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Original Research Article

Blocking NLRP3 inflammasome expression by RAS-like protein A mitigates neuropathic pain in chronic constriction injury rat models

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Abstract

Purpose: *To investigate the role of RAS-like protein A (RalA) in lipopolysaccharide-induced inflammatory regulation in primary microglia of chronic constriction injury (CCI)-induced neuropathic pain in rat models.*
Methods: *In vitro, overexpression (OE) of RalA was performed in rat microglia using transfection procedure, and then LPS was used to provoke the inflammatory phenotype. In vivo, the rat model of neuropathic pain was induced using CCI and treated with LV-RalA. Neuroinflammatory levels including the expressions of IL-1β, IL-6, and TNF-α were detected. Moreover, the expressions of NF-κB p65, thioredoxin-interacting protein (TXNIP) and NLR family pyrin domain-containing 3 (NLRP3) were examined in CCI rats and microglial cells. Finally, the functional evaluation was determined via mechanical allodynia and thermal hyperalgesia assays.*
Results: *The level of RalA decreased in the dorsal horn following CCI. OE of RalA in microglia after LPS insult and CCI-induced rat model significantly decreased the expressions of inflammation promoters ($p < 0.05$). Mechanistically, OE of RalA mitigated inflammatory response by inhibiting NF-κB/TXNIP/NLRP3 signaling pathway, thus attenuating neuropathic pain in microglial cells and CCI rats.*
Conclusion: *These results indicate that the OE of RalA plays a protective role in CCI-induced neuropathic pain via NF-κB/TXNIP/ NLRP3 axis. These findings may provide a promising therapeutic target for neuropathic pain.*

Keywords: *Neuropathic pain, NLRP3 inflammasome, NF-κB; RAS-like protein A, Microglia*

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INTRODUCTION

Neuropathic pain, as a common public health issue, has affected the population ranging from 7% to 10% in recent years [1]. The sensitization of central and peripheral neurons is the main cause of neurogenic pain due to continuous intensification of spinal dorsal horn inflammation [2-4]. Unfortunately, patients with chronic neuropathic pain suffer from severe mental distress including chronic depression, insomnia and even anxiety, which significantly reduces their quality of life [5]. However, there is an incomplete cognition in pathology and therapy of neuropathic pain.

Chronic constriction injury (CCI) is a basic procedure in the establishment of experimental models of neuropathic pain in rodents [6]. Therefore, it is important to explore the molecular mechanisms of CCI-mediated neuropathic pain and possible therapeutic targets for intervention. Previous studies [7,8] have demonstrated that nuclear factor-kappa B (NF-κB)-mediated inflammatory signaling is involved in the development of neuropathic pain. Correspondingly, blocking the NF-κB pathway has been shown to inhibit neuroinflammation and relieve neuropathic pain. Moreover, growing studies have confirmed that the thioredoxin interacting protein (TXNIP)/NLR family pyrin domain-containing 3 (NLRP3) inflammasome axis played a crucial role in inflammatory amplification as a downstream pathway of NF-κB in a variety of neurological diseases, including neuropathic pain [9,10]. TXNIP has been identified as a key regulator of NLRP3 generation in various inflammatory models [11], and the increased NLRP3 level has been found to aggravate the development of neuropathic pain in experimental animal models [12,13]. RalA plays an important role in cell biology as a RAS-like GTPase. In addition, an earlier study showed that RalA controls the functional activation of macrophages induced by a variety of inflammatory mediators [14]. Notably, RalA was found to inhibit IL-1β/IL-18 secretion by blocking the activation of NLRP3 in human THP-1 macrophages [15]. However, the specific mechanism by which RalA regulates the NF-κB/TXNIP/NLRP3 axis in neuropathic pain has not been elucidated. Therefore, CCI-mediated neuropathic pain in rats was used to explore the potential role of RalA in NF-κB induced neuroinflammation, which may provide strong theoretical support for better understanding of new therapeutic targets for neuropathic pain in the future.

EXPERIMENTAL

Ethical statement

All animal researches were approved by our Hospital Animal Center according to the Guide for the Care and Use of Laboratory Animals [16]. The aims are to minimize the number and the ordeals of experimental animals. This study was approved by the Animal Ethics Committee of the Animal Center of Chongqing Hospital of TCM (approval no. 0217-CNCQ-ACE-30217).

