Antinociceptive effects of Ginsenoside Rb1 in a rat model of cancer-induced bone pain

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Abstract. Ginsenoside Rb1 (GRb1) is a major ingredient of ginseng, a traditional medicine that has been used for thousands of years. Previous studies have reported that GRb1 had anti-inflammatory, antioxidant and neuroprotective effects. The current study aimed to evaluate the antinociceptive effects of GRb1 in a rat model of cancer-induced bone pain (CIBP) established by intratibial injection of Walker 256 cells. Intraperitoneal injection (i.p.) of GRb1 (5 and 10 mg/kg, but not 1 mg/kg) partially and transiently reversed the mechanical allodynia and thermal hyperalgesia in CIBP rats at 14 days following surgery when the pain behavior is established. Furthermore, repeated administration of GRb1 demonstrated persistent analgesic effect. Additionally, the protein expression and immunoreactivity of iba1, which is the maker of microglia, was significantly suppressed in CIBP rats treated with GRb1 (i.p., 10 mg/kg) from day 12 for three consecutive days compared with CIBP rats treated with a vehicle. Furthermore, upregulation of spinal interleukin (IL)-1β, IL-6 and tumor necrosis factor-α were also significantly inhibited by the treatment of GRb1 (i.p., 10 mg/kg) from day 12 for three consecutive days. Together, these results indicated that GRb1 may attenuate CIBP via inhibiting the activation of microglia and glial-derived proinflammatory cytokines.

Introduction

Cancer-induced bone pain (CIBP) is a common symptom in advanced cancer patients (1). Numerous types of cancer (e.g., breast cancer, prostate cancer) have a high preference to metastasize to bone, which disrupts the process of bone remodeling and leads to significant pain (2). Currently, there are several animal models of CIBP, which promote understanding of the mechanisms of CIBP (3-5). One of the animal models that used most commonly is intratibial injection of Walker 256 cells induced CIBP (6-8). Although marked advances have been made in recent years, the mechanism underlying CIBP remains largely unclear. Therefore, it is important to find novel therapeutic strategies for the management of CIBP.

Ginsenosides are the principle active constituents in ginseng, which has been used for thousands of years in traditional medicine (9). There are a number of ingredients isolated from ginseng including Rb1, Rg3, Rg1, and Rh1 (10). These ingredients have unique functions based on differences in the chemical structures (11). As a major ingredient of ginseng, Ginsenoside Rb1 (GRb1) exhibits a wide range of functions including anti-inflammatory, antioxidant, and neuroprotective effects (12,13). It was reported that GRb1 attenuated damage to cerebral cortex neurons by down-regulating nitric oxide, superoxide, and tumor necrosis factor (TNF)-α expression in hypoxia-activated microglia (14). A recent study provided evidence that GRb1 attenuated inflammatory pain induced by intraplantar injection of formalin by inhibiting neuronal phosphorylation of extracellular signal-regulated kinase (ERK) via regulating the nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor (NF)-κB pathways (15). Furthermore, other ingredients of Ginsenoside have also been reported to have antinociceptive effects (16-19). However, whether GRb1 can alleviate CIBP remains unknown. Therefore, the present study investigated the analgesic effect of GRb1 on a well-established rat model of CIBP established by intratibial injection of Walker 256 cells.

Several lines of evidence suggest that neuroinflammation in the central nervous system serves a vital role in the development and maintenance of CIBP (20,21). Neuroinflammation under CIBP condition is characterized by activation of glial cells (e.g., microglia and astrocyte) in the spinal cord and brain, which subsequently release proinflammatory cytokines (e.g., interleukin (IL)-1β, IL-6 and TNF-α). It is well-established

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that iba1, which is the maker of microglia, is significantly unregulated during CIBP (22,23). Additionally, inhibition of microglia activation or proinflammatory cytokines release by glial cells significantly attenuated CIBP (24). It was repeatedly reported that GRb1 demonstrated an anti-neuroinflammation effect in animal models including Alzheimer's disease and post-operative cognitive dysfunction (25,26). Therefore, the present study investigated whether GRb1 could suppress the activation of microglia and the production of proinflammatory cytokines in CIBP.

