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Calpain-2 Regulates TNF-α Expression Associated with Neuropathic Pain Following Motor Nerve Injury

Shao-Xia Chen,[a,b†] Guang-Jie Liao,[a,c†] Pei-Wen Yao,[a] Shao-Kun Wang,[a] Yong-Yong Li,[a] Wei-an Zeng,[b] Xian-Guo Liu[a] and Ying Zang[a*]

[a] *Pain Research Center and Department of Physiology, Zhongshan Medical School of Sun Yat-Sen University, 74 Zhongshan Rd. 2, Guangzhou 510080, PR China*

[b] *Department of Anesthesiology, Cancer Center, Sun Yat-Sen University, State Key Laboratory of Oncology in South China, Collaborative, Innovation Center for Cancer Medicine, 651 Dongfeng Road East, Guangzhou 510060, PR China*

[c] *Department of Pathology, The Red Cross Hospital of Yulin, 1 Jinwang Rd, Yulin 537000, PR China*

Abstract—Both calpain-2 (CALP2) and tumor necrosis factor-α (TNF-α) contribute to persistent bilateral hypersensitivity in animals subjected to L5 ventral root transection (L5-VRT), a model of selective motor fiber injury without sensory nerve damage. However, specific upstream mechanisms regulating TNF-α overexpression and possible relationships linking CALP2 and TNF-α have not yet been investigated in this model. We examined changes in CALP2 and TNF-α protein levels and alterations in bilateral mechanical threshold within 24 h following L5-VRT model injury. We observed robust elevation of CALP2 and TNF-α in bilateral dorsal root ganglias (DRGs) and bilateral spinal cord neurons. CALP2 and TNF-α protein induction by L5-VRT were significantly inhibited by pretreatment using the calpain inhibitor MDL28170. Administration of CALP2 to rats without nerve injury further supported a role of CALP2 in the regulation of TNF-α expression. Although clinical trials of calpain inhibition therapy for alleviation of neuropathic pain induced by motor nerve injury have not yet shown success, our observations linking CALP2 and TNF-α provide a framework of a systems' approach based perspective for treating neuropathic pain.

Key words: calpain-2 (CALP2), tumor necrosis factor-α (TNF-α), L5 ventral root transection (L5-VRT), dorsal root ganglias (DRG), spinal cord.

INTRODUCTION

We previously showed that L5 ventral root transection (L5-VRT) leads to bilateral mechanical hyperalgesia in Sprague–Dawley rats (SD) rat paws (Li et al., 2002, Wu, 2002, Xu et al., 2007, Xu et al., 2006; He et al., 2010; Wei et al., 2013a; Zang et al., 2015; Ouyang et al., 2016), and can be used to reveal the role of neuroimmune responses after nerve injury. Neuroimmune responses are critical in the initiation and development of pain (Tracey and Walker, 1995; Watkins et al., 1995; Moalem and Tracey, 2006; Scholz and Woolf, 2007). Tumor necrosis factor alpha (TNF-α) (Carswell et al., 1975; Bickels et al., 2002) plays important inflammatory roles in the nervous system. It is widely believed that TNF-α is one of the first cytokines released in the inflammatory cascade and is responsible for the generation of neuropathic pain (Shamash et al., 2002; Li et al., 2004; Xu et al., 2006; Clark, 2007; Constantin et al., 2008; Leung and Cahill, 2010; Zhang et al., 2010).

Mounting evidence suggests that following motor nerve injury, increased TNF-α may contribute to neuropathic pain by increasing excitability of uninjured DRG neurons (He et al., 2010; Chen et al., 2011). TNF-α has been shown to be important for peripheral and central sensitization in multiple different animal neuropathic pain models (Leung and Cahill, 2010). Nociceptive stimulation following spinal cord injury (SCI) provoked mechanical sensitivity by 24 h, which was associated with increased expression of TNF-α (Garraway et al., 2014). Overproduction of TNF-α was also implicated in synaptic structural alterations of spinal and hippocampal neurons associated with chronic pain and memory deficits (Liu et al., 2017). The administration of magnesium-l-threonate to normalize TNF-α activity was shown to mitigate vincristine-induced allodynia and hyperalgesia (Xu et al., 2017). In animal models, treatments targeting TNF-α can partially alleviate neuropathic pain (Leung and Cahill, 2010). Although increased TNF-α expression

*Corresponding author.
E-mail address: zangying@mail.sysu.edu.cn (Y. Zang).
† These authors contributed equally to this study.
Abbreviations: CALP2, calpain-2; DRGs, dorsal root ganglias; L5-VRT, L5 ventral root transection; NF-κB, Nuclear factor-κB; PFA, paraformaldehyde; SART, specific alternation of rhythm in temperature; SCI, spinal cord injury; TNF-α, tumor necrosis factor-α.

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following nerve injury is well documented, very little is known regarding the underlying mechanisms of this upregulation.

