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Ginsenoside Rh2 Ameliorates Neuropathic Pain by inhibition of the miRNA21-TLR8-mitogen-activated protein kinase axis

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Abstract

Ginsenoside Rh2 is one of the major bioactive ginsenosides in *Panax ginseng*. Although Rh2 is known to enhance immune cells activity for treatment of cancer, its anti-inflammatory and neuroprotective effects have yet to be determined. In this study, we investigated the effects of Rh2 on spared nerve injury (SNI)-induced neuropathic pain and elucidated the potential mechanisms. We found that various doses of Rh2 intrathecal injection dose-dependently attenuated SNI-induced mechanical allodynia and thermal hyperalgesia. Rh2 also inhibited microglia and astrocyte activation in the spinal cord of a murine SNI model. Rh2 treatment inhibited SNI-induced increase of proinflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-1 and IL-6. Expression of miRNA-21, an endogenous ligand of Toll like receptor (TLR)8 was also decreased. Rh2 treatment blocked the mitogen-activated protein kinase (MAPK) signaling pathway by inhibiting of phosphorylated extracellular signal-regulated kinase expression. Finally, intrathecal injection of TLR8 agonist VTX-2337 reversed the analgesic effect of Rh2. These results indicated that Rh2 relieved SNI-induced neuropathic pain via inhibiting the miRNA-21-TLR8-MAPK signaling pathway, thus providing a potential application of Rh2 in pain therapy.

Keywords

Ginsenoside Rh2, spared nerve injury, Chronic pain, miRNA-21, TLR8

Introduction

Ginseng (*Panax ginseng* C.A. Meyer) is a traditional Oriental herbal drug widely distributed in East Asia. Ginsenosides as the main active constituents have various pharmacological activities, such as anti-depressive,[1] antipruritic,[2] anti-inflammatory,[3] antiallergic[4] and anticancer[5] activities. Previous phytochemical and pharmacological investigations have demonstrated the antinociceptive effects of ginseng extracts in various pain models including those of abdominal,[6] neuropathic,[7] chronic[8] and incisional[9] pain. Several different mechanisms of action have been suggested to explain these effects, including reduced neural hypersensitivity, antagonism of adrenergic activation, and inhibition of microglial activation.[10–12]

Ginsenoside Rh2 is one of the major bioactive ginsenosides in *P. ginseng*, and has especially been used in the treatment of cancer.[5] In terms of structure, Rh2 can be divided into S-type and R-type configurations, among which 20(S)-Rh2 monomer is the main configuration isolated from medical herbs and plays a major role in anticancer activity [13] (Figure 1). Previous studies have revealed that ginsenoside Rh2 significantly inhibited

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lipopolysaccharide (LPS)-induced activation of BV2 cells and decreased production of inflammatory mediators via modulating the transforming growth factor-β1/Smad pathway.[14] However, the potential effect of Rh2 on neuropathic pain has not been investigated, and its mechanisms of action remain largely unknown.

Toll-like receptors (TLRs) are reported to play a critical role in the innate and adaptive immune responses.[15] After binding with ligands, TLRs initiate and regulate the inflammatory response via the release of cytokines.[16] TLR8 belongs to the TLR family and is important for viral single-stranded RNA recognition, neurite outgrowth and immune cell regulation.[17–19] Our previous study demonstrated that TLR8 was located in the endoplasmic reticulum, endosomes, and lysosomes of dorsal root ganglion (DRG) neurons, which was activated by endogenous miRNA-21 and contributed to the pathogenesis of spinal-nerve-injury-induced neuropathic pain.[20] Ginsenoside Rh2 was previously reported to mediate miRNA expression in human non-small cell lung cancer cells.[21] Thus, this study aimed to explore the effects of Rh2 on spared nerve injury (SNI)-induced neuropathic pain. Our results demonstrated that Rh2 specifically attenuated miRNA-21 to inhibit TLR8 activation and relieve nociceptive pain in SNI mice. Also, the anti-inflammatory effect of Rh2 was achieved by inhibiting the production of inflammatory cytokines through blocking the mitogen-activated protein kinase (MAPK) signaling pathways. This study provided a potential medicinal application of Rh2 in pain therapy.

Materials and Methods

Experimental animals and treatments

Adult male ICR mice (age: 8 weeks, weighing 26–30 g) were provided by the Experimental Animal Center at Nantong University. *Tlr8-/-* mice were developed by Cyagen (Suzhou, China). All experiments were approved by the Animal Care and Use Committee of Nantong University. Mice were kept in each cage in a 12-h light-dark cycle with free access to food and water. Animal treatments were performed in accordance with the guidelines of the International Association for the Study of Pain.

SNI-induced neuropathic pain model

The neuropathic pain model was induced by SNI. The operation was performed as described by Decosterd and Woolf.[22] After disinfecting the surgical site with alcohol and iodine, careful blunt dissection was performed through the biceps femoris muscle to expose the sciatic, sural, tibial and common peroneal nerves. The tibial and common peroneal nerves were tightly ligated with 6.0 silk suture and then completely severed in between, leaving the sural nerve intact. After transection, the muscle fascia and skin were sutured.

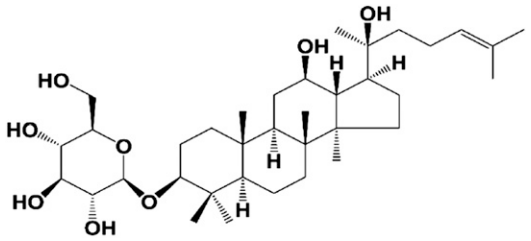

Figure 1. Chemical structure of ginsenoside Rh2.

Drugs and administration

Ginsenoside Rh2 (Figure 1) was purchased from Shanghai Source Leaf Biology Co. Ltd. and dissolved in 0.01 M phosphate-buffered saline (PBS) containing 0.4% dimethyl sulfoxide, with an intrathecal injection concentration of 100 μmol. VTX-2337 was purchased from Active Biochem (Hong Kong, China) with an intrathecal injection concentration of 100 ng. Intrathecal-injection was made with a 30 G needle between the L5 and L6 intervertebral spaces to deliver the reagents to the cerebrospinal fluid. Rh2 (100 μL) or 10 μL VTX-2337 was injected intrathecally.

