Shanzhiside methylester, the principle effective iridoid glycoside from the analgesic herb *Lamiophlomis rotata*, reduces neuropathic pain by stimulating spinal microglial β-endorphin expression

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ABSTRACT

*Lamiophlomis rotata* (L. rotata, Duyiwei) is an orally available Tibetan analgesic herb widely prescribed in China. Shanzhiside methylester (SM) is a principle effective iridoid glycoside of *L. rotata* and serves as a small molecule glucagon-like peptide-1 (GLP-1) receptor agonist. This study aims to evaluate the signal mechanisms underlying SM anti-allodynia, determine the ability of SM to induce anti-allodynic tolerance, and illustrate the interactions between SM and morphine, or SM and β-endorphin, in anti-allodynia and anti-allodynic tolerance. Intrathecal SM exerted dose-dependent and long-lasting (>4 h) anti-allodynic effects in spinal nerve injury-induced neuropathic rats, with a maximal inhibition of 49% and a projected ED50 of 40.4 μg. SM and the peptidic GLP-1 receptor agonist exenatide treatments over 7 days did not induce self-tolerance to anti-allodynia or cross-tolerance to morphine or β-endorphin. In contrast, morphine and β-endorphin induced self-tolerance and cross-tolerance to SM and exenatide. In the spinal dorsal horn and primary microglia, SM significantly evoked β-endorphin expression, which was completely prevented by the microglial inhibitor minocycline and p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580. SM anti-allodynia was totally inhibited by the GLP-1 receptor antagonist exendin(9–39), minocycline, β-endorphin antiserum, μ-opioid receptor antagonist CTAP, and SB203580. SM and exenatide specifically activated spinal p38 MAPK phosphorylation. These results indicate that SM reduces neuropathic pain by activating spinal GLP-1 receptors and subsequently stimulating microglial β-endorphin expression via the p38 MAPK signaling. Stimulation of the endogenous β-endorphin expression may be a novel and effective strategy for the discovery and development of analgesics for the long-term treatment of chronic pain.

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1. Introduction

*Lamiophlomis rotata* (Benth.) Kudo (*L. rotata, Duyiwei*) is a perennial herb from the Labiatae family that grows in the wild in the Qinghai-Tibet Plateau in northwestern China. For hundreds of years, *L. rotata* has been used as an analgesic and homeostatic agent in China and is listed in the Pharmacopoeia of China. The *L. rotata* aqueous extract, in the forms of pills, capsules, and softgel capsules, is widely prescribed in China to manage cancer pain (Li et al., 2002; Qing, 2001), postoperative and bone fracture pain (Chen, 2001; Tan et al., 2000; Zhai, 2000), and neuropathic pain (Tong, 2002). The *L. rotata* aqueous extract and iridoid glycosides extract have been shown to inhibit acetic acid-induced writhing response, formalin-induced tonic pain, peripheral nerve injury-induced neuropathic pain, and bone cancer pain in mice and rats. However, the herb was not effective in alleviating acute nociceptive pain such as formalin-induced acute nociception and thermally-induced nociception (Li et al., 2010; Zhu et al., 2014). *L. rotata* contains many iridoid glycosides and flavonoids, among which shanzhiside methylester (SM) and 8-O-acetyl-SM are two quality control ingredients (Fan et al., 2010; Li et al., 2008; Tan et al., 2007, 2011; Yi et al., 1991). Using an activity-tracking method, SM and 8-O-acetyl-SM were identified...
to be the principle effective iridoid glycosides in *L. rotata*, with nearly equal potency and efficacy (Zhu et al., 2014).

