Effect of reactive oxygen species of the psoas major muscle in complete Freund's adjuvant-induced inflammatory pain in rats

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Abstract

Lower limb pain is a common clinical disease that affects millions of people worldwide. It is found in previous studies that reactive oxygen species is closely related to neuropathic, cancer, chemotherapy, and inflammatory pain, which can be relieved by reactive oxygen species scavengers. Furthermore, acupuncture or electroacupuncture on the psoas major muscle has a great effect on adjuvant-induced arthritis and lower back pain. In our study, we investigated the function of reactive oxygen species scavengers locally injecting into the ipsilateral psoas major muscle on complete Freund's adjuvant-induced inflammatory pain. Our results demonstrated that in the development of complete Freund's adjuvant-induced inflammatory pain, early local continuous application of N-tert-Butyl-α-phenylnitrone (PBN, 1 and 5 mg/kg/0.2 ml) on the ipsilateral psoas major muscle effectively reduced mechanical and cold hyperalgesia. However, intraperitoneal injection of PBN (1 and 5 mg/kg) or local injection of PBN (1 and 5 mg/kg/0.2 ml) into contralateral psoas major muscle, ipsilateral quadratus lumborum, and ipsilateral erector spinae showed limited effect. In the developed inflammatory pain model, local injection of PBN into the ipsilateral psoas major muscle also alleviated pain and paw edema. In addition, reactive oxygen species level increased in ipsilateral psoas major muscle at seven days after complete Freund's adjuvant injection. In general, PBN reduces complete Freund's adjuvant-evoked inflammatory pain by inhibiting reactive oxygen species in the psoas major muscle.

Introduction

Reactive oxygen species (ROS), including hydroxyl radicals, superoxide radicals, nitric oxides, hydrogen peroxides, and peroxynitrites, are produced by aerobic cells during metabolism. Proper concentration of ROS can promote immunity, repair, survival, and growth of cells.[1] Under normal circumstances, the level of ROS is controlled by antioxidants such as superoxide dismutase enzymes, catalase, and glutathione peroxidase. While imbalance between prooxidant and antioxidant defenses occurs, excessive ROS causes oxidative stress.[2] Oxidative stress results in damage to DNA, lipids, and proteins.[3–5]

Increasing reports found that ROS played an important role in pain.[6–9] High intensity of exercise generates a lot of ROS, which causes an imbalance of ROS and antioxidants and then results in oxidative stress.[10] Muscle soreness after exercise is closely related to increased ROS level.[11] In chemotherapy pain,

ROS and its detoxification are considered to be one of the important pathogenic factors that damage peripheral sensory neurons.[12] Systemic application of

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ROS scavengers alleviates pain of animals in the models of inflammatory, neuropathic, cancer, and chemotherapy pain.[13–16]

Lower limb pain is a common clinical disease and traditional treatments for chronic lower pain include physical therapy, surgery, and medicine.[17–23] A study suggests that electroacupuncture on specific acupoints inhibits the inflammation of rats with adjuvant-induced arthritis, and one of the selected acupoints was Shenshu (BL 23) on which the needle puncture the psoas major muscle (PM).[24,25] Notably, smaller cross-sectional area, more fat infiltration, and higher activity of PM were found in long-term low back pain patients.[26] Furthermore, lower limb pain results in imbalance between left and right lower limbs. As the main muscle for leg flexion, PM promotes contraction when leg is lifted, and it is the contraction of PM provokes oxidative stress.[10,27] As for the relevant mechanism, it is not completely clear. We assumed that lower limb pain induces compensatory contraction of PM to produce ROS. Since there is a close connection between increase of ROS and pain, reducing the ROS in the PM is supposed to be a good way to alleviate the lower limb pain. In present study, we inject complete Freund's adjuvant (CFA) into the plantar of rat heel to develop inflammatory pain model. ROS scavengers are then injected into the ipsilateral psoas major muscle (iPM) locally to reduce ROS.

Materials and methods

Animals

Male Sprague Dawley rats, weighing 150 ± 20 g, were obtained from Guangdong Medical Laboratory Animal Center (Guangzhou, China). Animals were housed at an environment with controlled temperature ($22 \pm 2°C$) and humidity ($55 \pm 15\%$), on an alternating 12-:12-h light-dark cycle and with free access to water and food. Animal use and protocols were approved by the Committee on Ethical Use of Animals of Guangdong Provincial People's Hospital (Guangzhou, China) according to the National Institutes of Health Animal Use and Care Guidelines. All efforts were made to minimize animal suffering and reduce the number of used animals.

