Osthole, a herbal compound, alleviates nucleus pulposus-evoked nociceptive responses through the suppression of overexpression of acid-sensing ion channel 3 (ASIC3) in rat dorsal root ganglion

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Qiu-Lan He[*1ABCDEF], **Yuling Chen**[*2ABCDEF], **Jian Qin**[*3ABCD], **Sui-Lin Mo**[3ABCDE], **Ming Wei**[1ABCD], **Jin-Jun Zhang**[1ABCD], **Mei-Na Li**[1ABCD], **Xue-Nong Zou**[4DE], **Shu-Feng Zhou**[5ABCDEF], **Xiao-Wu Chen**[6ABCD], **Lai-Bao Sun**[1ABCDEFG]

* These authors contributed equally to this work

[1] Department of Anesthesiology, First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China
[2] Faculty of Medicine, University of New South Wales, Sydney, Australia
[3] Department of Traditional Chinese Medicine, First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China
[4] Institute of Orthopedic Research and Department of Spinal Surgery, First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China
[5] Department of Pharmaceutical Sciences, College of Pharmacy, University of South Florida, Tampa, FL, U.S.A.
[6] Department of General Surgery, Shunde First People's Hospital, Southern Medical University, Foshan, Guangdong, China

Summary

Background: Osthole (Ost), a natural coumarin derivative, has been shown to inhibit many pro-inflammatory mediators and block voltage-gated Na^+ channels. During inflammation, acidosis is an important pain inducer which activates nociceptors by gating depolarizing cationic channels, such as acid-sensing ion channel 3 (ASIC3). The aim of this study was to examine the effects of Ost on nucleus pulposus-evoked nociceptive responses and ASIC3 over-expression in the rat dorsal root ganglion, and to investigate the possible mechanism.

Material/Methods: Radicular pain was generated with application of nucleus pulposus (NP) to nerve root. Mechanical allodynia was evaluated using von Frey filaments with logarithmically incremental rigidity to calculate the 50% probability thresholds for mechanical paw withdrawal. ASIC3 protein expression in dorsal root ganglions (DRGs) was assessed with Western blot and immunohistochemistry. Membrane potential (MP) shift of DRG neurons induced by ASIC3-sensitive acid (pH6.5) was determined by $DiBAC_4$ (3) fluorescence intensity (F.I.).

Results: The NP-evoked mechanical hyperalgesia model showed allodynia for 3 weeks, and ASIC3 expression was up-regulated in DRG neurons, reaching peak on Day 7. Epidural administration of Ost induced a remarkable and prolonged antinociceptive effect, accompanied by an inhibition of over-expressed ASIC3 protein and of abnormal shift of MP. Amiloride (Ami), an antagonist of ASIC3, strengthened the antinociceptive effect of Ost.

Conclusions: Up-regulation of ASIC3 expression may be associated with NP-evoked mechanical hyperalgesia. A single epidural injection of Ost decreased ASIC3 expression in DGR neurons and the pain in the NP-evoked mechanical hyperalgesia model. Osthole may be of great benefit for preventing chronic pain status often seen in lumbar disc herniation (LDH).

key words: hyperalgesia • lumbar disc herniation (LDH) • osthole • acid-sensing ion channel 3 (ASIC3) • membrane potential

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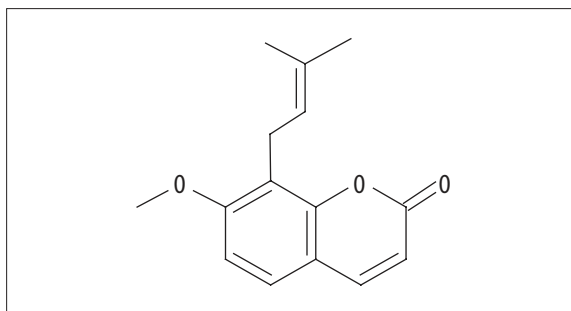**Figures:** 6
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Author's address: Lai-Bao Sun, Department of Anesthesiology, the First Affiliated Hospital, Sun Yat-sen University, 58 Zhongshan Er Lu, Guangzhou 510080, China, e-mail: sunlaibao88@hotmail.com and Shu-Feng Zhou, Department of Pharmaceutical Sciences, College of Pharmacy, University of South Florida, MDC 30, 12901 Bruce B. Downs Boulevard, Tampa, Florida 33612, e-mail: szhou@health.usf.edu

