Nrf2 Activation Attenuates Chronic Constriction Injury-induced Neuropathic Pain via Induction of PGC-1α-mediated Mitochondrial Biogenesis in the Spinal Cord

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Research Article

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Abstract

Background

Neuropathic pain is a debilitating disease with few effective treatments. Emerging evidence indicates the involvement of mitochondrial dysfunction and oxidative stress in neuropathic pain. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a potent regulator of antioxidant response system. In this study, we investigated whether RTA-408 (a novel synthetic triterpenoid under clinical investigation) could activate Nrf2 and promote mitochondrial biogenesis (MB) to reverse neuropathic pain and the underlying mechanisms.

Methods

Neuropathic pain was induced by chronic constriction injury (CCI) of the sciatic nerve. Pain behaviors were measured via the von-Frey test and Hargreaves plantar test. The L4-6 spinal cord was collected to examine the activation of Nrf2 and MB.

Results

RTA-408 treatment significantly reversed mechanical allodynia and thermal hyperalgesia in CCI mice in a dose-dependent manner. Furthermore, RTA-408 increased the activity of Nrf2 and significantly restored MB that was impaired in CCI mice in an Nrf2 dependent manner. Peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) is the key regulator of MB. We found that PGC-1α activator also exhibited a potent analgesic effect in CCI mice. Moreover, the antinociceptive effect of RTA-408 was reversed by the pre-injection of PGC-1α inhibitor.

Conclusions

Nrf2 activation attenuates chronic constriction injury-induced neuropathic pain via induction of PGC-1α-mediated mitochondrial biogenesis in the spinal cord. Our results indicate that Nrf2 may be a potential therapeutic strategy to ameliorate neuropathic pain and many other disorders with oxidative stress and mitochondrial dysfunction.

Introduction

Neuropathic pain arises due to a primary lesion or dysfunction affecting the somatosensory nervous system, which markedly impairs the patients’ quality of life and reduces individual productivity [1,2]. Unfortunately, the efficacy of pharmacologic treatment is limited and is associated with side effects and risks of abuse [3,4]. Therefore, identification of novel therapeutic strategies is considered to be a significant and unmet need.

Accumulating evidence indicates that oxidative stress and mitochondrial dysfunction are involved in various animal models of pathological pain [5-8]. Mitochondrial biogenesis (MB) is the process of
producing new functional mitochondria, which could restore mitochondrial function after various stimuli or injury [9-12]. The main regulatory factor of MB is peroxisome proliferator-activated receptor coactivator 1α (PGC-1α). PGC-1α displays its functions by increasing many transcription factors, including nuclear respiratory factors 1 and 2 (NRF1 and NRF2). NRF1 and PGC-1α coactivate the transcriptional function of mitochondrial transcription factor A (TFAM) that directly promotes transcription and replication of the mitochondrial genome [13]. The antioxidant response element (ARE) is a DNA regulatory element, mainly binding with nuclear factor erythroid-derived 2-like 2 (Nrf2) to activate these genes (such as heme oxygenase-1 (HO-1), NRF1, and Hmox1). Under physiologic conditions, Nrf2 is sequestered to the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1)-Nrf2 complex and ubiquitin degradation. However, oxidative stress triggers the dissociation of the Keap1-Nrf2 complex. Nrf2 then enters the nucleus and activates ARE to regulate the transcription of antioxidant-related genes [14-16]. Nrf2 is believed to be a master regulator of endogenous antioxidant defense and MB [17-19]. The principal role of Nrf2/ARE in MB has been revealed by HO-1 activity in several models [20,21]. Our previous study demonstrated that oltipraz and rosiglitazone significantly attenuate paclitaxel-induced neuropathic pain via induction of Nrf2/HO-1 signaling pathway in the spinal cord [22,23]. Moreover, several studies illustrated that Nrf2 promoted MB via the regulation of PGC-1α [24,25]. Thus, we suggested that Nrf2 may be an attractive therapeutic target to stimulate MB under neuropathic pain conditions in a PGC-1α dependent manner.

RTA-408, a novel Nrf2 activator, is a member of the synthetic oleanane triterpenoid compounds [26-29]. It is currently being used in several clinical trials for the prevention and treatment of a variety of diseases, including radiation-induced dermatitis (NCT02142959) [30], advanced solid cancers (melanoma or non-small cell lung cancer) (NCT02029729) [31], and Friedreich ataxia (NCT02255435) [32]. A previous study has shown that RTA-408 plays a critical role in mitigating radiation-induced bone marrow suppression by activating Nrf2 [33]. Notably, a recent study has demonstrated RTA-408 could stimulate MB and benefit patients with mitochondrial myopathy (NCT02255422) [34]. Another study demonstrated that activation of Nrf2 with RTA-408 exerted a neuroprotective and disease-modifying effect by inhibiting reactive oxygen species production, mitochondrial depolarization, and neuronal death [35]. Thus, this study investigated the promising effect of RTA-408 in the CCI model, and we hypothesized that RTA-408 may exert analgesic effects via induction of PGC-1α-mediated MB in the spinal cord in a mice model of chronic constriction injury (CCI).