Development of CFA-induced inflammatory paw pain

Two days before induction, the lower backs of all animals were shaved. Under mild anesthesia with sevoflurane, the left heel was disinfected with 75% alcohol. Fifty microliters of an equal volume mixture of 1 mg/ml of CFA (Sigma, St. Louis, MO, USA) and artificial cerebrospinal fluid (ACSF, Leagene, China) was subcutaneously injected into the plantar surface of the left heel with a 13-mm 29-gauge 1-ml syringe. The control group received an injection of equal volume of ACSF on the left paw. The syringe was pulled out 20 s after the injection to prevent liquid leakage. The model of CFA-induced pain was performed according to the method of Xie et al.[28]

Drug administration

Phenyl-N-tert-butylnitrone (PBN) was purchased from Sigma (St. Louis, MO, USA) and dissolved in 0.9% saline. The concentration and dose of PBN was determined according to previous studies[14,29] and our preliminary experiment results. To evaluate the ROS in the iPM, PBN, a ROS scavenger, was locally injected into iPM (1 and 5 mg/kg/0.2 ml) or systemic applied (1 and 5 mg/kg, i.p.) on the 1st, 3rd, 5th, and 7th day after CFA injection. The injection site of PM was determined by anatomy on the rat bodies. The syringe was inserted into the skin of the left side of the fourth lumbar spine at a 75° angle approximately 15 mm from the rear centerline with the needle tip directed to the centerline. Once the depth of the needle was inserted about 10 mm to reach the fourth lumbar vertebrae, and the needle was adjusted so that the needle stuck to the outside of the transverse process to access the PM. The injection location has been confirmed by ultrasound. To determine either iPM or other paravertebral muscles plays an important role on the analgesic effect. PBN was injected into contralateral psoas major muscle (cPM), paravertebral other muscles, ipsilateral erector spinae (iES), and non-PM in the same position as the psoas but did not reach the PM and ipsilateral quadratus psoas (iQL). The control group was injected with the same amount of saline. To assess the effect of ROS scavenger locally injected into the iPM on the established inflammatory pain model, PBN was locally injected into the iPM (10 mg/kg/0.2 ml) or systemic administrated (10 mg/kg, i.p.) seven days after CFA injection for a single dose. To determine whether repetitive treatment with PBN had a cumulative analgesic effect on CFA-induced pain, PBN was locally injected into the iPM (5 mg/kg/0.2 ml) or systemically applied (5 mg/kg, i.p.) every other day from the 8th to 14th day after CFA injection.

Behavioral tests

Tests were carried out under controlled temperature ($22 \pm 2°C$) and humidity ($55 \pm 15\%$) in a quiet and bright environment. The rats were placed in 18 cm $\times 22$ cm $\times 28$ cm plexiglass cages on a wire mesh (an aperture of 6 mm $\times$ 6 mm and a diameter of 0.8 mm). All of the animals were adapted to the environment for 1 h two days before the experiments and 30 min before tests on

every test day. Behavioral tests were performed at indicated days. When PBN was designed to injection on the test day, behavioral tests were performed 30 min after injection. Mechanical allodynia test was followed by cold allodynia and the interval between two tests was 30 min.

Mechanical allodynia was evaluated by measuring hind paw withdrawal threshold (PWT), which was tested by using the up-and-down method.[24,30] A series of standardized von Frey filaments (North Coast Medical Inc., Morgan Hill, CA) were applied to heel of the hind paws of the rats slightly curved for 6–8 s, 10 times with at least 5 s per stimulation interval. Positive response included acute withdrawal, biting, licking, or shaking of the limb. Once positive response occurred five or more than five times, next lower von Frey filament was used 5 min later, or a higher von Frey filament was applied. The lowest force (in grams) required to elicit five or more than five times positive responses was considered as the PWT.

Cold hyperalgesia of the rats was tested with acetone. The rats were placed in a plexiglass cage with a wire mesh at the bottom. After adapting to the environment for 30 min, a drop of 50 µl of acetone was applied to the heel surface of the hind paw and repeated three times with an interval of 5 min between applications.[31] Normal rats neglected the stimulation or had very small reaction, while the CFA-induced pain rats responded frequently and severely. Response to the acetone of the rat was assessed with scores, for example, 0 for no response, 1 for a flick, 2 for repeated flicks, and 3 for biting paws. The rats were observed for at least 20 s after applying the acetone. The observation time was increased by 20 s for animals that scored a 1 or 2.[32]

Paw measurements

The thickness and width of each heel was measured with a digital vernier caliper. For the thickness, one caliper was placed on the foot against the ankle and the other caliper was placed on the bottom of the heel. For the width, the widest point on the lateral and medial aspects of the heel was measured. Both width and thickness were measured three times and taken an average. Then, the width and thickness were multiplied as the cross-sectional area of the hind paw heel of the rats and the measurements of the contralateral hind paw heel at each time point was used as a baseline.[28]