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BACKGROUND

Chronic sciatica and low back pain induced by lumbar disc degeneration or herniation (LDH) is a common musculo-skeletal disease affecting about 5% of all individuals worldwide [1]. LDH refers to a process in which there is rupture of the anulus fibers and subsequent displacement of the central mass of the disc in the intervertebral space, common to the posterior or posterolateral aspect of the disc [2–7]. This disease most commonly occurs in individuals aged 30 to 50 years. Alterations in the vertebral endplate lead to loss of disc nutrition and disc degeneration. Aging, cellular apoptosis, abnormalities in collagen and proteoglycan, loads placed on the disc, and vascular ingrowth contribute to disc degeneration [8]. However, the pathophysiologic mechanism of painful radiculopathy caused by a herniated intervertebral disc remains unclear. Compression of the nerve has been shown to create edema formation and eventually cause intraneural inflammation and hypersensitivity [9,10]. This will result in increased mechanosensitivity of the nerve root with regard to compression and the induction of pain. It is now generally accepted that a combination of mechanical and abnormal biochemical events/pathways is involved in the generation of radicular pain [9]. Many chemical and inflammatory mediators such as phospholipase A_2, interleukins, and nitric oxide, induced by extruded or sequestrated intervertebral discs, are involved in the pathogenesis of painful radiculopathy in LDH [9,11]. In addition, family and twin studies have shown that genetic factors may play an important role in the development of LDH [12]. Two collagen IX alleles (*COL9A2* and *COL9A3*) have been associated with sciatica and lumbar disc herniation [13] and disc degeneration has been shown to be related to an aggrecan gene polymorphism, a vitamin D receptor and matrix metalloproteinase-3 gene alleles [14,15].

It is well established that alterations in nociceptors and elevated neuronal activity can lead to the development of inflammatory pain hypersensitivity. Tissue acidosis is commonly observed as a dominant contributor to hyperalgesia [16,17]. Acid-sensing ion channel (ASIC3) in dorsal root ganglion (DRG) neurons may play an important role in nerve root pain caused by LDH [18]. The ASIC family entails neuronal voltage-insensitive cationic channels activated by extracellular protons [19,20]. Around the LDH-induced inflammation tissue, the up-regulation of ASIC3 in DRG neurons might be an important integrator of allodynia [18]. ASIC3 could induce a transient inward current as a dominant sensor of pain responding to weak acidification, while a sustained current might be induced when pH drops to 5.0 [21].

Pharmacotherapy and regional nerve block is the cornerstone of management of LDH-associated pain. It seems likely that a combination of multi-modal and multi-disciplinary treatment is preferable. In traditional Chinese medicine, some herbal medicines such as Cusson and Angelica are considered to have great curative effects for LDH and arthritis. Osthole (7-methoxy-8-(3-methylpent-2-enyl) coumarin, Ost) (Figure 1) is extracted from *Cnidium monnieri* (L.), Cusson and *Angelica pubescens* maxin. To date, the antitumor [22], anticonvulsant [23] and memory-enhancing [24] activities of Ost have been demonstrated. Recently,

Figure 1. Chemical structure of osthole.

this herbal compound has also been found to exhibit potent anti-inflammatory properties through the inhibition of cyclooxygenase (COX), inducible-nitric oxide synthase (iNOS) and tumor necrosis factor-α (TNF-α) [25]. In a rat model of nucleus pulposus (NP)-evoked hyperalgesia, a single dose of epidural injection of Ost showed a potent antinociceptive effect [26]. However, its specific antinociceptive mechanism is unclear and controversial in different models of inflammatory pain [27].

We hypothesized that the regulation of ASIC3 was involved in the mechanism by which herbal compounds such as Ost induced an antinociceptive effect. As such, this study was designed to explore the effects of osthole on autologous nucleus pulposus (NP) – evoked hyperalgesia and ASIC3 overexpression in the rat dorsal root ganglion. The variation of membrane potentials (MP) of DRG neurons evoked by ASIC3-sensitive acid and the synergistic effect of Ost and Ami on pulposus-evoked hyperalgesia and ASIC3 expression was explored as well. Since amiloride (Ami) is a nonselective antagonist of ASICs and exhibits a moderate effect at high concentration in a mouse pain model [27]; the synergistic effect of Ost and Ami was also investigated in this study.

MATERIAL AND METHODS

Chemicals and reagents

Ost (analytical purity: 99%) was obtained from Nanjing TCM Institute of Materia Medica, Nanjing, China. Ami and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male Sprague-Dawley (SD) rats weighing 237.5±12.3 g were purchased from The Medical Laboratory Animal Center of Guangdong. A total of 151 SD rats were used, including 109 rats in the autologous NP-evoked hyperalgesia group (NP-rats), 21 sham-operated control rats and 21 blank control rats. All experiments were approved by the Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China. Rats were kept in colony cages with free access to food and water, under standardized housing conditions (12 hr light-dark cycle, temperature 22–24°C, relative humidity 55±5%). Experiments were carried out in compliance with the Experimental Animal Management Bill of the November 14th 1988 Decree No.2 of National Science and Technology Commission, Beijing, China.