Spinal glucagon-like peptide-1 (GLP-1) receptors were recently revealed to be a novel potential molecular target in experimental animals for the treatment of chronic pain secondary to inflammation, cancer, diabetes, and neuropathy (Gong et al., 2014a, 2014b; Fan et al., 2015; Zhu et al., 2014). The peptide exenatide and non-peptide WB4-24 have been shown to stimulate microglia in the spinal dorsal horn to express β-endorphin leading to antinociception (Gong et al., 2014b; Fan et al., 2015). β-Endorphin is an endogenous opioid peptide neurotransmitter that specifically activates post-synaptic μ-opioid receptors to produce analgesia (Bach, 1997; Petraschka et al., 2007) and induce analgesic tolerance (Bhargava, 1981a; Fichna et al., 2007). We illustrated that SM and β-O-acetyl-SM, as well as their iridoid glycoside derivatives (geniposide, geniposidic acid, loganin and catalpol), were small molecule orthosteric agonists of GLP-1 receptors and, along with *L. rotata*, produced anti-hypersensitivity in a variety of animal models of pain hypersensitivity by activating spinal GLP-1 receptors (Gong et al., 2014a; Zhu et al., 2014). However, in a study by Zhu et al. (2014), gavage administrations of the *L. rotata* aqueous extract over 7 days did not induce anti-nociceptive tolerance in a model of neuropathy, possibly implying that spinal β-endorphin may not be involved in *L. rotata* anti-allaodynia. It is important to determine whether *L. rotata* produces antinociception in neuropathy by stimulating β-endorphin expression or whether endogenously expressed β-endorphin does not induce anti-nociceptive tolerance in contrast to exogenous treatment with morphine or β-endorphin.

Mitogen-activated protein kinases (MAPKs) are a family of evolutionarily conserved molecules. After being activated by phosphorylation, they play a critical role in cell signaling, particularly in relation to microglial activation (Taves et al., 2013; Tsuda et al., 2004; Ledeboer et al., 2005). The MAPKs include p38, extracellular signal regulated kinase (ERK1/2), and c-Jun N-terminal kinase (JNK), representing three different signaling cascades (Ji and Suter, 2007; Pyo et al., 1999, 1998). We previously showed that minocycline was able to completely block exenatide- and WB4-24-stimulated β-endorphin expression (Gong et al., 2014b; Fan et al., 2015). Given that minocycline is likely a non-selective inhibitor of MAPKs, as it has been found to inhibit the activation of all of three MAPK subtypes (Cho et al., 2012; Hua et al., 2005; Mei et al., 2011; Nikodemova et al., 2006; Song et al., 2008; Tikkka and Koistinaho, 2001; Won et al., 2012), it would be worthwhile determining which subtype of MAPK activation is actually responsible for microglial GLP-1-receptor agonism-induced β-endorphin expression and anti-nociception. Therefore, we aimed to study the molecular mechanisms underlying SM anti-nociception in peripheral nerve injury-induced neuropathic rats. We determined the efficacy and potency of the anti-allaodynic effect of SM and its ability to induce anti-allaodynic tolerance. We also evaluated SM-induced β-endorphin expression in the spinal dorsal horn and primary microglial cells, its causal relationship to SM anti-allaodynia, and its MAPK signal mechanism. We finally illustrated the interactions between SM, exenatide, morphine, and β-endorphin in anti-allaodynia and anti-nociceptive tolerance.

2. Materials and methods

2.1. Animals

Male Wistar adult (160–250 g) and 1-day-old neonatal rats were purchased from the Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). The animals were housed in a temperature- and humidity-controlled environment on a 12-h light/dark cycle (lights on at 7:00 AM), given food and water *ad libitum*, and were acclimatized to the laboratory environment for 3–5 days before the study began. Experimental study groups were assigned randomly and the researcher was blind to the behavior testing. The number of animals was 6 in each group except one group where the number was 3 as indicated. All of the experiment procedures involving animals were approved and performed in accordance with the Animal Care and Welfare Committee of the Shanghai jiao Tong University.