Pain behavior analysis

The von Frey test was performed to measure paw mechanical sensitivity.[23] The mice were put in boxes on an elevated metal mesh floor and allowed 30 min for habituation before the examination. The plantar surface of the hind paw was stimulated with a series of von Frey hairs with logarithmically incrementing stiffness (0.02–2.56 g, North Coast). The 50% paw withdrawal threshold was determined using Dixon's up-down method.[24]

The Hargreaves test was used to detect paw thermal sensitivity.[25] The mice were put in a plastic box placed on a glass plate, and the plantar surface was exposed to a beam of radiant heat through a transparent glass surface. The baseline latencies were adjusted to 10–14 s with a maximum of 20 s as a cutoff to prevent potential injury.[26] All the behavioral experimenters were done by individuals that were blinded to the treatment or genotypes of the mice.

RNA isolation and quantitative real-time polymerase chain reaction for mRNAs and microRNAs

Total RNA from L4 or L5 DRG was extracted using a Total RNA Extraction Kit (Easy-spin™, Intron, USA) and reverse transcribed into cDNAs using GoScript™ Reverse Transcriptase (Promega, Madison, WI, USA). Tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and GAPDH were amplified by using DreamTaq DNA Polymerases (ThermoFisher, Waltham, MA, USA). For miRNA detection, small RNAs were extracted using the RNAiso kit (Takara, Shiga,

Japan), and 10 ng small RNA was reverse transcribed into cDNA using the One Step PrimeScript microRNA cDNA Synthesis kit (Takara). GAPDH and U6 small nuclear RNA were used as endogenous controls to normalize differences for mRNA and miRNA detection, respectively. Melt curves were performed upon completion of the cycles to ensure that nonspecific products were absent. Quantification was performed by normalizing target gene cycle threshold (Ct) values with corresponding GAPDH Ct (mRNA) or U6 Ct (miRNA), and then analyzed with the $2^{-\Delta\Delta Ct}$ method. The sequences of primers are shown in Table 1.

Table 1. The list of primer sequences designed for quantitative real-time RT-PCR.

Gene	Primers	Sequences (5′–3′)
TNF-α	Forward	GTTCTATGGCCCAGACCCTCAC
	Reverse	GGCACCACTAGTTGGTTGTCTTTG
IL-1β	Forward	TCCAGGATGAGGACATGAGCAC
	Reverse	GAACGTCACACACCAGCAGGTTA
IL-6	Forward	CCACTTCACAAGTCGGAGGCTTA
	Reverse	CCAGTTTGGTAGCATCCATCATTTC
miR-21	Forward	TAGCTTATCAGACTGATGTT
	Reverse	GGCCAACCGCGAGAAGATGTTTTTTTTT
U6	Forward	GCTTCGGCAGCACATATACTAA
	Reverse	CGAATTTGCGTGTCATCCTT
Gapdh	Forward	AAATGGTGAAGGTCGGTGTGAAC
	Reverse	CAACAATCTCCACTTTGCCACTG
Iba-1	Forward	ATGAGCCAAAGCAGGGATT
	Reverse	CTTCAAGTTTGGACGGCAG
GFAP	Forward	CCAAGATGAAACCAACCTGA
	Reverse	TCCAGCGATTCAACCTTTC

Immunofluorescence

For tissue immunofluorescence staining,[27] the spinal cord and L4–L6 DRG were dissected from 8-week-old ICR mice. The mice were killed at 1 h after drug injection. The samples were fixed with 4% paraformaldehyde and frozen in Tissue-Tek O.C.T. The samples were cut into 15-μm thick sections. The cryosections were incubated with blocking buffer [4% bovine serum albumin (BSA) in PBS with 0.01% Triton X100] for 2 h and with polyclonal Iba-1 (rabbit, 1:1000; Wako/Fujifilm, Richmond, VA, USA), GFAP (mouse,1:2,000; Millipore, Burlington, MA, USA), c-Fos (rabbit,1:1,000; Abcam, Cambridge, UK), or phosphorylated extracellular signal-regulated kinase (pERK) (rabbit, 1:5,000; Abcam) antisera overnight at 4°C. After being washed for 15 min, the samples were incubated with the secondary antibodies Donkey anti-mouse 488 (1:1,000; Jackson ImmunoResearch, West Grove, PA, USA), Donkey anti-rabbit 488 (1:1,000; Jackson ImmunoResearch). The fluorescence signals were checked and captured under a fluorescence microscope (Eclipse Ni-E; Nikon, Tokyo, Japan). The fluorescence images were analyzed with ImageJ (NIH, Bethesda, MD, USA). Analysis methods for fluorescence images have been described previously.[28] After selecting the fluorescence regions and choosing no fluorescence area as the background, ImageJ automatically generated the immunohistochemistry index. The number of c-Fos-positive neurons in the dorsal horn of the spinal cord ipsilateral to the SNI surgery site was counted as the level of neural activity. Three sections were randomly picked from each mouse for immunohistochemical analysis.

In situ hybridization

Expression of miR-21 in DRG was determined by using the mature mouse miR-21 (mmu-miR-21a-5p) detection probe as previously reported.[29] DRG sections were treated with proteinase K (BosterBio, Pleasanton, CA, USA) for 2 min at room temperature, and then washed in RNase-free PBS. After digestion with proteinase, sections were post-fixed with the 1% paraformaldehyde/PBS and washed in diethyl-pyrocarbonate-treated ultrapure water. After prehybridization and hybridization, sections were washed with 2× saline sodium citrate (SSC), 0.5× SSC and 0.2× SSC buffers. To identify the colocalization of miR-21 and neuronal nuclei (NeuN), the above sections under miR-21 in situ hybridization (ISH) were incubated overnight at 4°C with primary antibodies against NeuN. On the following day, secondary antibody was added and incubated for 2 h. The signal was detected with a fluorescence microscope (Eclipse Ni-E).