Methods

Animals

Adult male C57BL/6J mice weighed 23-25 g from Tongji Medical College, Wuhan, China were used. All mice were housed in a temperature- and humidity-controlled environment, under a 12:12 light-dark cycle, and with free access to a standard diet and tap water ad libitum. All behavioral tests were performed from 8:00 A.M. to 4:00 P.M. All experimental procedures were performed following the approval of the
ethics committee of the Animal Care and Use Committee of Huazhong University of Science and Technology.

**Drug administration**

RTA-408, purchased from Selleck (Houston, TX, USA), was dissolved in 10 % DMSO and 10 % Tween 80 in sterile saline. The Nrf2 inhibitor, trigonelline hydrochloride, purchased from Selleck (Houston, TX, USA), was dissolved in sterile saline. PGC-1α activator, ZLN005, purchased from MedChemExpress (Monmouth Junction, NJ, USA), was dissolved in 10 % DMSO and 10 % Tween 80 in sterile saline. PGC-1α inhibitor, SR-18292, purchased from MedChemExpress (Monmouth Junction, NJ, USA), was dissolved in 10 % DMSO and 10 % Tween 80 in sterile saline.

To determine whether treatment with RTA-408 or ZLN005 have analgesic effects in the CCI model, a single intrathecal (i.t.) injection of different doses of RTA-408 (1, 5, 10 μg) or ZLN005 (1, 5, 10 μg) was given on day 7 after CCI. The pain behavior tests were conducted before RTA-408 or ZLN005 injection and at 0 h, 0.5 h, 1 h, 2 h, 4 h, 8 h after RTA-408 or ZLN005 injection. To determine whether consecutive injections of RTA-408 or ZLN005 can reverse established CCI-induced neuropathic pain, RTA-408 or ZLN005 was given once daily for 5 consecutive days (from day 7 to day 11) after SNI establishment. The pain behavior tests were conducted on day 7 and 1 h after RTA-408 or ZLN005 injection each day. To determine whether the Nrf2 inhibitor trigonelline can reverse the analgesic effect of RTA-408, trigonelline (5 μg, i.t.) was given 30 min before RTA-408. The pain behavior tests were performed before trigonelline injection and 0.5 h, 1 h, 2 h, 4 h, and 8 h after RTA-408 injection. To determine whether the PGC-1α inhibitor SR-18292 can reverse the analgesic effect of ZLN005, SR-18292 (10 μg, i.t.) was given 30 min before ZLN005. The pain behavior tests were performed before SR-18292 injection and 0.5 h, 1 h, 2 h, 4 h, and 8 h after ZLN005 injection.

To assess the preventive effect of RTA-408 or ZLN005, RTA-408 (10 μg, i.t.) or ZLN005 (10 μg, i.t.) was given once daily from day 0 to day 2 after CCI. The pain behavior tests were performed before CCI and on days 1, 3, 7, and 14.

To determine whether the PGC-1α inhibitor SR-18292 can reverse the analgesic effect of RTA-408, SR-18292 (10 μg, i.t.) was given 30 min before RTA-408. The pain behavior tests were performed before trigonelline injection and 0.5 h, 1 h, 2 h, 4 h, and 8 h after RTA-408 injection.

**Intrathecal injection**

The lumber puncture methods were performed as previously described [25]. In brief, after determining the level of the hip bones with the non-dominant hand, the 30-gauge sterile needle was inserted vertically into the L5-6 vertebrae of conscious mice. The needle angle is inclined to approximately 30° when it connects the vertebrae, then insert the needle into the intervertebral space. An evident reflexive swing of the tail manifested a piercing of the dura, and 5 μl of drugs or vehicles were slowly injected (1 μl/min).

**CCI and behavioral tests**
Neuropathic pain was induced by CCI of the sciatic nerve in this study. Mice were anesthetized with 2% isoflurane via a facemask and were monitored during the surgery. The left common sciatic nerve of each mouse was exposed at the mid-thigh level, proximal to the trifurcation of the sciatic nerve was gently freed without stretching muscles and nerves. Three ligatures were loosely tied (5-0 chromic gut sutures) around it and 1 mm apart. In the sham-operated group, the same procedure was performed without injury of the nerves. Mice were randomly divided into the following groups: (1) Sham + Vehicle group: sham-operated mice with vehicle injection (5 μl, i.t.); (2) CCI + Vehicle group: CCI-injured mice with vehicle injection (5 μl, i.t.); (3) CCI + RTA group: CCI-injured mice with RTA-408 injection (1, 5, 10 μg, i.t.); (4) CCI + Trig group: CCI-injured mice with trigonelline hydrochloride (5 μg, i.t.) injection; (5) CCI + RTA + Trig group: CCI-injured mice with RTA-408 (10 μg, i.t.) combined with trigonelline hydrochloride (5 μg, i.t.) injection; (6) CCI + ZLN group: CCI-injured mice with ZLN005 injection (1, 5, 10 μg, i.t.); (7) CCI + SR group: CCI-injured mice with SR-18292 injection (10 μg, i.t.); (8) CCI + ZLN + SR group: CCI-injured mice with ZLN005 (10 μg, i.t.) combined with SR-18292 (10 μg, i.t.) injection; and (9) CCI + RTA + SR group: CCI-injured mice with RTA-408 (10 μg, i.t.) combined with SR-18292 (10 μg, i.t.) injection.