2.2. Reagents and drugs

SM was purchased from Chengdu Biopurify Phytochemicals (Sichuan, China) and its structure was confirmed using NMR spectroscopy and its purity was shown to be greater than 99.5% by the manufacturer. Minocycline and morphine hydrochloride were purchased from Yuanye Biotech (Shanghai, China) and Beijing Huikang Boyuan Chemical Tech (Beijing, China), respectively. CTAP and nor-BNI were obtained from Abcam (Cambridge, UK), SB203580 and U0126 were purchased from Selleck Chemicals (Houston, TX). Naltrindole was purchased from Tocris Bioscience (Bristol, UK). SP600125 was obtained from Sigma–Aldrich (St. Louis, MO). β-Endorphin was commercially synthesized and purified (≥90%) by China Peptides Co. (Shanghai, China). The rabbit antiserum containing polyclonal antibodies neutralizing β-endorphin was purchased from Abcam (Cambridge, UK). The manufacturer reported the antiserum to be specific to β-endorphin without cross-reactions to methionine-enkephalin, leucine-enkaphalin, γ-endorphin, α-endorphin, ACTH, or α-melanocyte stimulating hormone. All of the drugs and reagents were dissolved or diluted in 0.9% normal saline except SB203580, U0126, and SP600125, which were dissolved in 20% DMSO in saline.

2.3. Primary neuronal and glial cell culture (Gong et al., 2014b)

The isolated cortex of the 1-day-old neonatal rats was minced and then incubated with trypsin. Dissociated cells were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). For the neuronal culture, cell suspensions were plated in a 24-well plate coated with poly-l-lysine (100 μg/ml) (1 × 10^5 cells/well). Four hours later, the medium was changed to the Neurobasal medium containing B27 supplement and 0.5 mM of glutamine for further culture. All of the experiments were initiated 8–16 days after plating. The harvested neurons exhibited a purity greater than 90% as determined by the NeuN immunoreactivity. For the glial culture, cell suspensions were plated in a 75-cm^2 tissue culture flask (1 × 10^5 cells/flask for microglia and 2 × 10^6 cells/flask for astrocytes) coated with poly-l-lysine and maintained in a 10% CO₂ incubator at 37 °C. After an 8-day culture, microglial cells were prepared as floating cell suspensions by shaking the flask at 260 rpm for 2 h. The aliquots were transferred to plates, and the unattached cells were removed by the serum-free DMEM. The harvested microglia exhibited a purity greater than 95% as determined by the CD11b (OX42) immunoreactivity. Eight days later, astrocytes were prepared as floating cell suspensions by shaking the flask for 2 h and then incubating them with 0.05% trypsin-EDTA for 15 min to separate oligodendrocytes from astrocytes. After trypsin neutralization with the complete media, the floating cell suspensions were discarded. The nearly intact layer of astrocytes in the bed layer was then trypsinized and subcultured. The prepared astrocytes exhibited a purity greater than 90% as determined by the GFAP immunoreactivity.
2.4. RNA extraction, reverse transcription and real-time quantitative PCR (Zhao et al., 2010)  

The total RNA from primary cultures of neurons and glia was collected using the TRIzol reagent (Invitrogen, Grand Island, NY) (Zhang et al., 2013). The sample of the total RNA (1 μg) was reversely transcribed using the ReverTra Ace qPCR RT-Kit (Toyobo Co., Ltd., Japan). Real-time quantitative PCR was carried out with a Mastercycler ep realplex (Eppendorf, Germany) using the Realmaster Mix (SYBR Green I) (Tiangen, Beijing, China). The fold change was calculated using the 2^{-ΔΔCt} method after normalization to GAPDH. The used primers included the β-endorphin precursor proopiomelanocortin (POMC) exon 2–3 forward: 5′-CCC TCC TGC TTA AGA CCT CCA-3′, POMC exon 2–3 reverse: 5′-CTT CTT CCT CCG CAC GCC TCT-3′ (Busch-Dienstfertig et al., 2012; Sitte et al., 2007); and GAPDH forward: 5′-CCA AGG TCA TCC ATG ACA AC-3′, GAPDH reverse: 5′-TCC ACA GTC TTA GTC GC-3′ (Raghavendra et al., 2004).