Materials and methods

Animals. The present study used adult female Sprague-Dawley rats (180-200 g; Xi'an Jiaotong University, Xi'an, China). Rats were housed in a controlled lighting environment (12-h light/dark cycle) with *ad libitum* access to food and water. All the experimental protocols were approved by the Animal Care and Use Committee of Baoji Central Hospital.

Establishment of CIBP rat model. The CIBP rat model was established as previously described (27). Briefly, the rats were anesthetized by pentobarbital sodium [50 mg/kg, intraperitoneal (i.p.)]. The right leg of the rats was shaved, and the skin was disinfected with 75% (v/v) ethanol. Then, 10 μl volume of Walker 256 mammary gland carcinoma cells ($4x10^5$ cells) was injected into the right tibia of the rats. Sham rats were injected with of 10 μl volume of PBS into the right tibia. The injection site was sealed with medical glue to prevent leakage. Finally, the wound was disinfected with 75% (v/v) ethanol and sutured with 3-0 silk thread.

Behavioral tests. Mechanical allodynia was measured as previously described (28). Briefly, single rats were placed in a customized chamber and allowed to acclimate for 30 min prior to testing. Then, von Frey filaments (Stoelting Co., Wood Dale, IL, USA) were applied to the mid-plantar surface of hind paw in ascending order (0.4, 0.6, 1.4, 2, 4, 6, 8, 10 and 15 g). Each Von Frey hair was held for 6 to 8 sec with a 5 min interval between applications. Brisk withdrawal or paw flinching upon stimulus was considered as positive response. The lowest force of the filament required to elicit a positive response was considered to be the paw withdrawal threshold (PWT). Thermal hyperalgesia was measured as previously described (29). Briefly, single rats were placed in a customized chamber and allowed to acclimate for 30 min prior to testing. Then, the radiant heat source was delivered by a Plantar Analgesia meter (ITC Life Science Inc., Victory Blvd Woodland Hills, CA, USA) and focused onto the mid-plantar surface of hind paw. The heat source was turned off when the rat lifted the foot. The time from onset of radiant heat application to withdrawal of the rat's hind paw was defined as the paw withdrawal latency (PWL). A 25 sec cutoff was used to prevent tissue damage. The right hind paw was tested three times at an interval of 5 min. The behavioral tests were performed by an investigator blinded to the tested groups.

Drug administration. GRb1 was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and dissolved in normal saline. The appropriate dosage of GRb1 was determined by preliminary experiments and previous studies (30,31). To determine the analgesic effect of GRb1 on established CIBP, GRb1 (1, 5, and 10 mg/kg, i.p.) was treated at day 14 following surgery. Pain behaviors were measured at 15, 30, 45, 60, 75, 90 min following intraperitoneal injection of GRb1. To determine the analgesic effect of multiple administration of GRb1 on established CIBP, GRb1 (10 mg/kg, i.p.) was treated from day 12 for three consecutive days. To determine the effect of GRb1 on the activation of microglia and expression of proinflammatory cytokines, GRb1 (10 mg/kg, i.p.) was treated from day 12 for three consecutive days. The rats were sacrificed 30 min following the last injection of GRb1.

