INVOLVEMENT OF THE SPINAL NALP1 INFLAMMASOME IN NEUROPATHIC PAIN AND ASPIRIN-TRIGGERED-15-EPI-LIPOXIN A4 INDUCED ANALGESIA


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Abstract—Neuroinflammation plays an important role in nerve-injury-induced neuropathic pain, but the explicit molecular mechanisms of neuroinflammation in neuropathic pain remain unclear. As one of the most critical inflammatory cytokines, interleukin-1β (IL-1β) has been regarded as broadly involved in the pathology of neuropathic pain. The inflammasome caspase-1 platform is one primary mechanism responsible for the maturation of IL-1β. Lipoxins, a type of endogenous anti-inflammatory lipid, have proved to be effective in relieving neuropathic pain behaviors. The present study was designed to examine whether the inflammasome caspase-1 IL-1β platform is involved in chronic constriction injury (CCI)-induced neuropathic pain and in lipoxin-induced analgesia. After rats were subjected to the CCI surgery, mature IL-1β was significantly increased in the ipsilateral spinal cord, and the inflammasome platform consisting of NALP1 inflammasome, IL-1β maturation, and IL-1β signaling had been demonstrated in the CNS induced obvious pain behaviors (Sung et al., 2012). Further, blockade of IL-1 signaling has been shown to relieve nerve-injury-induced neuropathic pain (Kleibeuker et al., 2008). All these reports suggest a crucial role for IL-1β in the development of neuropathic pain, but the mechanisms underlying IL-1β production had not been clearly recognized, particularly in the CNS.

IL-1β is synthesized as an inactive cytoplasmic precursor and cleaved into the biologically active form by certain cysteine proteases in response to proinflammatory stimuli (Thornberry et al., 1992). The inflammasome caspase-1 platform is one of the primary pathways regulating the maturation of IL-1β (Martinon et al., 2002). Inflammasomes are groups of protein complexes that recognize diverse sets of inflammation-inducing stimuli such as pathogenic infection or tissue damage (Strowig et al., 2012). Recently, a newly discovered inflammasome family, the NACHT leucine-rich-repeat protein (NALP) inflammasome, composed of the NALP protein, caspase-1 inhibited NALP1 inflammasome activation, caspase-1 cleavage, and IL-1β maturation. These results suggested that spinal NALP1 inflammasome was involved in the CCI-induced neuropathic pain and that the analgesic effect of ATL was associated with suppressing NALP1 inflammasome activation.

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Key words: NALP1 inflammasome, interleukin, neuropathic pain, aspirin-triggered-15-epi-lipoxin A4, caspase.

INTRODUCTION

Chronic neuropathic pain presents as a common disease severely disrupting the patients’ quality of life (McCarberg and Billington, 2006). Although it is a complicated condition, neuroinflammation, including glial activation and the production of proinflammatory cytokines, has been widely regarded as one important mechanism of neuropathic pain (Myers et al., 2006). The proinflammatory cytokine interleukin-1β (IL-1β) has been shown to actively participate in the pathogenesis of neuropathic pain and to induce a secondary injury cascade during the course of disease (Thacker et al., 2007). Clinically, elevated IL-1β was observed in the cerebrospinal fluid (CSF) of patients with complex regional pain syndrome (Alexander et al., 2005), and the same pathology was observed in the spinal cord of rats with neuropathic pain (Amin et al., 2012). Previous studies had demonstrated that exogenous administration of IL-1β in the CNS induced obvious pain behaviors (Sung et al., 2012). Further, blockade of IL-1 signaling has been shown to relieve nerve-injury-induced neuropathic pain (Kleibeuker et al., 2008). All these reports support a crucial role for IL-1β in the development of neuropathic pain, but the mechanisms underlying IL-1β production had not been clearly recognized, particularly in the CNS.

