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Small interfering RNA-mediated knockdown of NF-κBp65 attenuates neuropathic pain following peripheral nerve injury in rats

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ABSTRACT

Recent reports show that the nuclear factor-κB (NF-κB) can control numerous genes encoding inflammatory and nociceptive mediators and play an important role in the development of central pain sensitization. The aim of the present study is to assess the role of NF-κB signal pathway and its downstream pro-inflammatory cytokines in the modulation of neuropathic pain, by using small interfering RNAs (siRNAs) technique, which has been shown to result in potent, long-lasting post-transcriptional silencing of specific genes. We developed a highly efficient method of lentivirus-mediated delivery of short-hairpin RNA (shRNA) targeting NF-κBp65 for gene silencing. This method successfully transduced LV-shNF-κBp65 into cultured spinal cord neurons in vitro and spinal cord cells in vivo, inhibited the expression of NF-κBp65 and pro-inflammatory factors (TNF-α, IL-1β and IL-6) and alleviated mechanical allodynia and thermal hyperalgesia for more than 4 weeks in chronic constriction injury (CCI) model of rats. Taken together, our results suggest that siRNA against NF-κBp65 is a potential strategy for analgesia. Furthermore, the lentiviral vector derived shRNA approach shows a great promise for the management of neuropathic pain and the study of functional NF-κBp65 gene expression.

1. Introduction

Neuropathic pain, characterized by hyperalgesia, allodynia and spontaneous pain, is usually accompanied by peripheral and central nervous system damage or dysfunction. Nerve injury leads to a rapid release of pain-related mediators, such as TNF-α, IL-1β, IL-6 and prostaglandins, resulting in inflammatory and immune responses, sensitization of central nervous system and facilitation of pain processing (Moalem and Tracey, 2006). The nuclear factor κB (NF-κB) may play a vital role in these changes as it was considered as an important factor in the regulation of pro-inflammatory cytokines and in the cytokine-induced production of major factors in inflammatory and immune responses (Makarov, 2000). The p50/p65 heterodimer, the most widely expressed NF-κB complex in the central nervous system, plays a more important role in regulation of gene expression than other hetero and homodimers (Baeuerle and Henkel, 1994). It has been reported that the expression and activation of NF-κBp65 is upregulated in the DRG neurons after partial sciatic nerve injury (Ma and Bisby, 1998). Our previously work has also indicated the extensive co-localization of NF-κBp65 with TNF-α in the spinal dorsal horn in CCI model of rats (Zhang et al., 2011). Moreover, intrathecal injection of antisense oligonucleotides against p65 subunit of NF-κB significantly alleviated mechanical allodynia and thermal hyperalgesia (Sun et al., 2006).

RNA interference, which is more potent than antisense oligonucleotides in down-regulating the level of mRNAs (Bertrand et al., 2002), has shown potential as a therapeutic strategy (Mello and Conte, 2004). Nociceptive molecular targets, such as TRPV1, Na(V)1.8, P2X3, NR1, TrkB, ephrinB2, DOR, NR2B and PKCγ, have been successfully knocked down by siRNA-mediated gene silencing techniques in recent years (Christoph et al., 2006; Dong et al., 2007; Dorn et al., 2004; Garraway et al., 2009; Guo et al., 2006; Kobayashi et al., 2007; Luo et al., 2005; Tan et al., 2005; Zou et al., 2011). Several approaches have been presented to interfere with the NF-κB pathway for pain relief in rodent models, such as antisense ODN against NF-κB or NF-κB inhibitors, pyrrolidinedithiocarbamate and SN50 (Ledeboer et al., 2005; Lee et al., 2004). However, few studies have focused on the role of RNA interference (RNAi) targeting p65 subunit of NF-κB in neuropathic pain.

The purpose of the present study was to construct a lentiviral vector encoding a specially designed shRNA against NF-κBp65 gene (LV-NF-κBp65) and observe the inhibitory effect of the shRNA in vitro and in vivo. Furthermore, we evaluated the analgesic effect of intrathecal delivery of LV-shRNA on CCI model of rats and probed the potential of LV-shRNA as a tool for functional investigation of NF-κBp65 in neuropathic pain.

