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·Original Article·

Toll-like receptor 4-mediated nuclear factor-κB activation in spinal cord contributes to chronic morphine-induced analgesic tolerance and hyperalgesia in rats

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ABSTRACT

Nuclear factor kappa B (NF-κB) in the spinal cord is involved in pro-inflammatory cytokine-mediated pain facilitation. However, the role of NF-κB activation in chronic morphine-induced analgesic tolerance and the underlying mechanisms remain unclear. In the present study, we found that the level of phosphorylated NF-κB p65 (p-p65) was increased in the dorsal horn of the lumbar 4–6 segments after intrathecal administration of morphine for 7 consecutive days, and the p-p65 was co-localized with neurons and astrocytes. The expression of TNF-α and IL-1β was also increased in the same area. In addition, pretreatment with pyrrolidinedithiocarbamate (PDTC) or SN50, inhibitors of NF-κB, prevented the development of morphine analgesic tolerance and alleviated morphine withdrawal-induced allodynia and hyperalgesia. The increase in TNF-α and IL-1β expression induced by chronic morphine exposure was also partially blocked by PDTC pretreatment. In another experiment, rats receiving PDTC or SN50 beginning on day 7 of morphine injection showed partial recovery of the anti-nociceptive effects of morphine and attenuation of the withdrawal-induced abnormal pain. Meanwhile, intrathecal pretreatment with lipopolysaccharide from *Rhodobacter sphaeroides*, an antagonist of toll-like receptor 4 (TLR4), blocked the activation of NF-κB, and prevented the development of morphine tolerance and withdrawal-induced abnormal pain. These data indicated that TLR4-mediated NF-κB activation in the spinal cord is involved in the development and maintenance of morphine analgesic tolerance and withdrawal-induced pain hypersensitivity.

Keywords: nuclear factor-κB; pro-inflammatory cytokines; Toll-like receptor 4; morphine tolerance; hyperalgesia

INTRODUCTION

Treatment of severe pain with morphine, the gold-standard opioid and a potent analgesic, is limited by the eventual development of analgesic tolerance and withdrawal-induced hyperalgesia and allodynia[1,2]. Although recent advances have been made in the neurobiological mechanisms underlying opioid-induced tolerance and hyperalgesia, these problems are still ineffectively managed in the clinic. At the cellular level, morphine exerts its effects *via* G protein-coupled receptors, primarily μ-opioid receptors, resulting in the activation of intracellular signal-transduction kinases[3]. Nuclear factor-κB (NF-κB) is one of the most diverse and critical transcription factors that may either directly or indirectly transmit opioid receptor-mediated signals to the nucleus, and it regulates NF-κB-dependent gene expression in immune and non-immune cells[4]. NF-κB is composed of homo- and heterodimers among five members of the Rel family: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. The p50/p65 complex is the

most common functional heterodimer in cells[5]. It is well established that the NF-κB family plays critical roles in inflammation, immunity, cell proliferation, and apoptosis[6], and has been implicated in the regulation of memory and neuroplasticity[7-9].

Numerous studies have shown that NF-κB activation in the dorsal root ganglia (DRG) and spinal dorsal horn contributes to the development of inflammatory and neuropathic pain[10-14]. Chronic administration of morphine also results in pain-related activation of neurons and glia, and induces the release of pro-inflammatory cytokines and chemokines[15-17]. The prevailing evidence shows that acute and chronic morphine treatment increases the expression of TNF-α, IL-1β, and IL-6 in activated glia in the DRG and spinal cord, which ultimately results in the decreased analgesic efficacy of morphine. The development and maintenance of morphine tolerance are effectively prevented by inhibition of the synthesis of these cytokines or by their neutralization with specific antibodies in the spinal cord[15,17-20]. NF-κB activation plays an important role in regulating the expression of TNF-α, IL-1β, IL-6, and other cytokines in immune cells[4]. Previous studies have also provided evidence that morphine treatment leads to NF-κB activation in cultured human NT2-N neurons[21]. The morphine-stimulated expression of pro-inflammatory cytokines and chemokines in immune cells is transcriptionally controlled by NF-κB[5]. This indicates that the NF-κB signaling pathway may also play a role in the morphine-induced release of pro-inflammatory cytokines by neurons and glia. However, the roles of NF-κB activation in the development of chronic morphine-induced analgesic tolerance and morphine withdrawal-induced hyperalgesia and the underlying mechanisms remain poorly understood. The present study was designed to answer these questions.

MATERIALS AND METHODS

Animal Preparation

Male Sprague-Dawley rats weighing 200–300 g were housed in separate cages with free access to food and water. The room temperature was maintained at 23 ± 2°C under a 12:12-h light-dark cycle. All experimental procedures were approved by the Animal Care and Use Committee of Zhengzhou University and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines for the experimental investigation of pain in conscious animals[22].

Intrathecal Catheter Implantation

Intrathecal (i.t.) catheter implantation was performed following the procedures described by Storkson *et al*.[23] with modifications. After the animal was anesthetized with sevoflurane, a midline skin incision was made in the lumbar region (L4–S1), and the intervertebral membrane between L5 and S1 was exposed. A needle was then used to puncture the membrane, and a polyethylene (PE-10) catheter was inserted into the subarachnoid space rostrally to reach the lumbar enlargement. The tube was fixed to the muscle, and the other end was tunneled rostrally underneath the skin to exit in the occipital region. The incisions were then closed in layers using 4-0 silk. The rats were allowed to recover for 7 days before behavioral testing or i.t. injections of drugs. The position of the catheter was checked postmortem. Animals that displayed any abnormal neurological signs were excluded from experiments.

Induction of Morphine Tolerance and Drug Delivery

Morphine tolerance was induced following the methods described by Lim *et al*.[24]. Briefly, to induce analgesic tolerance, rats were injected i.t. with 10 μg morphine twice daily for 7 consecutive days. Rats receiving i.t.saline (vehicle) (10 μL twice daily) for 7 days served as the control group. The analgesic effects of morphine were measured by the tail-flick test. Drugs were delivered as follows: in the pretreatment groups, all rats received i.t. injections of the NF-κB inhibitor PDTC (Sigma, St. Louis, MO) at 0.2 μg/10 μL and 0.5 μg/10 μL, or SN50 (Merck, Darmstadt, Germany) at 0.5 μg/10 μL once daily for 7 days, beginning 25 min prior to the morphine injections; in the post-treatment group, PDTC (0.5 μg/10 μL) or SN50 (0.5 μg/10 μL) administration began on day 7 of morphine injections, and continued once daily for 5 days. Another group of rats received i.t. injections of lipopolysaccharide derived from *Rhodobacter sphaeroides* (LPS-RS) (InvivoGen, San Diego, CA), an antagonist of toll-like receptor 4 (TLR4), at 20 μg/10 μL once daily for 7 days beginning 25 min prior to the morphine injections. The morphine hydrochloride was purchased from the Northeastern Pharmaceutical Group (Shenyang, China) and diluted in normal saline prior to use.

PDTC was freshly dissolved daily in normal sterile saline. SN50 was diluted in PBS to a concentration of 5 μg/μL and then freshly diluted daily in normal saline. The doses of PDTC and SN50 used in this study were based on a previous study[11]. The LPS-RS was diluted in PBS to a final concentration of 2 μg/μL, as in a previous report[25].