Western blotting

Western blotting was performed as described previously.[30] After the mice were killed and perfused with saline intracardially at 1 h after drug injection, the L4–L6 DRG were homogenized in lysis buffer containing phosphatase and protease inhibitors (Sigma-Aldrich, St Louis, MO, USA). The protein concentration was measured by BCA Protein Assay (Thermo Fisher Scientific). Protein samples (30 μg) were loaded in each lane of SDS-PAGE, and transferred to polyvinylidene difluoride membranes. After blocking with 5% BSA solution, the membranes were incubated with primary antibody against pERK or ERK (rabbit, 1:1,000; Cell Signaling Technology, Danvers, MA, USA), and GAPDH (mouse, 1:10,000; Millipore). The membranes were further incubated with secondary antibody (goat-anti-rabbit,1:10,000; LI-COR, Lincoln, NE, USA) and images were detected by Odyssey CLx system (Odyssey, LICOR, USA). The intensity of bands was statistically analyzed by ImageJ.

Statistics

Data are shown as mean ± SEM. The behavioral data were analyzed by two-way ANOVA. Differences between groups were compared using one-way ANOVA or Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Ginsenoside Rh2 attenuates SNI-induced pain

To test the antinociceptive effect of Rh2 in SNI-induced chronic pain, we first tested the effect of single intrathecal injection of Rh2 at a high concentration, but it did not affect neuropathic pain (data not shown). Then, we injected different doses of Rh2 daily for 6 consecutive days (Figure 2(a)). The development of mechanical allodynia and thermal hyperalgesia was evaluated for 10 days after surgery (Figure 2(b) and (c)). SNI significantly induced mechanical allodynia by decreasing the mechanical stimulus threshold from day 1 after surgery (Figure 2(b)). Intrathecal administration of Rh2 significantly attenuated mechanical allodynia at 100 μM but not at 1 μM (Figure 2(b)). Rh2 also attenuated SNI-induced thermal hyperalgesia. The antinociceptive effect of Rh2 continued until 10 days after SNI surgery (Figure 2(c)). These results suggested that Rh2 could relief SNI-induced mechanical allodynia and thermal hyperalgesia.

Ginsenoside Rh2 inhibits spinal microglia and astrocyte activation in the SNI model

Glia cells in the spinal cord dorsal horn (SCDH) play important roles in the development and maintenance of chronic neuropathic pain.[31,32] Therefore, we proceeded to explore the effect of Rh2 on astrocytic and microglial activation in SCDH of SNI model mice after drug (or vehicle) continuous treatment 6 days. We checked immunoactivity of glial fibrillary acidic protein (GFAP; an astrocyte marker) and ionized calcium binding adaptor molecule 1 (Iba-1; a microglia marker) in SCDH of vehicle or Rh2 treatment groups. We found strong increases in Iba-1 (or GFAP) immunoactivity (Figure 3(a), (b), (f), (g)) and the soma area (Figure 3(c), (h)) of Iba-1 (or GFAP) positive cells after SNI. However, Rh2 treatment significantly attenuated Iba-1 (or GFAP) immunoactivity (Figure 3(b), (g)). Quantification of morphological parameters showed that compared to controls, SNI surgery increased the microglia in soma area and reduced the process length in the SCDH, which was reversed by intrathecal Rh2(Figure 3(c), (d)), but not for astrocytes (Figure 3(h), (i)). qPCR of Iba-1 and GFAP confirmed the expression levels of Iba-1 and GFAP after SNI and decrease by Rh2 treatment (Figure 3(e), (j)). These results indicated that Rh2 treatment suppressed microglial and astrocytic activation to attenuate pain hypersensitivity induced by SNI.

Inhibitory effect of ginsenoside Rh2 on release of proinflammatory cytokines in the SNI model

TNF-α, IL-1β and IL-6 are important proinflammatory cytokines in mediating peripheral sensitization and neuropathic pain. The release of proinflammatory cytokines was increased after SNI.[33,34] To check whether the antinociceptive effect of Rh2 was associated with downregulation of proinflammatory cytokines, we measured expression of TNF-α, IL-1β and IL-6 in DRG after Rh2 continuous treatment 6 days. qPCR showed