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2. Materials and methods

2.1. Lentiviral vectors production

Three siRNAs targeting the cDNA sequence of rat NF-κBp65 (GenBank Accession NM_199267) were designed using the Ambion application, following previously published guidelines (Ui-Tei et al., 2004). The sequences of the three siRNAs were: 5-CCA TCA AGA TCA ATG GCT A-3″ (siRNA1), 5′-GAC ATT GAG GTG TAT TTC A-3′ (siRNA2) and 5′-GGA CCT ACG AGA CCT TCA A-3′ (siRNA3). An additional scrambled sequence was also designed as a negative control (NC) (5′-TTC TCC GAA CGT GTC ACG T-3′). Replication-deficient, self-inactivating lentiviral expressing vectors pFU-GW-shRNA-GFP (LV-shNF-κB p65 and LV-NC) were generated as follows. The cDNAs corresponding to the three siRNAs and NC were subcloned into the replication-deficient, self-inactivating lentiviral expression vector pFU-GW-RNAi-GFP (Shanghai Gene Chem Co, Ltd.). The resulting recombinant lentiviral vectors were designated as LV-shNF-κB p65 1, LV-shNF-κB p65 2, LV-shNF-κB p65 3 and LV-NC. To produce lentivirus, the 293T cells were transfected with 20 μg of either pFU-GW-shNF-κB p65 plasmid together with 15 μg of pHelper1.0 and 10 μg of pHelper2.0 packaging plasmids (Coleman et al., 2003). The culture medium was collected in 48 h after transfection, concentrated by ultracentrifugation, aliquoted, and stored at −80 °C until used. The titer of lentivirus was determined by hole-by-dilution titer assay (Deglon et al., 2000). Four days after a single exposure of 293T cells to the lentivirus, strong green fluorescence was shown in more than 90% of cells, indicating a high and stable transduction of the lentiviral vector system. The final titer of pFU-GW-shRNA-1, pFU-GW-shRNA-2, pFU-GW-shRNA-3 and pFU-GW-NC were 4×10^8 TU/ml, 5×10^8 TU/ml, 4×10^8 TU/ml and 2×10^9 TU/ml respectively.

2.2. Spinal cord neuron culture and immunofluorescence staining

Primary cultures of embryonic spinal cord neurons were prepared as previously described (Ahlemeyer and Baumgart-Vogt, 2005). Briefly, the spinal cords were dissected from embryonic day 16 (E16) rat. The spinal cord cells were dissociated with gentle trituration, plated at 150 neurons/mm^2 and grown in serum-containing medium (89.4% minimum essential medium, 10% horse serum, 0.6% glucose, supplemented with penicillin and streptomycin). To enrich the population of neurons, the serum containing medium was replaced by Neurobasal medium (Gibco/Invitrogen) supplemented with B-27 24 h after plating. Moreover, cytosine arabinoside (Ara-C, 5 μM) was added to these cultures on the 3rd day, which is replaced by fresh NB media on the 5th day. On the 6th day, cells were fixed in 4% paraformaldehyde/PBS for 20 min; washed in PBS for 3 times; blocked in 10% normal goat serum and incubated in a rabbit antibody against microtubule-associated protein 2 (MAP2) (1:100, Santa Cruz) overnight at 4 °C. Cells were washed in PBS and incubated in FITC-conjugated goat anti-rabbit IgG (1:100) for 1 h; washed in PBS 3 times and mounted in gel containing Hoechst 33342 (0.5 μM, Sigma). Hoechst 33342, which labels DNA, was used to stain all nuclei in the cultures. Purity of spinal neurons would be expressed as the MAP2 positive neurons related to the Hoechst33342 labeled cultures. Five randomly selected fields with more than 200 cells were chosen for each cover lip. As for staining with Hoechst 33328, cells were fixed, washed, and incubated with the dye following the instruction of the manufacture (Beyotime, Jiangsu, China).

2.3. Infection of primary cultured neurons and fluorescence imaging

Neurons were plated in 8-well dishes, which were coated with poly-D-lysine and laminin (Sigma-Aldrich, St. Louis, MO), and grown in Neurobasal media (Invitrogen, Carlasbad, CA) with supplements. Dissociated neurons were infected with lentivirus 1 h after plating at a multiplicity of infection (M.O.I.) = 3 TU/cell, yielding about 95% infection of neurons with no apparent toxicity. The GFP fluorescence in the cells was monitored with a fluorescent microscope (Leica, Germany) at 72 h post-infection.

2.4. SiRNA transfections

Cells were divided into 5 groups: blank control, which were incubated in neurobasal media only; negative control, which were transfected with NC and siRNA group (1–3): transfected with siRNA targeting NF-κB p65. After transfection for 72 h, the cells were incubated for another 1 h with LPS (1 ng/l, Sigma Chemical Co., St. Louis, Missouri, USA).

2.5. Real-time quantitative PCR

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). The amplification was performed at 95 °C for 2 min, followed by 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 $2^{-\Delta\Delta CT}$ method with normalization software. Primers utilized for the RT-PCR were as follows: NF-κB p65, 5 prime, 5′-GTG CAG AAA GAA GAC ATT GAG GTG-3′ and 3 prime, 5′-AGG CTA GGG TCA GCG TAT GG-3′; GAPDH, 5 prime, 5′-TGG AGA AAC CTG CCA AGT ATG A-3′ and 3 prime, 5′-TGG AAG AAT GGG AGT TGC TGT-3′.

