LIPOXIN A₄ ANALOG ATTENUATES MORPHINE ANTINOCICEPTIVE TOLERANCE, WITHDRAWAL-INDUCED HYPERALGESIA, AND GLIAL REACTION AND CYTOKINE EXPRESSION IN THE SPINAL CORD OF RAT


Department of Anesthesiology, Kunhua Hospital, The First People's Hospital of Yunnan Province, Kunming 650032, PR China
Department of Oral Medicine, Kunhua Hospital, The First People's Hospital of Yunnan Province, Kunming 650032, PR China

Abstract—Spinal neuroinflammation has been shown to play an important role in the development of morphine tolerance and morphine withdrawal-induced hyperalgesia. Lipoxins are endogenous lipoxigenase-derived eicosanoids that can function as "braking signals" in inflammation. The present study investigated the effect of 5 (S), 6 (R)-lipoxin A₄ methyl ester (LXA₄ME), a stable synthetic analog of lipoxin A₄, on the expression of antinociceptive tolerance and withdrawal-induced hyperalgesia in chronic morphine-treated rats. Chronic morphine administration through repeated subcutaneous injection induced the development of hyperalgesia and the expression of spinal antinociceptive tolerance to morphine. However, LXA₄ME treatment significantly attenuated the development of hyperalgesia and the expression of spinal antinociceptive tolerance to intrathecal morphine in both mechanical and thermal test. Moreover, the administration of LXA₄ME during the induction of morphine tolerance inhibited the activation of microglia and astrocytes; reduced the expression of proinflammatory cytokines interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α); upregulated the expression of anti-inflammatory cytokines IL-10 and transforming growth factor-β1 (TGF-β1); and inhibited nuclear factor-kappa B (NF-κB) activation at the L5 lumbar spinal cord. These results suggest that treatment of LXA₄ME provides a potential preventative or therapeutic approach for morphine tolerance and associated abnormal pain sensitivity. © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: lipoxin A₄, morphine, inflammation, tolerance.

Morphine is a potent opioid analgesic that is widely used for clinical pain management. However, repeated administration of morphine leads to tolerance and hyperalgesia, which limit its use as an antinociceptive agent. Although extensive re-search efforts in the area of morphine tolerance and morphine withdrawal-induced hyperalgesia have been made, the underlying mechanisms involved in these phenomena remain largely unknown. Recent evidence suggests that glial responses are involved in the development of morphine tolerance and morphine withdrawal-induced hyperalgesia (Song and Zhao, 2001; Raghavendra et al., 2002). Glial (astrocyte and microglia) activation, enhanced proinflammatory cytokines, and decreased anti-inflammatory cytokines were observed after chronic morphine treatment at the lumbar spinal cord of rats (Raghavendra et al., 2002; Horvath et al., 2010; Ramos et al., 2010). Inhibition of glial (astrocyte and/or microglia) activation or antagonizing the activity of proinflammatory cytokines (interleukin [IL]-1β, IL-6, and tumor necrosis factor [TNF]-α) reduced the expression of morphine tolerance and withdrawal-induced hyperalgesia in rats (Song and Zhao, 2001; Raghavendra et al., 2002; Johnston et al., 2004). Intrathecal IL-10 gene therapy can reduce hyperalgesia and allodynia in diverse pain facilitation states (Milligan et al., 2005).

