TNF-α expression, not iNOS expression, is correlated with NF-κB activation in the spinal cord of rats following peripheral nerve injury

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Previous studies have shown that pro-inflammatory cytokines were involved in the genesis and persistence of neuropathic pain. Nuclear factor kappa B (NF-κB) plays a crucial role in regulating pro-inflammatory cytokine gene expression. In this study, we examined the hypothesis that NF-κB would regulate the expression of spinal tumor necrosis factor α (TNF-α) and inducible nitric oxide synthase (iNOS) following chronic constriction nerve injury (CCI) in rats. CCI induced a significant upregulation of NF-κB in the ipsilateral spinal cord on postoperative Day 7 as revealed by Western blot, immunohistochemistry and real time polymerase chain reaction (real time-PCR). Moreover, TNF-α mRNA expression was markedly upregulated by CCI, whereas only a low level of iNOS mRNA was detected in the ipsilateral spinal cord. The immunohistochemical data indicated a pattern of colocalization between NF-κB and TNF-α within spinal cord dorsal horn. These results show that NF-κB activation is correlated with TNF-α expression, but not iNOS expression, in the spinal cord after peripheral nerve injury.

Key words: Neuropathic pain, chronic constriction nerve injury, NF-κB; TNF-α, iNOS.

INTRODUCTION

Neuropathic pain developing after peripheral nerve injury is associated with a complex network of molecules in the peripheral and central nervous systems. Numerous pro-inflammatory factors have been implicated in the pathophysiology of neuropathic pain, including tumor necrosis factor α (TNF-α) and inducible nitric oxide synthase (iNOS), contributing to hyperalgesia, allodynia and spontaneous pain (Zelenka et al., 2005; Sweitzer et al., 2001; De Alba et al., 2006). Nuclear factor kappa B (NF-κB) is a critical transcription factor for maximal expressions of many cytokines, including TNF-α and iNOS. Nerve injury such as partial ligation, complete transection or spinal cord compression triggers NF-κB activation (Sakaue et al., 2001; Pollock et al., 2005). Moreover, our previous studies have demonstrated intrathecal administration of antisense oligodeoxynucleotides (ODN) may alleviate allodynia and hyperalgesia through the NF-κB pathway (Sun et al., 2006). Therefore, it is believed that NF-κB contributes to the pathogenesis of neuropathic pain.

TNF-α, an important pro-inflammatory cytokine, plays a crucial role in neuropathic pain. Experimental studies have revealed that spinal application TNF-α induced long-term potentiation of C-fiber evoked field potentials in spinal dorsal horn, which is relevant to pathological pain (Liu et al., 2007). Topical application of TNF-α induces ectopic activity in nociceptive Aδ and C fibers (MacEwan, 2001).
2002). In addition, blocking the action of TNF-α can attenuate bilateral mechanical allodynia (Milligan et al., 2003).

The iNOS gene promoter includes binding sites for NF-κB which mediate iNOS transcription (Seo et al., 2009, Xie et al., 1994). Recent studies have suggested both a spinal and peripheral contribution of iNOS to mechanical hypersensitivity after L5 spinal nerve ligation (LaBuda et al., 2006).

While previous studies have shown the involvement of both TNF-α and iNOS in neuropathic pain, the relationship between the expression of spinal NF-κB and TNF-α/iNOS remains unknown. This study was devoted to elucidate the linkage between NF-κB and inflammatory factors TNF-α and iNOS. We examined the hypotheses that spinal NF-κB would regulate the expression of TNF-α and iNOS after chronic constriction nerve injury (CCI) and that the NF-κB pathway might play a role in the progression of neuropathic pain.

MATERIALS AND METHODS

Animals

A total of 104 male Sprague-Dawley rats weighing 220 to 280 g were used. The rats were housed in separated cages at constant temperature (22±2°C), with a 12:12 h light/dark cycle, and free access to food and water. Following the IASP guidelines for pain research in animals (Zimmermann, 1983), all experiment procedures were approved by the Animal Care and Use Committee at the Shandong University and in accordance with the University's guidelines for the care and use of laboratory animals. Rats were randomly divided into two groups (n=52): the sham group and CCI group.