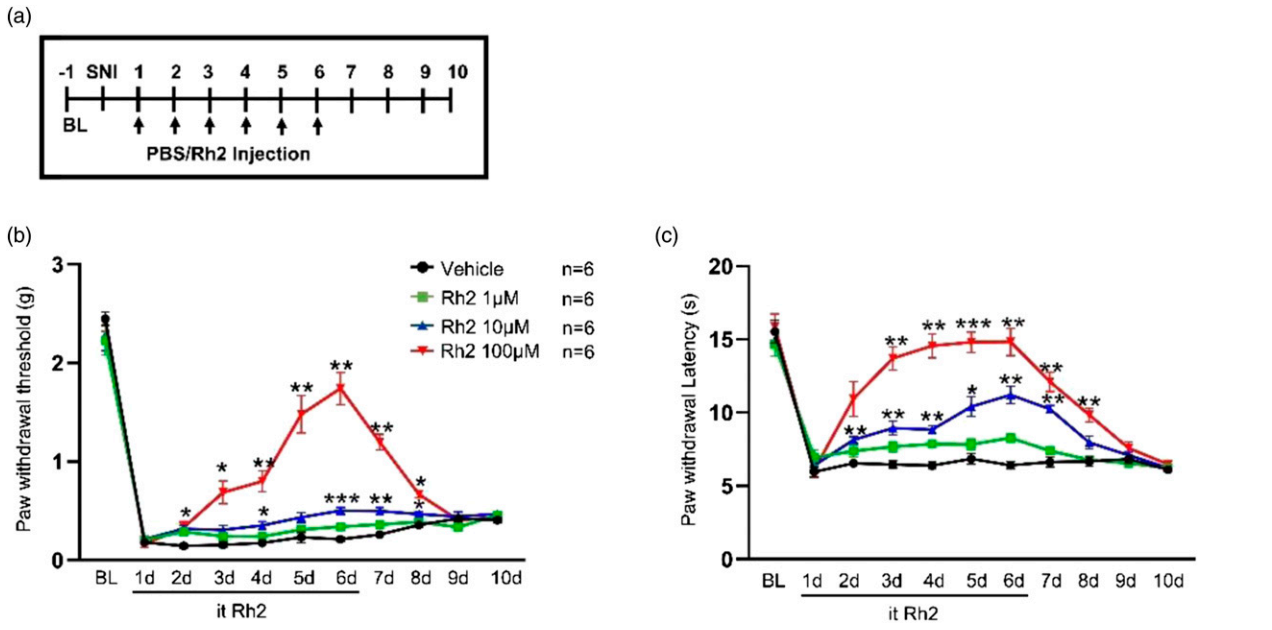

Figure 2. Intrathecal injection of Rh2 inhibits neuropathic pain in mice. (a) Schematic diagram of the timeline for SNI surgery, drug treatment, and behavioral testing. (b) Mechanical allodynia and (c) Thermal hyperalgesia of SNI mice treated with various doses of Rh2 or vehicle. Two-way ANOVA followed by Bonferroni's test was used for statistical comparison. n = 6 mice for each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

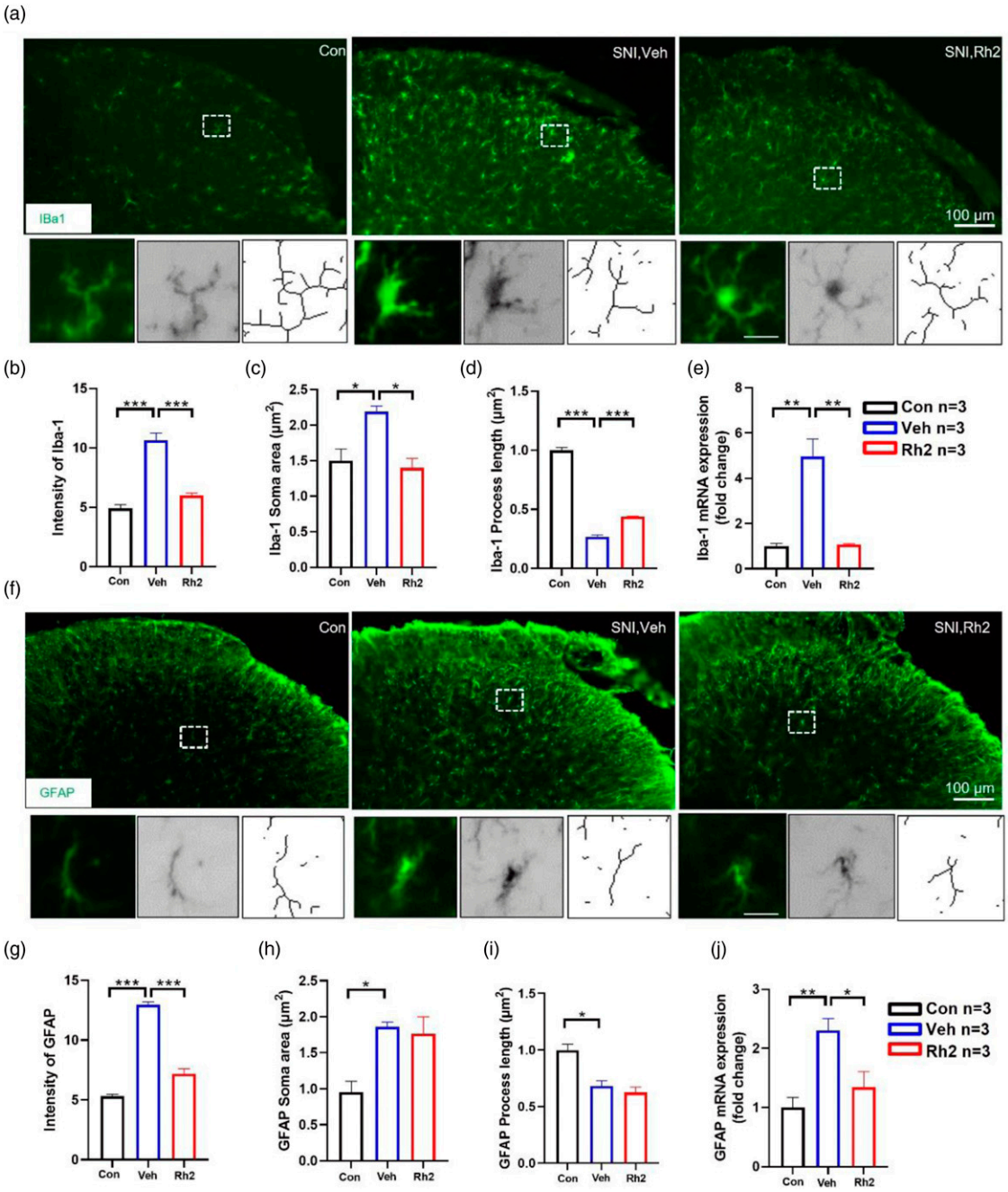

Figure 3. Ginsenosides Rh2 inhibits microglial and astrocytic activation in SNI model. (a and f) The immunohistochemistry showed that Rh2 treatment significantly decreased Iba-1(or GFAP) expression in the SDH of SNI mice after 6 days of continuous drug treatment. Scale bar=100μm. The insets showed the magnified fluorescent, binary, and skeletonized images of cropped cell corresponding to the white box. Scale bar=20μm. (b) Intensity of Iba-1. (c, d) Microglia morphological analysis showed that Rh2 reversed SNI-induced upregulation of Iba-1 positive cell area(c) and reduction of process length (d). (e) mRNA expression level of Iba-1. (g) Intensity, (h) Soma area and (i) Process length of GFAP cell. (j) mRNA expression level of GFAP. One-way ANOVA followed by the Bonferroni test. n = 3 for each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

that mRNA expression of these proinflammatory cytokines in the Rh2 treatment group was lower than in the vehicle group (Figure 4). These results indicated that the antinociceptive effect of Rh2 may act by preventing the expression of proinflammatory cytokines.