Mechanical allodynia was determined by measurement of paw withdrawal threshold (PWT) using Von Frey filaments as previously described [36,37]. In brief, mice were set in an elevated plexiglass cage for 30 min before the test to adapt to the environment. The tip of filaments was stuck upright to the plantar surface of the left hind paw of each mouse, with each force lasting 5 s. Ascending order of forces (0.02 g, 0.04 g, 0.16 g, 0.4 g, 0.6 g, 1.0 g, 1.4 g and 2 g) were used, starting with 0.02 g and ending with 2 g. Positive responses were defined as a quick paw lifting or licking. When a positive response was observed, mice were allowed to rest for 5 minutes before detecting the next descending von Frey filament. PWTs were decided as the lowest force after positive responses.

Thermal hyperalgesia was assessed by measurement of thermal withdrawal latency (TWL) using the Hargreaves plantar test (Ugo Basile, Comerio, VA, Italy) [38]. In brief, mice were placed in transparent separate compartments (10×10×15 cm) on a glass plate and kept quiet for 30 minutes before the test. Place the radiant heat source beneath the plantar surface of the left hind paw. Once the hind paw of mice was moved, the stimulation was shut off and the data was recorded. Each hind paw was repeated 3 times with a period of 5-6 minutes intervals. The final TWL was determined from the mean of three measurements. The cutoff time was 20 seconds to avoid tissue damage. The glass plate was cleaned between each interval. Animals exhibiting motor dysfunction were excluded from all the experiments.

Western blotting

Under deep 2.5% isoflurane anesthesia, the L4-6 spinal cord segments were excised rapidly at different time points. Total tissues were homogenized in cooled RIPA lysis buffer containing a cocktail of protease inhibitors and protein inhibitors on ice. After centrifuged at 12,000 rpm for 30 min at 4 °C, the supernatants were collected. The concentration of Nuclear and Cytoplasmic protein extractions (Nuclear and Cytoplasmic Protein Extraction Kit, Beyotime, Shanghai, China) was measured by the Bicinchoninic Acid (BCA) Protein Assay Kit (Boster, Wuhan, China) according to manufacturer's instructions. Then
protein extractions were separated by 10 % SDS-PAGE and transferred onto 0.45 μm Polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). Block PVDF membranes with 5 % non-fat milk or BSA at RT (room temperature) for 1.5 h and incubate the following primary antibodies at 4 °C overnight: anti-PGC-1α (A11971; Abclonal, Wuhan, China), anti-Nrf2 (16369-1-AP; Proteintech, Wuhan, China), anti-NRF-1 (ab175932; Abcam, Cambridge, UK), anti-TFAM (ab252432; Abcam, Cambridge, UK), anti-Histone H3 (GB11102; Servicebio, Wuhan, China). After washing with TBST 3 times, the membranes were incubated with goat anti-rabbit HRP conjugated secondary antibody (A21020, Abbkine, Wuhan, China) at RT for 1.5 to 2 h. Finally, the protein expression was detected using the SuperLumia ECL Kit (YEASEN, Shanghai, China) and quantified using chemiluminescence (Bio-Rad, CA, USA).

**Immunofluorescence staining**

Immunofluorescence staining was conducted as previously described [30]. Under deep 2.5% isoflurane anesthesia, mice were transcranial perfused with 40 ml cold-PBS followed by 40 ml 4% cold-parafomaldehyde (PFA). The L4-6 segments of the spinal cord were dissected out and post-fixed in 4% PFA overnight at 4 °C. After freezing at -80°C overnight, spinal cord sections were crosscut into 20-μm on a cryostat (CM1900, Leica, Heidelberg, Germany). After penetrated with 0.3% TritonX-100 for 40 min, sections were blocked with 10% donkey serum for 40 min at RT. Then incubate the following primary antibodies overnight at 4 °C: anti-Nrf2 (AF70006; Affinity, Wuhan, China), anti-neuronal nuclei antibody (NeuN; ab104224, Abcam, Cambridge, UK), anti-glial fibrillary acidic protein antibody (GFAP; 3670; Cell Signaling Technology, MA, USA) and anti-Iba1 antibody (ab5076; Abcam, Cambridge, UK). After washing 5 times with PBST, the sections were incubated with a mixture Alexa 488-conjugated donkey anti-rabbit secondary antibody (711-547-003; Jackson ImmunoResearch, PA, USA), Alexa 594-conjugated donkey anti-mouse secondary antibody (715-585-150; Jackson ImmunoResearch, PA, USA), or Alexa 594-conjugated donkey anti-goat secondary antibody (705-585-003; Jackson ImmunoResearch, PA, USA) for 2 h at RT. After washing 5 times with PBST, the spinal cord sections were detected with a fluorescence microscope (DP70, Olympus, Japan).