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Abbreviations: ALX, lipoxin A4 receptor; ASC, apoptosis-associated speck-like protein containing a caspase-activating recruitment domain; ATL, aspirin-triggered-15-epi-lipoxin A4; Boc2, butoxycarbonyl-Phe-Leu-Phe-Leu-Phe; CARD, caspase-activating recruitment domain; CCI, chronic constriction injury; CSF, cerebrospinal fluid; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; i.t., intrathecal; IgG, immunoglobulin G; IL, interleukin; MMP, metalloproteinase; NALP, NAcht leucine-rich-repeat protein; NeuN, Neuron-specific Nuclear Protein; PBS, phosphate-buffered solution; PWL, paw withdrawal latency ; SEM, standard error; TBST, tris-buffered saline with tween.
and the adaptor protein apoptosis-associated speck-like protein containing a caspase-activating recruitment domain (CARD) (ASC) was first found in humans and then implicated in multiple neuroinflammation-related disorders (Tschopp et al., 2003). Notably, studies have demonstrated that the NALP1 inflammasome was activated in spinal motor neurons after spinal cord injury (de Rivero Vaccari et al., 2008). In the peripheral mechanisms of complex regional pain syndrome, the NALP1 inflammasome also participated in the process of peripheral sensitization (Shi et al., 2011). However, whether the NALP1 inflammasome is activated in the spinal cord of neuropathic pain in rat has not been previously described.

Lipoxin and its exogenous analog aspirin-triggered carbon-15 epimer aspirin-triggered-15-epi-lipoxin A4 (ATL) belong to a class of eicosanoids that exhibit powerful anti-inflammatory effects and act as endogenous ‘brake signals’ in inflammation reactions (Serhan, 2005; Serhan et al., 2008). In the rat model of neuropathic pain and cancer-induced bone pain, intrathecal administration of ATL showed the longest and most potent analgesic effect compared to the two structural isomers of lipoxins, lipoxin A4 and lipoxin B4 (Hu et al., 2012). According to our previous research, ATL showed the most potent analgesic effect compared to the two structural isomers of lipoxins, lipoxin A4 and lipoxin B4 (Hu et al., 2012). In this study, we aimed to investigate whether intrathecal administration of ATL would inhibit NALP inflammasome activation and the subsequent IL-1β maturation in the spinal cords of rats with chronic constriction injury (CCI)-induced neuropathic pain.

The present study found that the spinal inflammasome-caspase-1 pathway was activated in the development of CCI neuropathic pain and inhibiting the product of activated inflammasome significantly attenuated the CCI pain behaviors. Moreover, the activated NALP1 inflammasome was found in both dorsal astrocytes and neurons, especially in the superficial laminae of the spinal dorsal horn. Finally, intrathecal administration of ATL relieved CCI-induced thermal pain behaviors and inhibited the activation of the NALP1 inflammasome, providing a new therapeutic target for neuropathic pain.

**EXPERIMENTAL PROCEDURES**

**Animals**

Experiments were performed on adult (180–200 g) male Sprague–Dawley rats. Animals were obtained from the Experimental Animal Center, Shanghai Institutes for Biological Science, Chinese Academy of Sciences. They were housed under a 12/12-h light/dark cycle at a room temperature of 23 ± 1 °C with food and water ad libitum. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983).

**CCI neuropathic pain model**

The rats were anesthetized with 10% chloral hydrate (i.p. 400 mg/kg), and the skin around the incision was disinfected with 75% ethanol. Then, the operation procedure was carried out according to the method of Bennett and Xie (1988). Briefly, the right common sciatic nerve was exposed by blunt dissection, and four loose ligatures (4–0 chronic catgut) were tied loosely around the sciatic nerve. Finally, muscle and skin were sutured in layers with silk suture. “Sham surgery” refers to the procedure of exposing the nerve as above but without any nerve ligation.

**von Frey test for mechanical allodynia**

Mechanical allodynia was measured using a series of von Frey filaments according to the method described by Dixon (1980). Before the test, the rats were placed into the plastic cage with a mesh floor to acclimate for at least 30 min. Then, an ascending series of von Frey filaments with logarithmically incremental stiffness (0.40, 0.60, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g) (Stoelting, Wood Dale, IL, USA) was used during the test. The mid-plantar surface of each hind paw was stimulated perpendicularly in the experiment. The testing contained five more stimuli after the first change in response occurred, and the final score was converted to a 50% von Frey threshold using the method described by Dixon.

**Hargreaves’ test for thermal hyperalgesia**

Thermal hyperalgesia was measured with an IITC Model 390 Paw Stimulator Analgesia Meter (Life Science Instruments, USA). The rats were placed separately into a clear plastic cage upon the floor of the window glass for an adaptation period of 30 min, and then radiant heat was applied to the plantar surface of the paw until the animal lifted its paw from the floor. The paw withdrawal latency (PWL), defined as the time from the onset of radiant heat application to withdrawal of the rat’s hind paw, was recorded as per a previously described method (Hargreaves et al., 1988).

