenter) of acute (1 dpi), subacute (7 dpi) and chronic phases (28 dpi) were harvested. The TRIzol reagent (Invitrogen, New Jersey, NJ, USA) was used to extract the total RNA from the spinal cords according to the manufacturer's instructions. A reverse transcription system (Promega, Madison, WI) was used to reverse transcribe RNA into cDNA. Real-time PCR was performed by using a SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7900 PCR detection system (Applied Biosystems, Foster City, CA). Parallel amplification of the housekeeping gene β-actin was used to normalize gene expression. The β-actin gene is unchanged under our experimental conditions. The primer sequences were: HK3 forward primer 5′-TGTTGTGTATTTATTACGGGAAGCC-3'; HK3 reverse primer 5′-ACATTCCGGAGTTCTTCCATATAGC-3'; β-actin forward primer 5′- AAGTCCCTCACCCTCCCAAAAG-3'; β-actin reverse primer 5′-AAGCAATGCTGTCACCTTCCC-3'. The relative expression level of target mRNAs was calculated using the $^{\Delta\Delta}$Ct method (Pfaffl, 2001) and expressed relative to the value in the sham group (designated as 1).

2.4. Western blot analysis

Another separate series of animals was used to detect the expression of HK3 at protein level by using Western blot analysis ($n = 6$ in every group). Briefly, the rats were intracardially perfused with 200 ml of saline under deep anesthesia (80 mg/kg pentobarbital, intraperitoneal) and the spinal cord segments (T8-T10) were dissected. The spinal cords were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 100 μg/ml PMSF, 4 μg/ml leupeptin and 1 μg/ml aprotinin (all from Sigma). After centrifugation at 16,000 g for 10 min at 4 °C, the supernatant was clarified and the protein concentrations were determined by using a BCA Protein Assay kit (Pierce, Rockford, IL, USA). For Western blotting, supernatants were diluted in sample buffer (50 mM DTT, 2% SDS, 62.5 mM Tris–HCl, pH 6.8, 10% glycerol and 0.1% bromophenol blue) and boiled for 5 min. Equal amounts of protein (20 μg) were electrophoresed on 8% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked at room temperature (RT) for 1 h with 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS) plus 0.1% Tween-20 (TBST), and incubated with primary antibodies at 4 °C overnight. Primary antibodies used were rabbit anti-beta-actin