Measurement of ROS

Total ROS level was determined by using 2′, 7′-dichlorofluorescein diacetate (DCFH-DA). iPM was obtained from rats at one and seven days after CFA or ACSF injection. Samples were made into single-cell suspensions and the specific operation was as follows. Samples were fully cut into pieces, then put into the incubator at 37°C after adding 0.2% type I collagenase (nine times volume of samples). The enzymolysis was stopped with cold phosphate-buffered saline (PBS) and filtrated with a 300-mesh cell strainer to get single-cell suspensions. Suspensions were incubated at 37°C for 30 min with 10 µM DCFH-DA (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Fluorescence was recorded at 500 nm (excitation) and 525 nm (emission) with BD AccuriC6 flow cytometer (Becton, Dickinson Company, USA). All the steps followed the instructions strictly. The level of ROS was expressed by fluorescence.

Statistical analysis

Data are expressed as mean ± standard error of the mean and analyzed using SPSS software. Analysis of variance (ANOVA) followed by Bonferroni post hoc test was used for behavior and paw edema data analysis. ROS level was analyzed by one-way ANOVA. $p < 0.05$ was considered statistically significant.

Results

Inflammatory pain induced by subcutaneous injection of CFA

In the present study, 50 µl of an equal volume mixture of 1 mg/ml of CFA and ACSF was subcutaneously injected into the plantar surface of the heel to develop inflammatory pain. We observed the rats for 21 days and PWT, cold withdrawal threshold, and paw cross-sectional area ratio were evaluated at baseline and on the 1st, 3rd, 7th, 14th, and 21st day after CFA injection. As shown in Figure 1, one day after CFA injection, rats expressed a significant decrease in the PWT and cold withdrawal threshold and a great increase in paw cross-sectional area ratio compared with the sham group that lasted for at least three weeks. These results suggest that CFA injection can result in obvious inflammatory pain and paw swelling.

Preventative effect of early treatment with PBN on the development of CFA-induced inflammatory pain