Western blot analysis. Briefly, the rats were deeply anesthetized by pentobarbital sodium (120 mg/kg, i.p.). Then, the L4-L6 spinal cord was removed and stored at -80˚C until use. The tissue samples were homogenized in lysis buffer containing PMSF and 0.02% protease inhibitor cocktail. The homogenates were centrifuged at 12,000 x g for 10 min at 4˚ to obtain the supernatants. Protein concentrations were determined by the Bradford method. Equivalent amounts of protein (50 μg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Then, the membranes were blocked with 5% non-fat milk for 2 h at room temperature (RT) and incubated overnight at 4˚C with primary antibodies for iba1 (1:1,000; Abcam, Cambridge, UK), IL-1β (1:1,000; Abcam), IL-6 (1:500; Sigma-Aldrich; Merck KGaA), TNF-α (1:2,000; EMD Millipore, Billerica, MA, USA) and GAPDH (1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA). The membranes were washed in tris buffered saline with tween 20 and then incubated with appropriate horseradish peroxidase-conjugated secondary antibody (1:5,000; Sigma-Aldrich; Merck KGaA). Bands were revealed using an ECL kit (EMD Millipore). The intensity of proteins was measured by image lab software and normalized to GAPDH. The protein intensity of control groups was set as 1.

Immunohistochemistry. Briefly, the rats were deeply anesthetized by pentobarbital sodium (120 mg/kg, i.p.) and perfused with 0.1 M PBS followed by 4% paraformaldehyde (PFA). The L4-L6 spinal cord segments were removed and post-fixed in 4% PFA for 4 h at 4˚C, then placed in a 30% sucrose solution at 4˚C for three days. The embedded samples were sectioned 30-μm thick in a cryostat and stored in PBS until use. The sections were blocked with 5% donkey serum and 0.3% Triton X-100 for 2 h at RT, then incubated overnight at 4˚C with the primary antibody against iba1 (1:200; Abcam). After washed in PBS for three times, the sections were incubated with Alexa Fluor 594-labeled donkey anti-goat secondary antibody (1:400, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Following immunostaining procedures, the sections were examined using a fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) at the same exposure time.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA,

USA). All data were presented as means ± standard error of the mean. Western blotting data was analyzed using one-way analysis of variance (ANOVA) with repeated measures followed by the Bonferroni post hoc test. Behavioral tests data was analyzed using two-way ANOVA with repeated measures followed by Bonferroni post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Time course of mechanical allodynia and thermal hyperalgesia induced by intratibial inoculation of Walker 256 cells. The PWT and PWL were at a similar baseline in all rats. Compared with the sham rats, the CIBP rats began to exhibit a decrease in PWT and PWL in the ipsilateral hind paw at 7 days following surgery, which indicates the development of mechanical allodynia and thermal hyperalgesia, respectively. Furthermore, the PWT and PWL progressively decreased from day 7 to day 21 (Fig. 1A and B). These results indicated that the model of CIBP was established successfully.

Analgesic effects of GRb1 on established CIBP. To determine the analgesic effect of GRb1 on established CIBP, GRb1 (1, 5, and 10 mg/kg; i.p.) was administered at day 14 following surgery. Pain behaviors were measured at 15, 30, 45, 60, 75 and 90 min following intraperitoneal injection of GRb1. At 14 days following surgery when the pain behavior is established, intraperitoneal injection of GRb1 (5 and 10 mg/kg, but not 1 mg/kg) partially and transiently reversed the mechanical allodynia and thermal hyperalgesia in CIBP rats (Fig. 1C and D). The analgesic effect of GRb1 started at 15 min following injection and lasted for ~1 h. However, CIBP rats treated with a vehicle exhibited no significant alterations regarding PWT and PWL.

To determine the analgesic effect of multiple administrations of GRb1 on established CIBP, GRb1 (10 mg/kg, i.p.) was given from day 12 for three consecutive days. Pain behaviors were measured at 30 min following i.p. injection of GRb1. It was demonstrated that repeated administration of GRb1 exhibited persistent analgesic effects as indicated by a significant upregulation of PWT and PWL (Fig. 1E and F).

Effects of GRb1 on pain threshold of naive rats. To determine whether treatment with GRb1 could affect the pain threshold of naive rats, naive rats were injected intraperitonally with GRb1 (1, 5, and 10 mg/kg). The behavioral tests demonstrated that none of the doses chosen effected the PWT and PWL in naive rats (Fig. 1G and H), suggesting that GRb1 did not affect the pain behavior in naive rats.