**Quantification of mitochondrial DNA (mtDNA) copy number**

Under deep 2.5% isoflurane anesthesia, the L4-6 spinal cord of mice was collected. The DNA was extracted using the Tissue Genomic DNA Extraction Kit (EP007, ELK Biotechnology, Wuhan, China) according to the manufacturer’s instructions. qPCR was performed with SYBR Green PCR SuperMix (ELK Biotechnology, EQ001, Wuhan, China). mtDNA copy number was calculated and quantified with the 2^{-ΔΔCt} method after comparison of a mitochondrial gene ND1(mtND1) to nuclear-encoded gene β-actin. The following primer sequences were used for PCR:

mtND1: sense: 5’-AACGTAGAATACGCAGCCG-3’, antisense: 5’-TTGATCGTAACGGAAGCGTG-3’;

β-actin sense: 5’-CTTCTATGAGCTGAGTCTCCCTTG-3’, antisense: 5’-GACAGGGGCTCCACTTAGACC-3’;

**Statistical analysis**
Data are expressed as means ± SEM and analyzed with GraphPad Prism version 6.0. Western blot results were analyzed using one-way ANOVA followed by Bonferroni post hoc test. Behavioral data (including PWT and TWL) was analyzed by two-way ANOVA with repeated measures, followed by Bonferroni post hoc analysis. P values less than 0.05 were indicated statistically significant. The investigator responsible for data analysis was blinded to the experimental design.

Results

RTA-408 attenuated CCI-induced pain behaviors

Before surgery, the von Frey test showed no significant differences in PWT among all groups. After CCI, PWT in the ipsilateral paws potently decreased from day 3 to day 14. The most severe decrease of PWT appeared on day 7 after CCI. (Figure 1A). Likely, Hargreaves plantar test showed no significant difference in TWL among all groups before surgery. The ipsilateral TWL potently attenuated from day 3 after surgery and remained on day 14 (Figure 1B). There was no significant change in PWT and TWL in the sham group during the experiment. These results indicated that CCI induced a marked decrease of PWT and TWL in a pattern of rapid-onset and long-lasting manner.

To assess the analgesic effect of RTA-408, a single injection of different doses of RTA-408 (1 µg, 5 µg, and 10 µg, i.t.) or vehicle (5 µl, i.t.) were given on day 7 after CCI. PWT and TWL tests were performed at 0 h, 0.5 h, 1 h, 2 h, 4 h, and 8 h after RTA-408 injection. As shown in Figures 1C and D, administration of 1 µg RTA-408 had no significant influence on PWT and TWL. However, 5 µg or 10 µg RTA-408 significantly reversed the mechanical allodynia and thermal hyperalgesia beginning at 0.5 h, peaking at 1 h, and lasting at least 4 h. Besides, there were significant differences between the 5 µg and 10 µg RTA-408, suggesting that i.t. RTA-408 could reverse mechanical allodynia and thermal hyperalgesia in a dose-dependent manner after CCI. Moreover, after CCI, repeated i.t. administration of RTA-408 to mice from day 7 to day 11 significantly reversed the established mechanical alldynia and thermal hyperalgesia without showing any signs of tolerance (Figure 1E and F).

Preventive effect of RTA-408 on the development of CCI-induced pain hypersensitivity

To determine the preventive effect of RTA-408, 10 µg RTA-408 was i.t. administered once daily from day 0 to day 2. The pain behaviors were tested before CCI and on day 1, day 3, day 7 and day 14 following CCI. As shown in Figure 1G and H, the PWT and TWL was significantly upregulated on day 1 and day 3, but not on day 7 and day 14. These results suggest that early administration of RTA-408 (10 µg, i.t.) from day 0 to day 2 delayed the onset of CCI-induced mechanical alldynia and thermal hyperalgesia.

Expression and cellular localization of spinal Nrf2 after CCI

Western blot analysis and immunohistochemistry were performed to determine the activation of Nrf2 in the spinal cord following CCI. As shown in Figure 2A, the expression of Nrf2 protein in the nuclear extracts was rapidly increased, beginning at day 1, and remained at high levels until the last observation on day
14 in the spinal cord dorsal horn following CCI. We then detect the co-localization of Nrf2 with the nucleus using immunofluorescence staining (Figure 2B). As predicted, the proportion of Nrf2 nuclei was increased following CCI in a time-dependent manner, which is consistent with our western blot data.

**Effects of RTA-408 on Nrf2 signaling in the spinal cord of CCI mice**

To determine whether RTA-408 could activate Nrf2 following CCI in the spinal cord, we treated CCI mice and sham mice with RTA-408 (10 μg, i.t.) once a day from day 7 to day 11, and examined the expression of Nrf2 protein in the nuclear extracts after the final administration. As shown in Figure 3, the expression of Nrf2 protein in the nuclear extracts was rapidly enhanced after RTA-408 administration in the CCI group. These results demonstrated that RTA-408 could significantly activate Nrf2 following CCI in the spinal cord.

To evaluate whether activation of the Nrf2 signaling pathway is responsible for the antinociceptive effects of RTA-408, Nrf2 inhibitor trigonelline was given 30 minutes before RTA-408 administration on day 7 following CCI. The threshold of PWT and TWL was tested before trigonelline administration and at 0.5 h, 1 h, 2 h, 4 h, and 8 h after administration [39]. As shown in Figure 4A and B, the alleviating effect of RTA-408 against CCI-induced pain hypersensitivity was completely prevented by trigonelline injection. Trigonelline did not change mechanical allodynia and thermal hyperalgesia in mice without RTA-408 treatment in the CCI group.