Autologous NP-evoked hyperalgesia model

In accordance with the methods of Kawakami et al. [11] and Ohtori et al. [28], 109 rats were anesthetized with 20% urethane (0.8 ml/100g by intraperitoneal injection). Laminectomies were performed, exposing the left L5 nerve roots and associated DRGs. Autologous nucleus pulposus (about 0.4 mg) was harvested from the 2 near-end intervertebral spaces of each tail and gently placed onto the exposed left L5 DRG. A PE-0503 catheter was put into the epidural space cranially where the exposure was performed. In 21 sham control rats, nucleus pulposus was harvested using the above procedures but it was not applied to the L5 DRG. The blank control rats were normal SD rats without surgery.

Drug treatment

For behavioral assessment and ASIC3 expression assay, Ost and Ami (100 μg/kg, Sigma-Aldrich, St. Louis, MO, USA) were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) and diluted in distilled water. The vehicle control was distilled water containing 0.1% DMSO. All the solutions were adjusted to a pH value of approximately 7.4. The drugs were injected through a PE-0503 catheter on day 6. The volume of injection was 50 μl. For the MP analysis, Ost and Ami were dissolved in DMSO and diluted in DMEM/F12. The vehicle control was DMEM/F12 containing 0.1% DMSO. All the tested drugs were adjusted to pH 7.4. Each rat's neurons were pretreated with the tested drugs 1 h before testing.

Mechanical pain threshold study

Before the behavioral study, a 72-h adaptation to the experimental condition was made for all the rats. Fifty-six NP rats were randomly assigned to receive 1% Ost, 2% Ost, 5% Ost, Ami, 2% Ost plus Ami, and DMSO alone. The thresholds of NP rats were measured on day 1 and days 1, 3, 5, 7, 10, 14, 17, 21 and 28 after NP application (day 0, the surgery day for NP application; day 6, the treatment day). Hyperalgesia was assessed using von Frey filaments with logarithmically incremental rigidity (0.41, 0.70, 1.20, 2.00, 3.63, 5.50, 8.50, and 15.1 g; Stoelting, Wood Dale, IL, USA) to calculate the 50% probability thresholds for mechanical paw withdrawal [18]. Filaments were applied to the pad of the left paw for 6–8 seconds in an "up-down" method [29]. Ost, Ami, Ost plus Ami, or DMSO was injected to the experimental and control rats on day 6 through the PE-0503 catheter. The tested drugs or vehicle control were given at the same volume of 50 μl with a pH value of approximately 7.4. The investigators were blind to all treatments.

Determination of ASIC3 protein expression in DRG neurons

There were 63 rats used for evaluation in this study, including 33 in the NP, 15 in the sham control and 15 in the blank control group. All rats were anesthetized using carbon dioxide and decapitated for exsanguination. The left L4-L5 DRGs were rapidly dissected and homogenized in 50 mM Tris HCl, pH 7.5/5mM EDTA/150 mM NaCl/1%Triton X-100/Complete protease inhibitors (Boster, Beijing, China). The protein concentration was measured using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were resolved in SDS-10% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore Inc., Billerica, MA, USA). After the membranes were blocked with 5% dry milk, they were incubated overnight at 4°C with anti-ASIC3 (1:1000, Abcam, Cambridge, MA, USA) or anti-β-actins (1:5000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibodies followed by extensive washes. A 1:5,000 dilution of anti-rabbit horseradish peroxidase-labeled antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added and incubated for another 1 h. Finally, blots were developed with enhanced chemiluminescence (ECL plus, Amersham Pharmacia, Piscataway, NJ, USA) and exposed onto X-films for 2–10 min. Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA) was used for the analysis of optic density of ASIC3 and β-actin. Multiple Western blots of ASIC3 were quantified by densitometric analysis. The expression levels of each group were normalized to the corresponding level of β-actin.

Immunohistochemistry

Fifteen rats were used, including 9 in the NP, 3 in the blank control and 3 in the sham control group. The rats were deeply anesthetized with sodium pentobarbital (40 mg/kg body weight, by intraperitoneal injection) and perfused transcardially with 500 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The L5 DRGs were resected and post-fixed with the same fixative for 30 min and then soaked in phosphate-buffered 20% sucrose overnight. Frozen sections (12 μm) were made on a cryostat and pretreated with a blocking solution containing 0.3% Triton X-100 and 3% normal goat serum (Sigma-Aldrich, St. Louis, MO, USA) for 1.5 h at room temperature. Sections were processed for ASIC3 immunohistochemistry analysis using an avidin-biotin complex technique that involved with rabbit polyclonal body to ASIC3 (1:1000, Abcam, Cambridge, MA, USA) for 48 h at 4°C, followed by incubation with goat anti-rabbit staining kits (FITC; 1:200; Boster, Beijing, China) for 2 h at room temperature. The sections were finally examined using a fluorescence microscope (Leica Co., Solms, Germany). We counted 10 serial sections exhibiting the greatest number of labeled cells in each rat. DRG neurons were counted at 100× magnification using a counting grid. The number of ASIC3 immunoreactive cells per 0.0225 mm^2 were counted and averaged for each animal.