Nociception Tests

The pain-related behavioral tests were performed according to our previously described methods[26,27]. All rats were acclimated to the testing environment for at least 3 days prior to baseline measurements. The tail-flick test was performed with an analgesia meter (PL-200, Tai-Meng, Chengdu, China) using baseline latencies of 6–7 s and a cutoff time of 13 s. At least three trials were conducted for each rat at intervals of 2 min, and the position of the radiant heat stimulus on the tail was changed for each trial. The percentage of maximal possible analgesic effect (%MPAE) was determined by comparing the latencies at baseline (BL) and after drug injection (TL) using the following equation: %MPAE = [(TL - BL) / (13 - BL)] × 100% (the constant 13 refers to the cutoff time). Mechanical sensitivity was assessed with Von Frey hairs, using the up-down method[28] and the paw-withdrawal threshold. Each stimulus consisted of 2–3 s application of the Von Frey hair to the plantar surface of the paw, and the inter-stimulus interval was 5 min. Quick withdrawal or licking of the paw in response to the stimulus was considered a positive response. Heat hypersensitivity was evaluated by testing the paw-withdrawal latency[29] using the analgesia meter. The rat was placed in a Plexiglas chamber on a glass plate. A radiant heat source beneath the glass floor was aimed at the plantar surface of a hindpaw. Three latency measurements were acquired for each hindpaw in each test session. The hindpaws were tested alternately, and the intervals between consecutive tests were >5 min. The three latency measurements were averaged on each side. To assess morphine withdrawal responses, some tests were performed on the second day between 08:00 and 10:00; i.e., 12 h after morphine injection. All behavioral tests were performed by an experimenter blinded to the experimental group.

Western Blotting

Western blotting was performed as in our previous procedures[30]. Briefly, the animals were sacrificed by decapitation at a designated time point. The L4–6 spinal dorsal horns and DRGs were harvested and placed temporarily in liquid nitrogen. Then the samples were homogenized in ice-cold lysis buffer (10 mmol/L Tris, 5 mmol/L EGTA, 0.5% Triton X-100, 2 mmol/L benzamidine, 0.1 mmol/L PMSF, 40 μmol/L leupeptin, 150 mmol/L NaCl, 1% phosphatase inhibitor cocktail II and III). The crude homogenate was centrifuged at 4°C for 15 min at 3 000 r/min, and the supernatant was collected. After the protein concentration was measured, the sample was heated for 5 min at 99°C, and 30–60 μg protein was loaded onto 10%–12.5% SDS-polyacrylamide gel. The proteins were electrophoretically transferred onto PVDF membranes. The membranes were blocked with 3% non-fat dry milk for 1 h and incubated overnight at 4°C with primary antibody, followed by incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:3 000; Bio-Rad, Philadephia, PA). The following primary antibodies were used: rabbit anti-phospho-p65-Ser^{346} (1:1 000; Cell Signaling Technology, Danvers, MA), rabbit anti-TNF-α (Cell Signaling Technology, 1:500), rabbit anti-IL-1β (Cell Signaling Technology, 1:500), rabbit anti-TLR4 (Sigma, 1:500), and mouse anti-β-actin (1:10 000; Sigma). The proteins were visualized using the chemiluminescence reagents provided with the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ), and exposed to film. The intensities of the blots were quantified by densitometry. The blot density of the control rats was set at 100%. The relative density values of the other groups were determined by dividing the values for these groups by that of the controls.

Immunohistochemistry

Immunohistochemistry was done following our previous procedures[26,27]. Briefly, after 5 or 7 days of i.t. injections of morphine, rats were deeply anesthetized with sevoflurane and perfused through the ascending aorta with normal saline, followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer. After perfusion, the lumbar (L4–6) spinal cord was removed and post-fixed in the same fixative for 3 h, and then the fixative was replaced by 30% sucrose in PBS over two nights. Transverse spinal sections (25 μm) were cut on a cryostat and prepared for immunofluorescence staining. Sections were randomly selected and put into different wells of a 24-well plate. After a PBS wash,

the sections were blocked with 5% goat serum in 0.3% Triton X-100 for 1 h at 37°C, and incubated with primary antibody overnight at 4°C. For double immunofluorescence staining, the sections were incubated in a mixture of primary antibody with a specific spinal cell marker antibody overnight and then incubated with a mixture of Cy^3- and FITC-conjugated secondary antibody for 1 h at 37°C. The stained sections were mounted on slides, examined under a fluorescence microscope (Nikon TE 2000-E, Melvile, NY) and images were captured with a CCD spot camera. The primary antibody was rabbit anti-phospho-p65-Ser^{346} (1:200; Cell Signaling Technology). The spinal cell-specific markers were monoclonal neuron-specific nuclear protein (NeuN) (neuronal marker, 1:1000; Chemicon, Billerica, MA), glial fibrillary acidic protein (GFAP) (astrocyte marker, 1:1 000; Chemicon), and OX42 (CD11b, microglia marker, 1:500; Chemicon). The specificity of antibodies was checked by omitting the primary antibody during staining as well as western blotting.

Statistical Analyses

Data are presented as mean ± SEM. Two-way ANOVAs with treatment (i.t. morphine or vehicle and NF-κB inhibitors) as the independent factors and time as the repeated factor were used for analyses of changes in %MPAE or paw-withdrawal threshold and latency on each side. A *post hoc* Newman–Keuls test was used when the ANOVAs indicated overall significance. Student's *t*-test with the Welch correction was used to analyze differences between two groups. The western blot data were analyzed with one-way ANOVAs followed by individual *post hoc* comparisons (Tukey's *post hoc* tests) when they showed a significant difference. P <0.05 was considered statistically significant. SigmaStat (Systat, San Jose, CA) or GraphPad Prism 4.0 (GraphPad Software Inc., La Jolla, CA) was used to perform all analyses.

RESULTS

Intrathecal Chronic Morphine Exposure Induced Activation of NF-κB and Expression of TNF-α and IL-1β in the Rat Spinal Dorsal Horn

As has been reported[24], the i.t. injection of morphine (10 μg) twice daily for 7 days significantly reduced the analgesic potency of morphine. Compared to that on day 1, the %MPAE of morphine was lower on day 5 (P <0.01) and was further reduced to baseline on day 7 (P <0.001) (Fig. 1A). Consistent with previous reports, repeated i.t. morphine injection over 7 days induced bilateral mechanical and thermal pain hypersensitivity on day 8. Compared with the saline group and the baseline values, paw-withdrawal thresholds (P <0.01, Fig. 1B) and paw-withdrawal latencies (P <0.05, Fig. 1C) were clearly decreased on day 8; i.e.,

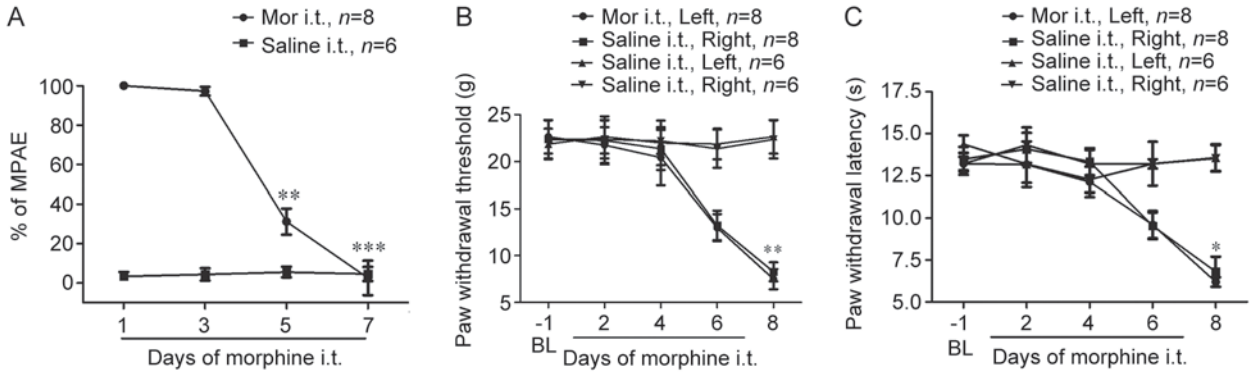

Fig. 1. Chronic intrathecal injection of morphine induced analgesic tolerance and pain-related hypersensitivity in rats. (A) The tail-flick test showed the development of morphine analgesic tolerance following repeated i.t. injections of morphine (10 μg/10 μL, twice daily for 7 consecutive days) (P <0.01, ***P <0.001 *vs* day 1 of morphine injection). (B) Chronic i.t. morphine induced the development of mechanical allodynia in both hindpaws. Compared to the saline group, morphine withdrawal induced reductions in paw-withdrawal thresholds (**P <0.01). (C) Chronic i.t. morphine induced the development of thermal hyperalgesia in both hindpaws. Compared to the saline group, morphine withdrawal also induced reduced paw-withdrawal latencies (*P <0.05). BL, baseline; i.t.: intrathecal injection; Mor, morphine; MPAE, maximal possible analgesic effect.**

one day after the cessation of morphine injections.