Lipoxins (LXs) are groups of endogenous anti-inflammatory lipid-based autacoids that could serve as "braking signals" in inflammation (Morris et al., 2006). Lipoxin A₄ (LXA₄) is one of the major physiological LX form generated by mammalian cells. LXA₄ exerts biological functions (including anti-inflammatory and antioxidant activities) through binding with high affinity to a G protein-coupled receptor (GPCR) termed LXA₄ receptor (ALXR), also known as formyl peptide receptor 2 (FPR2) (Chiang et al., 2005, 2006; Mitchell et al., 2007). LXA₄ also acts as a partial agonist to exert biological activities via interacting with cysteinyl leukotriene receptor 1 and 2 (Norel and Brink, 2004; Chiang et al., 2006). Furthermore, LXA₄ can also interact with nuclear receptor aryl hydrocarbon receptor, which triggers expression of suppressor of cytokine signaling 2 (Schaldach et al., 1999). LXA₄ has been shown to protect against various inflammatory disorders such as asthma, arthritis, pain, and ischemia (Svensson et al., 2007; Petasis et al., 2008; Ye et al., 2010; Sun et al., 2012b). Considering that LXA₄ is biosynthesized and rapidly enzymatically inactivated, 5 (S), 6 (R)-lipoxin A₄ methyl ester (LXA₄ME), a stable and more potent analog of LXA₄, was constructed (Serhan et al., 1995; Chiang et al., 2000). LXA₄ME depresses the activation of astrocytes and microglia, which are associated with neuronal damage during ischemia injury (Ye et al., 2010). It has been demonstrated that ALXR was expressed by spinal astrocytes and that spinal delivery of LXA₄ and its stable analogs attenuate inflamma-
tion-evoked hyperalgesia and edema (Svensson et al., 2007). A number of inflammatory mediators (i.e. IL-1β, IL-6, TNF-α, IL-10, transforming growth factor-β1 [TGF-β1], and nuclear factor-kappa B [NF-κB]) regulated by LXA4 are involved in the modulation of morphine tolerance (Pouliot and Serhan, 1999; Jozsef et al., 2002; Wu et al., 2008). Hence, the present study was designed to evaluate the possible beneficial effects of LXA4ME in the expression of morphine tolerance and morphine withdrawal-induced hyperalgesia in rats.

**EXPERIMENTAL PROCEDURES**

Chemicals

LXA4ME was obtained from Cayman Chemical (Ann Arbor, MI, USA). Antibodies against β-actin, Lamin B, and NF-κB (p65) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-macrophage antigen complex-1 (Mac-1) antibody was from Novus Biologicals (Littleten, CO, USA). Anti-glial fibrillary acidic protein (GFAP) antibody was purchased from Millipore (Billerica, MA, USA).

Animal preparation and intrathecal catheter implantation

Male Sprague-Dawley rats (Kunming Medical College, Kunming, PR China) weighing 250–300 g were used in the experiments. The rats were allowed to habituate to the housing facilities for 1 week before the experiments began. Intrathecal catheters were implanted as described by Yaksh and Rudy (Yaksh and Rudy, 1976). The rats were anesthetized with phenobarbital (50 mg/kg, i.p.). The occipital muscles were bluntly separated, and the cisterna membrane exposed. Intrathecal (i.t.) polyethylene catheters (PE-10) were inserted via an incision in the cisterna magna and advanced caudally to the lumbar enlargement of the spinal cord. The incision site was closed in layers, and the catheter was fixed firmly under the skin and sealed effectively. After the surgery, all rats were returned to home cages for recovery and housed individually and maintained on a 12-h light–dark cycle, with food and water freely available. Rats with neurological deficit or infection were excluded from experiments. All procedures were performed in accordance with guidelines set for the use of experimental animals by the local Committee on Animal Care and Use to minimize the number of animals used and their suffering.

Behavioral tests

The antinociceptive responses were determined by mechanical (Analgesy-Meter) and thermal (tail-flick) test paradigms (RWD Life Science Co., Ltd, Shenzhen, China). Mechanical nociceptive thresholds were evaluated as described by Stein et al. (Stein et al., 1990). Rats were gently held, and increasing pressure (maximum 250 g) was applied onto the dorsal surface of the ipsilateral hind paw. The paw pressure thresholds (PPT), the pressure required to elicit paw withdrawal, were determined. Thermal nociceptive thresholds were determined by the hot-water tail-flick test. The tails of rats were immersed into water (49 ± 0.5 °C), and the latency to a rapid flick was recorded. The morphine-induced antinociceptive response in mechanical and thermal tests was expressed as the % maximum possible effect (%MPE) using the formula (Raghavendra et al., 2004): 

\[
%\text{MPE} = \left( \frac{\text{WT} - \text{CT}}{\text{CO} - \text{CT}} \right) \times 100
\]

where WT = withdrawal latency (s) or threshold (g) after morphine/saline treatment, CT = latency before morphine/saline treatment, and CO = the cut-off value (i.e. 250 g for mechanical test and 15 s for the tail-flick test). All behavioral tests were blinded with respect to the groups.