2.6. Western blot analysis

Cells or the lumbar spinal enlargement (L_{4-6}) were removed 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 being blocked in TBS containing 5% skim milk and 0.1% Tween 20 for 2 h at room temperature, membranes were incubated overnight at 4 °C in the primary rabbit monoclonal anti-NFκB-p65 (1:1000, 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.

2.7. Animal preparations

Male Sprague–Dawley (SD) rats weighing 200–220 g (Experimental Animal Center of Shandong University) were kept at 24 ± 1 °C on a 12 h light–dark cycle and given free access to laboratory chow and water. Following the IASP guidelines for pain research in animals, all animal studies were approved by the Animal Care and Use Committee at the Shandong University and were in accordance with the University's guidelines for the care and use of laboratory animals.

2.8. Intrathecal catheter implantation

Intrathecal catheters were implanted as described by Yaksh and Rudy (1976). In brief, under anesthesia induced by chloral hydrate (300 mg/kg, i.p.), the occipital muscles were bluntly separated, and the cisternal membrane was exposed. Polyethylene catheters (PE-10) were inserted via an incision in the cisterna magna and advanced 7.0–7.5 cm caudally to the level of the lumbar enlargement. The

incision site was closed in layers and the catheter was fixed firmly under the skin and sealed effectively. The rats were allowed to recover for 3 days before the CCI test and monitored daily after surgery for signs of motor deficiency or infection.

2.9. Nerve injury surgery

CCI to the sciatic nerve was performed as previously described by Bennett and Xie (1988). Briefly, the rats were anesthetized by using chloral hydrate (300 mg/kg i.p.) and the left sciatic nerve was exposed at the mid-thigh level. Four chromic gut (4-0) ligatures were tied loosely proximal to the trifurcation of the sciatica's at 1.0 mm intervals. Sham surgery was performed by exposing the left sciatic nerve without ligation. The incision site was closed in layers and penicillin was administered i.m.

2.10. Virus injection

Virus (5 μl or 10 μl) was administered 3 days after CCI through the implanted catheter, which was flushed by using 10 μl or 5 μl of PBS. Rats were intrathecally delivered with NS, LV-NC, or LV-shNF-κBp65 plus PBS in a total volume of 15 μl. In order to confirm the delivery of LV-shNF-κBp65 into spinal cord cells, the L_{4-5} lumbar segment of the spinal cord was removed 7 days after IT administration and the uptake of labeled GFP by spinal cord cells was evaluated by fluorescence microscopy (Garraway et al., 2009). The protein expression of NF-κBp65 and pro-inflammatory factors (TNF-α, IL-1β and IL-6) in the spinal cord was measured by Western blot analysis and ELISA respectively 7 days after intrathecal delivery (6 animals in each group).

2.11. Protein estimation by ELISA

The ipsilateral hemi-spinal cords were removed and homogenized as described previously (George et al., 1999). In brief, spinal cord segments were pooled and homogenized in ice-cold phosphate-buffered saline (PBS), pH 7.4, containing protease inhibitors (1% Triton-X100, 1 mM/l PMSF, 10 μg/ml aprotinin, and 1 μg/ml leupeptin). After centrifuge at 10,000 *g* at 4 °C for 30 min, supernatant was aliquoted and stored at −80 °C for future protein quantification. Supernatants were assayed by the Rat TNF-α ELISA kit (BioSource Europe, SA), IL-1β (BioSource International, Inc., Camarillo, CA) and IL-6 (Jingmei Biotech Co. Ltd., Shenzhen, China) according to the manufacturer's instructions.

2.12. Evaluation of tactile allodynia and thermal hyperalgesia

Rats were transferred to a plastic chamber (20 cm × 25 cm × 15 cm) and allowed to acclimatize for 30 min before testing. Mechanical hyperalgesia 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. 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 paw withdrawal latency (PWL). Cut-off was set at 30 s. Intervals were 5 min between trials. Tests were performed 1 day before surgery (baseline) and 0, 4, 7, 14, 21 and 28 days after drug delivery.

2.13. Statistical analysis

All data are expressed as mean ± S.E.M. Statistical analysis between two samples was performed using Student *t* test. Statistical comparison of more than two groups was performed using one-way ANOVA followed by a Tukey post hoc test. The significance of any differences in behavioral data of different experimental groups was assessed using two-way ANOVA. $P<0.05$ was considered statistically significant.

3. Results

3.1. Spinal cord neuron culture and identification by immunofluorescence staining

In the spinal cord primary culture, the percentage of neurons reached a relative stable state in 6 days. This was confirmed by immunofluorescence staining (Fig. 1). Purity reached 86.90 ± 9.20% (n = 5). Spinal neurons were stained with Hoechst 33258 and examined under fluorescence microscope.