Western blotting revealed that the phosphorylation of NF-κB p65 (p-p65) increased in the dorsal horn beginning on day 3, reached a peak on day 5, and lasted until day 7 of morphine injection (Fig. 2A). Chronic i.t. morphine exposure also led to enhanced expression of TNF-α and IL-1β in the dorsal horn. Similar to p-p65, TNF-α significantly increased on day 1, reached a peak on days 3 and 5, and lasted until day 9 (Fig. 2A). Regarding IL-1β, a statistically significant increase was detected on day 5 and persisted until day 9 (Fig. 2A). In the L4 and L5 DRGs, although morphine injection caused slight increases in p-p65, TNF-α, and IL-1β on days 1 and 3, they did not reach statistical significance compared to the saline control group (Fig. 2B).

To further determine the cell-types involved in the morphine-induced NF-κB activation in the dorsal horn, we used double immunofluorescence staining. The results showed that p-p65 was co-localized with NeuN (a specific marker of neurons) (Fig. 3A–C) and GFAP (a specific marker of astrocytes) (Fig. 3D–F), but not with OX42 (a marker of microglia) (Fig. 3G–I) in the dorsal horn after 5 days of i.t. morphine administration.

Inhibition of NF-κB Activation Prevented the Development of Chronic Morphine-Induced Analgesic Tolerance and Morphine Withdrawal-Induced Hyperalgesia

Based on the above results, we further investigated the effects of NF-κB inhibition on the development of morphine analgesic tolerance *via* i.t. injections of PDTC, a potent inhibitor of NF-κB activation, 25 min prior to the morphine injections and continued for 7 days. The results revealed that the development of chronic morphine-induced analgesic tolerance was partially and dose- and time-dependently blocked by PDTC, as a significant increase in %MPAE in the high-dose PDTC group was present from days 5 to 7 of morphine injection compared to vehicle

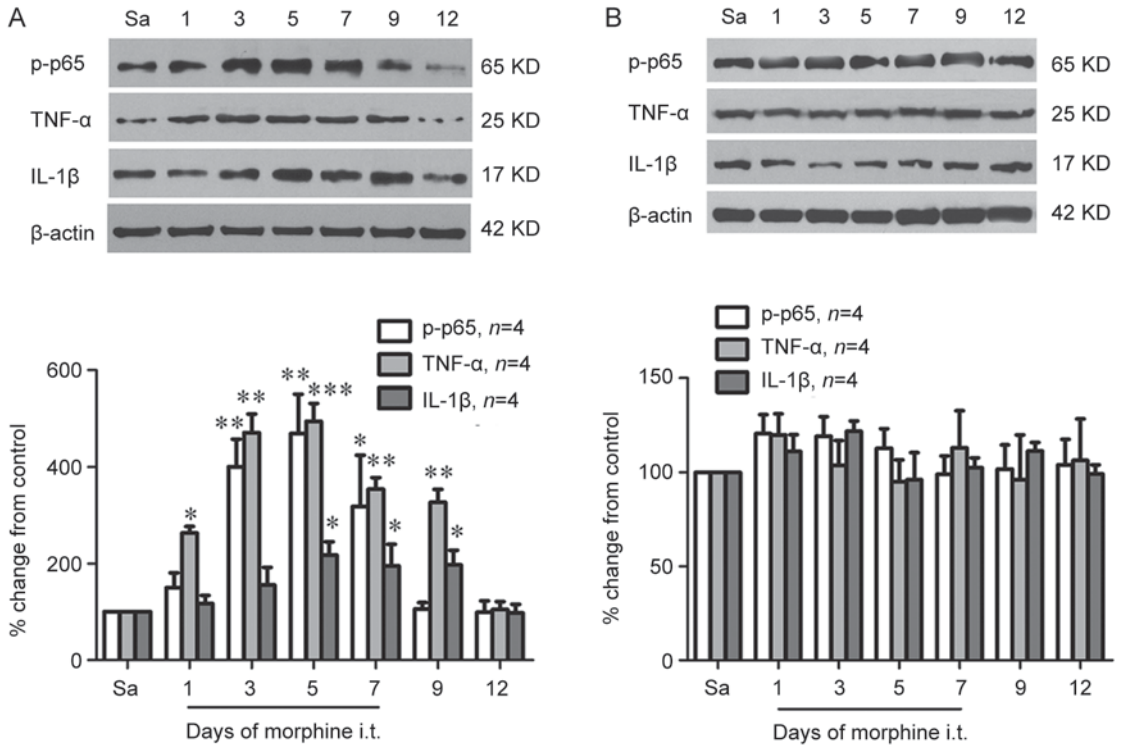

Fig. 2. Chronic intrathecal injection of morphine induced activation of NF-κB and upregulation of TNF-α and IL-1β expression in the spinal dorsal horn. (A, B) Upper panels: western blots showing the changes in p-p65, TNF-α, and IL-1β in the lumbar (L4–6) dorsal horn (A) and in the lumbar DRGs (B). Lower panels: statistical summaries of densitometric analysis. Compared with rats receiving i.t. saline for 7 days (Sa), i.t. morphine induced increased expression of p-p65, TNF-α, and IL-1β in the dorsal horn (A), but not in the DRG (B) (P* <0.05, ***P* <0.01, ****P* <0.001).**