Experimental design

Intrathecal drug administration was accomplished using microinjection syringe connected to the i.t. catheter in awake rats. Rats (n=100) received either saline or morphine (10 mg/kg, s.c., twice daily for 5 days) and were treated once daily (11 AM) with LXA4ME (0.1 and 1 μg/kg) or saline via i.t. catheter during induction of morphine tolerance. Chronic morphine withdrawal-induced hyperalgesia and allodynia in rats were examined 16 h after the last injection of s.c. morphine. Animals were treated with morphine (5 μg) acutely via i.t. catheter to study the expression of morphine tolerance after the recording of morphine withdrawal-induced hyperalgesia and allodynia. Acute antinociceptive activity of i.t. administered morphine in these rats was evaluated by Analgesy-Meter and tail-flick test. Behavior recorded before the acute administration of i.t. morphine served as the basal latency.

Spinal cord sample preparation

After behavioral testing, the heart perfusion of mice was performed by using saline under isoflurane anesthesia. Laminectomy was performed from the lower edge of the 12th thoracic vertebrae to sacral vertebrae, and then the L5 lumbar part of spinal cord was collected and frozen immediately in liquid nitrogen and stored at −80 °C until further study.

Quantitative real-time PCR

Total RNA was isolated from the L5 lumbar spinal cord using the TRIzol extraction method (CWBio Corporation, Beijing, PR China). The reverse transcription (RT) was carried out using the ReverTra Aid First Strand cDNA Synthesis Kit (Fermentas, Waldorf Baden, Germany). Real-time PCR analysis was performed on a Prism 7300 Sequence Detection System (Applied Biosystems, Foster, CA, USA). The gene-specific sequences of the primer pairs and probes used in the assays have been described previously (Raghavendra et al., 2004): GFAP, forward primer: 5′-TG-GCCACCCATTAACATGCAA-3′; reverse primer: 5′-CAGTTT-GGCGGGATAGTCA-3′; probe: 5′-CAAGCTTTCCCTCCG-GAAGCCG-3′; Mac-1, forward primer: 5′-CTGCCCTACGGA-TCCGTAAG-3′; reverse primer: 5′-CCTCTGCTCCAGGATT-GACATC-3′; probe: 5′-CCCCGGGACAATGCGCGAA-3′; GAPDH, forward primer: 5′-CCCCCAATGTATCCGTTGTG-3′; reverse primer: 5′-TAGCCCGGATAGTCCCCGCTT-3′; probe: 5′-TGCGCGCTTGGGAACTCGGC-3′.

Enzyme-linked immunosorbent assay (ELISA)

The L5 lumbar spinal cord was collected after behavioral testing. The tissue was pooled and homogenized in homogenization buffer (phosphate-buffered saline, pH 7.4, containing 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotonin, and 1 μg/ml leupeptin). Samples were spun at 15,000 g for 30 min at 4 °C. The supernatant was aliquoted and stored at −80 °C for future protein quantification. The concentrations of TNF-α (BioSource, Europe, SA), IL-1β (BioSource International, Inc., Camarillo, CA, USA), IL-6, IL-10, and TGF-β1 (Boster Biological Technology, Wuhan, China) were measured in the L5 lumbar spinal cord using specific ELISA kits according to the manufacturers’ instructions.