2.5. Measurement of the β-endorphin level (Fan et al., 2015)  

The β-endorphin levels in the spinal cord (L3–L5) homogenates and the culture medium from the primary neuronal and glial cells were determined using a commercial enzyme-linked fluoroscent immunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA) that had been validated in the linear range of 30–608 pg/ml. The relative fluorescence units were measured with a Fluorescence Microplate Reader (Thermo Labsystems, Vantaa, Finland) and the concentrations were determined through the calculation with a calibration curve run at the same time. Based on the manufacturer’s information, the cross-reactivity of the assay included α-endorphin (100%) and γ-endorphin (60%) but not met-enkephalin (0%) or leu-enkephalin (0%).

2.6. Immunoblotting (Gong et al., 2014b)  

The spinal lumbar enlargements were homogenized in a radioimmunoprecipitation analysis buffer containing the protease inhibitor phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Jiangsu, China). The protein concentrations of lysates were determined using the standard bicinchoninic acid protein assay (Beyotime Institute of Biotechnology). The homogenate was centrifuged at 12,000 rpm for 5 min at 4 °C. The proteins were separated by SDS-PAGE (12%) and then transferred to a polyvinylidene fluoride membrane by an electrophoretic method. The membrane was blocked in 5% skim milk in TBS containing 0.1% Tween 20 at room temperature for 1 h, and it was incubated with primary antibodies against phospho-p38, total p38, phospho-ERK1/2, total ERK1/2, phospho-JNK, or total JNK (1:1,000; rabbit with primary antibodies against phospho-p38, total p38, phospho-ERK1/2, total ERK1/2, phospho-JNK, or total JNK (1:1,000, mouse monoclonal, Protein Tech Group, Chicago) polyclonal, Cell Signaling Technology, Boston), with GAPDH ERK1/2, total ERK1/2, phospho-JNK, or total JNK (1:1,000, rabbit with primary antibodies against phospho-p38, total p38, phospho-

2.7. Intrathecal catheterization and injection in rats (Gong et al., 2011; Huang et al., 2012)  

Inhaled isoflurane anesthesia (4% for induction and 1% for maintenance) was administered and controlled by an anesthesia meter (Ugo Basile Gas Anesthesia System, Comerio, Italy) for rats. An 18-cm polyethylene catheter (PE-10: 0.28 mm i.d. and 0.61 mm o.d., Clay Adams, Parsippany, NJ) with a 13-μl volume was inserted into the spinal cord at the lumbar level. The placement of the catheter in the spinal cord was verified by administering 4% lidocaine (10 μl) with a 50-μl micro-injector (Shanghai Anting Micro-Injector Factory, Shanghai, China) after recovery from anesthesia. The lidocaine test was performed 5–7 days before the drug-testing sessions began. Only rats that exhibited no motor impairment before the intrathecal administration of lidocaine but experienced bilateral paralysis of their hindlimbs after lidocaine injection were selected for the study. For intrathecal injection of the control and test articles, 10 μl of each drug was injected followed by a normal saline flush (15 μl) with a 50-μl micro-injector.

2.8. Rat model of neuropathy induced by peripheral spinal nerve ligation (Zhang et al., 2013)  

Unilateral ligation of spinal nerves was applied to the rats under anesthesia immediately after the completion of intrathecal catheterization. The left L5 and L6 spinal nerves were isolated and tightly ligated with 6-0 silk thread. After ligation, the wounds were sutured and the rats returned to their home cages for recovery. Only those nerve-ligated rats with marked unilateral allodynia under mechanical stimulation (the hindlimb withdrawal thresholds of less than 10 g in the operated side) and no major motor impairment were selected for further study. Drug tests started 1–2 weeks after spinal nerve ligation.