Ginsenosides Rh2 attenuates miRNA-21 increase in DRG after SNI

The miRNA-21 was reported to be secreted from tumors as a ligand to TLR8.[35] Our previous study revealed that miRNA-

21 was involved in neuropathic pain development by activating TLR8 in DRG.[20] We investigated whether Rh2 affected miRNA-21 expression after SNI, by measuring expression of miRNA-21 in DRG by ISH and immunostaining (Figure 5(a)). Expression of miRNA-21 was significantly reduced after Rh2 treatment (Figure 5(b)), which was consistent with PCR results (Figure 5(c)), indicating that Rh2 manifested its effects on reducing chronic pain partially through suppression of miRNA-21 upregulation.

Ginsenoside Rh2 relieves neuropathic pain by suppressing TLR8 activation

Because Rh2 can suppress miRNA-21 expression, we confirmed the effect of Rh2 on TLR8 activation. Continuous intrathecal injection of Rh2 inhibited mechanical allodynia and hyperalgesia in SNI model mice. However, intrathecal injection of TLR8 agonist VTX-2337 reversed the analgesic effect of Rh2 but not in TLR8 knockout mice

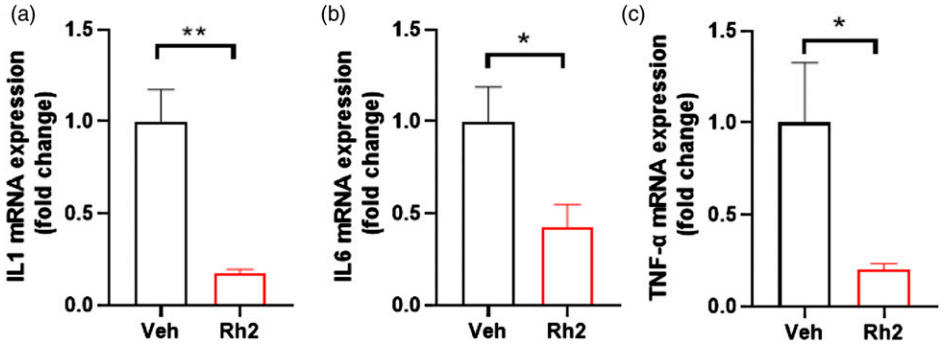

Figure 4. Intrathecal injection of Rh2 reduced expression of proinflammatory cytokines IL-1, IL-6 and TNF-α in SNI mice. (a - b) Effects of Rh2 on the mRNA expression levels of proinflammatory cytokines (a) IL-1, (b) IL-6 and (c) TNF-α. The Unpaired *t*-test was conducted for two group comparisons. n = 5 mice for each group. $^{*}p < 0.05$, $^{**}p < 0.01$.

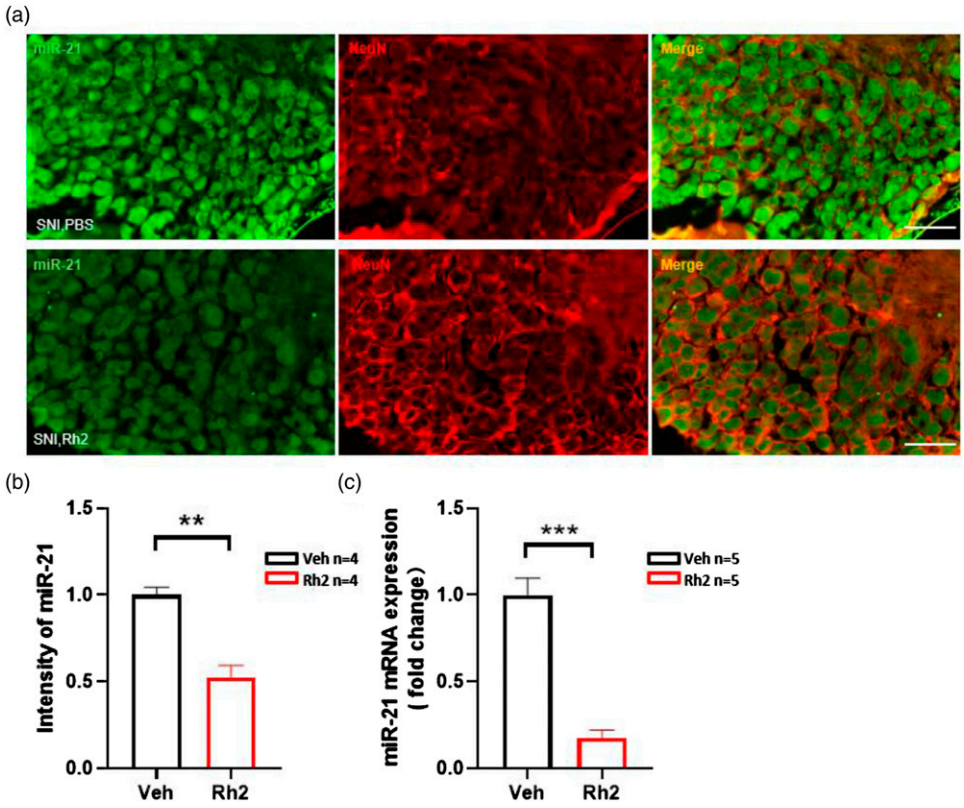

Figure 5. Expression of miRNA-21 was significantly reduced after Rh2 treatment. (a) Immunostaining for Neuron marker NeuN and ISH for miRNA-21 in DRG. Scale bar=100 μm. (b) Statistical data show the miRNA-21 staining intensity in the DRG. n = 4 for each group. (c) Expression level of miRNA-21. Unpaired *t*-test was performed for two group comparisons. n = 5 for each group, $^{**}p < 0.01$, $^{***}p < 0.001$.

(Figure 6(a)). These data confirmed that Rh2 attenuates SNI-induced pain hypersensitivity through inhibition of TLR8 activation.