Acid-induced MP change assessment

The left L4-L6 DRG neurons of rats were prepared on day 7. In brief, the DRG neurons were isolated by a standard enzyme protocol and cultured in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) plus 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Neurons from each rat were plated (1×10^5 cell/ml) on the bottom of each well (200 μl/well, 24-well plate) and maintained at 37°C in a humidified atmosphere containing 5% CO_2 up to 5 h. Then the neurons were labeled with a solution containing 50 nM bis (1,3-dibutyl barbituric acid) trimethine oxonol (DiBAC4$_{(3)}$, Sigma-Aldrich, St. Louis, MO, USA) for 20 min. After DiBAC4$_{(3)}$ staining, the neurons were washed by physiological salt solution (PSS, pH 7.4, 10 mM HEPES, 124 mM NaCl, 25 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, and 2 mM $CaCl_2$) for 30 sec followed by acidic PSS (added HEPES 15 mM, pH 6.5) stimulation through a glass micropipette at a rate of 1 ml/min for 60 sec. Dynamical measurements of the intracellular fluorescence intensity (F.I.) were performed using a fluorescence microscope. The values of F.I. were recorded every 5 sec.
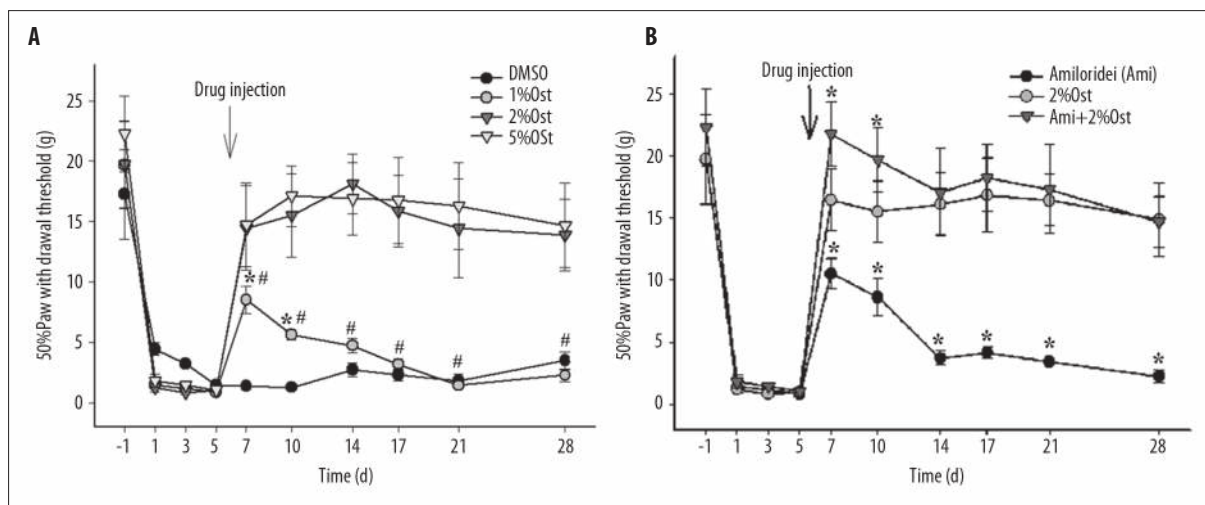
Figure 2. Mechanical pain threshold measured by von-Frey hair stimulation of the hind paw. Data from the paw withdrawal of all the groups are expressed as 50% of threshold and showed as means ± s.e.m. (n=8). NP was applied on the left L5 DRG on Day 0 and drugs were injected on Day 6. Autologous NP-evoked hyperalgesia are expressed as a decrease of threshold value. (**A**) There was no significant difference of the threshold values (about 1 g) among the 4 groups on Day 5. In 2% Ost and 5% Ost-treated groups, hyperalgesia were rapidly and persistently inhibited. In 1% Ost-treated group the nociceptive threshold only increased transiently and slightly. n=8, * $P<0.05$, compared with DMSO group; # $P<0.05$, compared with 2% Ost group. Two-way ANOVA with Bonferroni correction as post-hoc test. (**B**) Antinociceptive effect of 2% Ost was more potent than that of Ami (100 μg·kg⁻¹), while the antinociception of 2% Ost in combination with Ami (2% Ost + Ami) at early time points (on Day 7 and Day 10) was more potent than that of the pretreatment using 2% Ost only. * $P<0.05$, compared with 2% Ost group. Two-way ANOVA with Bonferroni correction as post-hoc test.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). All data are presented as means ±SEM unless otherwise stated. A Shapiro-Wilk test for normality was performed when appropriate. A value of $P<0.05$ was considered to be statistically significant. Statistical analysis was performed by 2-way ANOVA analysis, followed by Bonferroni-type multiple t-test for behavioral experiments and MP assessment experiments among groups over the time. Comparison of acid-induced MP change of each group was made by Student's paired t-test. Comparison of ASIC3 protein expression between groups was made by 1-way ANOVA analysis followed by Bonferroni-type multiple t-tests.

