

Role of receptor-interacting protein 1/receptor-interacting protein 3 in inflammation and necrosis following chronic constriction injury of the sciatic nerve

Shaofeng Pu^a, Shuangyue Li^b, Yongming Xu^a, Junzhen Wu^a, Yingying Lv^a and Dongping Du^a

Nerve damage often leads to nervous system dysfunction and neuropathic pain. The serine-threonine kinases receptor-interacting protein 1 (RIP1) and 3 (RIP3) are associated with inflammation and cell necrosis. This study aimed to explore the role of RIP1 and RIP3 in sciatic nerve chronic constriction injury (CCI) in mice. On a total of thirty mice, sciatic nerve CCI was performed. The paw withdrawal threshold was measured using Von Frey filaments. The mRNA expression and protein levels of inflammatory factors RIP1 and RIP3 in the dorsal root ganglion (DRG), spinal cord (SC) and hippocampus (HIP) were also determined. We found that paw withdrawal threshold was significantly reduced from the second day after the operation, and the levels of tumour necrosis factor- α and interferon- γ in DRG, SC and HIP were significantly increased on the eighth and 14th days in CCI mice. Furthermore, the downstream signalling molecules of RIP1 and RIP3, GTPase dynamin-related protein-1, NLR family pyrin domain containing-3 (NLRP3) and nuclear factor κ B-p65 were upregulated. Increased protein levels of programmed cell death protein 1,

which indicate cell death of peripheral and central nervous tissue, were induced by CCI of the sciatic nerve. Overall, this study showed that RIP1 and RIP3 were highly expressed in DRG, SC and HIP of the sciatic nerve in CCI mice and may be involved in chronic neuroinflammation and neuron necrosis. *NeuroReport* 29:1373–1378 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.

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^aDepartment of Pain Management, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai and ^bDepartment of Anesthesiology, Ningbo Urology and Nephrology Hospital, Ningbo, Zhejiang, People's Republic of China

Correspondence to Dongping Du, PhD, 600 Yi Shan Rd, Shanghai 200233, People's Republic of China
Tel: +86 216 436 9181 8896; fax: +86 216 436 9181 8330;
e-mail: dudp@sjtu.edu.cn

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Introduction

Nerve damage is usually caused by trauma, disease, or surgical intervention. Inflammatory responses and pain because of nerve damage can cause substantial distress in patients. Damage to the peripheral nervous system can induce inflammatory responses in the central nervous system and neuropathic pain in other nerves [1]. Chronic constriction injury (CCI) of the sciatic nerve, developed by Bennett and Xie [2], is a traditional model for studying neuropathic pain [3]. Immune cells infiltrate the dorsal root ganglia (DRG) and the spinal cord (SC) after CCI of the rat sciatic nerve [4]. Immune and glial cells release inflammatory mediators [5], such as tumour necrosis factor (TNF)- α [6] and interleukin 6 [7]. The nuclear factor (NF)- κ B pathway is also activated, causing persistent inflammatory responses [8] or neuropathic pain [9].

Using the CCI model, scientists have validated treatment effects of different drugs on neuropathic pain [10,11]. However, the specific roles of many molecules in neuropathic

pain, such as receptor-interacting protein (RIP)1 and RIP3, still require elucidation. RIP1 is a regulator of cellular stress and inflammatory responses [12,13], but its involvement in CCI is poorly understood. A study reported that blockade of RIP1 signalling by a cytomegalovirus RIP1 inhibited inflammatory reactions. Moreover, RIP1 was established as an essential component of the TNF receptor 1 signalling pathway, which mediates the activation of NF- κ B, mitogen-activated protein kinases and programmed cell death [14]. RIP3 is an apoptosis-inducing kinase [15] and regulates inflammation [16]. RIP1 usually forms a complex with RIP3. The RIP1–RIP3 complex regulates programmed necrosis and virus-induced inflammation [17]. The RIP1–RIP3 complex promotes inflammatory responses through activation of the NLRP3, which requires the participation of dynamin-related protein 1 (DRP1) [18,19]. However, it is unclear whether RIP1 and RIP3 exert proinflammatory or pro-necrosis effects in CCI of the sciatic nerve. In this study, we reproduced the sciatic nerve CCI model and investigated the roles of RIP1 and RIP3 in inflammation and nerve injury. Studies have shown that peripheral nervous system damage causes pathological changes in the DRG, SC and hippocampus (HIP) [20–22]. Also, the number of inflammatory

