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The inhibitor of calcium/calmodulin-dependent protein kinase II KN93 attenuates bone cancer pain via inhibition of KIF17/NR2B trafficking in mice

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ABSTRACT

The N-methyl-D-aspartate receptor (NMDAR) containing subunit 2B (NR2B) is critical for the regulation of nociception in bone cancer pain, although the precise molecular mechanisms remain unclear. KIF17, a kinesin motor, plays a key role in the dendritic transport of NR2B. The up-regulation of NR2B and KIF17 transcription results from an increase in phosphorylated cAMP-response element-binding protein (CREB), which is activated by calcium/calmodulin-dependent protein kinase II (CaMKII). In this study, we hypothesized that CaMKII-mediated KIF17/NR2B trafficking may contribute to bone cancer pain. Osteosarcoma cells were implanted into the intramedullary space of the right femurs of C3H/HeJ mice to induce progressive bone cancer-related pain behaviors. The expression of spinal t-CaMKII, p-CaMKII, NR2B and KIF17 after inoculation was also evaluated. These results showed that inoculation of osteosarcoma cells induced progressive bone cancer pain and resulted in a significant up-regulation of p-CaMKII, NR2B and KIF17 expression after inoculation. Intrathecal administration of KN93, a CaMKII inhibitor, down-regulated these three proteins and attenuated bone cancer pain in a dose- and time-dependent manner. These findings indicated that CaMKII-mediated KIF17/NR2B trafficking may contribute to bone cancer pain, and inhibition of CaMKII may be a useful alternative or adjunct therapy for relieving cancer pain.

1. Introduction

Pain is one of the most feared and debilitating symptoms in cancer patients. Approximately 62% to 86% of patients with advanced cancer experience significant pain, which illustrates that this problem has not been solved (van den Beuken-van Everdingen et al., 2007). In particular, patients with bone cancer experience more frequent and severe pain (Coleman, 2006; Mercadante and Fulfaro, 2007). However, the mechanisms of bone cancer pain still remain unclear (Colvin and Fallon, 2008). Thus, it is important to understand the underlying mechanisms of bone cancer pain in order to develop more efficacious therapies.

The N-methyl-D-aspartate (NMDA) receptor, which is an ionotropic glutamatergic receptor, is a voltage- and ligand-gated receptor, which demonstrates a high permeability to Ca^{2+} (Mori and Mishina, 1995; Nakanishi, 1992). These properties make it an essential component of synaptic plasticity via the activation of various intracellular signaling cascades. Numerous studies have demonstrated that the NMDA receptor is responsible for pain signal transduction and regulation induced by tissue injury, inflammation and peripheral nerve injury (Guo et al., 2002; Gu et al., 2010a,c; Qu et al., 2009; Zhang et al., 2012). Bone cancer pain is thought to exhibit inflammatory, neuropathic and tumorigenic components. Previous studies have suggested that the NMDA receptor, specifically NR2B subunit-dependent synaptic plasticity, in the pain pathway contributes to central sensitization, which refers to an increased synaptic excitability established in somatosensory neurons in the spinal cord and underlies the central mechanisms of bone cancer pain (Gu et al., 2010a; Matsumura et al., 2010; Ma et al., 2007; Zhang et al., 2012). Intrathecal administration of NR2B-selective NMDA receptor antagonists, such as ifenprodil and Ro25-6981, could be of great analgesic effects in various pain models (Gu et al., 2010a; Pedersen and Gjerstad, 2008). Thus, a series of studies focusing on NR2B, the key modulator in pain signal transduction, was recently performed.

NR2B is synthesized in the cell bodies of neurons and needs to be transported to the vicinity of synapse along microtubules. The functional NMDA receptor channels are subsequently assembled on the synaptic

Abbreviations: ANOVA, analysis of variance; CaMKII, calcium/calmodulin-dependent protein kinase II; CREB, cAMP-response element-binding protein; i.t, intrathecal; KN93, 2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; LTP, long-term potentiation; NMDAR, N-methyl-D-aspartate receptor; NR2B, NMDA receptor 2B subunit; p-CaMKII, phosphor-CaMKII; PWMT, paw withdrawal mechanical threshold; SG, substantia gelatinosa.

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membrane. KIF17, a member of the kinesin superfamily motor protein, transports and regulates NR2B in living hippocampal neurons (Guillaud et al., 2003; Hirokawa, 1998; Hirokawa and Takemura, 2004). Moreover, KIF17 and NR2B are co-regulated by the same transcription factor in neurons. The increase in expression of KIF17 is concurrent with NMDAR activation, particularly the up-regulation of NR2B subunit. (Dhar and Wong-Riley, 2011; Roberson et al., 2008). KIF17/NR2B trafficking may mediate synaptic changes and is required in multiple processes of learning and long-term memory formation in the mammalian brain (Yin et al., 2011); however, their role in pain is still unclear.