**Drug administration**

ATL (Merck, Darmstadt, Germany), was stored at –80 °C until it was diluted in saline immediately before use and administered via lumbar puncture as described before (Xu et al., 2006). Briefly, after the rats were anesthetized with 2% isoflurane, the drug was injected intrathecally (i.t.) at the L5-6 interspace using a 0.5-inch, 20-gauge needle. Correct subarachnoid positioning of the tip of the needle was confirmed by a tail- or paw-flick test. Then, the drug was delivered to the CSF through the needle with a single injection volume of ATL (100 ng or 200 ng in 20 µl). ATL was administered daily from day 3 to day 6 after the CCI operation.

The caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-CMK (Ac-YVAD-CMK) (Merck, Darmstadt, Germany) was dissolved in 10% dimethyl sulfoxide (DMSO) and administered intrathecally once a day from day 3 to day 6 after the CCI operation.

**ELISA**

The concentration of mature IL-1β in ipsilateral L4 and L5 spinal segments was measured with enzyme-linked
immunosorbert assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's protocol. Each sample was run in triplicate, and the concentrations were calculated from the kit's standard curve.

Western blot
The ipsilateral L4–L5 segments of the spinal cord were quickly removed and ultrasonically disrupted in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium deoxychol sulfate, sodium orthovanadate, sodium fluoride, ethylene diamine tetraacetic acid, leupeptin), followed by centrifugation at 12000 × g. The total protein level in the supernatants was measured using the Pierce bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Samples were separated on 12% acrylamide gels and then transferred onto polyvinylidene fluoride membranes. After blocking with 5% skim milk in tris-buffered-saline with tween (TBST) (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 1 h at room temperature, the membranes were incubated with the primary antibody, goat anti-IL-1β (1:5000: R&D System, Minneapolis, MN), rabbit anti-caspase-1 (1:1000: Millipore, Bedford, MA), horseradish peroxidase (HRP)-rabbit-anti-tubulin (1:2000: Cell Signal Technology, Beverly, MA, USA) or HRP-mouse-anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10000: KangChen, Shanghai, China) at 4 °C overnight. Then, the blots were washed in TBST and incubated in the appropriate secondary antibody, HRP-goat-anti-rabbit (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or HRP-donkey-anti-goat (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Western blot images were captured on an ImageQuant LAS4000 mini image analyzer (GE Healthcare, Buckinghamshire, UK), and the band levels were quantified using Image J software, version 1.42q.

Immunofluorescence
Three rats from each group were used in this experiment. The transverse sections of the L4–L5 spinal segments were cut in a freezing microtome (Leica CM1900, Munich, Germany) at a thickness of 30 μm. For double immunofluorescence, the spinal cord sections were incubated with (1) a mixture of rabbit anti-caspase-1 (1:1000: Millipore, Bedford, MA, USA) and mouse anti-NeuN (1:400, Millipore, Billerica, MA, USA), mouse anti-CD11b (1:300, Chemicon, Billerica, MA, USA), mouse anti-GFAP (1:1000, Thermo Scientific, Waltham, MA, USA) antibodies; or (2) a mixture of rabbit anti-NALP1 (1:200: Abcam, Cambridge, MA, USA) and rabbit anti-caspase-1 (1:1000: Millipore, Bedford, MA, USA). Whole tissue lysate prepared for immunoprecipitation (50 μg) was used as an input, and the non-immunized serum of the same isotype was used as the negative control.

Statistical analysis
The data are presented as the mean ± standard error (SEM), and all statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) 17.0 statistical software (SPSS Inc., Chicago, IL). The statistical significance of differences between groups was analyzed with Student’s t-test or a one-way analysis of variance (ANOVA) followed by the Bonferroni post-test. In all statistical analyses, P < 0.05 was considered the threshold for statistical significance.

RESULTS
CICI induced pain-like behavior in rats
In the Hargreaves test, CCI on rats’ right hind limbs provoked a significant decrease in the PWL of the ipsilateral hind paws from day 3 after the sciatic nerve injury that was stably sustained for 1 month (Fig. 1A, B). It also induced a profound decrease in the mechanical threshold over the same timeframe (Fig. 1C, D). However, there were no significant differences in the above pain behaviors between normal and sham surgery rats. Collectively, these data suggest that rats subjected to the CCI developed obvious thermal hyperalgesia and mechanical allodynia.