Effects of GRb1 on the activation of microglia in the spinal cord. It was reported that the activation of microglia in the spinal cord serves an important role in the development of CIBP and microglia inhibitor could alleviate CIBP (32). Therefore, whether treatment with GRb1 could affect the activation of microglia was investigated. GRb1 was intraperitoneally injected (10 mg/kg) from day 12 for three consecutive days. The rats were sacrificed 30 min following the last injection of GRb1. The protein expression of iba1, which is the maker of microglia, was significantly suppressed by the treatment with GRb1 in the spinal cord of CIBP rats (Fig. 2A and B). Furthermore, the immunohistochemistry results also confirmed that the immunoreactivity of iba1 was significantly decreased in the spinal cord of CIBP rats intraperitoneally injected with GRb1 (10 mg/kg) from day 12 for three consecutive days (Fig. 2C-J). Additionally, i.p. injection of GRb1 did not alter the protein expression and immunoreactivity of iba1 in the spinal cord of sham rats.

Effects of GRb1 on the expression of proinflammatory cytokines in the spinal cord. It is well established that upregulation of proinflammatory cytokines contributes to the development of CIBP (33). Consistent with previous studies, the results of the present study demonstrate that CIBP rats exhibited upregulated protein expression levels of IL-1β, IL-6, and TNF-α. GRb1 was intraperitoneally injected (10 mg/kg) from day 12 for three consecutive days. The rats were sacrificed 30 min following the last injection of GRb1. The protein expression of IL-1β, IL-6 and TNF-α was significantly suppressed by the treatment with GRb1 in CIBP rats compared with CIBP rats treated with a vehicle (Fig. 3).

Discussion

The present study provided evidence that intratibial injection of Walker 256 cells could induce mechanical allodynia and thermal hyperalgesia from day 7-21. Furthermore, i.p. injection of GRb1 (5 and 10 mg/kg, but not 1 mg/kg), which belongs to the family of triterpene glycosides, partially and transiently reversed the mechanical allodynia and thermal hyperalgesia in CIBP rats. Additionally, intraperitoneal injection of GRb1 did not affect the pain threshold in naive rats. Notably, i.p. injection of GRb1 (10 mg/kg) suppressed the activation of microglia and glial-derived proinflammatory cytokines including IL-1β, IL-6 and TNF-α. Together, these results suggested that GRb1 may alleviate CIBP via suppression neuroinflammation in the spinal cord.

Breast cancer has a high prevalence of metastasizing to the bone, which causes osteolytic lesions, hypercalcaemia, bone fractures, and significant pain (34,35). Currently, the available therapeutic strategies for CIBP includes localized radiation therapy, non-steroidal anti-inflammatory drugs, and opioids. However, at least half of these patients did not obtain adequate pain relief (36). More importantly, increasing doses of analgesic drugs due to analgesics tolerance leads to unsatisfied side effects including nausea and vomiting as well as addiction (37). Therefore, it is important to discover novel therapeutic strategy for the management of CIBP.

GRb1 is one of main active constituents isolated from a traditionally used herb named ginseng (38). Previous studies have demonstrated that GRb1 has multiple functions. For example, GRb1 serves a protective role in animal models of cerebral and intestinal as well as spinal cord ischemia/reperfusion injury (31,39-41). In addition, *in vitro* and *in vivo* studies have demonstrated that GRb1 has anti-inflammatory and anti-apoptosis effect (42-46). Additionally, treatment with GRb1 attenuates oxidative stress, suggesting an antioxidant effect of GRb1 (47-49). A recent study provided evidence that GRb1 attenuates inflammatory pain induced by intraplantar injection of formalin by inhibiting neuronal phosphorylation of ERK via regulating the Nrf2 and NF-κB pathways (15).