Microglia culture

Primary rat microglia cells were obtained from the Procell Life Technology Co., LTD (CP-R110, Wuhan, China). The cells were seeded in Dulbecco's Modified Eagle medium (DMEM, Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gbico, Rockville, MD, USA), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) and cultured at 37 °C with 5 % CO_2. Then the treated cells were activated inflammatory response using lipopolysaccharide (LPS, 100 ng/mL, Sigma, St. Louis, MO, USA) for 24 h when the confluency reached to 90 %.

Transfection of RalA gene

Microglia cells seeded in 6-well plate were cultured until they reached 80 % confluency, followed by transfection of RalA full-length cDNA loaded in plasmid provided by GenePharma Co. Ltd. (Shanghai, China). After 48h incubation at 37°C, the cells were used for the detection of reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Establishment of neuropathic pain

Sprague-Dawley (SD) rats (male, 8 week, 180 - 200 g) were housed in an adaptive environment (provided with 22 - 25°C, 50 - 60% humidity, and 12 h - 12 h light - dark cycle) and given available feed and tap water. They were randomly divided into Sham group, CCI group, CCI-NC group, and CCI-RalA group. The rats were intraperitoneally anesthetized using 3 % pentobarbital sodium (40 mg/kg), then the dissected rat's right thigh was dissected to expose the sciatic nerve. A length of approximately 7 mm of nerve was ligated in 4 places with 1 mm interval, followed by a brief twitch of the rats. The sham group used the same methods, except for the ligation. Then the wound was disinfected and sutured. After 14 days for CCI induction, the rats were treated with lentiviral vectors carrying RalA (LV-RalA, GenePharma, Shanghai, China) or scrambled vectors *via* tail intravenous injection. After 14 days, the rats were euthanized with excessive anesthesia *via* pentobarbital sodium, and then the dorsal horn of the spinal cord was collected for subsequent detection.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cells and tissues were treated using 1 mL TRIzol RNA extracted reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted using a PrimeScript RT Reagent Kit (TaKaRa, Shiga, Japan). The RNA measurement was performed using a chimeric dye SYBR® Premix Ex Taq ™ II kit (TaKaRa, Shiga, Japan). Amplification procedure was performed using a

Thermal Cycler Dice Real-Time System (TP800, TaKaRa, Shiga, Japan). The level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization using $2^{-\Delta\Delta Ct}$ method. The primer information was recorded in Table 1.

Table 1: Primer sequences used in qRT-PCR

Name	Sequences (5'-3')
RalA (Forward)	ATGTACGACGAGTTTGTAGAGGA
RalA (Reverse)	CCCGCTGTATCTAAGATGTCGAT
GAPDH (Forward)	AGGTCGGTGTGAACGGATTTG
GAPDH (Reverse)	GGGGTCGTTGATGGCAACA

Enzyme-linked immunosorbent assay (ELISA)

Tissue added with RIPA lysis (Solarbio, Beijing, China) and cell medium were and centrifuged for 10 min to gather the supernatant. Then 1 μL sample was used to verify protein concentration using BCA method. Further operations were conducted in accordance with the protocols of ELISA Kits (Invitrogen, Carlsbad, CA, USA). The optical density (OD) value was measured at a wavelength of 450 nm using a microplate reader (BioTek, Friedrichshall, Germany).

Immunofluorescence (IF) and immunohistochemical (IHC) staining

Paraffin sections of spinal cord tissue were conducted with antigen blocking using 3% bovine serum albumin (BSA) blocking buffer for 1h at room temperature. The sections were incubated with primary rabbit antibodies to NF-κB (1:200, Abcam, Cambridge, MA, USA), TXNIP (1:200, Proteintech, Rosemont, IL, USA), iNOS (1:1000, CST, Danvers, MA, USA), IBA-1 (1:500, Abcam, Cambridge, MA, USA), NLRP3 (1:100, Abcam, Cambridge, MA, USA), and Caspase-1 (1: 200, Abcam, Cambridge, MA, USA) at 4°C for 24 h and washed. Sections were next performed using fluorescence secondary antibodies for 1 h in the dark at room temperature or diaminobenzidine (DAB) treatment for coloration. Images were captured using a microscope system (FV1000, OLYMPUS, Tokyo, Japan).

Mechanical allodynia

Paw withdrawal threshold (PWT) to mechanical stimuli was assessed by pain gauge measurement (von Frey, CA, USA). The rats were placed individually in transparent plastic cages with a mesh floor. The von Frey filament was used perpendicularly to the rat sole by an increasing force, until a paw withdrawal was elicited. The force was recorded when the flexion reflex occurred.