Ginsenoside Rh2 inhibits SNI-induced pERK and c-Fos activation in DRG

We further determined whether the analgesic effects of Rh2 were associated with inhibition of the pERK signaling pathway. We used western blotting to check the expression of phosphorylation of ERK in DRG. Western blotting showed that expression of pERK was significantly increased after SNI surgery; however, Rh2 treatment decreased SNI-induced pERK upregulation (Figure 7(a) and 7(b)). We also used immunofluorescence to confirm this result, which was consistent with the western blotting results (Figure 7(c)–(e)). The c-Fos is encoded by the proto-oncogene *c-fos* and has been extensively used as a marker for neuronal activity in various regions including in the spinal cord.[36] We therefore sought to assess the change in neuronal activity of dorsal horn neurons of the L4–L5 spinal cord using c-Fos immunohistochemistry. c-Fos immunoreactivity was observed throughout the ipsilateral dorsal horn of the lumbar spinal cord after SNI (Figure 7(f)). However, the number of c-Fos-positive neurons in the dorsal horn was significantly lower in Rh2 treated mice (Figure 7(g)), which suggested that Rh2 reduced SNI-induced neural hyperactivity through inhibition of the MAPK signaling pathway.

Discussion

In the current study, we found a novel effect of Rh2 on neuropathic pain and its antinociceptive mechanism. Our results demonstrated that intrathecal administration of Rh2 relieved SNI-induced mechanical allodynia and thermal hyperalgesia. In addition, Rh2 administration attenuated SNI-induced microglia and astrocyte activation in the spinal cord and reduced miRNA-21 expression. Finally, we also found that Rh2 reduced SNI-induced neural hyperactivity through inhibition of the MAPK signaling pathway.

Previous studies have shown that ginsenoside Rh2 had anti-inflammatory and anticancer activities and acts against atopic dermatitis by modulating T helper type 2 differentiation.[37,38] However, the role of Rh2 in neuropathic pain has not yet been studied. We first demonstrated that repeated intrathecal injection of Rh2 attenuated SNI-induced thermal hyperalgesia and mechanical allodynia. These findings indicated that Rh2 had analgesic properties.

The Toll like receptor (TLR) family is a group of 13 members with various physiological as well as pathological functions (such as mediating innate immunity).[39] Recent research has investigated the functional role of TLRs in pain and itch.[40] Several TLRs are expressed in DRG neurons and glial cells under chronic pain conditions. TLR2 and TLR4 modulate glial activation in the spinal cord and contribute to the development of neuropathic pain.[41] TLR3 is expressed in small nociceptive DRG neurons, and regulates sensory neuronal excitability and spinal synaptic transmission.[42] TLR7 is co-expressed with transient receptor potential ankyrin subtype 1 protein (TRPA1)

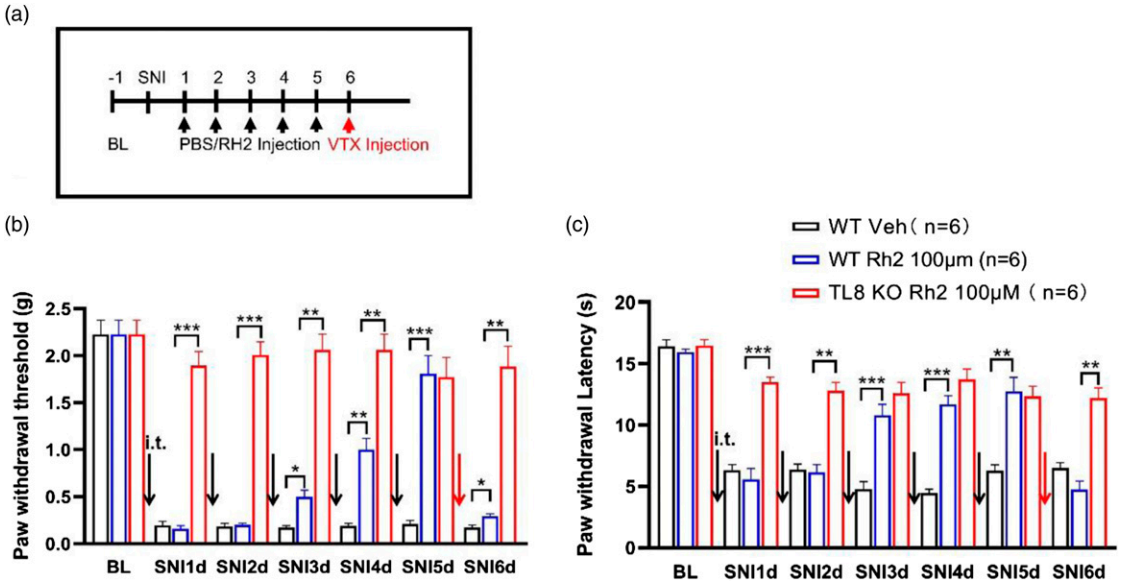

Figure 6. Rh2 relieved SNI-induced pain hypersensitivity through inhibition of TLR8. (a) Schematic diagram of the timeline for SNI surgery, drug treatment, and behavioral testing. (b) Mechanical allodynia and (c) Thermal hyperalgesia in WT or Tlr8$^{-/-}$ mice induced by VTX-2337(100ng). The mice were treated with of Rh2(100 μM) or vehicle. Two-way ANOVA followed by Bonferroni's test was used for statistical comparison. n = 6 mice for each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