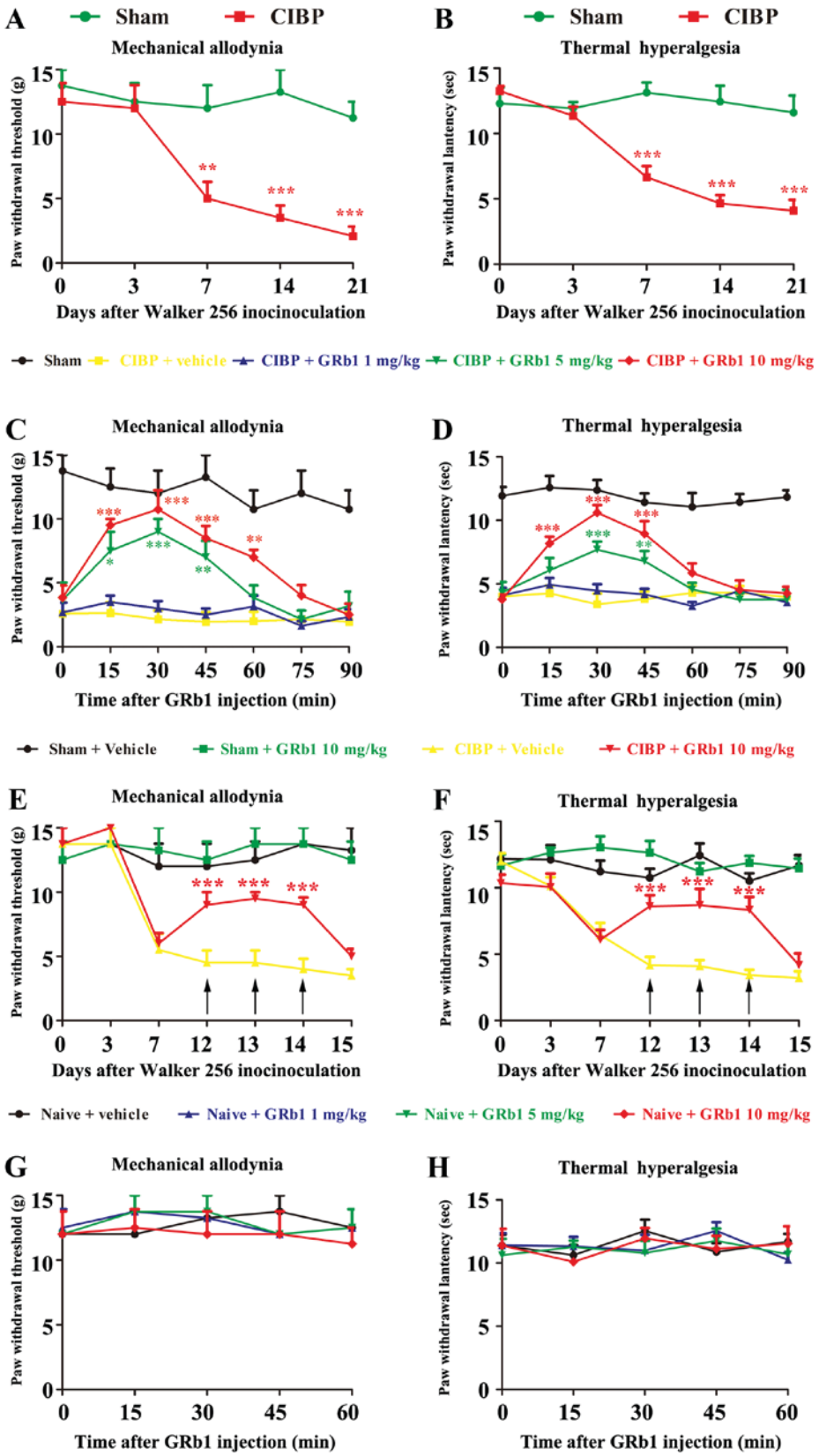

Figure 1. GRb1 attenuated established CIBP. A significant decrease of (A) PWT and (B) PWL was observed in CIBP rats compared with sham rats from day 7 to 21. **P<0.01, ***P<0.001 vs. the sham group. Data were presented as the mean ± SEM. n=6 rats in each group. At 14 days following surgery when the pain behavior is established, i.p. injection of GRb1 (5 and 10 mg/kg, but not 1 mg/kg) partially and transiently reversed the mechanical allodynia (C) and thermal hyperalgesia (D) in CIBP rats. *P<0.05, **P<0.01 and ***P<0.001 vs. the CIBP rats treated with the vehicle. Pain behaviors were measured at 15, 30, 45, 60, 75 and 90 min following i.p. injection of GRb1. Data were presented as the mean ± standard error of the mean. n=6 rats in each group. I.p. injection of GRb1 (1, 5 and 10 mg/kg) has no significant effects on PWT (E) and PWL (F) in naïve rats. Data were presented as the mean ± SEM. n=6 rats in each group. Repeated administration of GRb1 persistently elevated PWT (G) and PWL (H) in CIBP rats. ***P<0.001 vs. the CIBP rats treated with vehicle. GRb1 (10 mg/kg, i.p.) was treated from day 12 for three consecutive days. Pain behaviors were measured at 30 min following i.p. injection of GRb1. Data were presented as the mean ± SEM. n=6 rats in each group. PWT, paw withdrawal threshold; CIBP, cancer-induced bone pain; i.p., intraperitoneal; SEM, standard error of the mean; PWL, paw withdrawal lantency; GRb1, Ginsenoside Rb1.