We next examined the activity of Nrf2 following trigonelline co-injection. As shown in Figure 4C, the activity of Nrf2 enhanced by RTA-408 significantly suppressed by trigonelline treatment. Consistent with our western blot data, the immunofluorescence data showed that RTA-408 injection could further enhance Nrf2 activation following CCI, and cotreatment with trigonelline reduced the activation of Nrf2 in the spinal cord dorsal horn compared with RTA-408 treatment alone following CCI (Figure 5). Moreover, our double immunofluorescence staining showed that Nrf2 was mainly colocalized with neurons, and a minority in astrocytes and microglia in the spinal cord dorsal horn (Figure 5, Supplementary Figure 1, and Supplementary Figure 2). These results suggested RTA-408 could alleviate CCI-induced pain hypersensitivity through activation of Nrf2 in the spinal cord.

**Mitochondrial biogenesis in the spinal cord of CCI mice.**

An imbalance of redox biology disrupts mitochondrial bioenergetics, which is thought to lead to neuropathic pain [40,41]. PGC-1α is the principal regulatory factor of MB and is involved in the transcriptional control of several mitochondrial genes, including NRF1, and TFAM [13]. Thus, the time-course (day 1, day 3, day 7 and day 14) of CCI was conducted to test the expression of PGC-1α/NRF1/TFAM gene and mtDNA in the spinal cord. Western blot analysis showed that the expression of PGC-1α was significantly diminished at day 1 in the dorsal horn of the spinal cord following CCI. Moreover, the expression of NRF1 and TFAM was decreased from day 3 to day 14 (Figure 6A). To investigate the changes of mtDNA expression in the spinal cord of CCI mice, the L4-6 spinal cord was collected on day 1, day 3, day 7, and day 14 after CCI. RT-PCR results showed that expression of mtDNA
downregulated from day 1 compared to sham mice, which is consistent with changes in mitochondrial
genes (Figure 6B). These results indicated that mitochondrial dysfunction was induced by CCI in the
spinal cord.

**PGC-1α activator attenuated CCI-induced pain behaviors**

As the main regulatory factor of MB, PGC-1α was significantly decreased following CCI. To further assess
the role of PGC-1α in CCI-induced pain model, a single injection of PGC-1α activator ZLN005 (1 μg, 5 μg,
and 10 μg, i.t.) and vehicle (5 μl) were given on day 7 after CCI. PWT and TWL tests were performed at 0
h, 0.5 h, 1 h, 2 h, 4 h, and 8 h after RTA-408 injection. As shown in Figure 7A and B, a single i.t. injection of
ZLN005 (5 μg and 10 μg) significantly increased mechanical allodynia and thermal hyperalgesia
following CCI beginning at 0.5 h, peaking at 1 h and lasting for at least 2 h. However, there was no
significant difference between the two groups (5 μg and 10 μg). To determine the accumulative effect of
the PGC-1α activator, ZLN005 (1 μg and 10 μg) was i.t. injected from day 7 to day 11. As shown in Figure
7C and D, repeated injection of ZLN005 resulted in significant reversal of mechanical and thermal
hyperalgesia without signs of tolerance after CCI. These results suggested that ZLN005 significantly
reversed mechanical allodynia and thermal hyperalgesia following CCI.

**Preventive effect of PGC-1α activator on the development of CCI-induced pain hypersensitivity**

To determine the preventive effect of ZLN005, ZLN005 (10 μg, i.t.) was administered once daily from day
0 to day 2. The pain behaviors were tested before CCI and on day 1, day 3, day 7, and day 14 following
surgery. As shown in Figure 7E and F, mice pretreated with ZLN005 showed upregulated PWT and TWL
on day 1, day 3 and 7, but not day 14. These results suggested that early administration of ZLN005 (10
μg, i.t.) significantly delayed the onset of CCI-induced mechanical allodynia and thermal hyperalgesia.

To evaluate whether PGC-1α is involved in the antinociceptive effects of ZLN005, PGC-1α inhibitor SR-
18292 was given 30 minutes before ZLN005 administration on day 7 following CCI. The threshold of
PWT and TWL was tested before SR-18292 administration and at 0.5 h, 1 h, 2 h, 4 h, and 8 h after
ZLN005 administration. As shown in Figure 7G and H, the effect of ZLN005 against CCI-induced pain
hypersensitivity was completely prevented by SR-18292 injection. SR-18292 did not change mechanical
alldynia and thermal hyperalgesia in mice following CCI.

**Effect of RTA-408 on mitochondrial biogenesis in the spinal cord of CCI mice**

To determine the effect of RTA-408 on MB in the spinal cord of CCI mice, the L4-6 spinal cord was
extracted after the last injection of RTA-408 (10 μg) on day 11 and examined for mitochondrial genes and
mtDNA expression. As shown in Figure 8A, treatment with RTA-408 for 5 consecutive days (from day 7 to
day 11) significantly reversed the downregulation of PGC-1α/NRF1/TFAM compared to the CCI group.
Moreover, RTA-408-treated mice completely restored the down-regulation of mtDNA in the spinal cord
cased by CCI (Figure 8B). However, RTA-408 had no significant effect on mtDNA in the sham group.
Further, trigonelline significantly reversed the effect of RTA-408 in decreasing the expression of PGC-
1α/NRF1/TFAM, as well as downregulating the expression of mtDNA (Figure 9A and B). These results suggested RTA-408 treatment could restore mitochondrial bioenergetics via the Nrf2 signaling pathway in the CCI model.