2.9. Behavioral assessments of mechanical allodynia in rats (Zhu et al., 2014)  

To assess mechanical allodynia in rats, the hindlimb withdrawal thresholds evoked by stimulation of ipsilateral injured and contralateral hindpaws with a 2391 electrical von Frey hair (ITTC Life Science Inc.) were determined while the rats stood on a metal grid. The monofilament (with forces ranging from 0.1 to 90 g) was applied to the foot pad with increasing force until the rats suddenly withdrew their hindlimbs. The lowest force producing a withdrawal response was considered as the threshold. Triplicate measurements were made at intervals of approximately 30 s, and the three threshold values were averaged for each hindpaw at each time point.

2.10. Rat experimental procedures and design  

The dose—response of SM anti-allodynia study. Six groups of neuropathic rats received a single intrathecal injection of saline (10 μl) and 4 doses of SM (10, 30, 100 and 300 μg), respectively. The withdrawal thresholds to mechanical stimuli in both contralateral and ipsilateral hindlimbs were measured prior to and 0.5, 1, 2 and 4 h after injection.  

The intervention studies. In order to assess the blockade effects of a variety of antagonists on SM anti-allodynia, groups of neuropathic rats in each study received two intrathecal injections of the vehicle (10 μl) + SM (100 μg) and the antagonist + SM (100 μg), respectively. All of the second treatments were administered 0.5 h after the first treatment except the minocycline study in which the second treatment was given 4 h after. The withdrawal thresholds to mechanical stimuli in both contralateral and ipsilateral hindlimbs
were measured prior to and 0.5, 1, 2 and 4 h after injection. The time and dose regimens of the antagonists in the different studies were based on the following references: 1) the selective GLP-1 receptor antagonist exendin(9–39) (2 μg) (Fan et al., 2015; Gong et al., 2014a, 2014b); 2) the specific β-endorphin antisera (1:10 dilution) (Fan et al., 2015; Gong et al., 2014b); 3) the selective μ-opioid antagonist CTAP (10 μg), κ-opioid antagonist nor-BNI (100 μg) and δ-opioid antagonist naltrindole (5 μg) (Fan et al., 2015); 4) the specific microglial inhibitor minocycline (100 μg) (Hua et al., 2005; Mei et al., 2011); 5) the selective p38 MAPK activation inhibitor SB203580 (10 μg), ERK1/2 MAPK inhibitor SP600125 (10 μg) and JNK MAPK inhibitor U0126 (10 μg) (Jin et al., 2003; Wang et al., 2011; Zhuang et al., 2006).

The ex vivo spinal β-endorphin level measurement study. Four groups of neuropathic rats received two intrathecal treatments: 1) 10 μl of saline - 10 μl of saline; 2) 100 μg of minocycline + 10 μl of saline; 3) 10 μl of saline + 100 μg of SM; and 4) 100 μg of minocycline + 100 μg of SM. The second treatment was administered 4 h after the first treatment. The contralateral and ipsilateral spinal lumbar enlargements were obtained 0.5 h after the second injection and the β-endorphin levels were measured using the commercial ELISA assay.

The anti-allodynic tolerance study of SM, exendinatide, morphine, and β-endorphin. Ten groups of rats in two separate studies received multiple bi-daily intrathecal injections of 10 μl of saline, 100 μg of SM, 20 μg of morphine, 1 μg of β-endorphin, 100 ng of exendinatide, 100 μg of SM + 20 μg of morphine, 100 μg of SM + 1 μg of β-endorphin and 100 ng of exendinatide + 20 μg of morphine for 7 days. On the eighth day, the rats received consecutive intrathecal injection of SM (100 μg) and morphine (20 μg), SM (100 μg) and β-endorphin (1 μg), or exendinatide (100 ng) and morphine (5 μg).

