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Research Paper

Reactive oxygen species scavengers ameliorate mechanical allodynia in a rat model of cancer-induced bone pain

Ya-Qun Zhou[a,b,1], Dai-Qiang Liu[a,b,1], Shu-Ping Chen[a,b], Jia Sun[a,b], Xue-Rong Zhou[c], Heike Rittner[d], Wei Mei[a,b], Yu-Ke Tian[a,b], Hui-Xian Zhang[e], Fei Chen[f], Da-Wei Ye[c,*]

[a] Anesthesiology Institute, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[b] Department of Anesthesiology and Pain Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[c] Cancer Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[d] Department of Anesthesiology, University Hospital of Würzburg, Würzburg, Germany
[e] Department of Oncology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China
[f] Department of Oncology, Xiaogan Hospital Affiliated to Wuhan University of Science and Technology, Xiaogan, China

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ABSTRACT

Cancer-induced bone pain (CIBP) is a frequent complication in patients suffering from bone metastases. Previous studies have demonstrated a pivotal role of reactive oxygen species (ROS) in inflammatory and neuropathic pain, and ROS scavengers exhibited potent antinociceptive effect. However, the role of spinal ROS remains unclear. In this study, we investigated the analgesic effect of two ROS scavengers in a well-established CIBP model. Our results found that intraperitoneal injection of N-tert-Butyl-α-phenylnitrone (PBN, 50 and 100 mg/kg) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol, 100 and 200 mg/kg) significantly suppressed the established mechanical allodynia in CIBP rats. Moreover, repeated injection of PBN and Tempol showed cumulative analgesic effect without tolerance. However, early treatment with PBN and Tempol failed to prevent the development of CIBP. Naive rats received repetitive injection of PBN and Tempol showed no significant change regarding the nociceptive responses. Finally, PBN and Tempol treatment notably suppressed the activation of spinal microglia in CIBP rats. In conclusion, ROS scavengers attenuated established CIBP by suppressing the activation of microglia in the spinal cord.

1. Introduction

Cancer-induced bone pain (CIBP) is a frequent complication in patients suffering from bone metastases [1,2]. Currently, the World Health Organization (WHO) analgesic ladder remains the golden standard for the management of CIBP in clinic [3,4]. However, current therapeutic strategies often provided inadequate pain relief and are associated with numerous unavoidable side effects which limit their prolonged application [5,6]. Despite marked advance has been made regarding the mechanisms of CIBP, few effective drugs has been developed for the management of CIBP in the past decades. Therefore, further investigations are warranted to uncover the underlying mechanisms of CIBP.

Reactive oxygen species (ROS) are produced as a natural byproduct of normal metabolism and play an important role in cell signaling and homeostasis [7]. There are many types of ROS including hydroxyl radicals, superoxide radicals, nitric oxides, hydrogen peroxides and peroxynitrites [8]. Under normal conditions, the production of ROS is tightly regulated by antioxidant defense systems [9]. However, excessive ROS levels due to increased ROS production and/or decreased antioxidant defense ability leads to lipid peroxidation, protein oxidation, and nucleic acid oxidation [10]. Recently, emerging evidence suggested that ROS scavengers showed potent analgesic effect in rodent models of inflammatory pain and neuropathic pain [11–14]. However, the role of ROS in CIBP remains largely unknown. Therefore, the present study examined the antinociceptive effect of two ROS scavengers in a well-established CIBP model.

2. Material and methods

2.1. Animals

In the present study, we chose virgin female Sprague-Dawley rats (180–200 g, Tongji Medical College, Huazhong University of Science

* Corresponding author.
E-mail address: dy0711@gmail.com (D.-W. Ye).
[1] These authors contributed equally as first authors.

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and Technology, Wuhan, PR China) since female rats are more susceptible to Walker 256 mammary gland carcinoma cells. The animals were housed under controlled conditions (24 ± 0.5 °C, 12 h alternating light-dark cycle, with free access to water and food). All experimental protocols were approved by the Animal Care and Use Committee of Huazhong University of Science & Technology.

2.2. Establishment of CIBP models

Tumor cells were harvested from the ascitic fluid of rats that were inoculated with Walker 256 cells (4×10^7 cells/mL, 1 mL) into the abdominal cavity. Suspensions of tumor cells (4×10^7 cells/mL) in phosphate buffer saline (PBS) were prepared for injection using a hemocytometer. The model of CIBP was performed as described previously [15,16]. In brief, the right leg was disinfected after anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal, i.p.). Then, Walker 256 cells (4×10^7 cells/mL, 10 μL) were slowly injected into the tibial cavity using a 10 μL Hamilton syringe. 10 μL PBS was injected instead of tumor cells for the sham group. The injection site was sealed with bone wax once the syringe was removed. Finally, the wound was sealed with 3-0 silk thread.