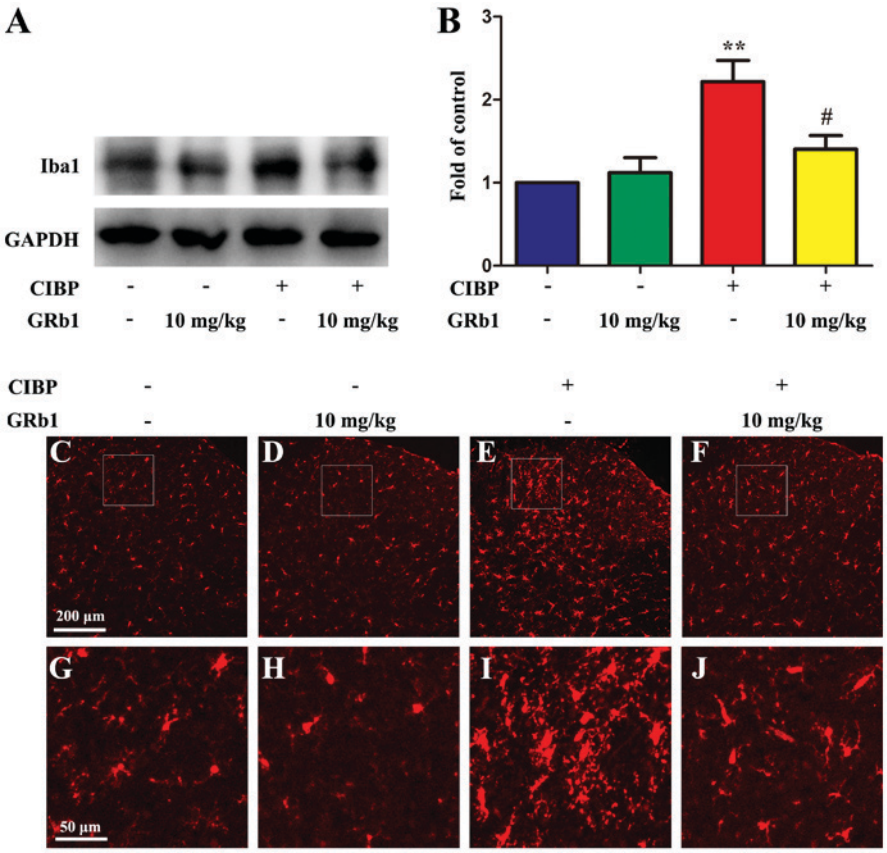

Figure 2. (A-J) GRb1 attenuated the activation of microglia in the spinal cord. (A and B) GRb1 was intraperitoneal injected (10 mg/kg) from day 12 for three consecutive days. The rats were sacrificed 30 min following the last injection of GRb1. The protein expression of iba1, which is the maker of microglia, was suppressed by the treatment with GRb1 in CIBP rats. **P<0.01 vs. the sham rats treated with a vehicle, $^{\#}$P<0.05 vs. the CIBP rats treated with vehicle. Data were presented as the mean ± standard error of the mean. n=6 rats in each group. GRb1 was intraperitoneal injected (10 mg/kg) from day 12 for three consecutive days. The rats were sacrificed 30 min following the last injection of GRb1. Microglia was inactivated in sham rats treated with (C and G) vehicle and (D and H) GRb1. (E and I) However, the immunoreactivity of iba1 in CIBP rats was significantly upregulated. (F and J) The activation of microglia was markedly suppressed by the treatment with GRb1 in CIBP rats in the spinal cord. n=6 rats in each group. GRb1, Ginsenoside Rb1; CIBP, cancer-induced bone pain.

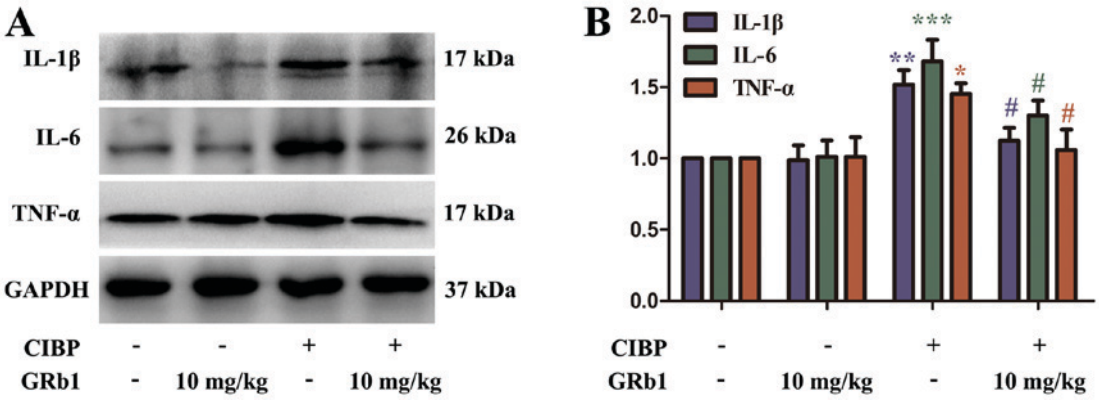

Figure 3. GRb1 suppressed the protein expression of proinflammatory cytokines in the spinal cord. (A and B) GRb1 was intraperitoneal injected (10 mg/kg) from day 12 for three consecutive days. The rats were