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cells at the injury site of the sciatic nerve and DRGs increased significantly 14 days after CCI [23]. Moreover, CCI leads to marked alterations in the SC [24], as well as in HIP [25]. Therefore, we focused on the expression of RIP1 and RIP3 in the DRG, SC and HIP.

Methods

Animals

A total of 30 C57BL/6 mice (male, 5–6 weeks, 19–24 g) were purchased from the Chongqing Tengxin Biological Technology Co., Ltd and housed under approved conditions with 12/12-h light/dark cycles. All mice had *ad libitum* access to food and water. The experimental protocols were approved by the Experimental Animal Care and Use Committee of Shanghai No. 6 People's Hospital (registration number: 2016-01116).

Induction of the chronic constriction injury model

Thirty mice were allocated randomly to sham or CCI groups. Mice in the CCI group were subjected to CCI surgeries as described previously [2]. In brief, after an intraperitoneal injection of 7% chloral hydrate (5 mg/kg), the right sciatic nerve was bluntly dissected using a surgical suture line (8-0) and was loosely ligated at four different segments. After observing slight contraction of the right posterior limbs, the muscles and skin were closed with sutures. Mice in the sham group were also subjected to blunt dissection of the right sciatic nerve, but without ligation. Three mice in each group were killed on both the second and the eighth day for sampling. Other mice were killed 14 days after surgeries.

Von Frey filaments test

The paw withdrawal threshold (PWT) was tested by a complete set of 20 Von Frey filaments (Stoelting, Dale Wood, Illinois, USA) every 2 days after the surgeries. In the mouse tests, filaments numbered 2 through 15 were used. The PWT of every mouse was calculated according to the following equation: $PWT = 10^{(F \times x + B)}$, where F is the PWT calculated in terms of filament number, $x = 0.224$ and $B = -4.00$ [26].

Enzyme-linked immunosorbent assay

After the mice were killed, the CRG, SC and HIP were harvested. The TNF- α and interferon (IFN)- γ levels in these tissues were examined using the ELISA kit (Elabscience, Texas, USA) according to the manufacturer's instructions. In brief, 10 mg tissue samples and 10 ml PBS were ground mechanically into a homogenate at 4°C. Supernatants were collected after 10 min, centrifuged at 5000 rpm and 10 μ l of each supernatant sample was used for detection. After incubation with biotinylated antibody (100 μ l/well) for 1 h at 37°C, the plates were washed five times in PBS and incubated with enzyme substrate for 30 min at 37°C and a chromogenic substrate 3,3',5,5'-tetramethylbenzidine solution for 15 min at 37°C in the dark. The reaction was then terminated. The optical density was read using a Microplate Reader (Thermo, Waltham, Massachusetts, USA) at 450 nm.

Real-time PCR

Total RNA was isolated from DRG, SC and HIP with Trizol (Invitrogen, California, USA) according to the manufacturer's instructions. Random RNA-specific primers were used to reverse transcribe 1 μ g of RNA to cDNA using the Bestar qPCR RT Kit (DBI). Gene expression levels were analysed using the Agilent Stratagene Mx3000P PCR machine. RT-PCR primers for RIP1: 5'-GCA CCA GCT GTC AGG GCC AG-3' and 5'-GCC CAG CTT TCG GGC ACA GT-3', for RIP3: 5'-TTT GGC CTG TCC ACA TTT CAG-3' and 5'-GGT TGG CAA CTC AAC TTC TCT T-3', for PD1: 5'-CCG CTC GAG CTC ACC ATG TGG GTC CGG CAG GTA CCC TGG-3' and 5'-AGA TCT TCC TCC TCC TCC TTG AAA CCG GCC TTC TGG TTT GGG-3', for TNF- α : 5'-CCC ACT CTG ACC CCT TTA CT-3' and 5'-TTT GAG TCC TTG ATG GTG GT-3, for IFN- γ : 5'-AGC AAC AGC AAG GCG AAA AA-3' and 5'-AGC TCA TTG AAT GCT TGG CG-3', and for GAPDH: 5'-ATG ACA TCAA GAA GGT GGT G-3' and 5'-CAT ACC AGG AAA TGA GCT TG-3'. The relative expression was analysed using the $2^{-\Delta\Delta C_t}$ method.