**PGC-1α was required for RTA-408 induction of mitochondrial biogenesis**

We have shown in previous studies that RTA-408 can restore PGC-1α expression following CCI. To determine whether Nrf2 could activate PGC-1α in the spinal cord following CCI, PGC-1α inhibitor SR-18292 was given 30 minutes before RTA-408 administration on day 7 following CCI. The threshold of PWT and TWL was tested before SR-18292 administration and at 0.5 h, 1 h, 2 h, 4 h, and 8 h after RTA-408 administration. As shown in Figure 10A and B, the effect of RTA-408 against CCI-induced pain hypersensitivity was completely inhibited by SR-18292 injection. SR-18292 did not change mechanical allodynia and thermal hyperalgesia in the CCI group. These results suggested that Nrf2 could activate PGC-1α in the spinal cord following CCI.

**Discussion**

This study demonstrated that (1) i.t. injection of a novel synthetic triterpenoid RTA-408 significantly reversed the mechanical allodynia and thermal hyperalgesia in a dose-dependent manner in CCI mice, which was reversed by the preinjection of Nrf2 inhibitor. (2) Early treatment with RTA-408 delayed the onset of CCI-induced mechanical allodynia and thermal hyperalgesia. (3) Nrf2 was significantly activated by CCI and further activated after RTA-408 treatment in the spinal cord, which was blocked by the preinjection of Nrf2 inhibitor. (4) Persistent mitochondrial dysfunction was induced by CCI in the spinal cord in a time-dependent manner. (5) i.t. injection of PGC-1α activator significantly inhibited the mechanical allodynia and thermal hyperalgesia in CCI mice, which was reversed by PGC-1α inhibitor. (6) Early treatment with ZLN005 significantly delayed the onset of CCI-induced mechanical allodynia and thermal hyperalgesia. (7) RTA-408 alleviated CCI-induced pain behaviors via stimulating MB, which was reversed by Nrf2 inhibitor. (8) Inhibition of spinal PGC-1α reversed the analgesic effect of RTA-408 after CCI. Together, these results suggested that RTA-408 may induce MB through the Nrf2 signaling pathway for the treatment of neuropathic pain in a PGC-1α dependent manner in the spinal cord.

Neuropathic pain is a common chronic pain that affects patients' quality of life. Neuropathic pain. However, no medication currently is available for the treatment and prevention of neuropathic pain. In this study, we first determined the analgesic effect of RTA-408, which is mediated by the activation of Nrf2. Our previous study has reported the crucial role of Nrf2 in treating paclitaxel-induced neuropathic pain [22]. However, the exact mechanisms are not yet fully elucidated. In this study, we focused on its analgesic actions for the treatment of neuropathic pain and explored the underlying mechanisms. In our study, a CCI mice model was successfully established to simulate clinical pathophysiological progress in neuropathic patients. The mechanical allodynia and thermal hyperalgesia were significantly lower after CCI, indicating the successful establishment of a CCI mice model in the present study. Our results demonstrated the activity of Nrf2 was significantly elevated in the spinal cord after CCI and Nrf2 was
mainly colocalized with neurons. These findings are consistent with our previous study showed that spinal Nrf2 was significantly increased and localized mostly in neurons in paclitaxel-induced neuropathic pain model [22]. These results indicated that CCI-induced rapid activation of the endogenous antioxidant defense system. However, Li et al. [39] illustrated that Nrf2 was only modestly activated in the DRGs of SNI models. This discrepancy may be due to the use of different tissues, different pain models, and different animals. Moreover, Nrf2 was further activated after treatment with RTA-408 (i.t. 10 μg) from day 7 to day 11, which was blocked by Nrf2 inhibitor. These data demonstrated that Nrf2 signaling is the primary target of RTA-408 for the treatment of pain hypersensitivity. To determine the preventative effect of RTA-408 in the development of CCI-induced neuropathic pain, the same dose of RTA-408 (10 μg) was given once daily in the early stage (from day 0 to day 2). The threshold of PWT and TWL was significantly elevated in RTA-treated CCI mice compared with vehicle-treated CCI mice on d 1 and d 3, but not on d 7 or d 14. We suggested that repeated treatment of RTA-408 may have a cumulative analgesic effect, leading to an increase in PWT and TWL on d 3. However, this cumulative analgesic effect may persist for no more than 1 week, leading to a decrease in PWT and TWL on d 7 and d 14. Similarly, our previous study demonstrated that repeated treatment of oltipraz in paclitaxel-induced neuropathic pain model has an accumulative analgesic effect on d 7, but not on d 14 or d 21 [22].

Nrf2 is not only a major regulator of redox homeostasis [42] but also involved in the transcriptional control of several mitochondrial genes, including NRF1, and TFAM [13]. It has been reported that Nrf2 participated in MB by activating AREs/PGC-1α signaling pathway [24,43,44]. Moreover, mitochondrial dysfunction has been observed in numerous neuropathic pain models [6,45]. In the present study, it has been observed that CCI caused a significant decrease in mtDNA and several mitochondrial genes, including PGC-1α, NRF1, and TFAM in a time-dependent manner.