To evaluate the effect of early treatment with PBN in the development of CFA-induced inflammatory pain, PBN (1 and 5 mg/kg/0.2 ml) was locally injected into the iPM on the 1st, 3rd, 5th, and 7th day after CFA injection. PWT, cold withdrawal threshold, and paw cross-sectional area ratio were conducted at baseline and on the 1st, 3rd, 7th, 14th, and 21st day. It is suggested in Figure 2, PBN (1 and 5 mg/kg/0.2 ml) local injection of the iPM significantly alleviated pain of CFA-induced model. However, it failed to decrease inflammation of
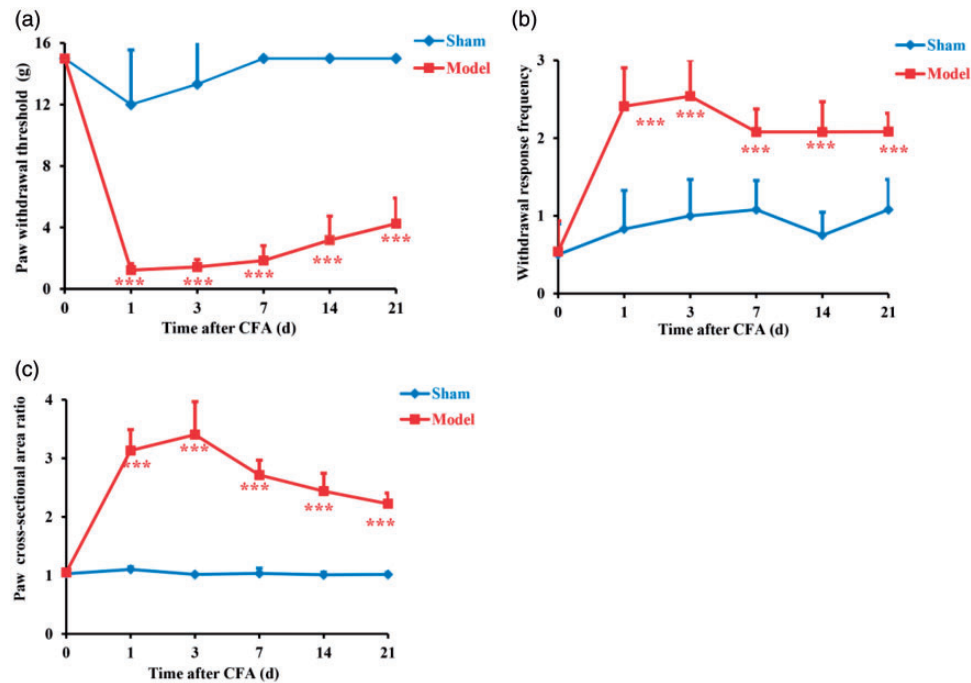
Figure 1. Time course of PWT, cold withdrawal threshold, and paw cross-sectional area ratio in sham and CFA-induced inflammatory pain rats. PWT, cold withdrawal threshold, and paw cross-sectional area ratio were tested at baseline and on the 1st, 3rd, 7th, 14th, and 21st day after CFA injection. PWT and cold withdrawal threshold significantly decreased, and paw cross-sectional area ratio was increased 1 day after CFA injection, and maintained for 21 days (***$p < 0.001$ compared with the sham group, n = 4 in sham group and 8 in model group). In contrast, sham rats showed no significant change in PWT during the 21-day observation period. CFA: complete Freund's adjuvant.
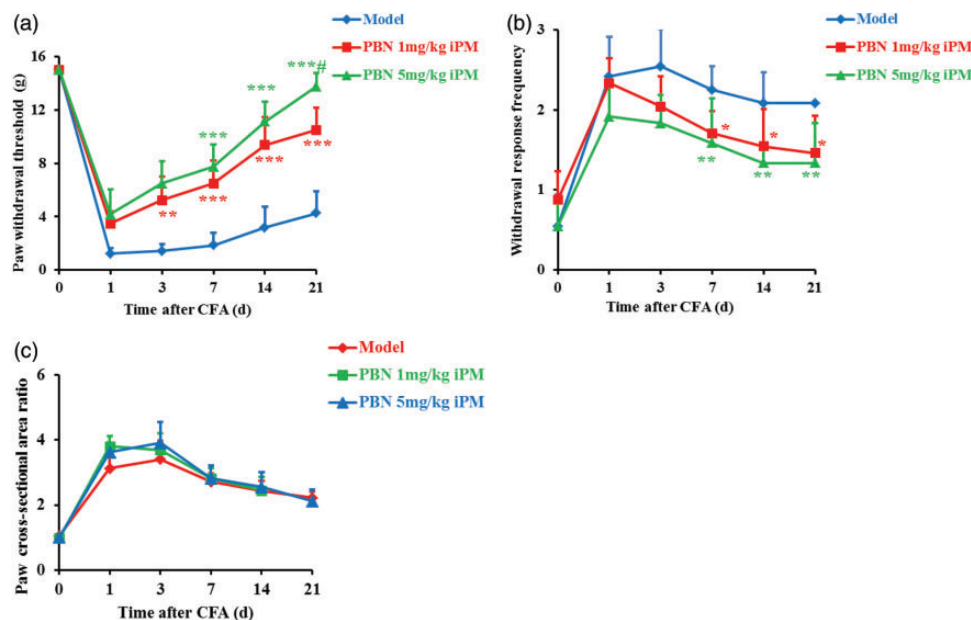

Figure 2. Preventative effect of early treatment with PBN on the development of CFA-induced inflammatory pain. PBN (1 and 5 mg/kg/ 0.2 ml) was injected locally into the iPM every two days from day 1 to day 7. PWT, cold withdrawal threshold, and paw cross-sectional area ratio were tested at baseline and on the 1st, 3rd, 7th, 14th, and 21st day. PWT and cold withdrawal threshold significantly increased, while paw cross-sectional area ratio was not improved (**$p < 0.01$, ***$p < 0.001$ compared with the model group, #$p < 0.05$ compared with the group treated with PBN 1 mg/kg, n = 8 in each group). CFA: complete Freund's adjuvant; iPM: ipsilateral psoas major muscle; PBN: N-tert-Butyl-α-phenylnitrone.

the inflammatory pain model. These results demonstrate that PBN injection into the iPM can prevent the development of pain on CFA-induced inflammatory pain in a dose-dependent manner.

Effects of early treatment of PBN on other paraspinal non-psoas major muscles or intraperitoneal injection on the CFA-induced inflammatory pain

To determine the function of ROS in the PM, PBN was intraperitoneally injected (5 mg/kg) or locally injected into paraspinal other non-psoas muscles (5 mg/kg/0.2 ml), cPM, iES, and iQL, every two days from day 1 to day 7. Other muscles' local injection or systemic treatment of PBN in the CFA-induced inflammatory rats showed slight or no change in PWT, cold withdrawal threshold, and paw cross-sectional area ratio (Figure 3). These results indicate that PBN was locally injected into the iPM but not other paraspinal muscles could relieve CFA-induced pain.