Western blot

The L5 lumbar spinal cord samples were homogenized in ice-cold solubilizing solution (20 mM Tris–HCl [pH 7], 25 mM β-glycerophosphate, 2 mM EGTA, 1% Triton X-100, 1 mM vanadate, 1 μg/ml aprotonin, 1 mM phenylmethylsulfon fluoride, 2 mM dithiothreitol) on ice for 40 min. The lysate was centrifuged at 15,000 rpm for 15 min. Supernatants were collected. Nuclear protein extracts were prepared according to the instructions provided with the NE-PER Nuclear and cytoplasmic Extraction Reagents kit (Pierce Biotech-
nology, Rockford, IL, USA). Protein concentrations were determined using Bio-Rad protein assay reagent (Hercules, CA, USA). An equal quantity of proteins was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membranes were incubated with GFAP, Mac-1, β-actin, Lamin B, and NF-κB p65 antibodies, washed and then incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG (KPL, Gaithersburg, MD, USA). The immunoblot was revealed with an ECL Western blot detection kit (Amersham Pharmacia Biotech, Buckinghamshire, England). Densitometry analysis was performed using ImageJ software.

Statistical analysis

All values are expressed as means±SD. Data were analyzed by ANOVA followed by Tukey–Kramer multiple comparisons test using SPSS software. Differences were considered statistically significant at a level of \( P<0.05 \).

RESULTS

LXA4ME attenuated the development of morphine withdrawal-induced hyperalgesia

As shown in Fig. 1, chronic morphine treatment (10 mg/kg, twice daily for 5 days, s.c.) induced withdrawal-induced thermal and mechanical hyperalgesia when recorded 16 h after the last injection. Similarly, chronic morphine treatment also developed mechanical allodynia to both 2 and 12 g of mechanical stimuli (Fig. 1). Chronic LXA4ME treatment (0.1 and 1 μg/kg i.t. for 5 days) had no effect on nociceptive threshold in the saline-treated rats. However, LXA4ME administration during the induction of morphine tolerance significantly attenuated morphine withdrawal-induced thermal and mechanical hyperalgesia (Fig. 1).

LXA4ME attenuated the expression of spinal antinoceptive tolerance to morphine

As shown in Fig. 2, antinoceptive tolerance to acute i.t. morphine (5 μg) developed in rats injected with saline/morphine (10 mg/kg, twice daily for 5 days, s.c.). Rats injected with LXA4ME (0.1 and 1 μg/kg i.t. for 5 days)/saline had no effect on the antinoceptive action of acute i.t. morphine (5 μg). However, LXA4ME treatment during the induction period of morphine tolerance (10 mg/kg, twice daily for 5 days, s.c.) significantly attenuated the expression of spinal antinoceptive tolerance to i.t. morphine (5 μg) in both mechanical and thermal test (Fig. 2).

LXA4ME repressed glial activation in morphine-tolerant rat spinal cords

To determine the effect of LXA4ME on spinal neuroinflammation in morphine tolerance, rats were killed for the real-time RT-PCR and Western blot assay. In the present study, we found that chronic administration of morphine (10 mg/kg, twice daily for 5 days) significantly increased the expression of mRNA and protein levels of GFAP and Mac-1 at L5 lumbar spinal cord compared with saline-treated rats (Figs. 3 and 4). LXA4ME (0.1 and 1 μg/kg i.t. for 5 days) had no effect on the expression of GFAP and Mac-1 in saline-treated rats. In contrast, LXA4ME treat-
In the present study, by using ELISA, we also observed significant increases in the protein levels of IL-1β, IL-6, and TNF-α at L5 lumbar spinal cord of chronic morphine-treated (10 mg/kg, twice daily for 5 days) rats compared with saline-treated rats (Fig. 5).

LXA₄ME (0.1 and 1 μg/kg i.t. for 5 days) had no effect on the expression of IL-1β, IL-6, and TNF-α in saline-treated rats (Fig. 5). However, LXA₄ME treatment during the induction of morphine tolerance significantly attenuated morphine-induced upregulation of IL-1β, IL-6, and TNF-α (Fig. 5).