CICI induced spinal IL-1β maturation
In the ipsilateral spinal cord of CCI rats, a significant increase in the maturation of inflammatory cytokine IL-1β was detected (Fig. 2). In the western blot experiments, pro IL-1β (36 kD), mature IL-1β (17 kD) and tubulin (55 kD) were detected on the same membranes at different time points. At 7 days after the CCI operation but not the sham surgery, western blot
signal for mature IL-1β was clear (Fig. 2A). The data from the ELISA tests, in which CCI caused a significant production of mature IL-1β at day 7 relative to normal rats (\(P < 0.05\)) (Fig. 2B), further confirmed the western blot results. Thus, these data indicated that CCI enhanced the processing of IL-1β precursors.

CCI induced spinal NALP1 inflammasome activation

Caspase-1 is one of the enzymes that process the inactive IL-1β precursors into their mature, active forms, and only the mature form of caspase-1 possesses this catalytic activity. To provide evidence for the involvement of caspase-1 in CCI-induced neuropathic pain, we studied the mature caspase-1 in the ipsilateral spinal cord at different time points (Fig. 3). CCI induced the activation of spinal caspase-1, characterized by an obvious increase in the mature caspase-1 (20 kD) at day 3 and day 7 after injury (\(P < 0.05\)) (Fig. 3A, B), but without any changes in the amount of pro-caspase-1 (40 kD) between groups.

Processing of caspase-1 into its mature form required the activation of the inflammasome. A significant aggregation of the protein components of the NALP1 inflammasome was detected in the IP test, including the adaptor protein ASC, pro-caspase-1 and the NALP1 protein (Fig. 3C). In the normal spinal cords, ASC was immunoprecipitated with anti-ASC, and a very low level of caspase-1 was present in this signaling complex, but NALP1 was not associated with ASC. Beginning on day 3 after the CCI operation, the composition of the complex changed obviously. Although the expression of pro-caspase-1 in the whole lysate did not change (Fig. 3A), the portion that forms the inflammasome significantly increased at day 3 and day 7 after CCI (Fig. 3C). Notably, there was also an obvious aggregation of the NALP1 protein with ASC induced by the CCI operation. The control IgG did not immunoprecipitate the inflammasome-associated proteins and thus served as a control. All these results support that the NLRP1 inflammasome caspase-1 platform was activated in the development of CCI neuropathic pain.
Effects of caspase-1 inhibitor on CCI neuropathic pain

To assess the involvement of inflammasome in the development of neuropathic pain, the caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-CMK (Ac-YVAD-CMK) was intrathecally administrated to the CCI rats. In the Hargreaves test, both 2 and 20 nmol Ac-YVAD-CMK significantly attenuated the CCI-induced thermal hyperalgesia compared to the control DMSO treatment ($P < 0.05$) (Fig. 3D). In the ELISA test, the CCI rats that received 20 nmol Ac-YVAD-CMK also displayed a significant reduction in the level of mature IL-1$\beta$ compared to the rats that received DMSO ($P < 0.05$) (Fig. 3E).

CCI induced NALP1 inflammasome activation in spinal cord neurons and astrocytes

Double immunofluorescence results showed that in the normal condition, the caspase-1 immunoreactivity within the dorsal horn of the ipsilateral spinal cord was mainly co-localized with the neuron marker Neuron-specific Nuclear Protein (NeuN) (Fig. 4A–C, G–I) and partly co-localized with the astrocyte marker glial fibrillary acidic protein (GFAP) but not with the microglia marker CD11b. However, after the rats received the CCI operation, the caspase-1 immunoreactivity colabeling with GFAP was increased at the superficial laminae of the spinal dorsal horn, which is closely linked with pain sensation (Fig. 4G–L). Because this experiment showed immunoreactivity for both pro- and mature caspase-1, the above results suggested that the CCI-induced mature caspase-1 was largely expressed in spinal cord dorsal astrocytes.

In addition to caspase-1, we also detected the possible expression of NALP1 protein in the spinal dorsal horn. Confocal images of spinal sections revealed low levels of NALP1 immunoreactivity in the spinal cords of normal rats (Fig. 5A–C, G–I), and the CCI surgery upregulated NALP1 immunoreactivity in the NeuN-positive and GFAP-positive cells (Fig. 5D–F, J–L). Moreover, most of the CCI-induced NALP1 immunoreactivity colabeled with GFAP was located at the superficial laminae of the dorsal horn (Fig. 5J–L).