Chronic constriction injury of the sciatic nerve model

Peripheral neuropathy was induced by chronic constriction injury of the sciatic nerve in the right hind paw, according to Bennett and Xie (1988). Briefly, the rats were anesthetized with chloral hydrate anesthesia (300 mg kg⁻¹ i.p.). The right sciatic nerve was exposed at the mid-thigh level, 4 ligatures were loosely tied with about 1 mm spacing (4-0 chromic gut) proximally to the trifurcation. Sham surgery was performed by exposing the right sciatic nerve without ligation. The incision site was closed in layers and penicillin was administered i.m.

Behavioral test

Rats were transferred to a plastic chamber (20 × 25 × 15 cm) and allowed to acclimatize for 30 min before testing. Mechanical allodynia was assessed using a series of von Frey filaments as described previously (Chaplan et al., 1994). Each filament was applied perpendicularly 5 times to the plantar surface of the hind paw pads (the ipsilateral/contralateral side of the nerve-ligated rats and the ipsilateral side of the sham-operated rats). Quick withdraw or licking of the paw in response to the stimulus was considered a positive response. The paw withdrawal threshold (PWT) was determined by sequentially increasing and decreasing the stimulus strength (the up-and-down method), and the data were analysed using the nonparametric method of Dixon (1980), and the smallest filament that evoked positive responses on three of five was taken as mechanical withdraw threshold. Thermal hyperalgesia was assessed using the paw withdrawal latency (PWL) to radiant heat according to the protocol of Hargreaves et al. (1988). A high intensity light beam was focused onto the plantar surface of the hind paw pads through the glass plate. The time when positive responses appeared was considered the PWL. Cut-off was set at 30 s. Intervals were 5 min between trials. Tests were performed at 1 day before and 1, 4, 7, 14 and 21 days after surgery.

Western blot

Rats (n=4 for each time point) were decapitated under chloral hydrate anesthesia at 1 day before and 1, 4, 7, 14 and 21 days after surgery, and the lumbar spinal enlargement (L₄-L₆) was removed. Each segment was separated into the ipsilateral and contralateral side and homogenized in SDS sample buffer containing a protease inhibitor cocktail (Sigma, USA). Protein samples were separated on an 8% SDS-polyacrylamide gel and transferred onto NC membrane (Millipore, Bedford, MA). After blocking with TBS containing 5% skim milk and 0.1% Tween 20 for 2 h at room temperature, membranes were incubated overnight at 4°C with primary rabbit monoclonal anti-TNFα (1:50, Santa Cruz Biotechnology, CA, USA) and iNOS (1:25, Cell Signaling Technology, USA). Membranes were then washed and incubated with secondary antibody (1:5000, Santa Cruz Biotechnology, CA, USA) for 1 h. After extensively washing, the protein bands were visualized by an enhanced chemiluminesence assay (Millipore, Bedford, MA) following the manufacturer’s instructions.

Real-time quantitative PCR

At the same time points, total RNA was extracted using Total RNA Isolation Reagent (Invitrogen, CA, USA) and 2 μg of RNA was reverse-transcribed in a 10 μl reaction using random primers and Transcriptor First Strand Synthesis Kit (Takara, Japan), both in accordance with the manufacturer's instructions. Amplification mixture (20 μl) contained 4 μl of CDNA, 5 μl of primers and 11 μl of Ex Taq SYBER Premix (Takara, Japan). Amplification was 95°C for 2 min, followed by a 40 cycles of 95°C for 30 s, 60°C for 1 min. All real-time-PCR were performed in triplicate to ensure quantitative accuracy. PCR was performed on ABI 7500HT instrument and data were analyzed based on the 2⁻ΔΔCT method with normalization software. The primers are listed in Table 1.

Immunohistochemistry

At 7 days after surgery, 8 rats were perfused transcardially with 200 ml phosphate-buffer saline (PBS,0.1 M, pH 7.4) followed by 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The lumbar spinal enlargement (L₄-L₆) was removed and postfixed in the same fixative for 4 h and then replaced with 30% sucrose until they sank. Transverse spinal sections (20 μm) were cut and stored at -20°C until processed for immunofluorescence. All sections were blocked with 15% goat serum in 0.3% Triton X-100 for 2 h at room temperature and incubated with a mixture of rabbit monoclonal anti-NFκB-p65 (1:25, Cell Signaling Technology, USA) and mouse polyclonal anti-TNFα (1:50, Santa Cruz Biotechnology, CA, USA)/mouse monoclonal anti-iNOS (1:50, Abcam Inc, UK) overnight at 4°C. Sections were rinsed and incubated for 1 h at room temperature in mixture secondary antibodies: goat anti-rabbit TRITC (1:100, Santa Cruz Biotechnology, CA, USA) and goat anti-mouse FITC (1:100, Santa Cruz Biotechnology, CA, USA). Images
were obtained under a fluorescence microscope (Leica DMRE, Heidelberg, Germany) and processed using Adobe Photoshop 8.0.3 (Adobe, San Jose, CA, USA).