3.2. Lentiviral vector transduction of shNF-κBp65 into neurons in vitro and in vivo

Strong green fluorescence was found in more than 95% cultured neurons 72 h after virus infection (M.O.I = 3), indicating a successful infection (Fig. 2A). On the other hand, no cell death or significant morphology change was shown indicating that the lentiviral vector had little toxic effect on cells. In terms of virus infection into the spinal cord, LV-shNF-κBp65 injection induced strong fluorescence in a large numbers of cell bodies (Fig. 2B). Fluorescence appeared primarily in the cytoplasm.

3.3. The down-regulation of NF-κB p65 mRNA and protein by lentivirus-mediated delivery of shRNA in neuronal cells in vitro

Real time-PCR and Western blot analysis were performed 72 h after infection to evaluate the level of NF-κBp65 mRNA and protein expressions. Our results showed that no significant inhibition in NF-κB p65 mRNA or protein expression was found in neurons treated either by LV-NC or LV-siNF-κBp65-1. In contrast, LV-shNF-κBp65-2 and LV-NF-κBp65-3 treatment induced 96.8% and 92.4% reduction, respectively, in NF-κBp65 mRNA ($P<0.01$, Fig. 3A). The protein expression levels were down regulated by 87.3% and 80.7%, respectively, in the two groups. ($P<0.01$, Fig. 3B).

3.4. The inhibition of spinal NF-κBp65 protein by intrathecal injection of LV-shNF-κBp65-2 in a dose-dependent manner

According to the results obtained in vitro, LV-shNF-κBp65-2, as the most effective one among the three siRNAs, was used for in vivo observation of gene silencing effect. The effect of siRNA on inhibiting NF-κBp65 expression was confirmed at protein level by western blotting (Fig. 4). Compared with the sham group, rats in CCI group exhibited significantly higher NF-κB p65 protein levels ($P<0.01$). Compared with the LV-NC group, the expression of NF-κB p65 protein was markedly attenuated after intrathecal injection of LV-shNF-κBp65, which was less in siRNA (10 μl) group (interference efficiency was 74.2%) than in siRNA (5 μl) group (interference efficiency was 66.5%)($P<0.05$). There was no significant difference between CCI group and LV-NC group ($P>0.05$).

3.5. The attenuated expression of TNF-α, IL-1β and IL-6 by intrathecal injection of LV-shNF-κBp65-2 in the spinal cord in vivo in a dose-dependent manner

ELISA was utilized to determine changes in TNF-α, IL-1β and IL-6 production in the spinal cord segments (Fig. 5). There were significant increases in the protein levels of TNF-α, IL-1β and IL-6 within L_{4-6} spinal segments in CCI group compared to sham group

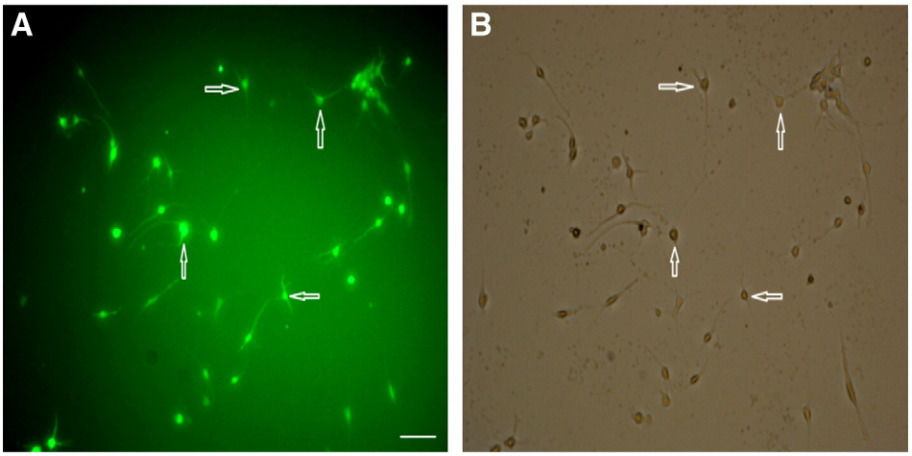

Fig. 1. The immunofluorescence staining for the identification of neuron purity.(A) White arrows indicated immunofluorescence staining of cultured spinal cord neurons. The green MAP-2 positive neurons and Hoechst33342-stained blue nuclei were merged by the Leica QWin automatic Image Analysis software. (B) White arrow showed the cultured spinal cord neurons in a bright field (bar = 100 μm).