Effect of a single dose of PBN on established CFA-induced inflammatory pain

To determine the effect of a single dose of PBN on established CFA-induced inflammatory pain, a single dose of PBN (10 mg/kg/0.2 ml) was locally injected into the iPM or intraperitoneally injected seven days after CFA injection. PWT was conducted at 0, 0.5, 1.5, 1, 2, 3, 4, 5, and 6 h after PBN treatment. It is shown in Figure 4, PWT increased by PBN local injection, but the statistical difference was not significant. However, systemic treatment of PBN failed to relieve mechanical hyperalgesia of CFA-induced inflammatory rats. These results reveal that a single dose of PBN has analgesic effect on the CFA-induced inflammatory pain.

Effect of repeated administration of PBN on established CFA-induced inflammatory pain

To determine whether repetitive treatment with PBN had analgesic effect on established CFA-induced inflammatory pain, PBN (5 mg/kg/0.2 ml) was locally injected into the iPM or intraperitoneally injected every other day from 8th day to 14th day. The behavioral tests and paw cross-sectional area ratio were conducted at baseline and 7 days, 14 days, and 21 days after CFA. Repeated injection of PBN (5 mg/kg/0.2 ml, iPM) notably reversed the mechanical and cold hyperalgesia in CFA-induced inflammatory pain (Figure 5). Moreover, paw swelling was relieved significantly and maintained for at least seven days. Nevertheless, intraperitoneal injection of PBN (5 mg/kg) showed no significant difference compared with model group in behavioral tests and
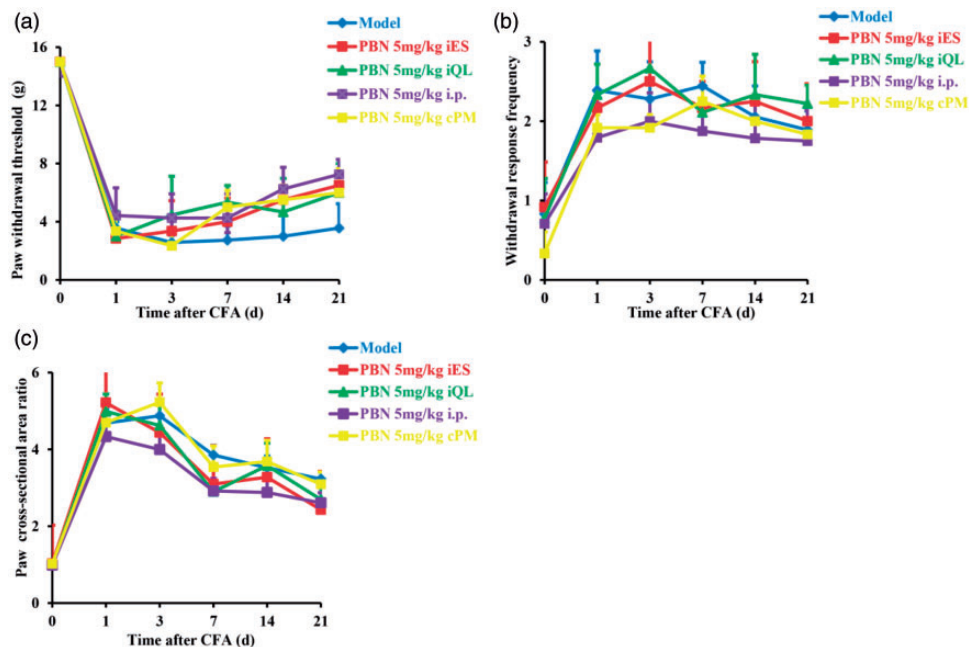