sacrificed 30 min following the last injection of GRb1. The protein expression of interleukin 1β, interleukin 6 and tumor necrosis factor α were suppressed by the treatment with GRb1 in CIBP rats. *P<0.05, **P<0.01, ***P<0.001 vs. the sham rats treated with a vehicle, $^{\#}$P<0.05 vs. the CIBP rats treated with a vehicle. Data were presented as the mean ± standard error of the mean. n=6 rats in each group. GRb1, Ginsenoside Rb1.

Other ingredients of Ginsenoside have also been reported to have antinociceptive effects (16-19). The present study investigated the analgesic effect of GRb1 on a well-established rat model of CIBP established by intratibial injection of Walker 256 cells. Consistent with previous study, the present study's behavioral results demonstrated that CIBP rats exhibited a significant decrease in PWT and PWL in the ipsilateral hind paw from day 7 to day 21. Furthermore, it was demonstrated that i.p. injection of GRb1 (5 and 10 mg/kg, but not 1 mg/kg) partially and transiently reversed the mechanical allodynia and thermal hyperalgesia in CIBP rats at 14 days following surgery when the pain behavior was established. In addition,

repeated administration of GRb1 showed persistent analgesic effect. However, treatment with GRb1 did not affect the pain threshold of naive rats. These results suggested that GRb1 may be an effective drug for the management of CIBP.