LXA₄ME upregulated IL-10 and TGF-β1 expression in rat spinal cords

As shown in Fig. 6, chronic administration of morphine (10 mg/kg, twice daily for 5 days) significantly decreased the expression of IL-10 and TGF-β1 at L5 lumbar spinal cord compared with saline-treated rats. LXA₄ME (0.1 and 1 μg/kg i.t. for 5 days) had no effect on the expression of IL-10 and TGF-β1 in saline-treated rats (Fig. 6). However, LXA₄ME treatment during the induction of morphine tolerance significantly attenuated morphine-induced downregulation of IL-10 and TGF-β1 expression (Fig. 6).

LXA₄ME inhibited NF-κB activation in the rat spinal cords

As shown in Fig. 7, NF-κB activity of the nuclear extracts of L5 lumbar spinal cord was measured by Western blot. NF-κB activity was induced in chronic morphine-treated (10 mg/kg, twice daily for 5 days) rats. However, LXA₄ME treatment during the induction of morphine tolerance significantly attenuated morphine-induced downregulation of NF-κB activity (Fig. 7).
Significantly attenuated morphine-induced upregulation of NF-κB activity in a dose-dependent manner.

**DISCUSSION**

The repeated administration of morphine is associated with significant problems, including the development of antinociceptive tolerance and hyperalgesia (Kamei et al., 2011). Several opioid and nonopioid systems are involved in the morphine tolerance and morphine withdrawal-induced hyperalgesia (Gregus et al., 2010). Recent studies showed that glial activation and subsequent immune responses at the lumbar spinal cord contribute to the development of morphine tolerance and morphine withdrawal-
induced hyperalgesia (Song and Zhao, 2001; Raghavendra et al., 2002; Johnston et al., 2004). Lipoxin A4 has anti-inflammatory actions \textit{in vivo} when administered to the site of inflammation or systemically by the i.v. or oral route (Serhan, 2005). The present study demonstrates that chronic administration of LXA4ME, a stable synthetic analog of LXA4, during the induction of morphine tolerance attenuated both: (a) the development of morphine withdrawal-induced hyperalgesia and allodynia and (b) the expression of antinociceptive tolerance to morphine and associated inflammatory responses at the lumbar spinal cord.

Lipid mediators, such as LXs, resolvins, and protectins, are chemical messengers that regulate inflammation, oxidative stress, and apoptosis (Bonnans and Levy, 2007; Farooqui, 2009; Spite and Serhan, 2010). Recent studies have shown that lipid mediators are involved in pain processing (Farooqui, 2009; Ren and Dubner, 2010; Xu and Ji, 2011). Intraplantar or i.t. administration of resolvin E1 or D1 (0.3–20 ng) reduced inflammatory pain behaviors (Xu et al., 2010). It also has been reported that i.t. administration of resolvin D1 (20–40 ng) prevents against postoperative pain (Huang et al., 2011). As for LXs, it has been shown that LXA4 and aspirin-triggered LX analog (0.3 nmol) inhibited inflammatory pain processing (Svensson et al., 2007). Intrathecal administration of LXA4 (10–100 ng) alleviated hyperalgesia and allodynia in chronic compression of dorsal root ganglia (Sun et al., 2012b). There is same efficacy between LXs and resolvins in chronic compression of dorsal root ganglia (Sun et al., 2012b). The dose of LXA4ME in the present study is about two times that of previous studies. However, it did not show toxic effects at the salutary doses used in the present study. Whether other lipid mediators, such as resolvins, have the inhibitory effects on the development of morphine tolerance is still unknown. It needs further study. Furthermore, further study would be necessary to determine optimal dose and therapeutic time of LXA4ME for prevention against morphine tolerance.