PGC-1α is a critical transcriptional coactivator that regulates MB [13]. Kashiwagi et. al. [46] first showed that recombinant PGC-1α (rPGC-1α) could increase mechanical allodynia and thermal hyperalgesia in morphine tolerance rats. In this study, we explored the effect of PGC-1α in CCI-induced pain mice. Our results found that repeated injection of PGC-1α activator ZLN005 (10 μg, i.t.) from day 7 to day 11 significantly reversed the established pain hypersensitivity in CCI mice. To explore the preventative of ZLN005 in the development of CCI-induced neuropathic pain, the same dose of ZLN005 (10 μg, i.t.) was administered in the early phase (from day 0 to day 2). Our results showed that mice pretreated with ZLN005 showed upregulated PWT and TWL on day 1, day 3 and 7, but not day 14. These results suggested that early administration of ZLN005 (10 μg, i.t.) significantly delayed the onset of CCI-induced mechanical allodynia and thermal hyperalgesia. Moreover, inhibition of spinal PGC-1α significantly reversed the analgesic effect of ZLN005, illustrating that PGC-1α plays a key role in pain hypersensitivity following CCI.

To further explore the role of PGC-1α mediated MB in the development of CCI-induced pain hypersensitivity, mitochondrial proliferative markers (including PGC-1α, NRF1, and TFAM) were examined after treatment with RTA-408. As expected, RTA-408 significantly reversed the downregulation of mtDNA and several mitochondrial genes, including PGC-1α, NRF1, and TFAM. Further, these protective effects of
RTA-408 were blocked by Nrf2 inhibitor. These results indicated that RTA-408 could stimulate MB via Nrf2 signaling in the spinal cord of CCI mice. Several studies have demonstrated the existence of regulatory loops between Nrf2 and PGC-1α in MB [47]. Whether Nrf2 is directly involved in regulating this process is still controversial. Thus, the PGC-1α inhibitor was preinjected 30 min before RTA-408 treatment in the CCI model to assess the mechanical allodynia and thermal hyperalgesia. Data showed that inhibition of spinal PGC-1α significantly reversed the analgesic effect of RTA-408, illustrating that Nrf2 regulated the expression of PGC-1α. These results suggested that RTA-408 stimulated MB in CCI mice through the Nrf2 signaling pathway, and the mechanism was dependent on PGC-1α.

In summary, our study provided evidence that RTA-408 could attenuate CCI-induced neuropathic pain via induction of PGC-1α-mediated MB in the spinal cord dorsal horn. Moreover, our results suggested the involvement of PGC-1α in modulating the development of neuropathic pain following CCI. Although the precise mechanism by which RTA-408 mediates neuropathic pain needs to be further investigated, our findings suggested a possible therapeutic pathway to stimulate MB while improving neuropathic pain hypersensitivity.

**Declarations**

**Authors’ contributions**

WM and YQZ conceived the project and supervised all experiments. JS, DYL and BYX analyzed data, prepared figures and wrote manuscripts. LQZ, JYW and SJG performed experiments on behavioral tests, western blot, and immunofluorescence. JYL and SZ revised manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

All experiments were approved by the Experimental Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology, and were in agreement with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Consent for publish**

Not applicable.

**Availability of data and materials**

The data that support the findings of the current study are available from the corresponding author upon reasonable request.

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Competing interests

The authors have no conflicts of interest to declare.

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Figures
Figure 1

Effect of RTA-408 on the treatment and prevention of mechanical pain and hyperalgesia after chronic constriction injury (CCI). (A, B) Mechanical allodynia and thermal hyperalgesia were used to detect the paw withdrawal threshold (PWT) and thermal withdrawal latency (TWL). PWL and PWT were conducted before CCI (day 0), and at day 1, day 3, day 7, and day 14 after surgery. (*P < 0.05, **P < 0.01, ***P < 0.001 compared with the Sham group, n = 5 per group). (C, D) A single injection of RTA-408 (RTA, 1 μg, 5 μg, and 10 μg/5 μl, i.t.) or vehicle (5 μl) was given on day 7 following CCI. (*P < 0.05, **P < 0.01, ***P < 0.001 compared with the Sham group, n = 5 per group).
compared with the CCI + Vehicle group, ###P < 0.001 compared with the group treated with RTA-408 (5 μg). In contrast, CCI + Vehicle group had no significant change in PWT and TWL (###P < 0.001 compared with the Sham + Vehicle group). No significant difference in the baseline thresholds was observed among all groups. (n = 5 per group) (E, F) RTA-408 (1 μg, 5 μg, and 10 μg/5 μl, i.t.) or vehicle (5 μl) was given for 5 consecutive days from day 7 to day 11. (***P < 0.001 compared with the CCI + Vehicle group, #P < 0.05, ##P < 0.01, ###P < 0.001 compared with the group treated with RTA-408 (5 μg)). In contrast, CCI + vehicle group had no significant change in PWT and TWL (###P < 0.001 compared with the Sham + Vehicle group) (n = 5 per group). (G, H) Preventive effect of RTA-408 on the development of pain hypersensitivity following CCI. RTA-408 (10 μg, i.t.) was given once daily from day 0 to day 2 after CCI. The pain behavioral tests were performed before CCI and on day 1, day 3, day 7, and day 14 after CCI. (*P < 0.05 compared with CCI + Vehicle mice, n = 5 per group).
Time-course of Nrf2 expression in the spinal cord after CCI. (A) Western blotting showed the time course of Nrf2 protein in the nuclear extracts (*P < 0.05, ***P < 0.001 compared with indicated group, n = 5 per group) (B) Representative fluorescent photomicrographs showed the time-course expression of Nrf2 and nuclei in the ipsilateral spinal cord dorsal horn following CCI. (Scale bar = 100 μm, n = 3 per group).
Effect of RTA-408 on the spinal expression of Nrf2 in the nuclear extracts of CCI mice. The spinal expression of Nrf2 in the nuclear extracts was significantly increased following CCI. RTA-408 administration further increased the spinal expression of Nrf2 in the nuclear extracts in the sham group and CCI group (**P < 0.01, ***P < 0.001 compared with indicated group, n = 5 per group).