RESULTS

**Antinociceptive effect of Ost on NP-evoked hyperalgesia**

NP was applied on the left DRG on day 0. NP rats were equally randomized into 4 groups and received epidural administration of 1%, 2%, 5% Ost and DMSO on day 6, respectively. Autologous NP-evoked hyperalgesia was expressed as a decrease of mechanical pain threshold value. There was no significant difference of the threshold values (about 1 g) among the 4 groups on day 5. In the 2% Ost-treated and 5% Ost-treated groups, the hyperalgesia was rapidly and persistently inhibited after the administration. The nociceptive threshold only increased transiently and slightly in the 1% Ost group from day 7 to day 10 (Figure 2A). Based on the results, the concentration of 2% was selected for the subsequent experiments. Then the antinociceptive effect of Ami, Ost and Ami plus Ost was compared in NP rats. The threshold values on day 7 were 10.54±1.20, 16.47±2.49, and 21.76±2.58

g for Ami, 2% Ost, and Ami plus 2% Ost groups, respectively, indicating that the antinociceptive effect of Ost was more potent than that of Ami ($P<0.05$). The combination of Ami and Ost resulted in a short-term (day 7 to day 10) but stronger antinociceptive effect than Ost ($P<0.05$) (Figure 2B).

Effect of Ost on ASIC3 protein expression in DRG neurons of NP rats

Western blots were performed using antibodies to ASIC3, recognizing a band at 59 kDa. The Western blotting analysis showed a significant increase of ASIC3 protein in DRG of NP rats compared with that in the blank and sham groups. The elevation of ASIC3 expression was delayed and gradual, reaching peak on day 7 and then decreasing slowly (Figure 3A). To investigate the effect of Ost on ASIC3 protein expression in DRG of NP rats, the drugs were administered on day 6 and the Western blotting analysis was performed on day 7 (NP was applied on the left DRG on day 0). The data show that Ost resulted in a decreased expression of ASIC3 protein, but not in a totally concentration-dependent manner. The ASIC3 protein expression in the Ami plus 2% Ost group was significantly lower compared with that in the 2% Ost group, indicating that the inhibitory effect of Ost on ASIC3 protein expression was enhanced by the combination treatment of Ami (Figure 3).