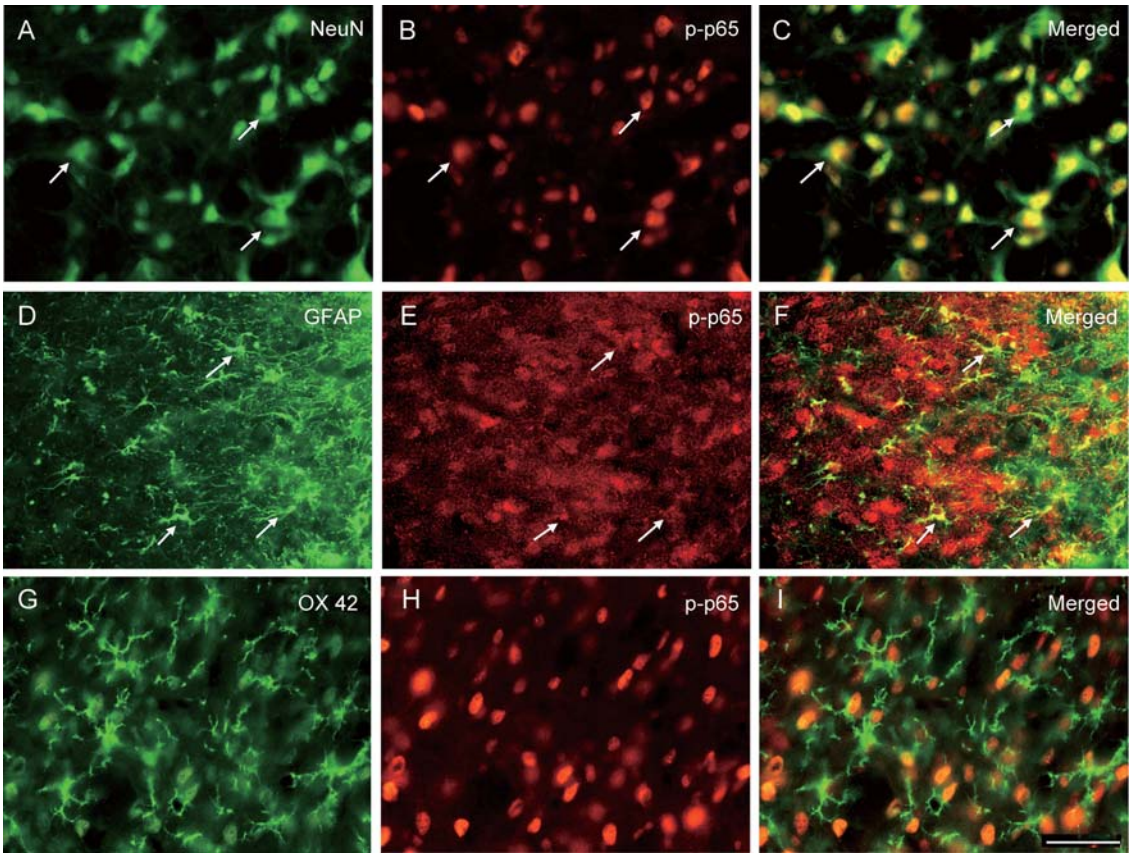

Fig. 3. Cell-types associated with NF-κB activation in spinal cord following chronic intrathecal injections of morphine. (A–I) Double immunofluorescence staining for p-p65 (red; B, E, and H) and NeuN (green; A); GFAP, an astrocyte marker (green; D); and OX42, a microglia marker (green; G). The pairs of images are merged in C, F, and I. The results showed co-localization of p-p65 with NeuN as well as GFAP (scale bar, 50 μm).

pretreatment (Fig. 4A). In the low-dose PDTC group, an increase in %MPAE was detected on day 7 compared with the vehicle + morphine group (Fig. 4A). Compared to the vehicle + morphine group, the rats that received i.t. injections of PDTC also exhibited increases in paw-withdrawal threshold and latency after the cessation of morphine administration on day 8. To confirm these results, we tested another inhibitor of NF-κB, SN50. The behavioral tests revealed that i.t. injection of SN50 effectively prevented the development of chronic morphine-induced decrease in analgesia (Fig. 4D) and alleviated the morphine withdrawal-induced mechanical allodynia and thermal hyperalgesia in both hindpaws (Fig. 4E and F). In addition, neither PDTC nor SN50 alone altered the basal %MPAE, paw-withdrawal threshold or paw-withdrawal latency compared to the saline injection group (Fig. 4).

Effects of NF-κB Inhibition on Established Analgesic Tolerance to Morphine

It remained unclear whether NF-κB inhibition can restore the analgesic effects of morphine after tolerance has been established. Thus, in this experiment, the NF-κB inhibitors PDTC and SN50 were used beginning on day 7 of morphine injections; i.e., at the time when analgesic tolerance had been completely induced. In the morphine + vehicle group, the %MPAE was further reduced following morphine injection (P <0.01 *vs* %MPAE on day 1, Fig. 5A and D). However, in the PDTC and SN50 treatment groups, the decreases in morphine's analgesic effects were partially reversed. Compared to the morphine + vehicle group, significant increases in the %MPAEs of the PDTC- and SN50-treated rats were detected on day 11 of morphine injection (P <0.05, Fig. 5A and D). Moreover, the decreases

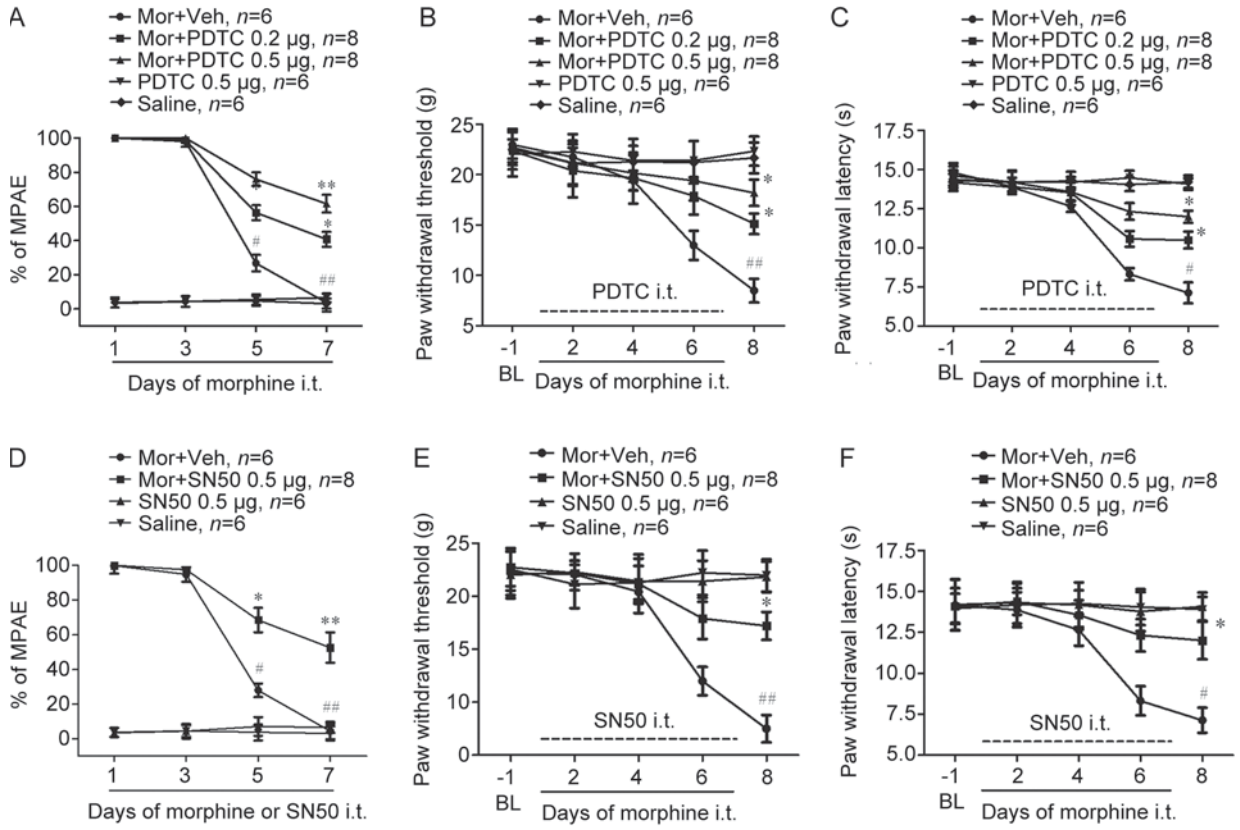

Fig. 4. NF-κB inhibition prevented the development of morphine analgesic tolerance and morphine withdrawal-induced allodynia and hyperalgesia. (A) Rats pretreated with intrathecal injections (i.t.) of pyrrolidinedithiocarbamate (PDTC, 0.2 or 0.5 μg/10 μL, 25 min prior to the morphine i.t. injections once daily for 7 consecutive days) exhibited delays in the development of morphine analgesic tolerance that were dose- and time-dependent (*P <0.05, **P <0.01 *vs* morphine + vehicle group; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs* %MPAE on day 1). (B, C) Effects of pretreatment with i.t. injections of PDTC on morphine withdrawal-induced reductions in paw-withdrawal threshold, which measures mechanical allodynia (B) and paw-withdrawal latency, which measures thermal hyperalgesia (C) ($^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs* baseline; *P <0.05 *vs* morphine + vehicle group. (D–F) Effects of pretreatment with i.t. injections of SN50 (0.5 μg/10 μL, 25 min prior to the morphine i.t. injections once daily for 7 consecutive days) on the development of morphine analgesic tolerance (*P <0.05, **P < 0.01 *vs* morphine + vehicle; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs* day 1, D) and morphine withdrawal-induced decreases in paw-withdrawal threshold (E) and latency (F) (*P <0.05 *vs* morphine + vehicle group; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs* baseline). Repeated i.t. injections of PDTC, SN50, or vehicle (saline) alone did not alter the basal %MPAE, paw-withdrawal threshold, or paw-withdrawal latency. Mor, morphine; Veh, vehicle (saline + PBS); BL, baseline.

in paw-withdrawal thresholds and paw-withdrawal latencies induced by morphine withdrawal were also reversed in rats that received PDTC (Fig. 5B and C) or SN50 treatment (Fig. 5E and F). Compared to the baseline values, i.t. injections of vehicle (saline) alone for 11 consecutive days did not alter the %MPAE, paw-withdrawal threshold, or paw-withdrawal latency (Fig. 5).