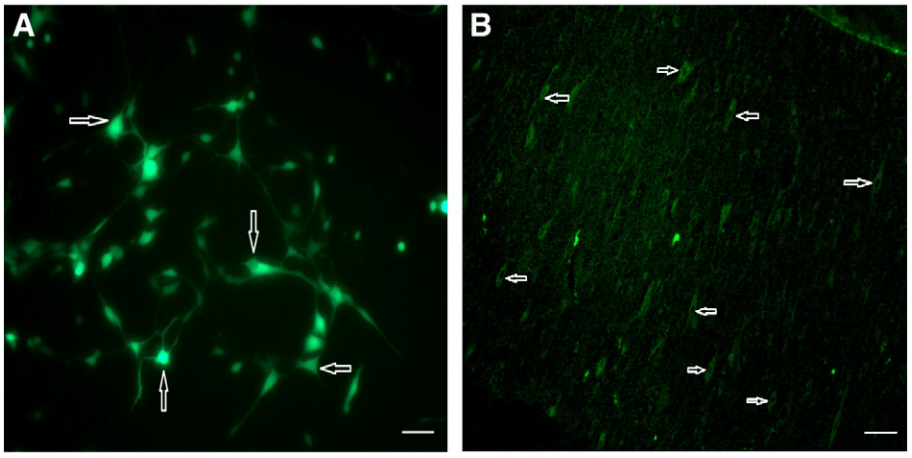

Fig. 2. Fluorescence microscopy images of neurons after infection with LV-shNF-κBp65 in vitro and in vivo.White arrows indicated GFP fluorescence in cultured spinal cord neurons under fluorescence microscopy (A) in vitro (bar = 50 μm) and in spinal cord cells of rats (B) (bar = 100 μm).

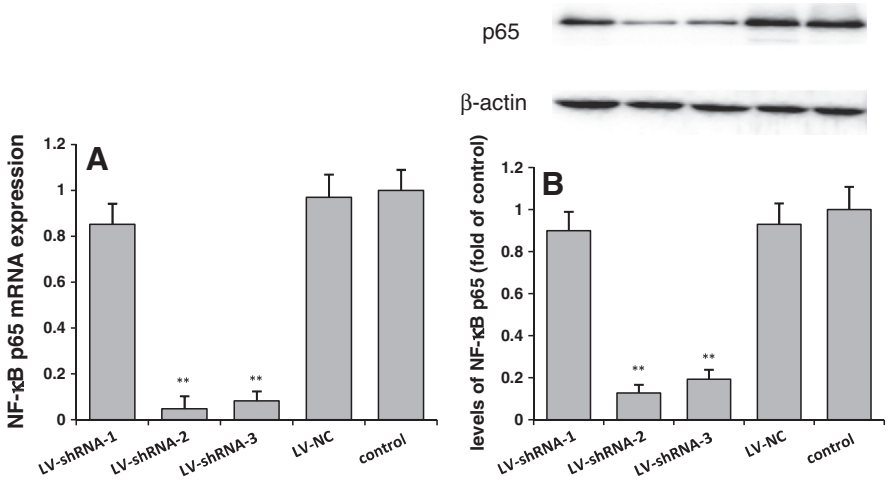

Fig. 3. Real-time PCR and Western blotting analysis of NF-κB p65 levels in cultured neurons in vitro.(A) LV-shNF-κB p65-2 and LV-shNF-κB p65-3 resulted in 96.8% and 92.4% inhibition, respectively, of the NF-κB p65 mRNA 72 h after infection. There was no significant down-regulation of NF-κB p65 mRNA by the LV-siNF-κBp65-1 and LV-NC treatments. GAPDH was used for normalization. The fold change of cytokine levels in sham group was set as 1 for quantifications. Data are presented as mean ± S.E.M. (**P<0.01 vs control group). (B) Western blot analysis showed a 87.3% and 80.7% down regulation of NF-κB p65 protein expression induced by of LV-shNF-κB p65-2 and LV-NF-κBp65-3 treatment, respectively. There was no significant down-regulation of NF-κB p65 protein by the LV-siNF-κBp65-1 and LV-NC treatment. The fold change of NF-κBp65 levels in control group was set as 1 for quantifications. Data are presented as mean ± S.E.M. (**P<0.01 vs control group).

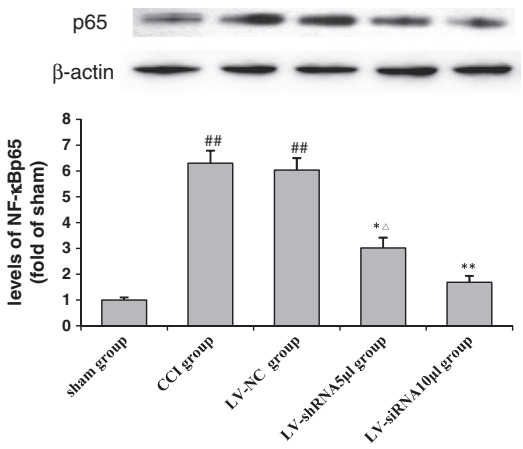

Fig. 4. Western blotting and the quantitative data of NF-κBp65 protein in ipsilateral spinal cord of rats.CCI significantly increased NF-κBp65 protein expression in the ipsilateral spinal cord. Intrathecal injection of LV-NF-κBp65-2 (5 μl and 10 μl), but not LV-NC, markedly reduced NF-κBp65 protein expression. The fold change of NF-κBp65 levels in sham group was set as 1 for quantifications. Data are presented as mean ± S.E.M. ($^{\#\#}P<0.01$ vs sham group;$^{*}P<0.05$ vs CCI group; $^{**}P<0.01$ vs CCI group; $^{\triangle}P<0.05$ vs LV-shRNA 10 μl group).