To determine the underlying mechanisms of the analgesic effect of GRb1 against CIBP the effect of GRb1 on neuroinflammation in the spinal cord was investigated. Emerging evidence has indicated that glial-derived neuroinflammation serves a fundamental role in the initiation and maintenance of CIBP (8,50-52). Pharmacological inhibition of the activation of microglia and glial-derived proinflammatory cytokines inhibited the pain behaviors in CIBP rats (53-56). In a rat model of Alzheimer's disease, Wang *et al* (26) demonstrated that GRb1 treatment improved learning and memory deficit via its anti-neuroinflammatory effect. In another study, Miao *et al* (25) demonstrated that i.p. injection of GRb1 mitigated isoflurane/surgery-induced cognitive impairment by downregulating the expression level of reactive oxygen species, TNF-α and IL-6 in the mice hippocampus. The results of the present study consistently demonstrated that the proteins expression and immunoreactivity of iba1, was upregulated in CIBP rats compared with the sham rats. Furthermore, i.p. injection of GRb1 (10 mg/kg) from day 12 for three consecutive days significantly suppressed the activation of microglia. Finally, the protein express levels of IL-1β, IL-6, and TNF-α in the spinal cord of CIBP rats was measured. The western blotting results of the present study demonstrated upregulated expression level of IL-1β, IL-6 and TNF-α in the spinal cord, which were significantly inhibited by the treatment of GRb1 (i.p., 10 mg/kg) from day 12 for three consecutive days. Together, these results indicated that GRb1 may attenuate CIBP via inhibiting the activation of microglia and glial-derived proinflammatory cytokines.

In conclusion, this study provided evidence that i.p. injection of GRb1 alleviated mechanical allodynia and thermal hyperalgesia in a rat model of CIBP. Mounting evidence showed that microglia inhibitors attenuated CIBP by suppressing the activation of microglia (6,57). Considering that GRb1 showed anti-neuroinflammation effect in animal models such as Alzheimer disease (26) and post-operative cognitive dysfunction (25), we explored whether GRb1 could suppressed the activation of microglia under CIBP condition. GRb1 was intraperitoneal injected (10 mg/kg) from day 12 after surgery for three consecutive days. The rats were sacrificed 30 min after the last injection of GRb1. Our results showed that repeated administration of GRb1 inhibited the activation of microglia. Consistently, repeated administration of GRb1 showed persistent analgesic effect. It is well-established that activated microglia in the spinal cord increase in number, change their morphology, and release proinflammarory cytokines such as IL-1β, IL-6 and TNF-α. Previous studies have demonstrated that microglia inhibitors could suppressed the expression of these proinflammarory cytokines under CIBP condition (33,58). Since we found repeated treatment with GRb1 suppressed the activation of microglia, we subsequently explored whether GRb1 could inhibited the expression of proinflammarory cytokines. It turns out that GRb1 did downregulated the expression of proinflammarory cytokines in CIBP rats. Therefore, we conclude that inhibiting the activation of microglia and glial-derived proinflammatory cytokines may be involved in the analgesic effect of GRb1 under CIBP condition. However, it must be acknowledged that there may be other mechanisms regarding the antinociceptive effect of GRb1 on CIBP. Further studies are warranted to investigate more detailed mechanisms underlying the analgesic effect of GRb1.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

FDY and JQY designed the experiments, conducted the experiments, analyzed the data, created the figures and wrote the manuscript. YCH conducted the experiments and analyzed the data. MPL, WJY and BZ performed the analysis with constructive discussions. XJL designed the experiments and contributed significantly to the revision of manuscript.

Ethics approval and consent to participate

All the experimental protocols were approved by the Animal Care and Use Committee of Baoji Central Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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