Figure 3. Effects of early treatment of PBN on paraspinal other non-psoas muscles or intraperitoneally injection on the CFA-induced inflammatory pain. PBN was intraperitoneally injected (5 mg/kg) and locally injected into paraspinal other non-psoas muscles (5 mg/kg/ 0.2 ml), cPM, iES, and iQL. PWT, cold withdrawal threshold, and paw cross-sectional area ratio were tested at baseline and on the 1st, 3rd, 7th, 14th, and 21st day. PWT showed slightly increase, while significantly no significant difference was observed in cold withdrawal threshold and paw cross-sectional area ratio compared with the model group. CFA: complete Freund's adjuvant; PBN: N-tert-Butyl-α-phenylnitrone; cPM: contralateral psoas major muscle; iES: ipsilateral erector spinae; iQL: ipsilateral quadratus psoas; i.p.: intraperitoneal.
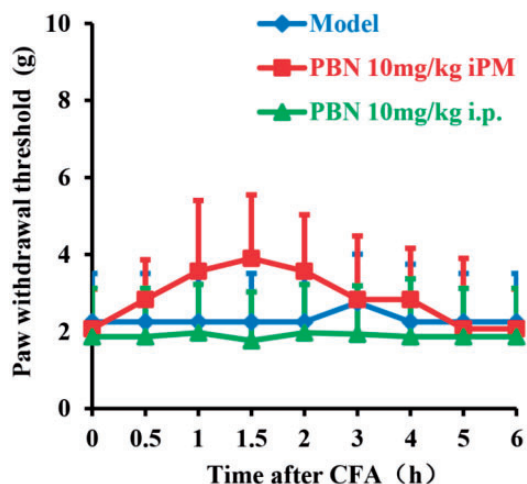
Figure 4. Effect of a single dose of PBN on established CFA-induced inflammatory pain. PBN (10 mg/kg/0.2 ml) was injected locally into the iPM or intraperitoneally injected at day 7. PWT were tested at 0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h after PBN treatment. IPM locally injection of PBN at dose of 10 mg/kg increased PWT of CFA-induced inflammatory rats, beginning at 0.5 h, peaking at 1.5 h, and lasting for at least 3 h, there is no statistical difference (p > 0.05 compared with the vehicle group, n = 6 in each group). However, intraperitoneal injection of PBN had no significant effect on the PWT (p > 0.05 compared with the vehicle group). CFA: complete Freund's adjuvant; PBN: N-tert-Butyl-α-phenylnitrone; cPM: contralateral psoas major muscle; iPM: ipsilateral psoas major muscle; i.p.: intraperitoneal.

paw cross-sectional area ratio. These results suggest that repetitive administration of PBN (5 mg/kg, iPM) also has analgesic effect on established CFA-induced inflammatory pain.

ROS levels of PM after CFA injection

It is found that ROS level in the iPM changed after CFA injection into the paw plantar. At 1 day after CFA injection, there were not many changes of ROS levels in the iPM. While after seven days, ROS increased by 73% in the iPM (Figure 6).

Discussion

Our study mainly indicates that ROS of the PM is involved in CFA-induced inflammatory pain in rats. ROS scavengers acting on the PM can effectively relieve CFA-induced pain and inflammation.

There is a strong connection between ROS and pain. Pain and paw edema can be evoked by injection of potassium superoxide KO_2 into the plantar of mice via production of ROS and cyclooxygenase-2.[33] Intramedullary injection of ROS donors, superoxide tert-butyl hydroperoxide (t-BOOH, an OH⁻ donor), or NaOCl (OCl⁻) elevated ROS levels, which resulted in increased excitability in the dorsal horn and mechanical hyperalgesia in normal
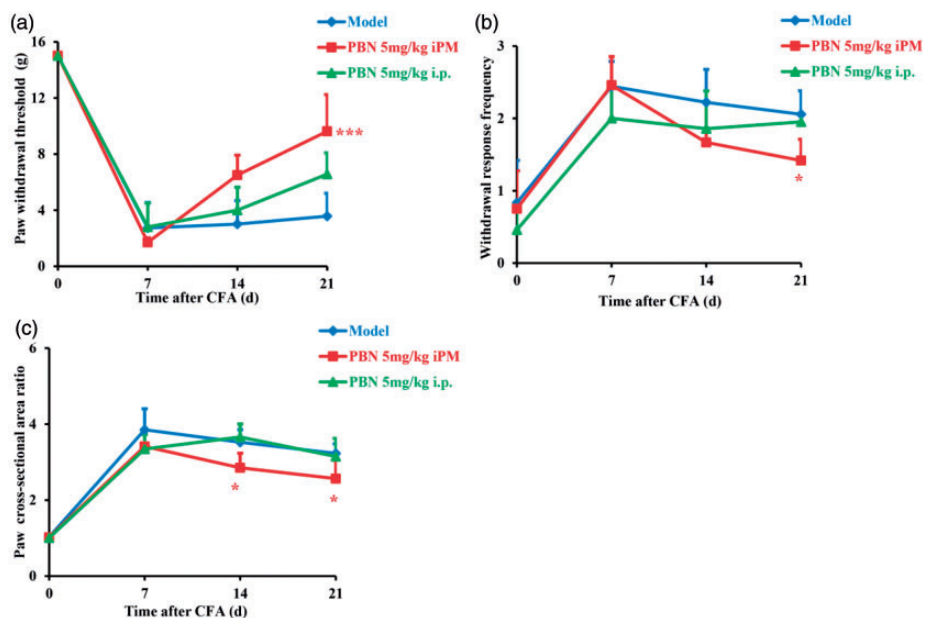