($P<0.01$). The increased TNF-α, IL-1β and IL-6 levels were down-regulated by intrathecal administration of LV-shNF-κBp65-2 (5 μl or 10 μl) ($P<0.05$). The cytokine levels in LV-shNF-κBp65 10 μl group decreased significantly compared with that in LV-shNF-κBp65 5 μl group ($P<0.05$). There was no significant difference between CCI group and LV-NC group.

3.6. The alleviation of CCI-induced mechanical allodynia and thermal hyperalgesia by intrathecal injection of LV-shNF-κBp65-2

The behavior of rats was observed following the injection of vectors. We have not found any motor disturbance or abnormal activity in rats during the 4 weeks study. CCI, but not sham surgery, produced significant mechanical allodynia and thermal hyperalgesia ($P<0.05$). The time course of PWT and PWL is presented in Fig. 6. Intrathecal injection of LV-shNF-κBp65-2 (5 μl or 10 μl), not LV-NC, significantly reduced allodynia and hyperalgesia in a dose-depended manner ($P<0.05$). The attenuation of allodynia and hyperalgesia persisted through the observation period of 28 days. The result correlated with the changes of NF-κBp65 and pro-inflammatory cytokine levels showed in Figs. 4 and 5. The delivery of LV-NC did not alter the latency of paw withdrawal compared to the responses of rats in CCI group.

4. Discussion

It is well recognized that the activation of NF-κB pathway is a key factor for the transmission and processing of nociceptive information. A number of inflammatory mediators (i.e., TNF-α, IL-1β, IL-6, NO and TGF-1β), which can activate NF-κB or can be activated themselves by NF-κB, are involved in the modulation of the neuropathic pain (Marchand et al., 2005). Endoneurial administration of NF-κB decoy at the site of nerve damage markedly attenuated thermal hyperalgesia and suppressed the mRNA expression of the inflammatory cytokines, iNOS and adhesion molecules (Sakaue et al., 2001). NF-κB decoy can be efficiently transduced into DRGs and significantly attenuate sciatic nerve pain in the lumbar disc herniation model of rats (Suzuki et al., 2009a, 2009b). Pretreatment with NF-κB inhibitor, PDTC, improved mechanical allodynia and down-regulated the over-expression of TNF-α and TNFR1 induced by peri-sciatic administration of rrTNF (Wei et al., 2007). In our study, we also observed the strong up-regulation of NF-κB, TNF-α, IL-1β and IL-6 mRNA levels in the dorsal spinal cord after CCI, which was consistent with the above reports.

In the family of pro-inflammatory cytokines, TNF-α is regarded as the trigger for a cascade of activation of cytokines (Leung and Cahill, 2010; Sacerdote et al., 2008). TNF-α–TNFR1 interaction leads to the NF-κB activation, which in turn induces transcription of genes encoding inflammatory and other pain-related mediators, such as TNF-α, IL-6 and COX-2 (Baud and Karin, 2001; Holmes et al., 2004; Ledeboer et al., 2005; O'Neill and Kaltschmidt, 1997). The levels of TNF-α and TNFR1 in dorsal root ganglion (DRG) and in spinal dorsal horn increased following peripheral nerve injury (Ohtori et al., 2004). Endoneurial or