2.1. Data evaluation and statistical analysis

The percentage of maximal possible effect (% MPE) was calculated using the formula: (post-drug threshold in the ipsilateral hindlimb – baseline threshold in the ipsilateral hindlimb)/(baseline threshold in the contralateral hindlimb – baseline threshold in the ipsilateral hindlimb) × 100. The % MPE values near 100 indicate normal mechanical thresholds (i.e., near contralateral thresholds) while values near 0 indicate allodynia (Bowersox et al., 1996). For the dose–response analysis, the parameters, i.e., minimum effect, maximum effect (Emax), median effective dose (ED50) and Hill coefficient (n), were fitted by non-linear least-squares curves to the relation Y = a + bX, where x = [D]n/[ED50 + [D]n), to give the ED50 value and b (Emax) yielding a minimum residual sum of squares of deviations from the theoretical curve (Gong et al., 2012; Zhang et al., 2013). The data were expressed as means ± SEM or with 95% confidence limits, and there were no missing data. The statistical significance was evaluated by one-way or two-way repeated measures analysis of variance (ANOVA). A post-hoc Student-Newman-Keuls test was conducted when the effect of the drug (dose) (for the one-way ANOVA, the factor was the drug [dose]; for the two-way ANOVA, the factors were the drug [dose], time and their interaction) was observed to be statistically significant. The probability was two-tailed-based and the P-value meeting the statistical-significance criterion was 0.05. All of the calculations and analyses were performed using the Prism program (version 5.01, GraphPad Software, San Diego, CA).

3. Results

3.1. SM reduced mechanical allodynia in neuropathic rats via activation of GLP-1 receptors

The anti-allodynic effect of intrathecal SM was examined in L5/L6 spinal nerve-ligated neuropathic rats. After intrathecal administration of normal saline, paw withdrawal thresholds and mechanical allodynia in control rats remained basically unchanged during the observation period of 4 h. Intrathecal injection of SM (10, 30, 100 and 300 μg) elevated ipsilateral paw withdrawal thresholds in a time- and dose-dependent manner without affecting contralateral paw withdrawal thresholds (Fig. 1A). The % MPE values of each dose of SM at 0.5 h after injection were calculated for the dose–response analysis. The Emax was 48% MPE and the projected ED50 was 40.4 μg (95% confidence limits: 32.4–50.4 μg) (Fig. 1B).

We previously showed that SM-induced anti-nociception in the rat formalin test was through the activation of spinal GLP-1 receptors (Zhu et al., 2014). In order to confirm the same mechanism underlying SM anti-allodynia in neuropathy, the selective GLP-1 receptor antagonist exendin(9–39) was used. As exhibited in Fig. 1C, intrathecal injection of SM (100 μg) produced marked anti-allodynia in ipsilateral paws. Although intrathecal injection of exendin(9–39) (2 μg) did not significantly alter the withdrawal responses in either contralateral or ipsilateral paws, it completely inhibited SM anti-allodynia in ipsilateral paws (P < 0.05 by two-way ANOVA followed by the post-hoc Student-Newman-Keuls test).

3.2. SM stimulated β-endorphin expression in a minocycline-sensitive manner

Minocycline is a specific inhibitor of microglial cells (but not neurons or astrocytes) (Tsuda et al., 2004; Ledeboer et al., 2005; Taves et al., 2013). To test whether SM stimulated spinal β-endorphin expression in a minocycline-sensitive manner, the β-endorphin levels were measured in the spinal cords of neuropathic rats and primary cultures of microglial cells, respectively, in the presence and absence of minocycline. Intrathecal injection of SM (100 μg) significantly increased the β-endorphin levels by 0.8 and 1.1 times in the homogenates of the contralateral and ipsilateral spinal cords obtained 0.5 h after injection. Although minocycline (100 μg) did not significantly alter the baseline level of β-endorphin, it completely inhibited SM-increased β-endorphin levels in both contralateral and ipsilateral spinal cords (P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test) (Fig. 2A).