Figure 5. Effect of iPM repetitive repeated administration of PBN on established CFA-induced inflammatory pain. PBN (5 mg/kg) was injected locally into the iPM or intraperitoneally injected every two days from day 8 to day 14. PWT, cold withdrawal threshold, and paw cross-sectional area ratio were tested at baseline and on the 7th day, 14th day, and 21st day. IPM repeated administration of PBN significantly reversed PWT, cold withdrawal threshold, and paw cross-sectional area ratio (*p < 0.05, ***p < 0.001 compared with the sham group, n = 8 in each group). However, intraperitoneal injection of PBN failed to change the behavioral tests and paw cross-sectional area ratio. CFA: complete Freund's adjuvant; iPM: ipsilateral psoas major muscle; PBN: N-tert-Butyl-α-phenylnitrone; i.p.: intraperitoneal.
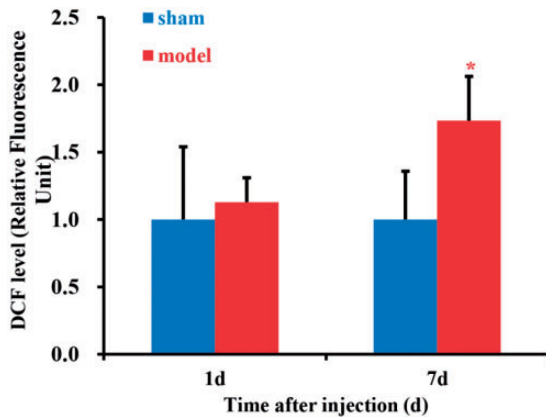
Figure 6. ROS levels of psoas major muscle after CFA injection. A total of 36 rats were distributed into two groups with 18 rats in each group. First group of rats were injected subcutaneously with 50 μl saline into the left paw plantar, and the other group received 50 μl CFA and ACSF equal volume mixture. Six rats from each group were killed on the 1st day and 7th day after CFA injection. Levels of ROS in iPM were measured by oxidative conversion of H2DCFDA to fluorescent DCF. ROS in the iPM increased significantly at seven days after CFA injection. (*p < 0.05 compared with the sham group, n = 6 in each group). DCF: 2′,7′-dichlorofluorescein.

rats.[34] At the same time, ROS is also closely related to persistent pain. For example, microenvironment of cells under low pressure may foster dysfunction of cells and trigger pain. And severe ROS can even lead to cell death and degradation.[35] ROS levels in cows with chronic lameness can be found elevated significantly.[36] In our experiments, increased ROS levels were detected in the iPM (Figure 6).

ROS involvement in inflammatory and neuropathic pain has been increasingly reported,[13–16] but it is not entirely clear which sites produce ROS. Singh et al.[37] have demonstrated ROS level increased in paw skin as well as spinal cord in CFA-induced hyperalgesia. Wang et al.[9] found that intravenous or intrathecal administration of superoxide dismutase mimetic, M40403 inhibited inflammation, and hyperalgesia of carrageenan-induced pain in rats. Thus, superoxides play an important role in the nociceptive signaling cascade both peripherally and centrally. However, many studies indicated that ROS plays a critical role in neuropathic pain, mainly through spinal mechanisms.[8,38,39] Gao et al. demonstrated that ROS take a part in N-methyl-D-aspartate receptor activation, an essential step in central sensitization, in neuropathic and capsaicin-induced pain models.[39] A study provided evidence that ROS regulated phosphorylation and cell-surface localization of a-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors, which was important for dorsal horn neuron sensitization and pain.[8] Electron delivery complex inhibitors antimycin A and rotenone intrathecal injection produced

significant pain responses in normal mice, which were effectively reversed by systematic injection of ROS scavengers, PBN, suggesting that superoxide produced by mitochondria in the spinal cord is involved in this process.[38] In the present study, there was a significant increase of ROS level in the psoas major muscle (Figure 6). Besides, treatment of ROS scavenger PBN in psoas major muscle can alleviate CFA-induced mechanical and cold hyperalgesia (Figures 2, 4, and 5). Our results suggested that ROS in the psoas major muscle were involved in CFA-induced inflammatory pain.