Western blotting

Tissues of the DRG, SC and HIP were lysed with lysis buffer (Cell Signaling Technology, Danvers, Massachusetts, USA), and the concentrations were detected using a BCA Protein Assay kit (Thermo Fisher Scientific, Rockford, Illinois, USA). Total proteins (30 μ g) were separated and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, Massachusetts, USA). Membranes were blocked with 5% nonfat dry milk for 1 h and incubated with the primary antibodies overnight. The membranes were incubated with the following primary antibodies: anti-RIP1 (1:1000 dilution; Cell Signaling Technology), anti-RIP3 (1:1000 dilution; Cell Signaling Technology), anti-PD1 (1:1000 dilution; Abcam, Cambridge, Massachusetts, USA) and anti- β -actin (1:1000 dilution; Abcam) overnight at 4°C. After incubation with the secondary antibody, the results were detected using an ECL chemiluminescence kit (Beyotime, Shanghai, China).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5 (GraphPad, San Diego, California, USA). Group differences in behaviour results and serum cytokines at different time points were analysed by repeated-measures two-way analysis of variance (group \times time). Individual protein expression and mRNA were analysed using Student's t -test. Differences were considered significant when P less than 0.05.

Results

Chronic constriction injury promoted neuroinflammatory responses

The PWT was not significantly different from before surgeries (0 day) to the first day after surgeries, whereas a

significant decline was observed in the CCI group at later timepoints (2, 4, 6, 8, 10, 12 and 14 day) ($F_{8, 144}=41.34, P<0.001$; Fig. 1a). The levels of inflammatory factors in the DRG, SC and HIP were altered. Both mRNA and protein levels of TNF- α on the eighth and fourteenth days after surgeries were significantly increased in DRG, SC and HIP of the CCI group compared with those of the sham group (DRG: day 8, $P=0.0051$, day 14, $P=0.00057$; SC: day 8, $P=0.0090$, day 14, $P=0.0018$; HIP: day 8, $P=0.0055$, day 14, $P=0.0019$; Fig. 1b. and DRG: day 8, $P=0.016$, day 14, $P=0.0036$; SC: day 8, $P=0.012$, day 14, $P=0.0073$; HIP: day 8, $P=0.032$, day 14, $P=0.012$; Fig. 1d.). The mRNA and protein levels of IFN- γ showed a trend similar to that of TNF- α (DRG: day 8, $P=0.00244$, day 14, $P=0.00029$, SC: day 8, $P=0.00049$, day 14, $P=0.00096$, HIP: day 8, $P=0.00424$, day 14, $P=0.00288$, Fig. 1c; DRG: day 8, $P=0.053$, day 14, $P=0.0021$, SC: day 8, $P=0.016$, day 14, $P=0.0060$, HIP: day 8, $P=0.023$, day 14, $P=0.078$, Fig. 1e).