However, neither caspase-1 nor NALP1 immunoreactivity was observed in the CD11b-positive cells in the dorsal horn of either normal rats or CCI rats (data not shown), indicating that no NALP1 inflammasome existed in the microglia. Together, these results suggested that the NALP1 inflammasome activated in the astrocytes might play an important role in the development of CCI neuropathic pain.

Effects of repeated treatment with ATL in CCI rats

ATL has exhibited its anti-inflammatory properties in many animal models (Romano, 2010). To detect whether ATL has an analgesic effect on CCI-induced neuropathic pain and to further reveal the underlying mechanism of its effect, we administered ATL to CCI rats at two different doses from day 3 to day 6 after the nerve injury. In the Hargreaves test, 200 ng ATL significantly reduced the CCI-induced thermal hyperalgesia compared to the control saline treatment ($P < 0.05$), but 100 ng ATL showed no obvious pain-relieving effect on CCI rats ($P > 0.05$) (Fig. 6A). In western blots, there was also a significant reduction in the level of mature IL-1$\beta$ (17 kD) after the CCI rats received 200 ng ATL (Fig. 6B), and the statistical analysis of the ELISA experiments further demonstrated the inhibitory effect of ATL on CCI-induced IL-1$\beta$ maturation ($P < 0.05$, vs. model + saline group as control) (Fig. 6C). The above data showed that ATL could alleviate CCI neuropathic pain and inhibit the production of mature IL-1$\beta$ in the ipsilateral spinal cord of CCI rats.

Effects of ATL on CCI-induced NALP1 inflammasome activation

We assumed that ATL suppressed the maturation of IL-1$\beta$ involving of the NALP1 inflammasome caspase-1 platform. To verify this hypothesis, we detected the mature caspase-1 by western blot and the combination of NALP1 inflammasome components via IP. The CCI rats that received 200 ng ATL displayed a significant reduction in the level of mature caspase-1 (20 kD) compared to the rats that received saline as control ($P < 0.05$) (Fig. 7A, B), and there was no significant
Fig. 3. CCI induced the activation of NALP1 inflammasome and the effects of caspase-1 inhibitor on CCI neuropathic pain are shown. (A, B) The expression level of mature caspase-1 was significantly elevated in the ipsilateral spinal by day 3 after the CCI operation (normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) but without any changes in pro-caspase-1. The data are expressed as the mean ± SEM (*P < 0.05 vs. normal group, †P < 0.05 vs. sham surgery group). (C) Immunoprecipitation with ASC of lysates obtained at day 1, day 3, and day 7 after CCI. ASC immunoprecipitates were blotted for ASC, caspase-1, NALP1. Input lane: whole tissue lysate. Control lane: non-immunized serum. (D) In contrast to intrathecal administration of 10% dimethyl sulfoxide (DMSO), which exhibited no effect, repeated injection of either 2 or 20 nmol Ac-YVAD-CMK statistically increased the PWL in the ipsilateral hind paw at day 7 after the CCI operation. The data are expressed as the mean ± SEM (*P < 0.05 vs. Model + DMSO group) (B) 20 nmol Ac-YVAD-CMK, but not DMSO, significantly reversed the CCI induced high expression of mature IL-1β in the ELISA test. The data are expressed as the mean ± SEM (*P < 0.05 vs. normal group, †P < 0.05 vs. Model + DMSO group).
change in the expression of the pro-caspase-1 between different treatments (Fig. 7A). In the IP experiments, repeated ATL treatment also reversed the increased aggregation of ASC, NALP1 and caspase-1 within the NALP1 inflammasome complex (Fig. 7C). All of these data demonstrate that ATL inhibited the CCI-induced NALP1 inflammasome activation.

**DISCUSSION**

In our study, we observed for the first time that the NALP1 inflammasome system participated in the development of neuropathic pain. CCI initiated the activation of the NALP1 inflammasome, leading to the cleavage of pro-caspase-1, upregulation of the adaptor protein ASC and the maturation of IL-1β. The components of the NALP1 inflammasome complex were visible in the astrocytes and neurons of the spinal cord dorsal horn. Repeated intrathecal administration of ATL to the CCI rats significantly relieved the CCI-induced pain behaviors and suppressed the NALP1 inflammasome activation, suggesting a potential mechanism underlying the analgesic effect of ATL.