Nuclear factor-κB (NF-κB) regulates transcription of multiple target genes including TNF-α (Pahl, 1999; Tegeder et al., 2004). Calpain is a calcium-dependent cysteine protease that can degrade I Kappa B Alpha (IκBa) and thereby activate NF-κB (Shumway, 1999). Calpain is activated in bilateral L4-L6 dorsal root ganglias (DRGs) soon after L5-VRT (Zang et al., 2015) and is involved in early inflammatory cytokine regulation (Vosler et al., 2008). Calpain-1 (CALP1) and CALP2 are the main calpain subtypes in the central nerve system (CNS), with CALP2 predominating in axons. They exhibit similar biochemical and catalytic properties but require different calcium concentrations for activation (E. D. 2002, Croall and Ersfeld, 2007).

Calpains are implicated in neuropathic pain. Pretreatment with calpain inhibitors reduced LPA-induced hyperalgesia and allodynia (Xie et al., 2010). Spinal inhibition of calpain or silencing of CALP1 blocked potassium chloride cotransporter-2 (KCC2) cleavage induced by nerve injury and N-methyl-D-aspartate (NMDA), thereby attenuating neuropathic pain (Zhou et al., 2012). Treatment with a calpain inhibitor also yielded anti-inflammatory and anti-hyperalgesic effects in the zymosan-induced paw inflammation model (Kunz et al., 2004). Recent studies reported that the calpain inhibitor MDL28170 chronically blocked the persistent sodium current (INaP), thus promoting functional recovery after SCI (Brocard et al., 2016). Neurotropin prevented specific alternation of rhythm in temperature (SART) stress-induced calpain activation in the mesencephalon of rats and had an analgesic effect on the hyperalgesia induced by SART stress (Fujisawa et al., 2017). The expression level of calpain markedly increased very early after sciatic nerve transection (Glass et al., 2002) or SCI, especially in motoneurons (Banik et al., 1997; Springer et al., 1997a). Our previous work showed very early activation of CALP2 (but not CALP1) following L5-VRT in bilateral L4–L6 dorsal root ganglion (DRG) neurons, contributing to the induction of allodynia (Zang et al., 2015). The mechanism by which CALP2 induces TNF-α overexpression following motor nerve injury to produce hyperalgesia remains unclear. Here, we show that TNF-α protein levels in DRG and spinal cord increased significantly 30 min after L5-VRT and were suppressed by the calpain inhibitor MDL28170. Furthermore, TNF-α levels could be elevated by CALP2 administration in the absence of injury to DRGs. Our observations reveal a role of CALP2 in elevating TNF-α levels in DRG and spinal cord after motor nerve injury, opening potential novel approaches to targeting TNF-α-induced effects leading to neuropathic pain.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague–Dawley rats (150–180 g) were used in accordance with institutional guidelines as approved by the animal care and use committee of the Animal Experimental Center, Sun Yat-sen University, China (License number SCXK (yue) 2008–0002). Animals were maintained on a 12-h light/12-h dark cycle in a temperature-controlled area with food and water provided *ad libitum*. Rats were acclimatized for at least one week prior to initiating experiments.

L5 ventral root transection (L5-VRT)

The L5-VRT model was performed as previously described (Li et al., 2002; Zang et al., 2010). Briefly, surgery was performed on rats under inhalation anesthesia consisting of 1–3% isoflurane. L5 hemilaminectomy was performed to expose the L5 nerve root. The ventral root was pulled out gently with fine forceps and transected 2–3 mm proximal to the DRG, and a small portion (2 mm) of the root was dissected. The animals in the sham operation group underwent the same procedure minus transection of the nerve. A complete hemostasis was confirmed and the wound was sutured.

Behavioral testing for allodynia

Baseline testing was performed 1 day prior to surgery or drug treatment, following at least 2 days of daily habituation to the testing environment. Mechanical allodynia was assessed by measuring the paw withdrawal threshold, using von Frey filaments of logarithmically incremental stiffness (0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0 and 15.0 g). The 2.0-g stimulus was first applied to the mid-plantar of both rat hindpaws. In the event of paw withdrawal absence, the next stronger stimulus would be chosen. On the contrary, a weaker stimulus would be applied. By repeating this procedure, the stimulation strength was determined by interpolation, which corresponded to a 50% response rate (Chaplan et al., 1994). Each stimulus consisted of a 5–6 s application of the von Frey filament perpendicular to the mid-plantar surface of the hindpaw with a 5-min interval between stimuli, in an ascending order of filament stiffness until elicitation of a flexion response. Brisk withdrawal or licking of the paw following the stimulus was defined as a positive response.

Immunohistochemistry

Immunofluorescence analysis was performed as previously described (Wei et al., 2013c) (He et al., 2010). Briefly, rats were anesthetized with 20% urethane, then perfused transcardially with saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The fifth lumbar (L5) spinal cord segments were removed and post-fixed with the 4% PFA for 3 h, then transferred to 30% sucrose in PBS overnight. Sample sections (25-μm thickness) were prepared on gelatin-coated glass slides using a freezing microtome (LEICA CM3050S, Germany). All sections were blocked with 3% donkey serum for 1 h at room temperature, and incubated with anti-TNF-α antibody (1:200, Bioworld, USA) or anti-CALP2 antibody (1:100, Abcam) over two nights at 4 °C. For double immunofluorescence staining, sections were incubated with a mixture of anti-TNF-α antibody (1:200, Bioworld, USA) and anti-CALP2 antibody (1:50,

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