**Figure 4**

Effect of Nrf2 inhibitor on pain hypersensitivity and the spinal expression of Nrf2 in the nuclear extracts.

(A, B) The analgesic effect of RTA-408 (10 μg, i.t.) in CCI mice was completely inhibited by the Nrf2 inhibitor trigonelline (Trig, *P < 0.05, **P < 0.01, ***P < 0.001 compared with the Sham + Vehicle group, #P < 0.05, ##P < 0.01, ###P < 0.001 compared with the CCI + RTA + Trig group, n = 5 per group). (C) The spinal expression of Nrf2 in the nuclear extracts was significantly increased following CCI (***P < 0.001 compared with the Sham + Vehicle group). RTA-408 administration further increased the spinal expression of Nrf2 in the nuclear extracts in the CCI group (***P < 0.001 compared with the CCI + Vehicle group, n = 5 in each group), which was significantly inhibited by the preinjection of trigonelline (***P < 0.001 compared with the CCI + RTA-408 group, n = 5 per group).
Figure 5

Distribution of Nrf2 in the spinal cord following CCI. Nrf2 was mainly colocalized with neurons (NeuN, a neuronal marker, red) in the spinal cord dorsal horn. (Scale bar = 200 μm, n = 3 per group).
Figure 6

Time-course of mitochondrial protein and mtDNA content in the spinal cord following CCI. The spinal cord was collected and analyzed for (A) mitochondrial protein expression and (B) mtDNA content following CCI. (*P < 0.05, **P < 0.01, ***P < 0.001 compared with the Sham group).
Figure 7

Effect of ZLN005 on the treatment and prevention of mechanical pain and hyperalgesia after CCI. (A, B) A single injection of ZLN005 (1 μg, 5 μg, and 10 μg/5 μl, i.t.) or vehicle (5 μl) was given on day 7 following CCI. (***P < 0.001 compared with the CCI + Vehicle group). In contrast, CCI + Vehicle group had no significant change in PWT and TWL (###P < 0.001 compared with the Sham + Vehicle group). No significant difference in the baseline thresholds was observed among all groups. (n = 5 per group) (C, D) ZLN005 (1 μg and 10 μg/5 μl, i.t.) or vehicle (5 μl) was given for 5 consecutive days from day 7 to day 11. Treatment with ZLN005 (10 μg) significantly reversed PWT and TWL in CCI mice (***P < 0.001 compared...
with the CCI + Vehicle group). In contrast, CCI + Vehicle group had no significant change in PWT and TWL (###P < 0.001 compared with the Sham + Vehicle group) (n = 5 per group). (E, F) Preventive effect of ZLN005 on the development of CCI. ZLN005 (10 μg, i.t.) was given once daily from day 0 to day 2 after CCI. The pain behavioral tests were performed before CCI and on day 1, day 3, day 7, and day 14 after CCI. Treatment with ZLN005 (10 μg, i.t.) significantly elevated the PWT and TWL at day 3 and day 7 after CCI. However, no significant difference was observed on day 14. (*P < 0.05, **P < 0.01, ***P < 0.001 compared with CCI + Vehicle mice) (n = 5 per group). (G, H) The analgesic effect of ZLN005 in CCI mice was completely inhibited by the PGC-1α inhibitor SR-18292 (SR, ***P < 0.001 compared with the Sham + Vehicle group, ###P < 0.001 compared with the CCI + ZLN + SR group, n = 5 per group).
Figure 8

Effect of RTA-408 on mitochondrial protein and mtDNA content and in the spinal cord following CCI. The spinal cord was extracted and analyzed for (A) mitochondrial protein expression of PGC-1α, NRF1, and TFAM and (B) mtDNA content. (*P < 0.05, **P < 0.01, ***P < 0.001 compared with indicated group, n = 5 per group).
Figure 9

Effect of trigonelline on mitochondrial protein and mtDNA in the spinal cord following CCI. The spinal cord was extracted and analyzed for (A) mitochondrial protein expression of PGC-1α, NRF1, and TFAM and (B) mtDNA content. (**P < 0.01, ***P < 0.001 compared with indicated group, n = 5 per group).
Figure 10

Effect of PGC-1α inhibitor on the analgesic effect of RTA-408. The analgesic effect of RTA-408 in CCI mice was completely inhibited by the PGC-1α inhibitor SR-18292 (***P < 0.001 compared with the Sham + Vehicle group, ###P < 0.001 compared with the CCI + RTA + SR group, n = 5 per group).

Supplementary Files

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