The immunohistochemistry assay showed that ASIC3 was expressed predominantly in the small and medium neurons (Figure 4). The number of ASIC3-positive cells was increased in DRG from NP group compared with that of the sham group (NP group, 42±11; sham group, 11±5; $P<0.05$). The number of labeled nuclei was decreased in the Ost group (23±7, $P<0.05$) and the Ami-Ost group (17±8, $P<0.05$) as compared with the NP group.
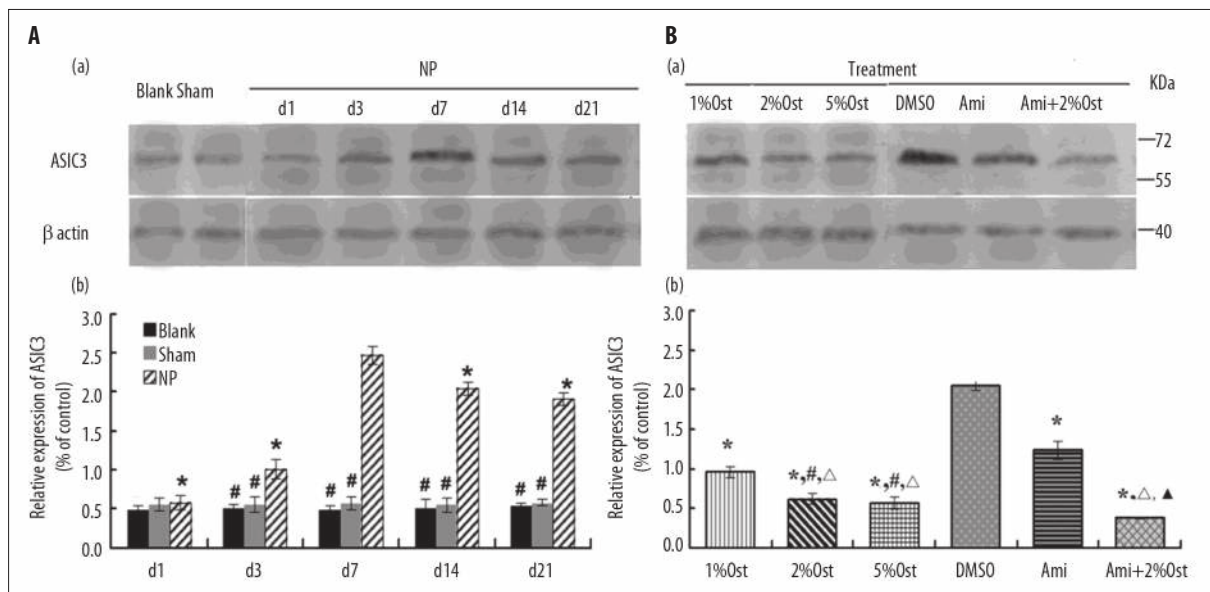
Figure 3. Western blot analysis of ASIC3 proteins in cell lysates of DRGs. A. Time course of change in ASIC3 expression in DRGs. (**A**) The protein expression of ASIC3 in DRGs of blank, sham and NP rats; (**B**) The relative densitometric values showed significant increase of ASIC3 protein in DRGs of NP rats compared with that in blank and sham group. Data represent mean ± s.e.m. from 6 individual analyses. * *P*<0.05, compared with the value of NP rats on Day 7. # *P*<0.05 compared with the value of NP rats at the same time points. B. Effect of Ost & Ami on ASIC3 protein expression in DRGs of NP rats. (**A**) The ASIC3 protein expression in DRG of NP rats treated with Ost, Ami & vehicle control on Day 7. (**B**) There was no significant difference in ASIC3 expression between 2% Ost and 5% Ost groups, both of which showed a lower level of ASIC3 compared with 1% Ost group. The expression of ASIC3 protein of the Ami group was lower than that of the DMSO group (vehicle control) but higher than that of 2% Ost group. Combination of Ami and 2% Ost resulted in a significant decrease of ASIC3 expression. * *P*<0.05, compared with the value of DMSO group; # *P*<0.05, compared with the value of 1%Ost group; △ *P*<0.05, compared with the value of Ami group; ▲ *P*<0.05, compared with the value of 2%Ost group. One-way ANOVA.
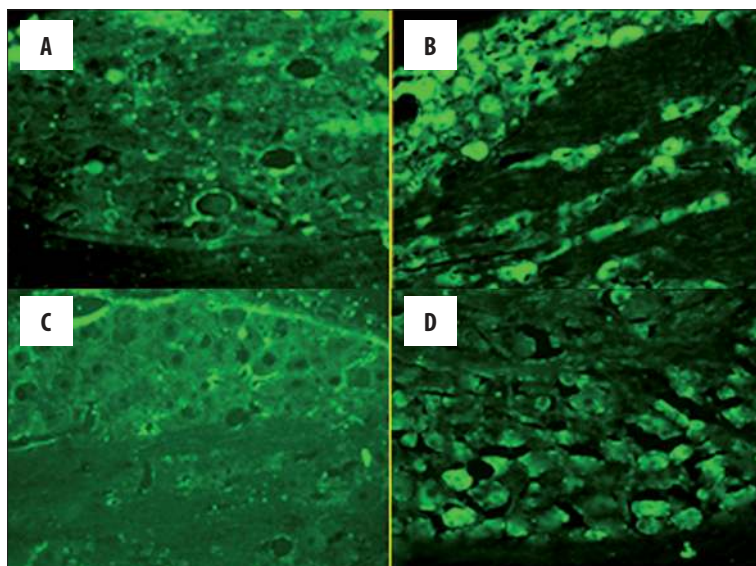

Figure 4. Representative images of immunofluorescence detection of ASIC-IR cells in DRG sampled from three rats per group on Day 7. Positive ASIC3-IR cells were stained with FITC (green) in (**A**) DRG from the rat of sham group; (**B**) DRG of NP rat; (**C**) DRG of NP rat after treatment of Ost in combination with Ami; (**D**) DRG from rat of Ost-treated group. Magnification, ×100.

Effect of Osthole on acid-induced changes of MP

On day 7, DRG neurons from blank, sham and NP groups were sampled and aliquoted into 3 wells. When extracellular pH was shifted from 7.4 to 6.5, the F.I. of NP neurons increased rapidly and significantly compared with that of the blank control and sham control neurons at the same time-points (Figures 5, and 6A).