Thermal hyperalgesia

Paw withdrawal latency (PWL) was used to measure the heat sensitivity of rats responding to radiant heat. The center of the plantar surface of the hind paw was exposed to a radiant heat source. The heating setting was controlled at 10 sec intervals, and the intervals between consecutive tests of the hind paws were more than 3 min.

Statistical analysis

Statistical Product and Service Solutions (SPSS) 19.0 (Chicago, IL, USA) are utilized to do statistical analysis. Statistical significance between two groups was assessed by the student *t*-test, while analysis of variance (ANOVA) followed by Tukey's post hoc test was used to analyze various groups. Data comparisons at different time points were conducted using repeated-measures ANOVA followed by Bonferroni post hoc test. Differences considered statistically significant at $p < 0.05$.

RESULTS

RalA expression decreased during neuroinflammatory response

The RNA levels of RalA in the spinal cord of CCI rats within 9 days and in the inflammatory microglia activated by LPS within 72 hours were measured. The results showed that the expression level of RalA in the spinal cord of CCI rats continued to decrease, which was consistent with the trend of inflammatory microglia (Figure 1 A and B). Moreover, the ELISA was used to detect pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α in the spinal cord of CCI rats within 9 days and in the inflammatory activated microglia within 72 h, it was found that the continuously increased expression appeared in the microglia *in vivo* and *in vitro* of CCI rats (Figure 1 C - H).

OE of RalA gene attenuates LPS-induced microglial inflammation via restraining TXNIP-NLRP3 inflammasome axis

To further confirm the regulatory mechanism of RalA in neuroinflammation, we transfected RalA gene to establish microglia that overexpressed RalA, and used LPS for inflammatory phenotypic activation.

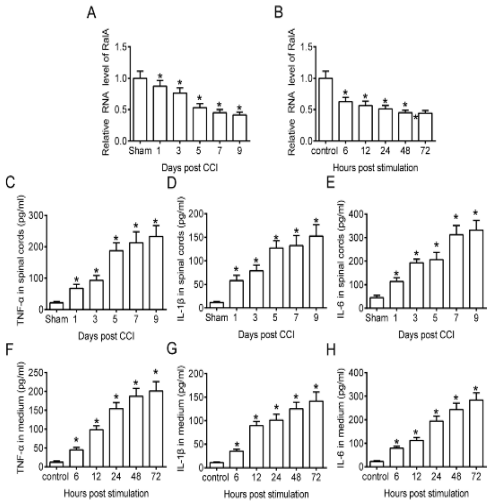

Figure 1: Decrease of RalA expression involved in neuroinflammatory response in CCI rats and microglial cells. (A) The PCR results of RalA levels in spinal cords at 1, 3, 5, 7 and 9 days post CCI. (B) The PCR results of RalA levels in LPS-treated microglia at 6, 12, 24, 48 and 72 hours post stimulation. The levels of TNF-α (C), IL-1β (D), and IL-6 (E) in spinal cords at 1, 3, 5, 7 and 9 days post CCI. The protein expressions of TNF-α (F), IL-1β (G), and IL-6 (H) in microglia medium at 6, 12, 24, 48 and 72 hours post stimulation. "*" p<0.05 *vs.* Sham or control group with statistical significance.

The PCR results showed that the expression of RalA was significantly increased in microglia transfected with RalA gene (Figure 2 A). Furthermore, the ELISA results showed that the levels of proinflammatory cytokines including IL-1β, IL-6, and TNF-α produced by RalA overexpressed microglia after LPS stimulation were significantly reduced (Figures 2 B - D). Immunofluorescence was used to determine the expression of RalA and NF-κB p65 in the microglia of each group. A significant decrease of RalA expression accompanied by a significant increase in NF-κB p65 level was in the LPS group. However, in the microglia with supplemented RalA gene, RalA expression was significantly higher than that in the LPS group, and NF-κB p65 expression was significantly lower than that in the LPS group (Figure 2 E). Moreover, immunofluorescence was used to measure downstream NF-κB p65 expression levels of TXNIP and NLRP3.The results showed that TXNIP in microglia significantly increased after inflammatory activation, while the level of NLRP3 significantly increased. However, TXNIP expression was significantly decreased in microglia overexpressed by RalA, while the expression of NLRP3 was down-regulated compared with microglia treated by LPS (Figure 2 F).