To further confirm the above effects of NF-κB inhibition on established morphine tolerance and the abnormal pain induced by morphine withdrawal, a single dose of PDTC (0.5 μg) or SN50 (0.5 μg) was injected i.t. on day 7 of morphine administration. The results showed that neither PDTC nor SN50 reversed the reduction of %MPAE (Fig. 6A) on day 7. The decreased paw-withdrawal threshold and latency induced by morphine withdrawal on day 8 were not changed significantly by the single-dose injections (Fig. 6B and C), indicating that the effects of PDTC and SN50 on the NF-κB signaling pathway are time- and dose-dependent.

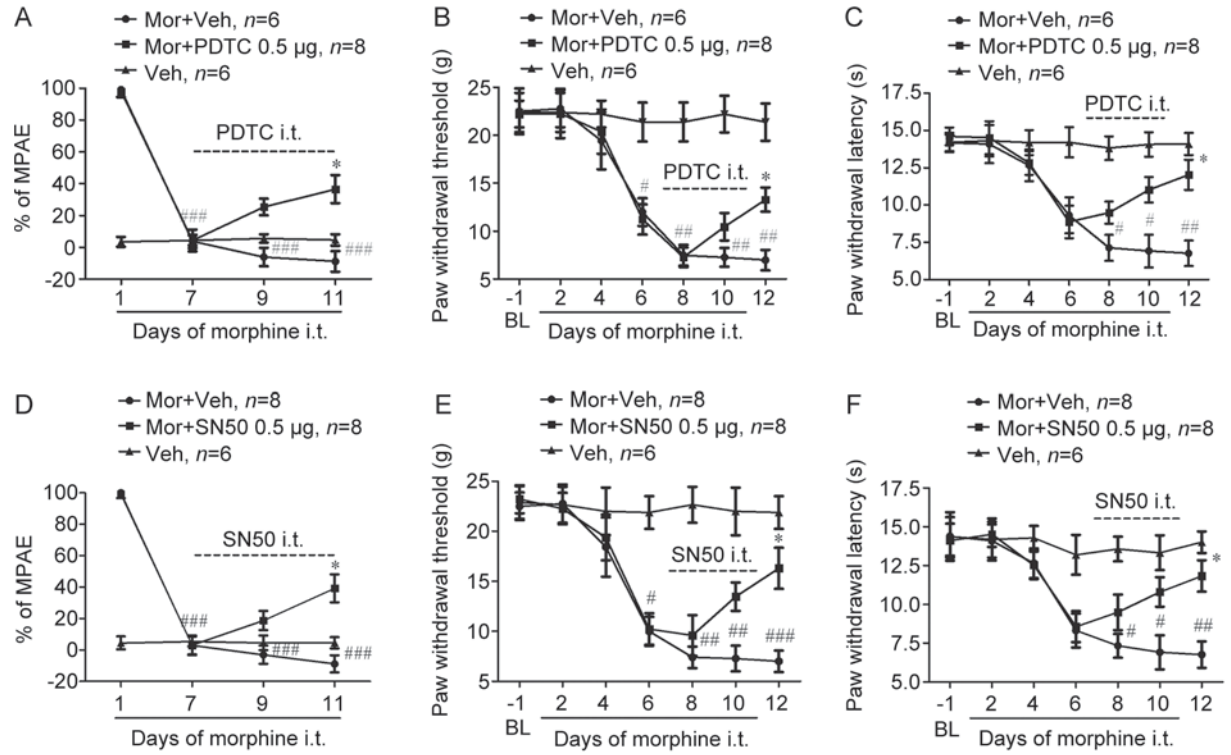

Fig. 5. Effects of NF-κB inhibition on established morphine antinociceptive tolerance and morphine withdrawal-induced abnormal pain. (A–C) Effects of PDTC (0.5 μg/day for 5 consecutive days) beginning on day 7 of morphine i.t. injections on established morphine analgesic tolerance (A), and morphine withdrawal-induced mechanical allodynia (B) and thermal hyperalgesia (C). Compared to the morphine + vehicle group, the rats that received PDTC exhibited a partial reversal of the decrease in %MPAE that was induced by chronic morphine exposure (*P <0.05 *vs* morphine + vehicle group; $^{###}P$ <0.01 *vs* %MPAE on day 1) (A). Rats post-treated with i.t. PDTC exhibited a reversal in the reduction of morphine withdrawal-induced paw-withdrawal threshold (B) and paw withdrawal latency (C) (*P <0.05 *vs* morphine + vehicle group; $^{#}P$ <0.05, $^{##}P$ <0.01 *vs* baseline. (D–F) Effects of SN50 (0.5 μg/day for 5 consecutive days) beginning on day 7 of i.t. morphine on established morphine analgesic tolerance (D), and morphine withdrawal-induced mechanical allodynia (E) and thermal hyperalgesia (F). Compared to the morphine + vehicle group, the rats that received SN50 exhibited partial reversals of the decrease in %MPAE (D) and the reductions of paw withdrawal threshold (E) and latency (F) (*P <0.05 *vs* morphine + vehicle group; $^{#}P$ <0.05, $^{##}P$ <0.01, $^{###}P$ <0.001 *vs* baseline). Repeated i.t. injection of vehicle (saline) alone did not alter the basal %MPAE, paw-withdrawal threshold or paw-withdrawal latency (A–F). BL, baseline; Mor, morphine; Veh, vehicle (saline + PBS).

NF-κB Inhibition Reduced the Expression of TNF-α and IL-1β in the Rat Spinal Dorsal Horn That Was Induced by Chronic Intrathecal Morphine

Previous studies have shown that the NF-κB signaling pathway plays an important role in the regulation of pro-inflammatory cytokine expression in immune cells[6]. So, we further examined the effects of i.t. injections of PDTC on the expression of TNF-α and IL-1β in the spinal dorsal horn after chronic i.t. morphine exposure. The results revealed that i.t. injections of morphine + vehicle for 7 days induced increases in the expression of p-p65, TNF-α, and IL-1β in the dorsal horn (P <0.05 *vs* the vehicle group, Fig. 7). However, PDTC + morphine (i.t. PDTC at 0.5 μg once daily for 7 days, 25 min before the morphine injection) reduced the phosphorylation of NF-κB p65 and the expression of TNF-α and IL-1β in the dorsal horn compared to the morphine + vehicle group (Fig. 7). As above, i.t. injection of PDTC alone did not alter the basal expression of p-p65, TNF-α, or IL-1β in the spinal cord.