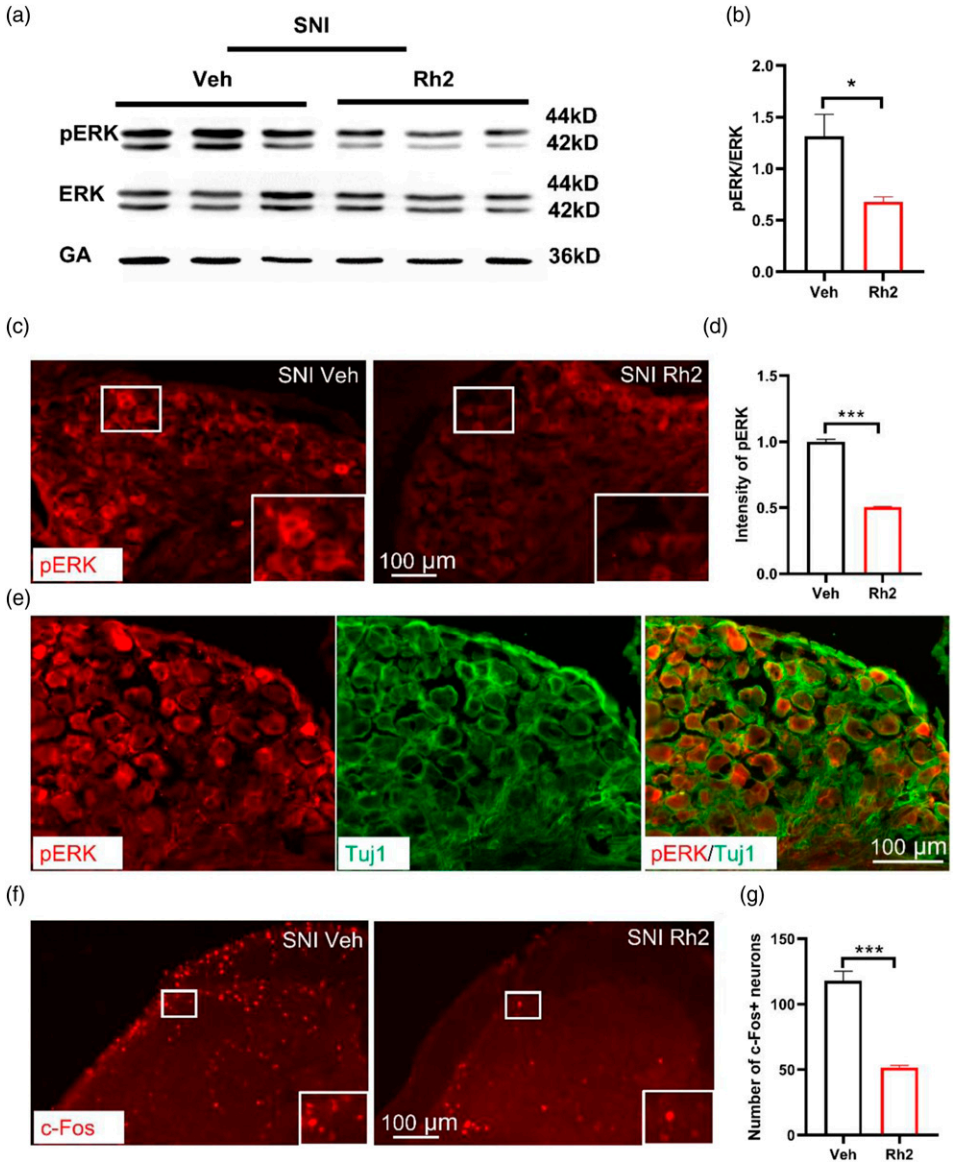

Figure 7. Intrathecal injection of Rh2 inhibits pERK and c-fos activation. (a and b) pERK expression of SNI model mice treated or untreated with Rh2. pERK expression was increased in DRG after SNI surgery, and inhibited by Rh2. n=3 for each group, The Unpaired *t*-test was conducted for two group comparisons. *$p < 0.05$. (c) Immunostaining of pERK in the DRG; (d) Statistical data show the pERK staining intensity in the DRG, n = 3 for each group, The Unpaired *t*-test was conducted for two group comparisons. ***$p < 0.001$. (e) Co-expression of pERK and a neuronal marker, β-tublin (Tuj1) in the DRG at 6 days after SNI. (f) Immunostaining of c-Fos in the SDH; (d) Statistical data show the number of pERK positive neurons, n = 3 for each group, The Unpaired *t*-test was conducted for two group comparisons. ***$p < 0.001$. Scale bar=100 μm.

in DRG neurons and involved in both pain and itch processes.[43] TLR8 is thought to affect inflammation and neuronal apoptosis.[44] Our previous research also showed that TLR8 was mostly expressed in small DRG neurons and mediated spinal-nerve-injury-induced neuropathic pain.[20] Here, intrathecal injection of VTX, an agonist of TLR8, induced pain hypersensitivity in mice, which was inhibited by Rh2 administration, suggesting that the antinociceptive effect of Rh2 may be mediated by TLR8 inhibition.

Under the condition of nerve injury, activation of glial cells leads to the development of neuroinflammation, which is responsible for induction and maintenance of chronic pain.[45] We found that Rh2 reduced microglial and astrocytic activation induced by SNI. Besides, when the microglia and astrocytes received pain signals, inflammatory factors such as TNF-α, IL-1β and IL-6 were secreted to maintain or develop the pathology of pain. TNF-α, IL-1β and IL-6, which are proinflammatory cytokines, have been implicated in