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine protein kinase that is widely distributed in the organelles of central and peripheral neurons. CAMKII has been implicated in synaptic plasticity and the formation of central sensitization in neuropathic pain (Soderling, 2000; Wang et al., 2011). Activation of NMDA receptors induces Ca^{2+} influx. Increased intracellular Ca^{2+} triggers a signaling cascade, which includes the phosphorylation and activation of CaMKII. Autophosphorylation of CaMKII at Threonine 286 (Thr286) serves as a biomarker for the activation of CaMKII. Subsequently, activated CaMKII phosphorylates multiple proteins and enzymes, including the NMDA receptor, which forms a positive feedback signal and results in Ca^{2+}-mediated central sensitization in spinal dorsal horn neurons after chronic constriction injury (CCI) (Dai et al., 2005). However, the role of CaMKII in bone cancer pain remains unknown. Wong et al. demonstrated that KIF17 is over-expressed mainly in the postnatal forebrain in KIF17 transgenic mice using the CaMKII promoter. In addition, the NMDA receptor-dependent behavioral patterns were altered in mice with overexpression of KIF17 (Wong et al., 2002). Further studies suggested that phosphorylation of KIF17 via CaMKII is critical for the release of NR2B-containing vesicles transported by KIF17 (Guillaud et al., 2008). The kinesin-based dendritic transport of NR2B is regulated by CaMKII signaling pathways, and this process may be a potential mechanism underlying emotion and cognition (Yuen et al., 2005).

The present study investigated the hypothesis that CaMKII-dependent KIF17/NR2B trafficking may play an important role in the formation of central sensitization, and contribute to the mechanism of bone cancer pain in the spinal cord.

2. Experimental procedures

2.1. Animals

All experiments were approved by the Animal Care and Use Committee at the Medical School of Nanjing University and were in compliance with the European Communities Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to reduce the number of animals used in this study. Experiments were performed on male C3H/HeJ mice (20–25 g, 4–6 weeks old; Weitong Lihua Laboratory Animal Technology Co., Ltd., Beijing, China; SCXK JING 2000-0009). The mice were housed in groups of five per cage and fed with food pellets and water was provided ad libitum. All animals were maintained in a temperature-controlled (21 ± 1 °C) room with 12-h alternating dark/light cycles.

2.2. Cell culture and implantation

Osteosarcoma NCTC 2472 cells (American Type Culture Collection, ATCC, 2087787) were incubated and subcultured in NCTC 135 medium (Sigma-Aldrich, St. Louis, USA) with 10% horse serum (Gibco, Carlsbad, CA) at 37 °C in an atmosphere of 5% CO_2 and 95% air (Thermo Forma, Ohio, USA), and passaged twice a week according to the recommendations provided by ATCC.

The mouse model of bone cancer pain was generated as previously described by Schwei et al. (1999). On day 0, the mice were anesthetized with an intraperitoneal injection of 50-mg/kg pentobarbital sodium (1% in normal saline), and a superficial incision was made in the skin above the right articulatio genu using eye scissors. Gonarthrotomy was performed, which exposed the femur condyles. A light depression was made using a dental bur. A 30-gauge needle was used to perforate the cortex, and a 25-μl microsyringe was used to inject a volume of 20-μl α-minimum essential medium (α-MEM) containing no or 2×10^5 NCTC 2472 cells into the intramedullary space of the femur, which corresponded to sham or tumor-bearing mice, respectively. Subsequently, the injection hole was sealed using dental amalgam, followed by copious irrigation with normal saline. The wound was then sutured closed.

2.3. Drug preparation and Intrathecal injection

KN93 (2-[N-(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) (Enzo Life Sciences, USA) was dissolved in 0.9% saline and intrathecally administered at a total volume of 5 μl. For vehicle treatment, 0.9% saline was used. On day 14 after the inoculation, the mice were treated with KN93 or vehicle, respectively.

Intrathecal (i.t.) injections were performed manually between the L5 and L6 lumbar space in unanesthetized mice according to a previous method described by Hylden and Wilcox (1980). The injection was performed using a 25-gauge needle attached to a glass microsyringe. Each mouse was injected with a volume of 5 μl. The accurate placement of the needle was confirmed by a quick "flick" of the mouse's tail.

2.4. Experimental protocol

2.4.1. Experiment 1: pain behaviors over time

All mice were tested for pain-related behaviors during a 2-week period: day 0 before the operation and days 3, 5, 7, 10 and 14 after the operation in both tumor-bearing mice (n = 8) and sham (n = 8) mice.

2.4.2. Experiment 2: measurement of the expression levels of p-CaMKII, t-CaMKII, KIF17 and NR2B in the spinal cords of tumor-bearing mice

To determine whether bone cancer altered the expression of p-CaMKII, t-CaMKII, KIF17 and NR2B in the L3–L5 spinal cord, which receives sensory inputs from the sciatic nerve, tissue samples were obtained from tumor-bearing mice at days 0, 5, 7, 10, 14 and sham mice at day 14 for Western blotting analyses.

2.4.3. Experiment 3: effects of i.t. injection of KN93 on pain behaviors

In this study, 40 mice were randomly divided into five groups (n = 8): tumor-bearing mice receiving vehicle (group T), sham mice receiving vehicle (group S), tumor-bearing mice receiving KN93 (15 nmol, group K1), tumor-bearing mice receiving KN93 (30 nmol, group K2) and tumor-bearing mice receiving KN93 (60 nmol, group K3). After the pain-related behaviors were observed at day 14, the mice were intrathecally administered with either KN93 or vehicle. Pain-related behaviors were measured at 1, 2, 4 and 24 h after administration. The data measured prior to administration were regarded as the baseline data.

2.4.4. Experiment 4: effects of i.t. injection of KN93 on the expression of p-CaMKII, t-CaMKII, KIF17 and NR2B in the spinal cord

To determine whether KN93 altered the expression of p-CaMKII, t-CaMKII, KIF17 and NR2B in the L3–L5 spinal cord, tissue samples were obtained from mice of each group at 1, 2, 4 and 24 h after administration for Western blotting analyses.

2.5. Pain-related behaviors

All tests were performed during the light phase. Prior to each test, the mice were allowed to acclimatize for at least 30 min. All behavioral

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