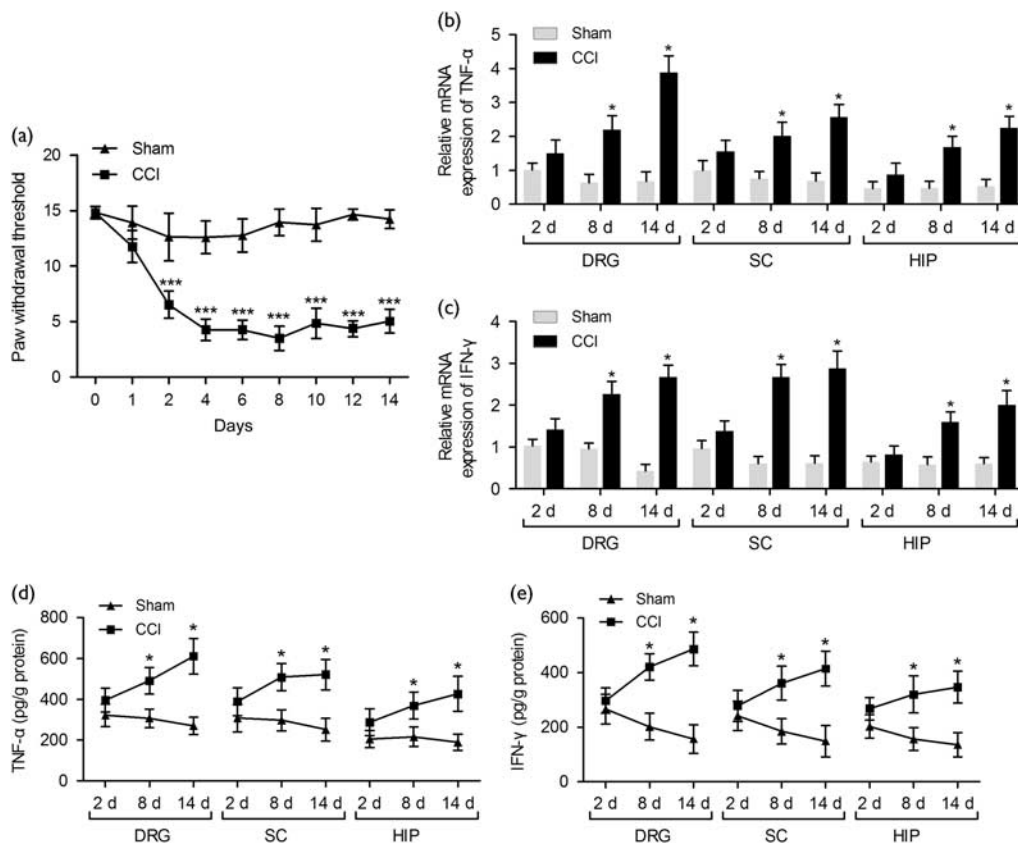
Upregulated expression of receptor-interacting protein 1/receptor-interacting protein 3

We analysed the mRNA expression of *RIP1* and *RIP3* with RT-PCR, and protein levels of RIP1 and RIP3 by western blotting. In the DRG, SC and HIP, the levels of *RIP1* mRNA were significantly increased at 14 d after ligation (DRG: $P=0.0018$, SC: $P=0.0010$, HIP: $P=0.0031$; Fig. 2a). *RIP3* was significantly upregulated in the CCI group compared with the sham group (DRG: $P=0.015$, SC: $P=0.0019$, HIP: $P=0.0066$; Fig. 2b). Results of western blotting showed that protein levels of RIP1 and RIP3 were also increased by CCI (Fig. 2c–e).

Activation of inflammasome and nuclear factor- κ B signalling

RIP1 and RIP3 modulate inflammasome signalling [27] and regulate the target protein DRP1. Analysis of DRP1 expression showed that DRP1 was induced in the DRG (Fig. 3a), SC (Fig. 3b) and HIP (Fig. 3c). NLRP3, a key protein of inflammasome signalling, was also observed in