To test the effects of Ost on acid-induced changes of MP, DRG neurons from 8 NP rats were treated with 1% Ost (100 µg), 2% Ost (200 µg), 5% Ost (500 µg), Ami (10 µg), Ami (10 µg) plus 2% Ost (200 µg), or DMEM/F12 containing 0.1% DMSO. Both Ost and Ami were found to inhibit the acid-evoked increase of MP. Effects of Ost (1%, 2% and 5%) showed a concentration-dependent inhibitory effect. Combination of Ami and 2% Ost was still the most potent pretreatment, inducing an almost complete
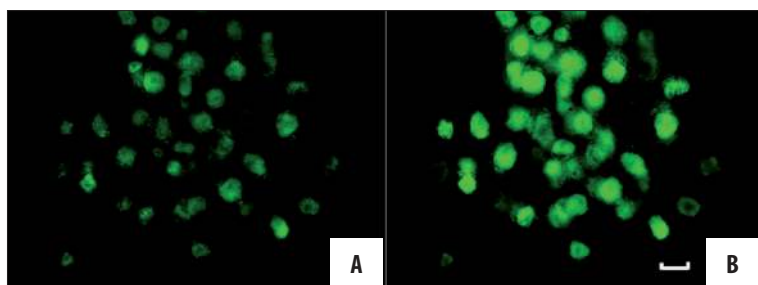
Figure 5. Fluorescent photomicrograph of DGR neurons in NP rat. (**A**) DRG neurons were incubated in DiBAC4$_{(3)}$ solution with PSS (pH 7.4) before acidic stimulus. (**B**) The F.I. of DRG neurons was increased after extracellular stimulus of acidic PSS (pH 6.5) for 5 sec. Bar, 40 μm.
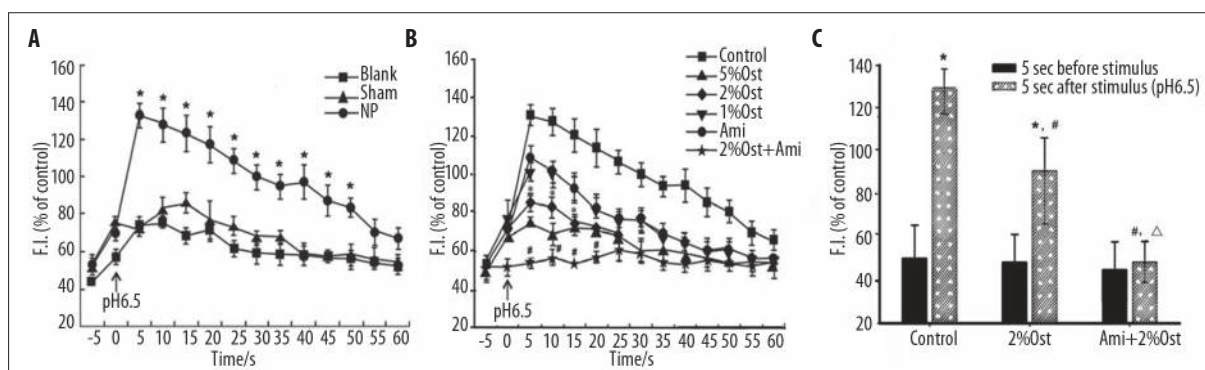
Figure 6. Acid-evoked MP changes of DRG neurons. Neurons were stimulated with acidic PSS (pH 6.5) at a rate of 1 mL/min and F.I. were detected with DiBAC4$_{(3)}$ at 5 sec intervals. (**A**) Data of blank, sham and NP group are obtained from nine individual experiments (three wells per rat × three rats per group). * $p<0.05$, compared with the corresponding values of blank neurons at the same time points. (**B**) Effects of six pretreatments were represented with mean ± s.e.m. from eight individual experiments (once per rat × eight rats). * $p<0.05$, compared with the corresponding values of control neurons (vehicle control) at the same time points; # $p<0.05$, compared with the corresponding values of 2% Ost-pretreated neurons at the same time points. (**C**) Histograms of relative F.I. of DRG neurons before (dark) and after low pH stimulation (light). Values were shown as% F.I. increase (Δ F.I.) relative to control neurons (neurons from Blank rats before stimulus). * $p<0.05$, compared with the value of control neurons 5 s after acidic stimulus; # $p<0.05$, compared with the value of 2% Ost-pretreated neurons 5 s after acidic stimulus. Two-way ANOVA with Bonferroni correction as post-hoc test was used for Figure 5A and B, paired-sampled t test was used for Figure 5C.

inhibition in the change in F.I. stimulated by acidic PSS (Figure 6B).

Discussion

The most prominent observation from the present study is the inhibitory effect of epidural Ost on ASIC3 overexpression in a NP-evoked inflammatory pain rat model, whose hyperalgesia was markedly relieved by Ost. Depolarization of DRG neurons induced by ASIC3-sensitive acid was prevented following pretreatment of Ost. The inhibitory effect of Ost was increased when combined with the non-specific ASIC3 antagonist Ami.

According to previous observations, LDH-associated mechanical allodynia is thought to be related to abnormal spontaneous activity of DRG neurons, caused by excess release of inflammatory mediators, decreased blood circulation of DRG, and local accumulation of protons [9,11]. ASIC3 has been reported to be abundantly present in middle and large sensory neurons of DRG, 50% of which are activated when pH decreases to 6.5. The increase of ASIC3 mRNA levels in inflammatory tissues suggests that ASIC3 modulation of sensory neurons is important for the production of inflammatory pain [30–32]. To determine the alteration of ASIC3 expression in an inflammatory pain model, a time-course analysis of ASIC3 expression was performed

in this study. The peak time of ASIC3 expression was at day 7 after NP application, which is consistent with findings of other studies on the up-regulation of ASIC3 [18,21,33].