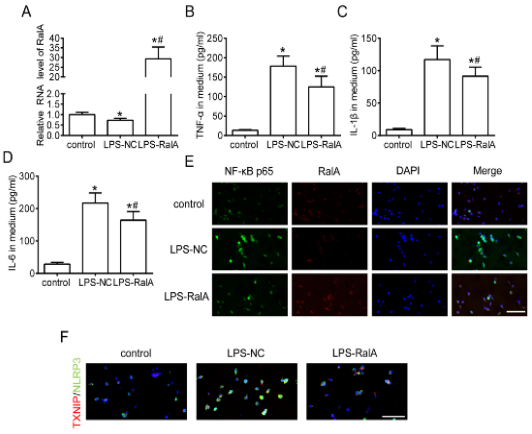

Figure 2: Supplement of RalA gene attenuates LPS-induced microglial inflammation *via* restraining TXNIP-NLRP3 inflammasome axis. (A) The RNA level of RalA in control microglia, LPS-NC treated microglia and LPS-RalA treated microglia at 24 h post stimulation. The protein expressions of TNF-α (B), IL-1β (C), and IL-6 (D) in microglia medium at 24 h post stimulation. (E) IF staining of NF-κB p65 (green) and RalA (red) expressions in control microglia, LPS-NC treated microglia and LPS-RalA treated microglia at 24 h post stimulation; amplification:400×, bar=100 μm. (F) IF staining of NLRP3 (green) and TXNIP (red) expressions in control microglia, LPS-NC treated microglia and LPS-RalA treated microglia at 24 h post stimulation; amplification:400×, bar=100 μm. "*"P < 0.05 *vs.* control group and "#" p<0.05 *vs.* LPS-NC

Overexpression of RalA reduces microglial accumulation and pro-inflammatory cytokines release in CCI rats

RalA level was detected by PCR, showing that RalA expression in spinal cord of CCI rats injected with RalA gene was higher than that of CCI rats (Figure 3 A). Besides, it was found that RalA overexpression reduced the levels of inflammatory factors including IL-1β, IL-6, and TNF-α in the spinal cord of CCI rats (Figures 3 B - D). Moreover, it was found that CCI caused a large number of microglia cells to accumulate, and iNOS was highly expressed in the dorsal horn area of the spinal cord, indicating the activation of pro-inflammatory phenotype. However, a significant reduction in the inflammatory phenotype of microglia was found after excessive RalA overexpression (Figure 3 E).

Increased expression of RalA mitigates neuropathic pain via blocking NLRP3/caspase-1 pathway

IHC staining results showed that NLRP3 and Caspase-1 were expressed in large areas of the dorsal horn.

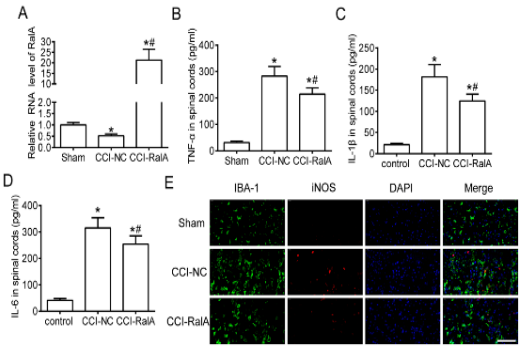

Figure 3: Overexpression of RalA reduces microglial accumulation and pro-inflammatory cytokines release in CCI rats. (A) The RNA level of RalA in Sham group, CCI-NC group and CCI-RalA group at 14 days post CCI. The protein expressions of TNF-α (B), IL-1β (C), and IL-6 (D) in spinal cords at 14 days post CCI. (E) IF staining of IBA-1 (green) and iNOS (red) expressions in Sham group, CCI-NC group and CCI-RalA group at 14 days post CCI; amplification:400×, bar=100 μm. "*" $p<0.05$ *vs.* Sham group and "#" $p<0.05$ *vs.* CCI-NC group

However, increased RalA expression reduced NLRP3 and Caspase-1 expressions in the spinal cords (Figure 4 A). In addition, PWT and PWL assays showed that mechanical hyperalgesia and thermal hyperalgesia were increased following RalA overexpression in CCI rats (Figure 4 B and C). These indicate that overexpression of RalA reduces neuroinflammatory levels by blocking NLRP3, leading to improved neurogenic behavior.