Effects of Intrathecal Injection of LPS-RS on NF-κB Activation in the Spinal Cord and the Development of Morphine Analgesic Tolerance

A recent study revealed that TLR4 partially mediates the

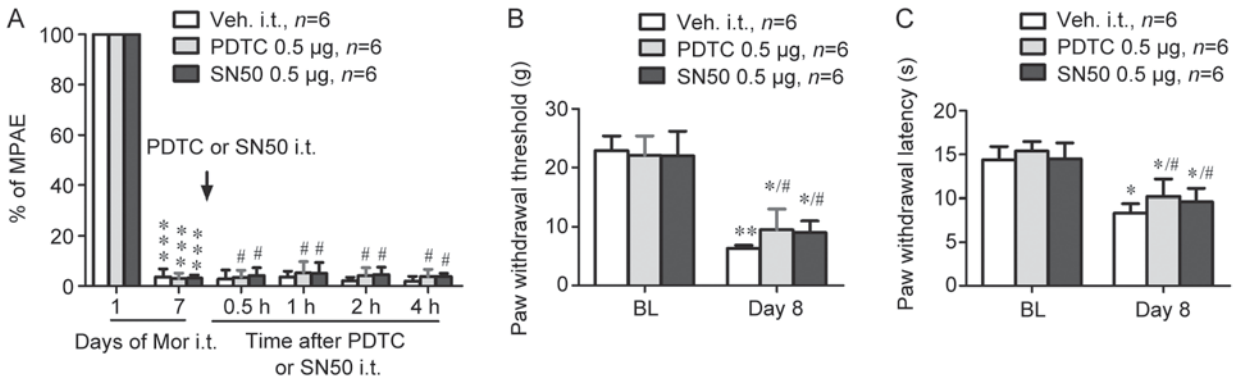

Fig. 6. Effects of single intrathecal injection of PDTC or SN50 on established morphine tolerance and morphine withdrawal-induced mechanical allodynia and thermal hyperalgesia in rats. (A) %MPAE was not altered after the rats received a single injection of PDTC or SN50 on day 7 of morphine administration ($^{\#}P$ >0.05 *vs* vehicle group; ***P <0.001 *vs* day 1). (B, C) Paw-withdrawal threshold (PWT) and paw-withdrawal latency (PWL) after morphine withdrawal on day 8. Neither PDTC nor SN50 produced significant increases in PWT or PWL compared to the vehicle group ($^{\#}P$ >0.05 *vs* vehicle group; *P <0.05,P <0.01 *vs* baseline) BL, baseline.**

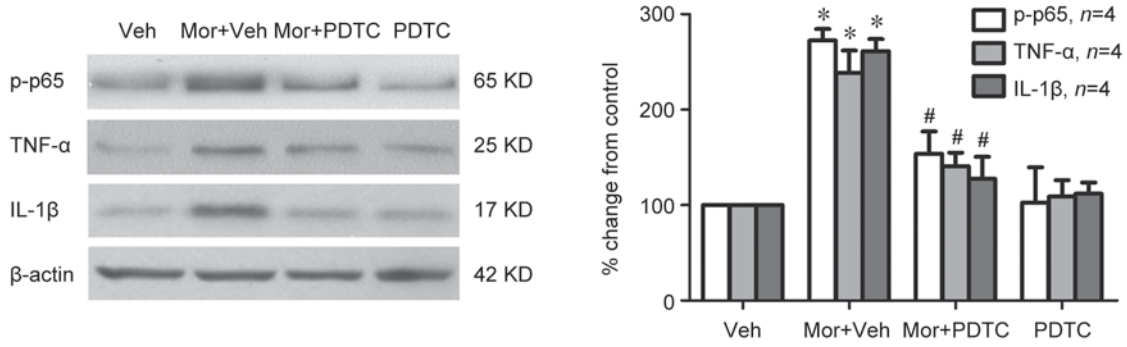

Fig. 7. Intrathecal injection of PDTC prevented the activation of NF-κB and suppressed the expression of TNF-α and IL-1β in the dorsal horn following intrathecal injection of morphine. Left panel: western blots of p-p65, TNF-α, and IL-1β in the vehicle + morphine and PDTC + morphine groups (PDTC 0.5 μg/day i.t. 25 min prior to the morphine injection over 7 days). Right panel: statistical summary of the densitometric analysis. Compared to the vehicle alone group, repeated i.t. injection of morphine + vehicle (saline) induced increases in the expression of p-p65, TNF-α, and IL-1β (*P <0.01). However, in rats that received morphine + PDTC, the activation of NF-κB was blocked, and the expression of TNF-α and IL-1β was inhibited ($^{\#}P$ <0.05 *vs* morphine + vehicle group). PDTC alone did not alter the basal expression of p-p65, TNF-α, or IL-1β. Veh, vehicle (saline).

analgesic effect of morphine[31], but whether it mediates the chronic morphine-induced NF-κB activation in the spinal cord remained unclear. Here, we found that i.t. injection of LPS-RS (20 μg) once daily for 7 days prevented the development of morphine analgesic tolerance and morphine withdrawal-induced abnormal pain. Compared to the morphine + vehicle group, the %MPAE in the morphine + LPS-RS group significantly increased on days 5 to 7 (Fig. 8A), and the paw-withdrawal threshold (Fig. 8B) and latency (Fig. 8C) also increased on day 8, one day after morphine withdrawal. Western blotting showed that i.t. injection of LPS-RS significantly blocked the increase of p-p65 expression induced by morphine in the dorsal horn (Fig. 8D).

DISCUSSION

Previous studies have shown that opioid receptors are

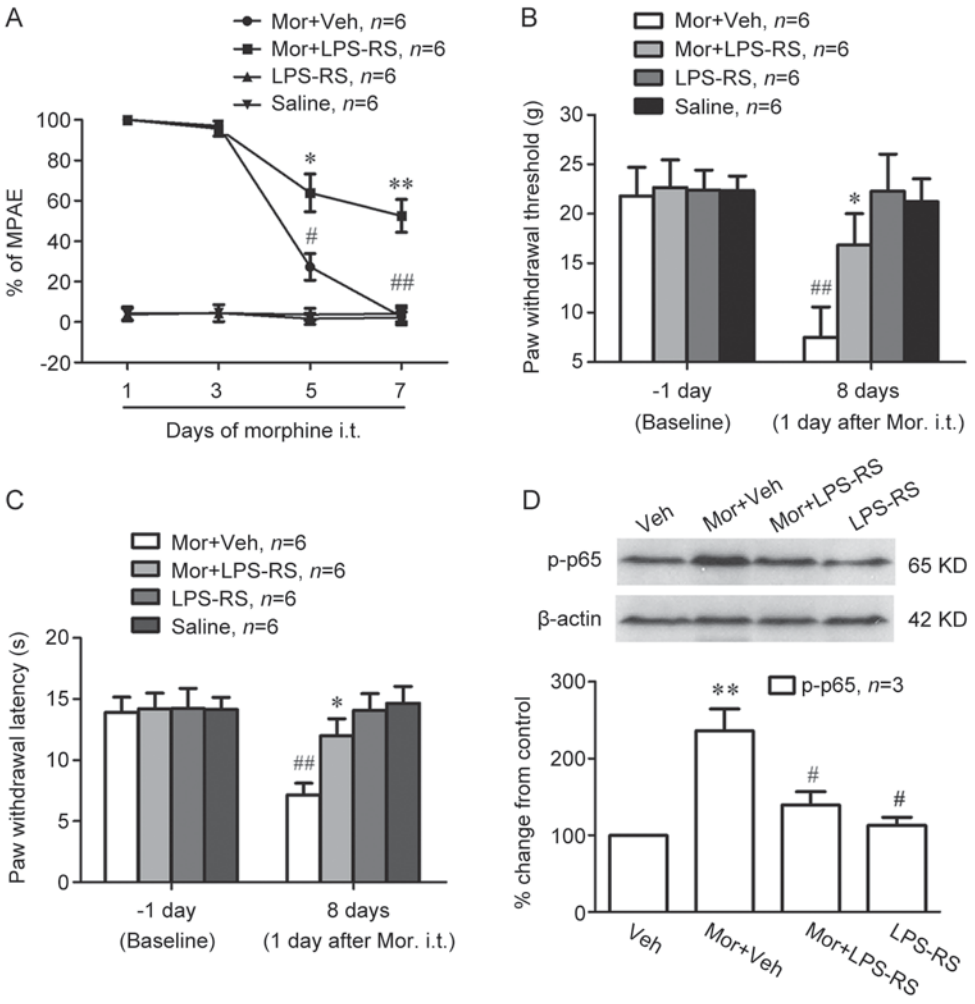

Fig. 8. Intrathecal injection of LPS-RS prevented the development of morphine analgesic tolerance and morphine withdrawal-induced abnormal pain, and blocked the activation of NF-κB in the spinal cord. (A) Effect of intrathecal injection (i.t.) of LPS-RS (20 μg/day 25 min prior to the morphine injection over 7 days), an antagonist of TLR4, on the development of morphine analgesic tolerance (*P <0.05, **P <0.01 *vs* corresponding time point in morphine + vehicle group; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 *vs* %MPAE on day 1). (B, C) Effects of LPS-RS on morphine withdrawal-induced mechanical allodynia (B) and thermal hyperalgesia (C) (*P <0.05 *vs* morphine + vehicle group on day 8; $^{\#\#}P$ <0.01 *vs* baseline value of morphine + vehicle group). (D) Effect of LPS-RS on chronic morphine-induced expression of NF-κB p-p65 in spinal cord (P <0.01 *vs* vehicle and morphine + LPS-RS group; $^{\#}P$ >0.05 *vs* vehicle group). Mor, morphine; Veh, vehicle (PBS).**