In addition, incubation of SM (300 μM) with primary cultures of microglial cells significantly increased the β-endorphin level by 1.3 times in the culture medium collected 2 h after incubation. Although the pretreatment (1 h prior to) with minocycline (60 μM) (Nutile-McMenemy et al., 2007) did not alter the baseline β-endorphin level, it completely suppressed the stimulatory effect of SM on the β-endorphin release (P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test). In contrast, SM did not stimulate β-endorphin release in primary cultures of either neurons or astrocytes (Fig. 2B). SM also significantly increased the POMC mRNA expression by 3.1 times in primary microglial cells collected 2 h after incubation. Although minocycline completely inhibited SM-stimulated POMC expression, it did not have significant effect on the baseline expression (P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test). In contrast, SM did not significantly increase the POMC gene
Fig. 1. Anti-allodynic effects of intrathecal injection of shanzhiside methylester (SM) on mechanical allodynia in spinal nerve ligation-induced neuropathic rats (A). Neuropathic rats received a single intrathecal administration of saline and four doses of SM (10, 30, 100, and 300 μg). The ipsilateral and contralateral hindlimb withdrawal thresholds to mechanical stimuli were measured. B. Dose-response analysis of the anti-allodynic effect of SM at 0.5 h after injection, best projected using the nonlinear least-squares method. C. Blockade effects of intrathecal injection of the GLP-1 receptor antagonist exendin(9-39) on SM anti-allodynia in neuropathic rats. Exendin(9-39) was injected 0.5 h before intrathecal SM treatment. The data are presented as means ± SEM (n = 6 per group). * Denotes statistical significance compared with the saline + SM group (P < 0.05 by two-way repeated-measures ANOVA followed by the post-hoc Student-Newman-Keuls test).

Fig. 2. Stimulatory effects of shanzhiside methylester (SM) on β-endorphin expression in the spinal cord of spinal nerve ligation-induced neuropathic rats (A) and primary cultures of microglia (B, C). For neuropathic rats, spinal lumbar enlargements were obtained 0.5 h after the second intrathecal injection of SM, which was undertaken 4 h after the first minocycline injection. For primary cultures of neurons, astrocytes and microglia derived from naive neonatal rats, the culture medium and primary cells were collected 2 h after SM incubation. The β-endorphin level and expression of the β-endorphin precursor proopiomelanocortin (POMC) gene were determined using a specific fluorescent immunoassay kit and real-time quantitative PCR, respectively. Minocycline was incubated for 1 h before SM treatment in microglial cells. The data are presented as means ± SEM (n = 6 per group of rats or n = 3 per treatment in primary cells with two independent repeats). a and b Denote statistical significance compared with the saline control and SM treatment groups, respectively (P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test).
expression in primary cultures of either neurons or astrocytes (Fig. 2C).

3.3. SM stimulated microglial β-endorphin expression via p38 MAPK activation

By using the selective inhibitors of the subtypes of MAPK phosphorylation, we tested whether SM stimulated microglial β-endorphin expression through specific activation of p38 MAPK. Treatment with SM (300 μM) significantly increased the POMC mRNA expression by 9.5 times in primary cultures of microglial cells collected 2 h after incubation. The pretreatment (1 h prior to) with the selective p38 MAPK activation inhibitor SB203580 (50 μM) (Pyo et al., 1999) did not significantly alter the baseline expression but completely prevented SM-increased POMC expression (P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test) (Fig. 3A). In contrast, neither the pretreatment with the JNK MAPK activation inhibitor SP600125 (50 μM) (Han et al., 2001) (Fig. 3B) nor JNK MAPK activation inhibitor SP600125 (50 μM) (Kim et al., 2014) (Fig. 3C) significantly attenuated the baseline or SM-increased POMC expression.