Statistical analysis

All data are shown as means ± SEM, and comparisons between groups were carried out by one way ANOVA. Differences with p < 0.05 were considered statistically significant.

RESULTS

Behavioral studies

Neuropathic rats experienced significant mechanical allodynia and thermal hyperalgesia (p < 0.05) in the ipsilateral, nerve-ligated side. The allodynia peaked at Day 7 with paw withdrawal thresholds (PWT) < 7 g, when compared to the contralateral side or the ipsilateral side of sham-operated rats (PWT > 22 g). The hyperalgesia peaked at Day 4 with paw withdrawal thresholds (PWL) < 8 s, while in the contralateral side or in sham-operated rats (PWL > 16 s). The attenuation of allodynia and hyperalgesia persisted through the observation period of 21 days. There was a significant difference in terms of PWT and PWL between the nerve-ligated side and sham-operated side (p < 0.05), but not between the contralateral side and sham-operated side (p > 0.05) (Figure 1).

Western blot of NF-κB protein expression

Our studies showed that CCI induced a significant upregulation of NF-κB in the spinal cord ipsilateral but not contralateral to CCI, which occurred 4 to 7 days after CCI and correlated with the development of neuropathic pain behaviors in CCI rats. There was no significant difference in NF-κB expression between the contralateral side of CCI rats and the ipsilateral side of sham-operated rats (Figure 2).

Real-time PCR of NF-κB, TNF-α and iNOS mRNA

CCI markedly activated NF-κB in the ipsilateral spinal cord compared to sham rats. The expression of NF-κB mRNA began to increase at 1 day (p > 0.05) after operation, increased significantly at 4 day (p < 0.05) and reached a peak at 7 day (p < 0.01), but remained elevated for the remainder of the experiment (Figure 3A). The expression of TNF-α mRNA began to increase at 4 day (p < 0.05) and reached a peak at 7 day (p < 0.01), this was followed by a gradual decrease, returning to the same level compared to the sham group at 21 day (Figure 3B). The expression of NF-κB and TNF-α mRNA increased significantly in the ipsilateral spinal cord, compared with the contralateral side (p < 0.05) and the sham group (p < 0.05), while no such alterations on iNOS expression could be seen in the ipsilateral spinal cord (Figure 3C).

Double-immunofluorescence histochemical staining of TNF-α, iNOS and NF-κB

Rats were decapitated at 7 day postoperatively and the L2-L5 spinal cords were removed for double-immuno-fluorescence histochemical staining. The distribution of NF-κB and TNF-α immunoreactivity indicated an increased expression in the spinal cord ipsilateral to CCI (Figure 4). Within the superficial laminae of ipsilateral spinal cord dorsal horn, a significant pattern of colocalization was detected between NF-κB and TNF-α and lots of NF-κB and TNF-α positive cells could be seen (Figure 4A-C), compared with sham-operated (Figure 4D-F) spinal cord dorsal horn. We found significant numbers of NF-κB positive cells in the spinal cord ipsilateral to CCI (Figure 5B), while no iNOS positive

<table>
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<tr>
<th>Name</th>
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<td>iNOS</td>
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Table 1. The prime sequence for RT-PCR.
Figure 1. The paw withdrawal threshold (PWT) and the paw withdrawal latency (PWL) in CCI model of rat. (A) Paw withdrawal thresholds (PWT) to stimulation with Von Frey hair filaments of nerve-ligated (lig) hind paws, at 1 day before operation up to 21 day post-operation. Withdrawal threshold to mechanical stimuli decreased in the ipsilateral side of the nerve-ligated rats, PWT < 7 g at 7 day post-operation, but not in the contralateral side or in sham-operated rats PWT > 22 g. Data are presented as mean ± SEM. (▲p < 0.05 vs. the sham side; +p < 0.05 vs. the ligated-control side.) (B) Paw withdrawal latency (PWL) to radiant heat stimulation of nerve-ligated (lig) hind paws. Withdrawal threshold to heat stimuli decreased in the ipsilateral side of the nerve-ligated rats, PWL < 8 s at 4 day post-operation, while in the contralateral side or in sham-operated rats PWL > 16 s. Data are presented as mean ± SEM. (▲p < 0.05 vs. the sham side; +p < 0.05 vs. the ligated-control side).