expressed in the spinal cord[3,32,33]. After chronic or acute exposure to morphine, activated neurons and glia exhibit increased expression of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6[19,34,35], and chemokines[36,37]. These morphine-induced changes play an essential role in the development and maintenance of morphine analgesic tolerance and morphine withdrawal-induced pain-related hypersensitivity[20]. In the present study, repeated i.t. injection of morphine was used to induce tolerance. Behavioral tests revealed that the rats also developed allodynia and hyperalgesia following morphine withdrawal. In addition, NF-κB was activated in the lumbar spinal dorsal horn after the rats received i.t. morphine injections, and the activated NF-κB co-localized with spinal neurons and astrocytes. The NF-κB activation was reflected by the increased expression of p-p65, as NF-κB p65 requires

phosphorylation prior to its binding to a specific target gene in the nucleus[38,39]. To further investigate the effects of NF-κB activation on the development and maintenance of morphine analgesic tolerance, we examined the effects of pre- and post-administration of PDTC or SN50, potent inhibitors of NF-κB with different mechanisms. PDTC suppresses the release of the inhibitory subunit IκB from the latent cytoplasmic form of NF-κB[40], and thereby indirectly inhibits NF-κB activation. SN50 is a cell-permeable synthetic peptide that is known to inhibit NF-κB activation directly by blocking the translocation of the active NF-κB complex into the nucleus[41]. Our results showed that NF-κB inhibition prevented the development of chronic morphine-induced analgesic tolerance, alleviated morphine withdrawal-induced allodynia and hyperalgesia, and partially reversed the established analgesic tolerance.

It is well known that NF-κB has diverse and complicated effects on the immune and nervous systems[5]. In immune cells, NF-κB activation plays a key role in regulating the expression of TNF-α, IL-1β, IL-6, and other pro-inflammatory cytokines after specific extracellular stimulation[6]. In contrast, TNF-α and IL-1β trigger a classical pathway that involves activation of the IKK complex, which leads to the phosphorylation and subsequent ubiquitination and degradation of IkBa, which in turn allows NF-κB to translocate to the nucleus and promote target gene expression[42]. Notably, NF-κB activation can also be modulated by G-protein-coupled receptors, such as the μ-opioid receptor, through a variety of upstream events including the cAMP/PKA/CREB, PI3K/Akt/IKK, and PLC/PKC/IkK pathways[12,43]. Recent studies have shown that, in cultured astrocytes from mice, exposure to morphine plus HIV Tat is sufficient to activate NF-κB and cytokine production[44]. Based on a number of previous studies showing that NF-κB regulates the expression of TNF-α, IL-1β, and IL-6 in immune cells and investigating the effects of TNF-α and IL-1β on NF-κB activation in glial cells[45], it has been postulated that a positive feedback loop exists between pro-inflammatory cytokine expression and NF-κB activation[11]. Here, we found that chronic i.t. morphine induced NF-κB activation and increased the expression of TNF-α and IL-1β in the rat spinal dorsal horn. Furthermore, the significant increase in the expression of TNF-α occurred prior to the activation of NF-κB. This finding implies that, in addition to direct stimulation by morphine, TNF-α might act as a second pathway to promote the activation of NF-κB after morphine injection. We also found that i.t. injection of PDTC not only led to the inhibition of NF-κB but also suppressed the expression of TNF-α and IL-1β in the spinal cord. These findings indicate that NF-κB activation-mediated morphine analgesic tolerance is facilitated partly by the regulation of TNF-α and IL-1β expression in the dorsal horn. We also found that the morphine-induced NF-κB activation and increased TNF-α expression occurred prior to a significant decrease of morphine analgesic tolerance. Intrathecal injection of a single dose of PDTC on day 7 of morphine injection did not restore the decreased analgesic effect. These results imply that NF-κB activation and TNF-α mediate morphine tolerance in an indirect manner, by triggering the activation of intracellular kinases or gene expression. It is well documented that the promoter regions of the IL-1β, IL-6, and TNF-α genes contain NF-κB binding sites and that initiation of the transcription of these genes is controlled by NF-κB[6]. As activated NF-κB mediates the expression of diverse inflammatory and immune-response mediators, the attenuation of morphine tolerance and morphine withdrawal-induced hyperalgesia after PDTC and SN50 administration may be associated with the inactivation of NF-κB and inhibition of the synthesis of its downstream pro-inflammatory cytokines (IL-1β, TNF-α, and other factors). Recent studies have revealed that TLR4 partially mediates the analgesic effects of acute and chronic morphine in the spinal cord[31]. Intrathecal administration of TLR4-targeted siRNA inhibits the production of IL-1β and TNF-α in the spinal cord and alleviates cancer pain in rats[46]. Here, we found that chronic morphine-induced analgesic tolerance and morphine withdrawal-induced abnormal pain were partially prevented by i.t. injection of LPS-RS, a specific antagonist of TLR4. Meanwhile, the morphine-stimulated activation of NF-κB was also blocked by the LPS-RS administration. This indicates that, besides the μ-opioid receptor, TLR4-mediated NF-κB activation in the spinal cord may partially mediate the effects of chronic morphine-induced analgesic tolerance.

In conclusion, we provide new evidence that TLR4 mediates NF-κB activation in the spinal dorsal horn after chronic morphine exposure, and that the NF-κB activation

contributes to the development and maintenance of morphine analgesic tolerance and morphine withdrawal-induced abnormal pain behaviors in rats. The underlying mechanisms may involve the regulation of pro-inflammatory cytokine (TNF-α and IL-1β) expression in the spinal cord.

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REFERENCES

[1] Bekhit MH. Opioid-induced hyperalgesia and tolerance. Am J Ther 2010, 17: 498–510.

[2] Mao J, Price DD, Mayer DJ. Mechanisms of hyperalgesia and morphine tolerance: a current view of their possible interactions. Pain 1995, 62: 259–274.

[3] Lopez-Gimenez JF, Milligan G. Opioid regulation of mu receptor internalisation: relevance to the development of tolerance and dependence. CNS Neurol Disord Drug Targets 2010, 9: 616–626.

[4] Chen YL, Law PY, Loh HH. Nuclear factor kappaB signaling in opioid functions and receptor gene expression. J Neuroimmune Pharmacol 2006, 1: 270–279.

[5] Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. Cell 2002, 109: S81–96.

[6] Li Q, Verma IM. NF-kappaB regulation in the immune system. Nat Rev Immunol 2002, 2: 725–734.

[7] Mattson MP. Insulating axons via NF-kappaB. Nat Neurosci 2003, 6: 105–106.

[8] Mattson MP. NF-kappaB in the survival and plasticity of neurons. Neurochem Res 2005, 30: 883–893.

[9] Kaltschmidt B, Kaltschmidt C. NF-kappaB in the nervous system. Cold Spring Harb Perspect Biol 2009, 1: a001271.