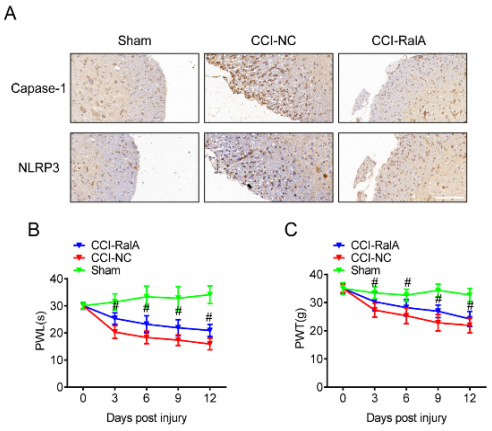

Figure 4: Increased expression of RalA mitigates neuropathic pain *via* blocking NLRP3/caspase-1 pathway. (A) IHC staining of NLRP3 and Caspase-1 expressions in spinal dorsal horn in Sham group, CCI-NC group and CCI-RalA group at 14 days post CCI; amplification:400×, bar=100 μm. Representative PWL (B) and PWT (C) tests in in Sham group, CCI-NC group and CCI-RalA group at 0, 3, 6, 9 and 12 days post-CCI

DISCUSSION

Deletion of multiple genes has been shown to play an important role in the development of neuropathic pain. In the current study, the results show that the absence of RalA both in CCI rats and inflammatory microglia leads to the activation of neuroinflammation. The results showed that the expressions of a variety of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α was significantly increased, with a decreased RalA expression both in spinal cords of CCI rats and LPS-treated microglia. Therefore, decreased expression of RalA may be closely related to neuroinflammatory processes. TXNIP, as a downstream factor of NF-κB p65, mediates oxidative stress, inhibits cell proliferation and induces apoptosis by inhibiting the function of the thioredoxin system [16]. Besides, TXNIP is a key molecule in the inflammatory process that leads to the death of insulin-producing cells in the pancreas [17]. Recent studies have found that TXNIP mediates the generation of NLRP3 inflammasome and increases inflammation level [18]. Here, the results similarly show that TXNIP-NLRP3 played an active role in microglia-mediated neuroinflammation. More importantly, overexpressed RalA inhibited TXNIP-NLRP3 level in LPS-induced microglia, which might further reduce neuroinflammatory damage.

Neuroinflammation is one of the main influencing aspects in the pathology of neuropathic pain, and inflammatory activation and aggregation of microglia in the dorsal horn of the spinal cords are important characteristics of neuroinflammatory aggravation of neuropathic pain [19]. *In vivo*, the obtained data showed significantly fewer pro-inflammatory microglia in the dorsal horn of spinal cords with reduced numbers in rats that overexpressed RalA. Correspondingly, the reduced number of microglia naturally reduced the level of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in the spinal cords. In addition, NLRP3 and its downstream Caspase-1 expressions in the spinal dorsal horn play a positive role in inflammatory response. Accumulation of NLRP3 promotes the activation and maturation of caspase-1. Therefore caspase-1 possesses the ability to cleave pro-IL-1 and pro-IL-18 to increase the amplification effect of inflammation [20].

Inhibition of caspase-1 expression has been shown to impair neuroinflammation in a variety of neurological diseases, including neuropathic pain [21-24]. It was found that the overexpression of RalA significantly reduced the expression of NLRP3 and Caspase-1 in the spinal dorsal horn of CCI rats, which also reasonably explains the

reduced neuroinflammation in the spinal dorsal horn. According to all the results, the primary findings in the current study are exhibited as follows: *in vitro*, the results suggest that RalA may be involved in neuroinflammatory and neurogenic pain processes, and RalA plays an inhibitory role in neuroinflammation by regulating the NF-κB/TXNIP/NLRP3 axis; *in vivo*, RalA overexpression reduces the inflammatory aggregation of microglia and the release of proinflammatory factors in CCI rats. Due to the good control of inflammation in CCI rat spinal cord by overexpression of RalA, mechanical allodynia and thermal hyperalgesia in rats were significantly attenuated after transfection of RalA, thus improving neurogenic behavior. The study proves that rescue of RalA level reduces microglial inflammation after CCI-induced neuropathic pain; nevertheless, further studies are required to systematically elaborate on other regulated mechanism of RalA during neuropathic pain.

CONCLUSION

The findings of this study show that RalA plays an anti-neuroinflammatory role in CCI-mediated neuropathic pain by inhibiting NF-κB/TXNIP/NLRP3 pathway. Thus, RalA is a potential therapeutic target for the management of neurogenic pain.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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