Fig. 1



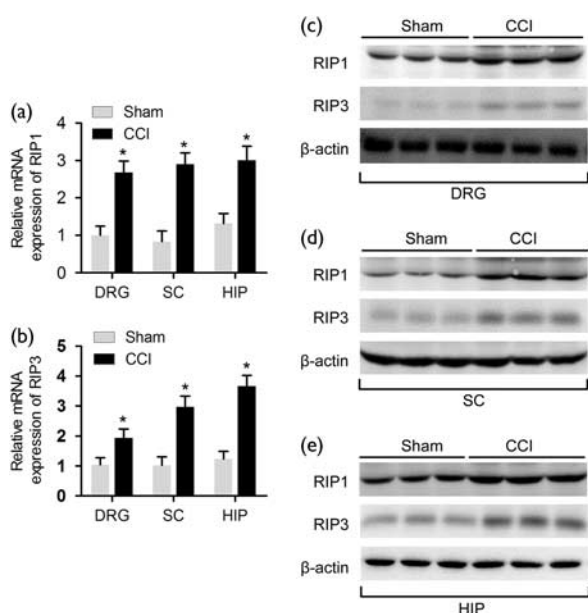
Chronic constriction injury (CCI) promoted the release of tumour necrosis factor (TNF)- α and interferon (IFN)- γ . A total of 30 mice were used to induce a CCI model through ligation of the sciatic nerve. (a) The paw withdrawal threshold (PWT) was measured using Von Frey filaments. (b, c) The mRNA expressions of TNF- α and IFN- γ in the dorsal root ganglion (DRG), spinal cord (SC) and hippocampus (HIP) were measured by RT-PCR. (d, e) Protein levels of TNF- α and IFN- γ in the DRG, SC and HIP were measured by enzyme-linked immunosorbent assay. $n = 10$. * $P < 0.05$, *** $P < 0.001$, versus the Sham group.

the DRG, SC and HIP. As shown in Fig. 3, the protein levels of NLRP3 in the DRG, SC and HIP were increased 14 days after the operation. Analysis of protein levels of NF- κ B p65 by western blotting showed that NF- κ B p65 was upregulated in the DRG, SC and HIP (Fig. 3a–c).

Increased PD1 protein

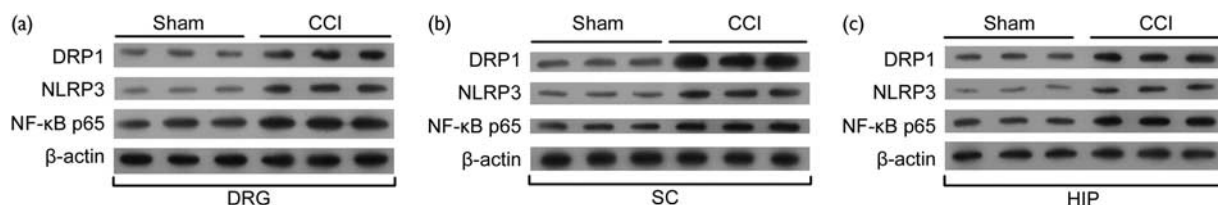
To assess programmed cell death induced by CCI, the protein levels of PD1 in the DRG, SC and HIP were measured. PD1 was increased in all three areas in the CCI group compared with the sham group (Fig. 4a–c). High levels of PD1 protein reflected that CCI promotes the cell death in these three nerve tissues.

Fig. 2



Sciatic nerve chronic constriction injury (CCI) upregulated mRNA expression of *RIP1* and *RIP3* and increased the protein level of receptor-interacting protein (RIP)1/RIP3 in dorsal root ganglion (DRG), spinal cord (SC) and hippocampus (HIP). The mRNA expression of *RIP1* (a), *RIP3* (b) and western blotting bands of RIP1/RIP3 in the dorsal root ganglion, spinal cord and hippocampus on the 14th day after the operation. $n=3$. * $P<0.05$, versus the Sham group.

Fig. 3



Sciatic nerve chronic constriction injury (CCI) activated inflammasome and nuclear factor (NF)- κ B signalling. Fourteen days after ligation, the protein levels of dynamin-related protein (DRP)1, NLR family pyrin (NLRP3) and NF- κ B p65 in the dorsal root ganglion (a), spinal cord (b) and hippocampus (c) were measured by western blotting.