Compared to the sham surgery, the thresholds for both thermal and mechanical pain in CCI rats were significantly decreased by day 3, reached their minimums at day 7 and lasted until 1 month after the CCI operation (Fig. 7A). From day 3 to day 7, the spinal NALP1 inflammasome was activated and further induced the cleavage of pro-caspase-1, which suggested that the NALP1 inflammasome platform might participate in the development of neuropathic pain. However, the increased level of mature IL-1β, as the product of the NALP1 inflammasome, was only detected at 7 days after the injury. This may be because the expression level of mature IL-1β at day 3 did not reach the level of detection and because the exact time point of IL-1β maturation was not recorded in our research.

IL-1β, acting as an important mediator of the inflammatory response, participates in numerous nervous system diseases (Dinarello, 2009). However, the only biologically active form of IL-1β is the mature 18-kDa molecule, generated by enzymatic cleavage, a function generally ascribed to caspase-1 (Thornberry et al., 1992; Cunha et al., 2010). Aside from caspase-1,
there are other proteases capable of cleaving the IL-1β precursor, such as the matrix metalloproteinases (MMPs) (Schönbeck et al., 1998). In a previous study, MMP-9 showed a rapid and transient upregulation in injured dorsal root ganglion primary sensory neurons at day 1 after the rats were subjected to the spinal nerve ligation, without the activation of caspase-1 (Kawasaki et al., 2008). However, in our research using the CCI model rats, we have demonstrated that mature caspase-1 was significantly activated in the ipsilateral spinal cord from day 3 after the operation. This suggested that MMP-9 and caspase-1 might regulate IL-1β cleavage at different phases of neuropathic pain. The inflammasome activated in humans is formed by members of the NALP protein family, such as NALP1 and NALP3, and the inflammasome components activated in the neuroinflammation process in our model may be different members of the NALP inflammasome family (Pedra et al., 2009). Our study revealed that the CCI injury induced a significant aggregation of NALP1 and caspase-1 proteins at the adaptor protein ASC, which suggested that the NALP1 inflammasome was activated during the neuropathic pain. However, whether the NALP3 inflammasome was activated in this model must be verified with further experiments. Previous studies had reported that in murine macrophages, the NALP1 inflammasome could be activated by the anthrax lethal toxin, which thus far has been the only toxin identified to activate NALP1 (Boyden and Dietrich, 2006; Liao and Mogridge, 2009). However, the precise CCI-induced stimulus that activated the NALP1 inflammasome in dorsal astrocytes and neurons remained unclear. Other studies have shown that decreased levels of intracellular K+ resulted in caspase-1 activation and IL-1β release in cultured primary astrocytes and neurons (de Rivero Vaccari et al., 2008; Silverman et al., 2009). These findings might suggest that this mechanism mediates the CCI-induced NALP1 activation.

In this study, we first showed that the CCI-induced NALP1 inflammasome was located within the astrocytes of the spinal cord dorsal horn. Much previous research has shown that the NALP1 inflammasome participates in CNS disorders and is mainly present in neuronal cells

Fig. 5. NALP1 proteins were present in spinal cord neurons and astrocytes. Confocal images displayed the immunofluorescence of ipsilateral NALP1 protein (red) with NeuN (green, A–F), GFAP (green, G–L), respectively. In normal animals, NALP1 immunoreactivity was scarce. By 7 days after CCI injury, increased NALP1 staining was present in neurons and astrocytes. The NALP1 immunoreactivity that colabeled with GFAP was primarily located at the superficial laminae of dorsal horn (arrow). Scale bars = 100 μm. (C', F', I', L') were high magnification images of C, F, I and L. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 6. ATL attenuated the CCI-induced thermal hyperalgesia and inhibited the maturation of IL-1β. (A) In contrast to the intrathecal administration of saline, which exhibited no effect, repeated injection of 200 ng ATL statistically increased the PWL in the ipsilateral hind paw at day 7 after the CCI operation. (B, C) 200 ng ATL, but not saline, significantly reversed the CCI-induced high expression of mature IL-1β in western blot and ELISA tests, respectively. The data are expressed as the mean ± SEM (*P < 0.05 vs. normal group. #P < 0.05 vs. saline group as control).