cells could be detected in the superficial laminae of ipsilateral spinal cord dorsal horn in both CCI group and sham-operated group (Figure 5A and D).

DISCUSSION

The present data demonstrated that chronic constriction...
Figure 2. The changes of NF-κB expressions in spinal cord of CCI and sham-operated rats. Western blot showed a significant increase of NF-κB protein expression in the ipsilateral side, which peaked at Day 7. The contralateral side and sham-operated side exhibited lower levels of protein expression than the ipsilateral side (n=4). Data are presented as mean ± SEM. (†p < 0.05, ††p < 0.01 vs. baseline (protein expression at 1 day before operation; *p < 0.05, **p < 0.01 vs. the ligated-control side; +p < 0.05, ++p < 0.01 vs. the sham side).

Injury accompanied by mechanical allodynia and thermal hyperalgesia causes profound alterations of NF-κB and TNF-α in the lumbar dorsal horn 7 days after CCI. A significant increase of NF-κB expression occurred in the L4-L6 spinal cord, ipsilateral to the ligated side. There was a pattern of colocalization between NF-κB and TNF-α in the same spinal region and the changes of TNF-α expression likewise correlated with NF-κB distribution detected by immunohischemistry. No alteration in iNOS expression was detected in the L4-L6 spinal cord. These findings suggested NF-κB may be correlated with the expression of TNF-α, but not iNOS in the spinal cord following sciatic nerve ligation. NF-κB is a critical transcription factor for maximal expression of many cytokines. CCI triggers the activation of NF-κB, which regulates the expression of the downstream inflammatory factor TNF-α. This implies that NF-κB pathway might play a role in the procession of neuropathic pain.

The role of NF-κB signaling pathway in neuropathic pain has been studied in several experimental rodent models, such as chronic constriction injury (LaBuda et al., 2006), partial lesion of sciatic nerve or its root (Park et al., 2002, Pérez et al., 2004; Shir and Seltzer, 2001; Seltzer et al., 1990; Kim and Chung, 1992). For example, Ma and Bisby (1998) have demonstrated that NF-κB expression and activity is upregulated in DRG neurons after partial
Figure 3. The mRNA expressions of NF-κB, TNF-α, iNOS in CCI rats. Quantitative evaluation of NF-κB (A), TNF-α (B), iNOS (C) expressions in the spinal cord from the contralateral, ipsilateral sides of nerve-ligated rats and sham-operated rats (n=4). Data are presented as mean ± SEM. (*p < 0.05, **p < 0.01 vs. baseline (gene expression at 1 day before operation; #p < 0.05, ##p < 0.01 vs. the ligated-control side; ++p < 0.05, +++p < 0.01 vs. the sham side).

Sciatic nerve injury. These authors report a progressive and significant increase in NF-κB in the ipsilateral lumbar dorsal horn after spinal nerve ligation (SNL), and the spinal pro-inflammatory factors generated in the affected spinal cord from onset of nerve injury facilitates this process (O’Rielly and Loomis, 2008). Moreover, NF-κB inhibitors can attenuate pain-related behaviors. Pretreatment with NF-κB decoy ODN significantly reduced mechanical allodynia and thermal hyperalgesia in CFA-induced pain model (Lee et al., 2004). Together, spinal
Figure 4. Colocalization of NF-κB and TNF-α (A-F). Displayed panels are representative immunohistochemical images from CCI and sham-operated rats at 7 day post-operation showing the distribution of NF-κB (red; Panel B, ipsilateral; Panel E, sham) and TNF-α (green; Panel A, ipsilateral; Panel D, sham) in the spinal cord dorsal horn. Images between Panels A and B, D and E were merged in Panels C and F. Arrows indicate the immunoreactive positive cells. Note that a majority of NF-κB positive cell profiles were also TNF-α positive as shown in the merged images (Panel C). Scale bar: 100 μm.

cord NF-κB plays a critical role in the procession of neuropathic pain.