**Fig. 6.** The expression of anti-inflammatory cytokines at the L5 lumbar spinal cord after chronic morphine treatment and upregulation by LXA4ME. Rats received chronic saline (S), chronic LXA4ME (1 μg/kg) (L10), chronic morphine (MO), chronic morphine plus LXA4ME (0.1 μg/kg) (MO+L1), and chronic morphine plus LXA4ME (1 μg/kg) (MO+L10) for five consecutive days. On day 6, L5 lumbar spinal cord was harvested after behavioral test. The content of IL-10 and TGF-β1 at L5 lumbar spinal cord was measured by ELISA respectively. Values are mean±SEM. * $P<0.05$ vs. chronic saline (S) and # $P<0.05$ vs. chronic morphine-treated group (MO) ($n=5$ group).

**Fig. 7.** LXA4ME inhibited NF-κB activation induced by chronic morphine treatment. (A) Rats received chronic saline (S), chronic LXA4ME (1 μg/kg) (L10), chronic morphine (MO), chronic morphine plus LXA4ME (0.1 μg/kg) (MO+L1), and chronic morphine plus LXA4ME (1 μg/kg) (MO+L10) for five consecutive days. On day 6, L5 lumbar spinal cord was harvested after behavioral test. Nuclear extracts were prepared from the tissues of the L5 lumbar spinal cord for Western blotting with NF-κB(p65) antibody. (B) Densitometric quantifications from representative immunoblots of NF-κB(p65), normalized with Lamin B. Values are mean±SEM. * $P<0.05$ vs. chronic saline (S) and # $P<0.05$ vs. chronic morphine-treated group (MO) ($n=5$ group).
Studies showed that morphine activates spinal glial activity, enhances production of TNF-\(\alpha\) and nitric oxide, and inhibits microglial chemotaxis (Chao et al., 1994, 1997; Magazine et al., 1996; Song and Zhao, 2001). In addition, morphine tolerance and its withdrawal-induced hyperalgesia were associated with spinal microglial and astroglial activation (Raghavendra et al., 2002). The inhibition of spinal glial activation by propentofylline or fluorocitrate reversed the development of morphine tolerance in rats (Song and Zhao, 2001; Raghavendra et al., 2004). In accordance with the findings mentioned previously, our research showed that i.t. LXA\(_4\)ME significantly inhibited the expression of spinal antinociceptive tolerance to morphine and withdrawal-induced hyperalgesia. This prevention was correlated with the anti-inflammatory effects of LXA\(_4\)ME, which is based on the observation that LXA\(_4\)ME suppresses morphine-induced spinal glial activation.