Fig. 7. ATL suppressed the CCI-induced NALP1 inflammasome activation. (A, B) The expression of mature caspase-1 was increased at day 7 after the CCI operation, and administration of 200 ng ATL but not of 100 ng ATL significantly reversed this elevation. The data are expressed as the mean ± SEM (*P < 0.05 vs. normal group. #P < 0.05 vs. saline group as control). (C) Immunoprecipitation with ASC of lysates obtained from CCI-treated rats that received repeated ATL treatment or a saline-only control. ATL (200 ng) reversed the CCI-induced aggregation of NALP1 inflammasome proteins.
In the age-induced inflammatory response, the NALP1 inflammasome was activated in the hippocampal neurons that were involved in the age-related cognitive decline (Mawhinney et al., 2011). In the spinal cords of rats with spinal-cord injury, the molecular platform of the NALP1 inflammasome was present in the motor neurons of the anterior horn (de Rivero Vaccari et al., 2008). In the CNS, the neurons were generally regarded as not responsible for the inflammatory reactions, so the inflammasome-induced IL-1β release from neurons might act as a cell signal associated with the communication between neurons and glial cells (Sonetti and Peruzzi, 2004). However, other reports showed that NALP1 was present in both dentate gyrus neurons and astrocytes after ethanol stimulus (Zou and Crews, 2012). After the rats were subjected to CCI, we also discovered that NALP1 was activated in astrocytes and was primarily located in the superficial laminae of the dorsal horn. During the neuropathic pain, the inflammation occurring at the superficial laminae was a key point for pain signal transmission and processing, and astrocytes also played an important part in the neuroinflammatory responses (Woolf and Salter, 2000; Kohn et al., 2003; Jha et al., 2012). Thus, the activated NALP1 inflammasome in astrocytes may be responsible for the pro-inflammatory cytokine release and the promotion of neuroinflammation in neuropathic pain.

Currently, one of the strategies applied to treating neuropathic pain is to use anti-inflammatory drugs. Lipoxins, a class of arachidonate-derived lipid mediators, have been regarded as an endogenous braking signal in the inflammation response (Serhan et al., 2008). Lipoxin and its analog ATL elicit cellular responses and regulate inflammation in vivo by activating a specific receptor, the lipoxin A4 receptor (ALX) (Chiang et al., 2006; Romano, 2010). According to previous work from our lab, the analgesic effect of ATL on CCI rats could be blocked by the ALX antagonist butoxycarbonyl-Phe-Leu-Phe-Leu-Phe (Boc2), suggesting that the therapeutic effect of ATL in our model was mediated by ALX. In the spinal cord dorsal horn of CCI rats, we also found that the spinal ALX was expressed in astrocytes and neurons but not in microglia. When we administered ATL to the CCI rats, the signaling pathway downstream of ALX that mediated the inhibitory effects of ATL on the activation of the NALP1 inflammasome was still unknown. Recently, studies have shown that lipoxins can downregulate the phosphorylated mitogen-activated protein kinases (Svensson et al., 2007; Luo et al., 2013) and reduce the expression of NF-κappa B (Sun et al., 2012). However, whether these signaling pathways participate in the CCI-induced activation of the NALP1 inflammasome and mediate the effect of ATL is ongoing work in our laboratory.

In summary, the NALP1 inflammasome was activated in the spinal cord of CCI rats, and ATL administration had a remarkable curative effect on CCI-induced neuropathic pain. Accompanying the improvement in pain behavior, ATL also significantly suppressed the NALP1 inflammasome. All these results suggest that the inflammasome-caspase-1 platform may be a possible mechanism underlying the pain-relieving effects of ATL. Additionally, the inflammasome platform might offer a new target for the treatment of chronic neuropathic pain and other CNS inflammatory diseases.

**AUTHOR CONTRIBUTIONS**

Qian Li carried out the major part of the study. Qian Li and Zhi-Fu Wang performed the animal surgery and the behavioral tests. Qian Li and Yu Tian carried out the immunoprecipitation study. Qian Li drafted the manuscript. Shen-Bin Liu, Wen-Li Mi and Hong-Jian Ma carried out part of the immunofluorescence study. Gen-Cheng Wu, Jun Wang and Jin Yu revised the manuscript. Yan-Qing Wang conceived and designed the study. All authors read and approved the final manuscript.

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