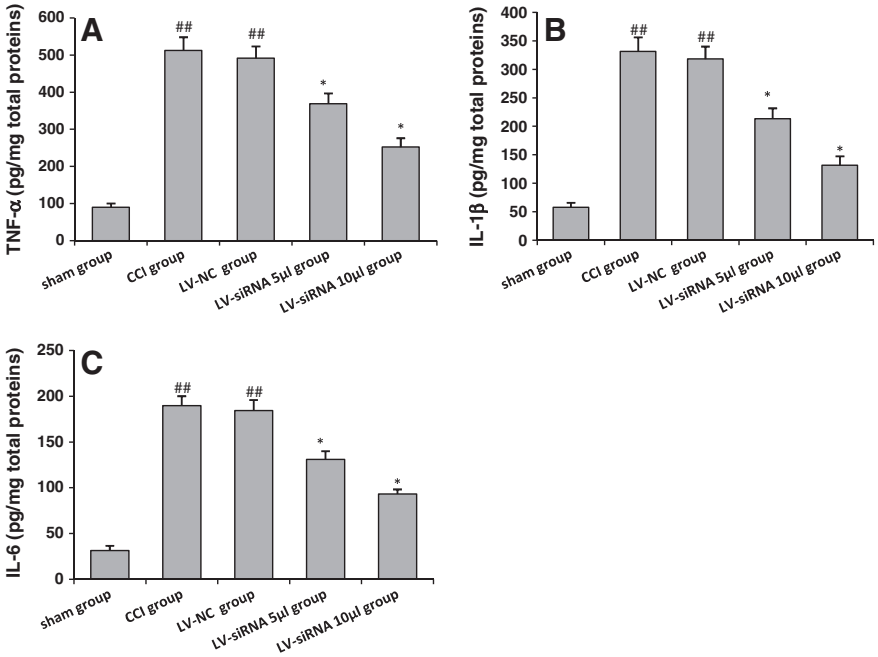

Fig. 5. ELISA for quantization of TNF-α, IL-1β and IL-6 proteins in ipsilateral spinal cord of rats.CCI significantly increased the expression of TNF-α, IL-1β and IL-6 proteins in the ipsilateral spinal cord. Intrathecal injection of 5 μl or 10 μl LV-shRNA, but not LV-NC, decreased the over-expression of TNF-α, IL-1β and IL-6 proteins in a dose-dependent manner in the ipsilateral spinal cord. Data are presented as mean ± S.E.M. ($^{\#\#}P<0.01$ vs sham group;$^{*}P<0.05$ vs CCI group; $^{**}P<0.01$ vs CCI group; $^{\triangle}P<0.05$ vs LV-shRNA 10 μl group).

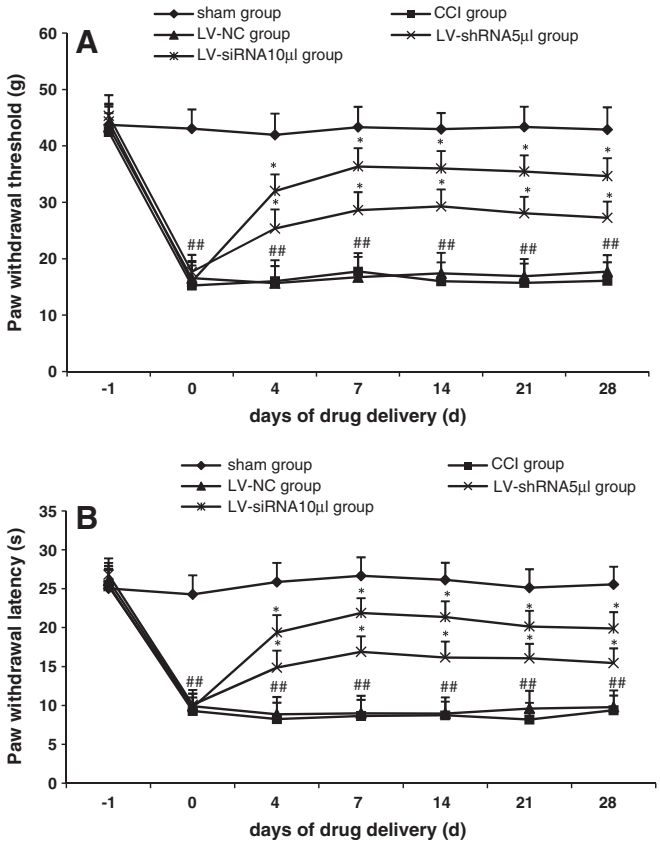

Fig. 6. The analgesic effect of intrathecal injection of LV-shNF-κBp65 in CCI rats.CCI produced significant mechanical allodynia and thermal hyperalgesia in rats. LV-shNF-κBp65 treated group caused a markedly increase in pain thresholds to mechanical and thermal stimulation. LV-shNF-κBp65 (10 μl) treated group resulted in more significant increase in pain thresholds as compared with LV-shNF-κBp65 (5 μl) treated group. PMWT and PWTL were measured 1 day before CCI (baseline) and 0, 4, 7, 14, 21, 28 days after intrathecal administration. '−1' is the time point of the basic pain threshold 1 day before CCI in rats, '0' is the time point before drug delivery (3 days after CCI), '4' is the time point 4 days after drug delivery (7 days after CCI). (8 animals in each group.) Data are presented as mean ± S.E.M. (*P<0.05 vs CCI group; △P<0.05 vs LV-shRNA 10 μl group).