Many studies have shown that ROS scavengers can effectively relieve kinds of pain, such as inflammatory,[9,40] neuropathic,[7,15] cancerous,[14] and chemotherapy pain,[16] since ROS is associated with the development and maintenance of pain. Zhou et al. reported that intraperitoneal injection of PBN and Tempol significantly suppressed established mechanical allodynia in cancer-induced bone pain (CIBP), and repetitive administration showed cumulative analgesic effect. However, pretreatment of PBN and Tempol failed to prevent the development of CIBP.[14] Mahmoud's study has shown that Tempol, a membrane-permeable radical scavenger, relieved carrageenan-induced hyperalgesia and paw edema.[40] Kim et al. demonstrated that systemic injection of a PBN relieved mechanical allodynia of spinal nerve ligation rats in a dose-dependent manner. Moreover, preemptive or repeated intraperitoneal injection of PBN had obvious effects, while intrathecal or intracerebroventricular administration turned out to be less effective.[15] In their another study, a single dose or multiple intraperitoneal injection of PBN ameliorated paclitaxel-induced pain, and early treatment also had a remarkable preventative effect.[16] In our study, single dose or continuous injection of CFA was effective for CFA-induced inflammatory pain (Figure 4). Repetitive local injection of PBN into iPM for four times every other day from day 1 to day 7 after CFA injection could effectively relieve CFA-induced inflammatory pain (Figure 2). And for the developed inflammatory pain, four times local administration of PBN into iPM from day 8 to day 14 after CFA made great decrease in mechanical and cold hyperalgesia (Figure 5).

In our study, obvious paw swelling was found after CFA injection (Figure 1). Paw edema did not differ from model group after early administration of PBN into the iPM for four times, but was dramatically reduced on rats locally injected of PBN (5 mg/kg/0.2 ml) into the iPM from day 8 to day 14 every other days. While early treatment of PBN (5 mg/kg/0.2 ml) from day 1 to day 7 failed to relieve paw swelling. Edema, a crucial sign of inflammation, is reduced only in the developed CFA-induced inflammatory pain. Inflammation reaction takes part in production of pain. A previous study evaluated that only

neuronal nitric oxide synthase deficient mice have a significant decrease in pain behavior after CFA injection into the paw, whereas there are no major differences to endothelial NOS and inducible NOS (iNOS)-deficient mice genotypes. Furthermore, only in mice lacking iNOS was found a reduction in paw swelling after CFA.[41] According to our results, preventative local injection of PBN into the iPM did not lighten paw edema in the developing of CFA-induced inflammatory pain. However, for the developed inflammatory pain, local injection of PBN into the iPM was effective to alleviate pain partly via reducing inflammatory of paw.

As far as we know, this is the first study to explore the connection between the ROS in the psoas major muscle and lower limb pain. We found that injection of very low doses of PBN (1, 5, and 10 mg/kg) into the psoas major muscle could significantly relieve lower limb pain (Figures 2, 4, and 5). Higher doses (50 and 100 mg/kg) were used to relieve pain by intraperitoneal injection.[14,29] Intraperitoneal injection of 5 mg/kg PBN did not allieviate the pain induced by CFA in the current study. This suggests that it is a kind of local effect other than systemic action. We detected the ROS levels in the iPM and found that seven days after CFA injection, there was a great increase compared to the sham group. PBN, a ROS scavenger, effectively reduce the ROS in the psoas major muscle. Therefore, lower limb pain induced by ROS in the psoas major muscle is alleviated. Our research is innovative in trying to relieve the pain through local injection of low dose of ROS scavenger into the psoas major muscle. As for intraperitoneal injection, and local injection of other lower back muscles, like iQL, iES, and cPM, the same dose of PBN was less effective (Figure 3).

A study by Xie et al. has proved that cutting the ipsilateral Lumbar 4 (L4) and Lumbar 5 (L5) gray rami reduced pain induced by local dorsal root nerve inflammation and CFA-induced paw inflammation.[28] Almarestani et al. have demonstrated that injection of CFA into the plantar surface of paw promotes the proliferation of sympathetic nerve fibers and hyperalgesia.[42] The L4 and L5 sympathetic gray ramus entered the spinal nerves through the psoas major muscle according to anatomy.[43] In our study, we have shown that ROS in the psoas major muscle is associated with the development and maintenance of CFA-induced inflammatory hyperalgesia and paw edema. We can infer that ROS of psoas major muscle regulates the sympathetic nerve which promotes inflammation and hyperalgesia, while the specific mechanism needs to be further explored.

In summary, this study revealed that continuous local injection of PBN into the iPM could effectively inhibit CFA-induced paw mechanical, hypoalgesia, and edema, in the early and final stages. But local injection of PBN into the cPM, iQL or iES, and intraperitoneal injection could only produce slight or no relief. ROS level in the iPM was significantly increased after CFA injection. The effects of analgesia and anti-inflammation of PBN might be due to reducing ROS in the psoas major muscle. It is known that ROS in the PM is involved in the development and maintenance of CFA-induced paw inflammatory pain, and treatment of eliminating ROS in psoas major might alleviate clinical lower limb pain.

Declaration of Conflicting Interests

Funding

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