Ost, mainly extracted from *Cnidium monnieri* (L.), has showed anti-inflammatory and analgesic effects when used systemically [34] or locally at the site of inflammation [27]. The antinociceptive effect of Ost might result from the potent anti-inflammatory effect [25], nerve block action, and inhibition of central nervous system activity. Our study focused on epidural usage of Ost and found that the antinociceptive effect was persistent and almost complete, but was not concentration-dependent. The behavioral assessment results of the present study are consistent with our earlier work, which found that epidural administration of Ost could alleviate the mechanical hyperalgesia following NP application to DRG, suggesting its clinical analgesic activity.

Ami is a non-specific but potent antagonist of ASIC3, which proved its antinociceptive effect on thermal and mechanical hypersensitivity [35,36]. In the present study using the NP rat model, Ami was used to evaluate the activity of Ost in absence and presence of inhibition of AISC3. Although the effect of a single epidural injection of Ami was not as potent as Ost, the inhibitory effect of Ost on hyperalgesia and ASIC3 expression was strengthened by Ami. To the best of our knowledge, ours is the first study to compare the efficacy of Ost with Ami on

antinociception and down-regulation of ASIC3. We hypothe-sized that the decrease of ASIC3 protein might be closely cor-related with Ost's analgesic effect; however, the pathological significance of such as association has not been established. The mechanism of Ost treatment may be explained, at least partly, by other mechanisms such as modulation of COX-2, phospholipase A$_2$, nitric oxide and 5-HT, which are all poten-tially involved in DRG local inflammation.

DiBAC4$_{(3)}$ is a voltage-sensitive fluorescent dye used for semi-quantitative measurement of MP [36,37]. It can enter depo-larized cells, exhibiting enhanced fluorescence. Increased de-polarization results in more influx of the anionic dye, with a concurrent increase in F.I.; therefore the F.I. increase of NP rat's DRG neurons indicates an elevated amplitude of acid (pH 6.5)-evoked depolarization. Ost is observed to partial-ly block the acid-evoked F.I. shift, indicating that the ampli-tude of depolarization is inhibited to some extent. In previ-ous studies, activation of ASIC3 channel has been reported to be able to directly induce the action potential (AP) gener-ation [18,38,39]. Based on this data, we propose the paranor-mal activity of ASIC3 channel might partly underlie the rapid-ly inactivating and sustained change of MP in DRG neurons.

To our knowledge, there are few reports about the possible impact from Ost on the MP of neurons. In rat hippocampal synaptosomes, Ost could not alter the resting synaptosomal membrane potential or 4-aminopridine-mediated depolar-ization [40]. However, our present study shows that about 50% of acid-evoked depolarization amplitude (expressed as ΔF.I.) of NP rats' DRG neurons could be inhibited by Ost, and the inhibition of Ost on MP was enhanced by Ami.

The conformity of the findings in the behavior study, ASCI3 protein analysis and MP detection indicate that the inhibi-tory effect of Ost on ASIC3 might correlate with its antino-ciception. However, other mechanisms might be involved, and some possible channels and proteins deserve consider-ation. Firstly, in the recent studies on neuroblastoma cells of mice, Ost has been proved to block voltage-gated Na}$^+$ channels intracellularly with state- and frequency-depen-dence, and to cause a concentration (0.3–100 mM)-depen-dent inhibition on voltage-dependent L-type Ca^{2+} current (I(Ca, L)) [12,13]. Secondly, Ost may alter the extent of acid-sensitivity through transient receptor potential vanil-loid 1 (TRPV1) [41] by disrupting the PKC pathway [40]. Further study is needed to compare the antalgic effect of Ost between ASIC3$^{-/-}$ rats and wild-type rats, and to direct-ly observe the structural and functional reactivity of ASIC3 channel to Ost.

CONCLUSIONS

In the current study, we systematically investigated the ef-fect of Ost on hyperalgesia behavior, ASIC3 protein expres-sion, and MP shift of DRG neurons in an LDH rat model. Ost showed the capability to ameliorate NP-associated hy-peralgesia for at least 3 weeks. ASIC3 expression and MP de-creased after administration of Ost; the mechanism of pain relief by the epidural injection using Ost may be through partial blockade of ASIC3 production in the DRG cells. The potency and cheapness of Ost may make it a practical choice to prevent the chronic pain state often seen in pa-tients suffering from LDH with continuing inflammation

and acidosis. Hence, further refinement is needed in or-der to explore the potential of Ost as a pain killer for LDH.

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