Inflammatory cytokines play an important role in inflammatory response in morphine tolerance and its withdrawal-induced hyperalgesia. The chronic administration of morphine leads to upregulation of proinflammatory cytokines IL-1\(\beta\), IL-6, and TNF-\(\alpha\) (Raghavendra et al., 2004; Tai et al., 2006). It has been reported that the administration of IL-1\(\beta\), IL-6, or TNF-\(\alpha\) induces hyperalgesia and allodynia in rodents (Minami et al., 2006). Among various proinflammatory cytokines, TNF-\(\alpha\) is considered as the trigger for activation of cytokines. Spinal delivery of TNF-\(\alpha\) increased the allodynia and hyperalgesia and blockade of TNF-\(\alpha\) had the opposite effect (Svensson et al., 2005; Zelenka et al., 2005; Hao et al., 2007; Sasaki et al., 2007; Youn et al., 2008). Etanercept, the TNF-\(\alpha\) antagonist, restored the antinociceptive effect of morphine in morphine-tolerant rats (Shen et al., 2011). In addition, herpes simplex virus vector-based gene transfer of tumor necrosis factor soluble receptor enhanced the antinociceptive effect of acute morphine and delayed the development of chronic morphine tolerance in rats (Sun et al., 2012a). IL-1\(\beta\) and IL-6 are also potent proinflammatory cytokines involved in morphine tolerance. Intrathecal administration of either IL-1\(\beta\) receptor antagonist or neutralizing anti-rat IL-6 antibodies significantly decreased mechanical allodynia in neuropathic pain models of rats (Arruda et al., 2000; Sweitzer et al., 2001). The selective inhibition of IL-1\(\beta\) or collective inhibition of proinflammatory cytokines attenuated the development of morphine tolerance and its withdrawal-induced hyperalgesia (Raghavendra et al., 2002; Johnston et al., 2004). Accordingly, a theory that blockade of proinflammatory cytokine expressions may have an important role in the preservation of morphine tolerance and its withdrawal-induced hyperalgesia has been presented. LXA\(_4\) and its analog have a potent ability to inhibit the production of proinflammatory cytokines and chemokines via the inhibition of transcription factors such as NF-\(\kappa\)B and activator protein 1 (Gewirtz et al., 2002; Jozset et al., 2002; Sodin-Semrl et al., 2004; Wu et al., 2005, 2008; Bonnans et al., 2007). The expressions of IL-1\(\beta\) and IL-6 were decreased by LXA\(_4\) in cultured pulmonary microvascular endothelial cells or glomeruli of nephritic rats (Wu et al., 2007, 2008). LXA\(_4\) suppressed TNF-\(\alpha\) expression in a murine model of zymosan-induced arthritis (Conte et al., 2010). In the present study, we found that LXA\(_4\)ME significantly inhibited the upregulation of these proinflammatory cytokines (IL-1\(\beta\), IL-6, and TNF-\(\alpha\)) induced by repeated morphine administration. These results further supported the theory mentioned previously and probably indicated that i.t. delivery of LXA\(_4\)ME could attenuate the expression of spinal antinociceptive tolerance to morphine and withdrawal-induced hyperalgesia through its anti-inflammatory and pro-resolving properties.

Several studies have shown that proinflammatory cytokines could influence the development of antinociceptive tolerance and hyperalgesia through regulating spinal cord synaptic transmission and activity of NMDA and AMPA receptors (Meller et al., 1996; Guo et al., 2007; Chen et al., 2010). IL-1\(\beta\), IL-6, and TNF-\(\alpha\) could enhance excitatory synaptic transmission and potentiate NMDA- and AMPA-induced currents in the spinal cord (Kawasaki et al., 2008; Schafer and Sorkin, 2008; Ren and Dubner, 2010). Spinal chemokine CCL2 led to thermal hyperalgesia by sensitizing NMDA and AMPA receptors through activating glial responses and releasing IL-1\(\beta\) (Baamonde et al., 2011). It has also been reported that the activation of AMPA and/or NMDA receptor is correlated with morphine tolerance and hyperalgesia (Guo et al., 2009). In this study, LXA\(_4\)ME reduced levels of IL-1\(\beta\), IL-6, and TNF-\(\alpha\) in the spinal cords. However, whether LXA\(_4\)ME could regulate spinal cord synaptic transmission and activity of NMDA and AMPA receptors needs further study.

The outcome of morphine-induced inflammatory responses mainly depends on the imbalance between the activation of proinflammatory cytokines cascade and the induction of anti-inflammatory cytokines. Anti-inflammatory cytokines have been shown to prevent hyperalgesic responses in a wide range of exaggerated pain states (Kaana et al., 1998; Brewer et al., 1999; Laughlin et al., 2000). Expression of anti-inflammatory cytokines IL-10 and TGF-\(\beta\)1 was decreased after chronic morphine treatment (Pacifici et al., 2000; Messmer et al., 2006). Previous reports have shown that both exogenous administration and gene transfer of IL-10 potentiated acute morphine analgesia and attenuated the development of tolerance, hyperalgesia, and allodynia (Johnston et al., 2004; Lin et al., 2010). Moreover, the inhibitory effects of IL-10 and TGF-\(\beta\)1 on proinflammatory cytokines production have already been reported in a number of inflammatory models, including asthma, pancreatitis, hepatitis, and ischemia (Moore et al., 2001; Yoshimura et al., 2010). It has been reported that LXA\(_4\)ME protected brain and reduced inflammation in a rat model of focal cerebral ischemia reperfusion through upregulating the expression of IL-10 and TGF-\(\beta\)1 in ischemic cortex (Ye et al., 2010). In the present study, LXA\(_4\)ME upregulates the expression of IL-10 and TGF-\(\beta\)1 at L5 lumbar spinal cord, which indicates that LXA\(_4\)ME attenuated morphine tolerance partly through these anti-inflammatory cytokines.

Nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) is a critical transcription factor in inflammatory processes and autoimmune diseases. NF-\(\kappa\)B released from I\(\kappa\)B translocates into nucleus, where
it enhanced the transcription of cytokines. The pivotal role of activation of NF-κB in inflammation during the induction of morphine tolerance has been well elucidated. It is reported that i.t. injection of antisense oligonucleotides to p65 subunit of NF-κB significantly alleviated hyperalgesia and allodynia in CCI models of rats (Sun et al., 2006). The inhibition of NF-κB activity attenuated the development of morphine antinociceptive tolerance (Wang et al., 2010). In the present study, chronic morphine treatment caused magnitude of NF-κB activation, which ran parallel with a marked increase in IL-1β, IL-6, and TNF-α. However, NF-κB activation at L5 lumbar spinal cord was suppressed by LXA4ME treatment followed by the decrease of IL-1β, IL-6, and TNF-α. These observations were in concordance with reports that LXA4 activated ALXR and inhibited NF-κB activity in epithelial cells and human leukocytes (Jozsef et al., 2002). Intrathecal LXA4 induced antiinociception and decreased expression of NF-κB and proinflammatory cytokines after chronic dorsal root ganglia compression in rats (Sun et al., 2012b). Proinflammatory cytokines, including IL-1β, IL-6, and TNF-α were proposed to be involved in the development of tolerance and hyperalgesia after chronic morphine (Raghavendra et al., 2004; Shen et al., 2011). Previous studies have shown that the promoter region of IL-1β, IL-6, and TNF-α gene contains NF-κB binding sites and is controlled by NF-κB to initiate gene transcription (Li and Verma, 2002, Liou, 2002). 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 LXA4ME administration is possibly associated with the inactivation of NF-κB and inhibition of its downstream proinflammatory cytokines (IL-1β, IL-6, TNF-α, and other factors).

It has been reported that aspirin-triggered LXA4 attenuates LPS-induced production of NO, IL-1β, and TNF-α by inhibiting activation of NF-κB and MAPKs in BV-2 microglial cells (Wang et al., 2011). LPS is the classical agonist of toll-like receptor 4 (TLR4). These results suggest that there is an interaction between LXA4 and TLR4 cascade. Recent studies also have shown that morphine induces spinal proinflammatory cytokines through activating TLR4 signaling (Hutchinson et al., 2008a,b). Pharmacological blockade of TLR4 signaling in vivo attenuates development of antinociceptive tolerance and hyperalgesia (Hutchinson et al., 2010). In the present study, we found that LXA4ME reduced morphine-induced expression of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) in the spinal cords. These results suggest that the inhibitory effects of LXA4ME on morphine antinociceptive tolerance and withdrawal-induced hyperalgesia are not by interacting with the downstream signaling of opioid receptors, but rather by interacting with the TLR4 cascade.

CONCLUSION

In conclusion, LXA4ME has a profound effect on attenuating the development of antinociceptive tolerance and hyperalgesia following chronic administration of morphine. The underlying mechanism involved inhibiting NF-κB activation, upregulating the expression of anti-inflammatory cytokines (IL-10 and TGF-β1), and reducing the expression of proinflammatory cytokines (IL-1β, IL-6, and TNF-α). Accordingly, these results provide insights into a novel lipid cascade regulating morphine tolerance and morphine withdrawal-induced hyperalgesia.

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