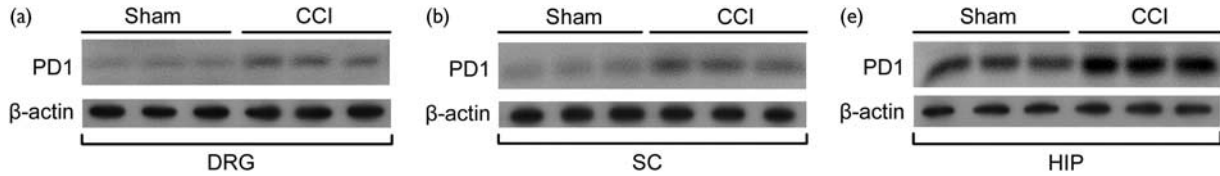
Discussion

Sciatic nerve CCI can cause neuroinflammation and neuropathic pain because of pathological changes in the central and peripheral nerves. In this study, we reproduced the sciatic nerve CCI model and analysed the levels of TNF- α , IFN- γ , RIP1 and RIP3 in the DRG, SC and HIP. Our results suggest that CCI of the sciatic nerve in mice may increase the expression of RIP1 and RIP3, which play a role in integrating neuroinflammatory responses and necrosis in CCI. Inflammasome signalling was activated by RIP1/RIP3/DRP1 in the DRG, SC and HIP, which may induce chronic inflammatory responses and necrosis. Moreover, the high levels of PD1 protein imply that CCI promotes cell death in these brain regions.

The levels of TNF- α and IFN- γ were also significantly increased. TNF- α induces several events, including activation of transcription factor NF- κ B and programmed cell death [28]. TNF- α is mainly secreted by macrophages [29]. Increased TNF- α levels in the DRG, SC and HIP imply that macrophages may infiltrate these regions, in agreement with previous findings [30]. TNF- α facilitates excitatory transmission in the acute phase after nerve injury and contributes towards neuropathic pain [6]. Our results showed that both mRNA and protein levels of TNF- α were significantly increased at 8 days after ligation in a time-dependent pattern. Changes in TNF- α at the early stage of injury may induce immune cell infiltration, including macrophages and T-cell. IFN- γ was also upregulated at 8 and 14 days after surgeries. Accumulation of IFN- γ can activate spinal microglia and drive neuropathic pain [31].

An inflammatory cascade was also induced by TNF- α and IFN- γ . Both TNF- α and IFN- γ activate RIP1 and RIP3 [32–34]. The cellular kinase RIP1 is situated at the converging point of several pathways [35]. In this study, RIP1 and RIP3 protein levels were markedly increased in the sciatic nerve CCI group compared with the sham group. We hypothesize that increased TNF- α and IFN- γ secretion induced the expression of RIP1 and RIP3, which subsequently upregulated the expression of DRP1 and NLRP3 [19], thus promoting inflammasome formation.

Fig. 4



The sciatic nerve chronic constriction injury (CCI) increased the protein level of PD1. Fourteen days after ligation, the protein levels of PD1 in the dorsal root ganglion (a), spinal cord (b) and hippocampus (c) were measured by western blotting. DRG, dorsal root ganglion; SC, spinal cord; HIP, hippocampus.

In CCI, DRP1 and NLRP3 protein levels were increased in the DRG, SC and HIP. NLRP3 inflammasome activation can induce neuropathic pain [36]. As a caspase-1-activating complex, the NLRP3 inflammasome promotes interleukin-1 β secretion and triggers necrosis [37]. We observed NF- κ B signalling activation in the CCI group, which may have been induced by RIP1 [38]. Increased NF- κ B activity may promote inflammatory factor release. Thus, CCI-induced RIP1 and RIP3 may promote an inflammatory cascade and cell death in the nervous system.

Conclusion

In conclusion, we show that levels of TNF- α and IFN- γ were increased in a sciatic nerve CCI model at the 8th day after ligation. Continuous accumulation of TNF- α and IFN- γ may activate RIP1 and RIP3 in the DRG, SC and HIP. RIP1 and RIP3 may induce chronic neuroinflammation and necrosis, possibly through the NLRP3 inflammasome and NF- κ B signalling pathways. This study showed that RIP1 and RIP3 were highly expressed in DRG, SC and HIP of CCI mice, and may play an important role in neuropathic pain.

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Shaofeng Pu, Shuangyue Li, and Dongping Du conceived and designed the study. Shaofeng Pu drafted the statistical analysis plan. Shaofeng Pu, Yongming Xu, and Yingying Lv drafted the original protocol. Junzhen Wu participated in study coordination. All authors have read and approved the final version of the manuscript.

Conflicts of interest

There are no conflicts of interest.

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