Numerous studies have shown that TNF-α plays a critical role in central sensitization (Watkins et al., 2001), contributing to the development of neuropathic pain (Wieseler-Frank et al., 2005; Sommer and Kress, 2004). Peripheral nerve injury leads to the upregulation of TNF-α and TNFR1 in DRG and in spinal cord (Ohtori et al., 2004; Xu et al., 2006). Intrathecal administration of TNF-α antagonists markedly attenuates pain behaviors caused by spinal nerve transaction (Ma and Bisby, 1998) and sciatic inflammatory neuropathy (Milligan et al., 2003). Our research demonstrates that expression of TNF-α increases after surgery, coinciding with the development of mechanical and thermal hyperalgesia, in agreement with previous studies showing that spinal inflammatory cytokines can mediate the development of neuropathic pain. NF-κB activation may amplify/perpetuate the inflammatory response. Activated NF-κB translocates into the nucleus and leads to transcription of a wide variety of relevant effected genes, such as IL-1β, IL-6, TGF-β (Karin and Ben-Neriah, 2000; Tergaonkar, 2006). Pretreatment with NF-κB inhibitor, PDTC, prevented allodynia and attenuated the upregulation of TNF-α and TNFR1 induced by peri-sciatic administration of rrTNF (Wei et al., 2007). Repeated administration of mirtazapine induced a robust suppression of TNF-α and IL-1β by inhibiting NF-κB activation (Zhu et al., 2008). TNF-α administrated locally or generated at the site of injury was transported retrogradely, leading to NF-κB activation through binding with TNFR1 expressed in DRGs and in spinal dorsal horn, which in turn induced transcription of genes encoding proinflammatory cytokines and other mediators, such as TNF-α, IL-6, COX-2, and so on (O’Neill and Kaltzschmidt, 1997; Holmes et al., 2004; Baud and Karin, 2001; Ledeboer et al., 2005). Endogenous TNF-α might be continuously produced via activated NF-κB pathway, which contributed to pain-related behavior changes. These findings supported our results that NF-κB pathway was correlated with the expression of TNF-α in the spinal cord after sciatic nerve injury.

In our experiments, we speculated that the expression of TNF-α mRNA decreased significantly at 14 day post-operation up to 21 day, while NF-κB remained elevated throughout the experiment. The mechanism behind the changes still remains unclear. One hypothesis is that a concentration-related negative feedback or interplay with TNF receptors, which regulate a number of signalling processes including kinase or phosphatase activation, lipase stimulation, and protease induction and attenuate the pain hypersensitive response (MacEwan, 2002).

Peripheral nerve injury may result in hyperexcitability of spinal dorsal horn neurons (central sensitization) through on-going afferent discharges, leading to NO production (Pitcher and Henry, 2002). Previous studies have focused on iNOS expression in the injured peripheral nerve. For instance, CCI induced local iNOS expression in both macrophages and Schwann cells within and distal
to the injury site (Levy and Zochodne, 1998), which play a significant role in Wallerian degeneration and subsequent nerve regeneration processes (Ramer et al., 1997). Only peripherally-expressed iNOS was involved in the development of neuropathic pain in CCI model of rats (De Alba et al., 2006). iNOS expressed by glia was thought to be involved in mechanisms of hyperalgesia in models of spinal cord injury (Sakaue et al., 2001). In our study, we detected iNOS mRNA expression in the spinal cord to observe the relationship between spinal NF-κB and iNOS. Our data showed no significant difference at observation time points. These results indicated that the activation of spinal NF-κB is not correlated with iNOS expression in CCI model of rats. The mechanism is unclear and the difference of iNOS expressions between peripheral nervous system and central nervous system needs to be further studied.

**Conclusion**

These findings suggest that spinal NF-κB is involved in the process of neuropathic pain induced by peripheral nerve injury. NF-κB activation is correlated with TNF-α expression, but not iNOS expression in the spinal cord after chronic sciatic nerve injury. Further understanding of this relationship may provide insights for the development of novel therapeutic strategies for the treatment of neuropathic pain.

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