spinal delivery of TNF-α can induce the mechanical allodynia and thermal hyperalgesia in rats (Youn et al., 2008) and blockade of TNF-α has the opposite effect (Hao et al., 2007; Sasaki et al., 2007). IL-1β and IL-6 are also potent pro-inflammatory cytokines involved in neuropathic pain (Eliav et al., 2009). Reports have showed that intrathecal injection of either IL-1β receptor antagonist or neutralizing anti-rat IL-6 antibodies can significantly decrease allodynia and hyperalgesia in neuropathic pain models of rats (Arruda et al., 2000; Sweitzer et al., 2001). Thus, it will be a novel strategy to focus on targeting systems that affect a series of nociceptive mediators, rather than either reducing the production of a single factor or blocking their site of action. In our study, we designed shRNA sequences targeting NF-κBp65 to investigate whether knockdown of NF-κBp65 could be effective in treating pain and whether its downstream pro-inflammatory factors could be decreased after blockage of NF-κB pathway. Our results demonstrated that intrathecal injection of shRNA-NF-κBp65 markedly attenuated mechanical allodynia and thermal hyperalgesia for at least 4 weeks and suppressed the over-expression of spinal TNF-α, IL-1β and IL-6 in CCI model of rats in a dose-dependent manner. We propose that the analgesic effect of siRNA-NF-κBp65 might be mediated, at least partly, through the prevention of TNF-α, IL-1β and IL-6 products.

RNAi, which can induce loss-of-function phenotypes by post-transcriptional silencing of gene expression, has the ability to design precisely targeted approaches for almost any gene in the absence of gene product function and protein structure information (Dorsett and Tuschl, 2004; Uprichard, 2005). SiRNA sequences can be designed into short hairpin RNA (shRNA), which are stem-loop RNA structures, and be packaged in viral vectors and delivered easily into target tissues in vivo or in vitro, especially into central nervous system without major off-target effects (Azkur et al., 2005; Hong et al., 2006). The potential therapeutic effect of RNAi is already being tested in clinical trials (Shrey et al., 2009). SiRNA targeting NF-κB pathway has been shown to exert their biological effect on a number of disorders, ranging from cancer to transplantation medicine and autoimmune diseases (Tas et al., 2009). In our experiment, we designed three siRNA sequences targeting NF-κBp65 and observed the difference of gene silencing effect among them in vitro. The results showed that LV-siRNA-2 held the highest potential to knock down the NF-κBp65 gene. When the LV-siRNA-2 was used in vivo in the CCI model in rats after intrathecal administration, a markedly antihyperalgesic and antiallodynic effect in a dose-dependent manner was revealed. Furthermore, we noticed that intrathecal delivery of LV-NF-κBp65 alleviated, although not completely, the nociceptive behavior in CCI rats. The results indicate that NF-κB signaling might play a partial role in the development of neuropathic pain. Literature has reported that NF-κB-independent pathways, such as mitogen-activated protein kinase (MAPK), are involved in the spinal plastic changes (Hagenacker et al., 2010; Zhuang et al., 2005). For example, the p38 MAPK can be activated following peripheral nerve injury and plays a role in the control of IL-1β production (Wu et al., 2004).

Key properties of an ideal gene delivery tool are safety (including low toxicity), stability, cell type specificity, and markers (Song and Yang, 2010). Viral vector-mediated RNAi has already been shown effective in inhibiting gene expression in a number of diseases (Couto and High, 2010). Compared with other delivery methods, such as direct administration via minipump (Dorn et al., 2004), using the transduction reagent, i-Fect™ (Tan et al., 2005), or ultrasound gene transfer technique (Suzuki et al., 2009a, 2009b), viral-mediated gene transfer is currently believed to be the most efficient system for delivering therapeutic proteins in vivo (Adriaansen et al., 2006; Vervoordeldonk et al., 2008). Lentiviral vectors also have a relatively limited host-inflammatory response and potential to yield sustained (in theory life-long) gene silencing (Manjunath et al., 2009). In our study, we observed high levels of GFP in cultured neurons and spinal cord cells, suggesting the successful infection of the recombinant lentiviral vector in vitro and in vivo. Our studies also showed a significant down-regulation of NF-κBp65 in vitro and in vivo after transfecting of LV-shNF-κB p65, as a further proof of the successfully transduction of the desired target cells.

Furthermore, the general behavior of rats, such as weight, intake of food and water and reactive ability of the rats were observed after LV-shRNA injection during the whole study. There were no significant difference among the LV-shRNA, LV-NC and NS treated groups. We also only found minor injury of the spinal parenchyma and little behavioral alterations in rats after LV-shRNA administration. Of course, more signs should be observed in further study, such as motor coordination and balance, learning and memory etc., as for a more objective conclusion of the safety of intrathecal LV-shRNA. These results suggest a potential clinical use of shRNA expression vectors as a gene therapy approach to neuropathic pain.

In conclusion, results from present study confirm the role of NF-κB pathway and its downstream pro-inflammatory factors in CCI induced neuropathic pain. In addition, we have also demonstrated that NF-κBp65 might be a feasible RNAi target in molecular pain management and lentiviral vector delivery strategy could be a suitable approach for future studies on gene functions of NF-κB p65.

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