2.3. Drug administration

N-tert-Butyl-α-phenylnitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 0.9% saline. The dose of drugs was chosen according to our preliminary results and previous reports [17,18]. To determine whether ROS scavengers could alleviate CIBP in advanced phase, a single dose of PBN (10, 50, 100 mg/kg, i.p.) or Tempol (10, 100, 200 mg/kg, i.p.) was given on day 14 (14 days) after the establishment of CIBP models. To determine whether repetitive treatment with ROS scavengers had a cumulative analgesic effect on CIBP, PBN (100 mg/kg, i.p.) or Tempol (200 mg/kg, i.p.) was given once daily from 14 days to 18 days. To determine whether early treatment with ROS scavengers could suppress the development of CIBP, PBN (100 mg/kg, i.p.) or Tempol (200 mg/kg, i.p.) was given once daily from 1 days to 5 days. To determine whether ROS scavengers could affect the mechanical sensitivity of naive rats, PBN (10, 50, 100 mg/kg, i.p.) or Tempol (10, 100, 200 mg/kg, i.p.) was given once daily for 5 consecutive days.

2.4. Behavioral tests

Mechanical allodynia was evaluated by measuring ipsilateral hind paw withdrawal threshold (PWT) in response to von Frey filament stimuli as described previously [19,20]. Briefly, animals were put in individual chambers on a metal mesh floor and allowed to habituate for 30 min. Von Frey filaments were applied to the mid-plantar of the right hind paw for 6 s per filament or until paw withdrawal in ascending order of forces (1, 1.4, 2, 4, 6, 8, 10, and 15 g), starting at 2 g von Frey filament. Positive responses were defined as abrupt paw withdrawal, lickings, and shaking. Once a positive response occurred, the paw was re-tested after a 5 min rest, starting with the next lower von Frey filament. Once no response was observed, the next higher von Frey filament was applied after a 5 min rest. The PWT was defined as the lowest force (in grams) required to elicit a positive response. The investigator who performed the behavioral tests were blinded to the experimental design.

2.5. Immunohistochemistry

Briefly, rats were deeply anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and then intracardially perfused with PBS followed by 4% ice-cold paraformaldehyde (PFA). The L4-L5 segments of spinal cord were removed and post-fixed using the same fixative. The embedded samples were sectioned 20 μm thick in a cryostat (CM1900, Leica, Germany). The sections were penetrated with 0.3% Triton X-100 for 15 min and blocked with 10% donkey serum for 1 h at room temperature (RT). Then, the sections were incubated with goat anti-ionized calcium-binding adapter molecule 1 (Iba1) antibody (microglial marker; 1:300; ab5076; Abcam) overnight at 4 °C. After been washed three times in PBS, the sections were incubated with Alexa Fluor 594-conjugated donkey anti-goat secondary antibody (1:500; 705-585-003; Jackson ImmunoResearch) for 2 h at RT. The sections were then captured using a fluorescence microscope (DM2500, Leica, Germany). The Iba1-immunolabeled surface areas were measured in laminae I-IV of the spinal cord dorsal horn using Image Pro Plus software as described previously [15]. Quantification of the immunoreactivity was accomplished by calculating the percentages of immunostaining ([positive immunofluorescent surface area]/[total measured picture area] × 100). Four rats of each group were used for statistical analysis. The investigator performed the image analyses was blinded to the experimental design.

2.6. Statistical analysis

Data are expressed as mean ± SEM and analyzed using the GraphPad Prism version 5.01 for Windows (Graph Pad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was used for immunochemistry data. Two-way ANOVA with repeated measures, followed by Bonferroni post hoc test was used for PWT. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Mechanical allodynia induced by intratibial injection of Walker 256 cells

In this study, we used a well-established rat model of CIBP by intratibial injection of Walker 256 cells. To observe the development of mechanical allodynia, ipsilateral PWTs were evaluated at baseline and 3 days, 7 days, 14 days and 21 days. Similar baseline PWTs were observed among all groups. As shown in Fig. 1, the PWTs of ipsilateral hind paw were significantly decreased from 7 days to 21 days in CIBP rats. In contrast, naive and sham rats showed no significant change in PWTs during the 21-day observation period. These results indicate that mechanical allodynia is developed after intratibial injection of Walker 256 cells.

3.2. Analgesic effect of a single dose of PBN on established CIBP

To determine whether PBN could alleviate CIBP in advanced phase, a single dose of PBN (10, 50, 100 mg/kg, i.p.) was given on 14 days.

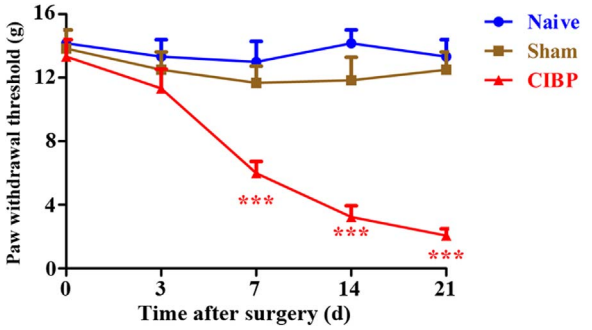

Fig. 1. The time course of paw withdrawal threshold (PWT) in naive, sham and cancer-induced bone pain (CIBP) rats. The ipsilateral PWTs were significantly decreased from day 7 to day 21 after surgery in CIBP rats (***$p < 0.001$ compared with the naive group, n = 6 in each group). In contrast, naive and sham rats showed no significant change in PWTs during the 21-day observation period.