[10] Fu ES, Zhang YP, Sagen J, Candiotti KA, Morton PD, Liebl DJ, et al. Transgenic inhibition of glial NF-kappa B reduces pain behavior and inflammation after peripheral nerve injury. Pain 2010, 148: 509–518.

[11] Ledeboer A, Gamanos M, Lai W, Martin D, Maier SF, Watkins LR, et al. Involvement of spinal cord nuclear factor kappaB activation in rat models of proinflammatory cytokine-mediated pain facilitation. Eur J Neurosci 2005, 22: 1977–1986.

[12] Niederberger E, Geisslinger G. The IKK-NF-kappaB pathway: a source for novel molecular drug targets in pain therapy? FASEB J 2008, 22: 3432–3442.

[13] Tegeder I, Niederberger E, Schmidt R, Kunz S, Guhring H, Ritzeler O, et al. Specific Inhibition of IkappaB kinase reduces hyperalgesia in inflammatory and neuropathic pain models in rats. J Neurosci 2004, 24: 1637–1645.

[14] Zang Y, He XH, Xin WJ, Pang RP, Wei XH, Zhou LJ, *et al.* Inhibition of NF-kappaB prevents mechanical allodynia induced by spinal ventral root transection and suppresses the re-expression of Nav1.3 in DRG neurons *in vivo* and *in vitro*. Brain Res 2010, 1363: 151–158

[15] Watkins LR, Hutchinson MR, Johnston IN, Maier SF. Glia: novel counter-regulators of opioid analgesia. Trends Neurosci 2005, 28: 661–669.

[16] Wen YR, Tan PH, Cheng JK, Liu YC, Ji RR. Microglia: a promising target for treating neuropathic and postoperative pain, and morphine tolerance. J Formos Med Assoc 2011, 110: 487–494.

[17] Hutchinson MR, Coats BD, Lewis SS, Zhang Y, Sprunger DB, Rezvani N, *et al.* Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. Brain Behav Immun 2008, 22: 1178–1189.

[18] Shen CH, Tsai RY, Wong CS. Role of neuroinflammation in morphine tolerance: effect of tumor necrosis factor-alpha. Acta Anaesthesiol Taiwan 2012, 50: 178–182.

[19] Berta T, Liu T, Liu YC, Xu ZZ, Ji RR. Acute morphine activates satellite glial cells and up-regulates IL-1beta in dorsal root ganglia in mice via matrix metalloprotease-9. Mol Pain 2012, 8: 18.

[20] Johnston IN, Milligan ED, Wieseler-Frank J, Frank MG, Zapata V, Campisi J, *et al.* A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. J Neurosci 2004, 24: 7353–7365.

[21] Wang X, Douglas SD, Commons KG, Pleasure DE, Lai J, Ho C, *et al.* A non-peptide substance P antagonist (CP-96,345) inhibits morphine-induced NF-kappa B promoter activation in human NT2-N neurons. J Neurosci Res 2004, 75: 544–553.

[22] Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983, 16: 109–110.

[23] Storkson RV, Kjorsvik A, Tjolsen A, Hole K. Lumbar catheterization of the spinal subarachnoid space in the rat. J Neurosci Methods 1996, 65: 167–72.

[24] Lim G, Wang S, Zeng Q, Sung B, Yang L, Mao J. Expression of spinal NMDA receptor and PKCgamma after chronic morphine is regulated by spinal glucocorticoid receptor. J Neurosci 2005, 25: 11145–11154.

[25] Li Y, Zhang H, Zhang HM, Kosturakis AK, Jawad AB, Dougherty PM. Toll-Like Receptor 4 Signaling Contributes to Paclitaxel-Induced Peripheral Neuropathy. J Pain 2014, 15: 712–725.

[26] Xu JT, Zhao JY, Zhao X, Ligons D, Tiwari V, Atianjoh FE, *et al.* Opioid receptor-triggered spinal mTORC1 activation

contributes to morphine tolerance and hyperalgesia. J Clin Invest 2014, 124: 592–603.

[27] Xu JT, Xin WJ, Zang Y, Wu CY, Liu XG. The role of tumor necrosis factor-alpha in the neuropathic pain induced by Lumbar 5 ventral root transection in rat. Pain 2006, 123: 306–321.

[28] Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 1994, 53: 55–63.

[29] Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988, 32: 77–88.

[30] Xu JT, Zhao X, Yaster M, Tao YX. Expression and distribution of mTOR, p70S6K, 4E-BP1, and their phosphorylated counterparts in rat dorsal root ganglion and spinal cord dorsal horn. Brain Res 2010, 1336: 46–57.

[31] Hutchinson MR, Zhang Y, Shridhar M, Evans JH, Buchanan MM, Zhao TX, *et al.* Evidence that opioids may have toll-like receptor 4 and MD-2 effects. Brain Behav Immun 2010, 24: 83–95

[32] Hameed H, Hameed M, Christo PJ. The effect of morphine on glial cells as a potential therapeutic target for pharmacological development of analgesic drugs. Curr Pain Headache Rep 2010, 14: 96–104.

[33] Hutchinson MR, Bland ST, Johnson KW, Rice KC, Maier SF, Watkins LR. Opioid-induced glial activation: mechanisms of activation and implications for opioid analgesia, dependence, and reward. ScientificWorldJournal 2007, 7: 98–111.

[34] Raghavendra V, Rutkowski MD, DeLeo JA. The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. J Neurosci 2002, 22: 9980–9989.

[35] Merighi S, Gessi S, Varani K, Fazzi D, Stefanelli A, Borea PA. Morphine mediates a proinflammatory phenotype via mu-opioid receptor-PKCvarepsilon-Akt-ERK1/2 signaling pathway in activated microglial cells. Biochem Pharmacol 2013, 86: 487–496.

[36] Verge GM, Milligan ED, Maier SF, Watkins LR, Naeve GS, Foster AC. Fractalkine (CX3CL1) and fractalkine receptor (CX3CR1) distribution in spinal cord and dorsal root ganglia under basal and neuropathic pain conditions. Eur J Neurosci 2004, 20:1150–1160.

[37] Milligan ED, Sloane EM, Watkins LR. Glia in pathological pain: a role for fractalkine. J Neuroimmunol 2008, 198: 113–120.

[38] Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-kappaB and IkappaB protein. implications in cancer and inflammation. Trends Biochem Sci 2005, 30: 43–52.

[39] Vermeulen L, De WG, Notebaert S, Vanden BW, Haegeman G. Regulation of the transcriptional activity of the nuclear factor-kappaB p65 subunit. Biochem Pharmacol 2002, 64: 963–970.

[40] Schreck R, Meier B, Mannel DN, Droge W, Baeuerle PA. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. J Exp Med 1992, 175: 1181–1194.

[41] Lin YZ, Yao SY, Veach RA, Torgerson TR, Hawiger J. Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J Biol Chem 1995, 270: 14255–14258.

[42] Liou HC. Regulation of the immune system by NF-kappaB and IkappaB. J Biochem Mol Biol 2002, 35: 537–546.

[43] Ye RD. Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. J Leukoc Biol 2001, 70: 839–848.

[44] El-Hage N, Bruce-Keller AJ, Yakovleva T, Bazov I, Bakalkin G, Knapp PE, *et al.* Morphine exacerbates HIV-1 Tat-induced cytokine production in astrocytes through convergent effects on [Ca(2+)](i), NF-kappaB trafficking and transcription. PLoS One 2008, e4093.

[45] Sawaya BE, Deshmane SL, Mukerjee R, Fan S, Khalili K. TNF alpha production in morphine-treated human neural cells is NF-kappaB-dependent. J Neuroimmune Pharmacol 2009, 4:140–149.

[46] Liu S, Liu YP, Song WB, Song XJ. EphrinB-EphB receptor signaling contributes to bone cancer pain via Toll-like receptor and proinflammatory cytokines in rat spinal cord. Pain 2013, 154: 2823–2835.