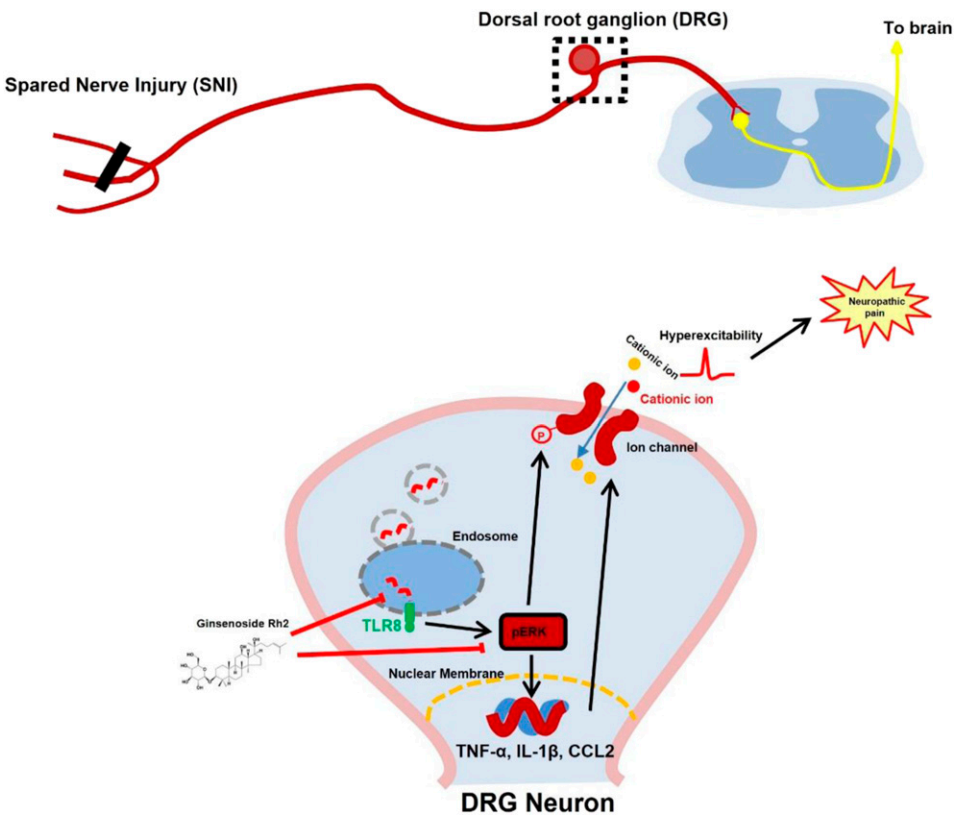

Figure 8. A mechanism of ginsenoside Rh2 relief of neuropathic pain. Ginsenoside Rh2 suppress TLR8 activation in DRG by reducing miRNA-21, and inhibiting SNI-induced pERK upregulation. The suppression of MAPK pathway leads to downregulation of proinflammatory cytokines (including TNF-α and IL-1β) and chemokines, and reduction of ion channel hyperexcitability, followed by relief of neuropathic pain.

neuropathic pain.[46] Our results showed that expression of TNF-α, IL-1β and IL-6 was significantly increased in DRG neurons after SNI and inhibited by Rh2 treatment. IL-1β expression is increased in TRPV1-positive DRG neurons following peripheral neuroinflammation. IL-6 is upregulated in DRG neurons or spinal cord following nerve-injury-induced neuropathic pain.[47] Therefore, Rh2 may relieve SNI-induced allodia and hyperalgesia though inhibition of neuroinflammation mediated by TNF-α, IL-1β and IL-6.

The miRNAs are small, noncoding RNAs, 19–24 nt in length. They can negatively regulate gene expression by canonical binding to their target mRNAs or direct interaction with proteins.[48] Recent array studies have shown that nerve injury changes expression of a variety of miRNAs in the DRG. Some miRNAs are thought to modulate nociception. For example, miR-let7b has a pronociceptive effect via mediation of neuron–neuron cross-excitation. miR-let7b is released by DRG neurons and activates TRPA1 channels to depolarize sensory neurons.[49] miRNA-21 is also upregulated in ipsilateral DRG neurons after peripheral nerve injury, which is associated with ipsilateral mechanical hypersensitivity. Downregulation of miRNA-21 prevents the development of ipsilateral mechanical hypersensitivity and decreases the number of inflammatory macrophages in DRG.[50,51] Consistent with other previous studies, we also found an increase in miRNA-21 expression in DRG neurons of SNI model mice. As an endogenous ligand of TLR8, mrRNA-21 bound to TLR8 in DRG neurons and activated downstream pathways to cause neuroinflammation. Yeung et al. found that miRNA-21 secreted by exosomes can be transferred from one cell to another.[52] Thus, we also do not exclude miRNA-21 secretion from nearby neurons. Ginsenoside Rh2 is reported to regulate miRNAs in different kinds of disease models. Wu et al. found that expression of miRNA-21 in several human glioma cell lines such as U251, T98MG and A172 was decreased after treatment with ginsenoside Rh2. An et al. also showed that ginsenoside Rh2 downregulated miRNA-21 in human non-small cell lung cancer A549 cells as part of its anticancer mechanism.[21] In the current study, we found a significant decrease in miRNA-21 in DRG neurons and relief of pain hypersensitivity after intrathecal treatment with Rh2. The possible antinociceptive mechanisms of Rh2 may because Rh2 can downregulate miRNA-21 that is induced by nerve injury, and this further inhibits TLR8 activation to relieve pain.

MAPKs, including c-Jun N-terminal kinase, p38 MAPK and ERK, are a family of serine/threonine protein kinases that transduce extracellular stimuli into intracellular post-

translational transcriptional responses.[53] A variety of extracellular stimuli activate intracellular MAPKs by phosphorylation, which modulates the intracellular responses that drive different downstream signaling. Nerve injury induces activation of ERK. In this study, we observed upregulation of pERK expression in SNI and alleviation of neuropathic pain by Rh2 was accompanied with downregulation of pERK, suggesting that the analgesic effects of Rh2 may correlate with inactivation of the MAPK signaling pathway.

In summary, the current findings supported the idea that Rh2 suppresses TLR8 activation through inhibition of miRNA-21 after SNI. The anti-inflammatory effect of Rh2 was achieved by inhibiting production of inflammatory cytokines through blocking the MAPK signaling pathway (Figure 8). Future studies will focus on how Rh2 modulates miRNA-21 and other possible pathways in nociceptive regulation. As Rh2 is involved in SNI-induced pain regulation, our study provided valuable information for its clinical application. Collectively, our findings suggested that Rh2 may exert its analgesic effect through inhibition of the miRNA21–TLR8–MAPK signaling axis.

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Author contributions

Yuan-Yuan Fu and Hao-Lin Song performed RT-PCR and animal behaviors, Jian-Ke Chen and Si-Yuan Song performed immunostaining and Western-blot and data analysis. Huan-Jun Lu initiated, designed the study. Huan-Jun Lu and Zhi-Jun Zhang wrote the manuscripts.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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