3.4. SM anti-allodynia was through spinal β-endorphin expression

The β-endorphin neutralizing antiserum was applied to neuropathic rats to test the causal role of spinal β-endorphin expression in SM anti-allodynia. Intrathecal injection of SM (100 μg) produced time-dependent anti-allodynia in ipsilateral paws. Pretreatment (0.5 h earlier) with the β-endorphin antiserum (1:10 dilution) did not alter the basal withdrawal responses in either paws, but completely blocked SM anti-allodynia in ipsilateral paws (P < 0.05 by two-way ANOVA followed by the post-hoc Student-Newman-Keuls test) (Fig. 4A).

β-Endorphin is an endogenous ligand for μ-opioid receptors (Lendeckel et al., 2009). We tested which subtype of the opioid receptors was associated with SM anti-allodynia. As shown in Fig. 4B, intrathecal injection of SM (100 μg) led to time-dependent anti-allodynia in the ipsilateral paws. Although intrathecal injection (0.5 h earlier) of the selective μ-opioid receptor antagonist CTAP (Steinmiller and Young, 2008) did not significantly affect the withdrawal responses in either contralateral or ipsilateral paws, it completely inhibited SM anti-allodynia in ipsilateral paws (P < 0.05 by two-way ANOVA followed by the post-hoc Student-Newman-Keuls test). However, pretreatment with either the κ-opioid receptor antagonist nor-BNI (Beardsley et al., 2010) or δ-opioid receptor antagonist naltrindole (Drower et al., 1991) did not significantly inhibit SM anti-allodynia.

3.5. SM anti-allodynia was through microglial p38 MAPK activation

We used the specific microglial inhibitor minocycline to test the causal role of microglial p38 MAPK activation in SM anti-allodynia. As shown in Fig. 5A, intrathecal injection of SM (100 μg) produced time-dependent anti-allodynia in ipsilateral paws. Pretreatment (4 h prior to) with intrathecal minocycline (100 μg) was not anti-allodynic, but was able to completely block SM anti-allodynia (P < 0.05 by two-way ANOVA followed by the post-hoc Student-Newman-Keuls test).

The selective inhibitors of the subtypes of MAPK activation were also applied to neuropathic rats to determine which MAPK subtype was responsible for SM anti-allodynia. Intrathecal injection of SM (100 μg) led to time-dependent anti-allodynia in the ipsilateral paws. Although intrathecal injection (0.5 h earlier) of the p38 MAPK activation inhibitor SB203580 (10 μg) did not significantly affect the baseline withdrawal responses in either contralateral or ipsilateral paws, it completely prevented SM anti-allodynia in ipsilateral paws.
In contrast, neither the JNK MAPK activation inhibitor SP600125 (10 μg) nor ERK1/2 MAPK activation inhibitor U0126 (10 μg) significantly decreased SM anti-allodynia (Fig. 5B).

We further tested whether SM and the peptidic GLP-1 receptor agonist exenatide specifically activated p38 MAPK phosphorylation. Intrathecal treatment with SM (100 μg) and exenatide (100 ng) markedly activated p38 MAPK phosphorylation in the homogenates of the lumbar enlargements obtained 1 h after injection (P < 0.05 by one-way ANOVA followed by the post-hoc Student-Newman-Keuls test). SM did not have any significant effects on the total p38 MAPK expression (Fig. 6A, B). In contrast, the same dose of SM and exenatide did not significantly alter either phospho-ERK1/2 or phospho-JNK signaling, or their total protein expression (Fig. 6C, D, E, F).

3.6. SM did not induce self-tolerance to anti-allodynia or cross-tolerance to morphine or β-endorphin

Given that SM induces anti-allodynia via the expression of β-endorphin, which is known to induce anti-nociceptive tolerance (Bhargava, 1981b), we tested whether chronic SM treatment induced self-tolerance to anti-allodynia or cross-tolerance to morphine or β-endorphin. As shown in Fig. 7A and B, multiple bi-daily intrathecal injections of SM (100 μg) over 7 days did not induce tolerance to anti-allodynia in ipsilateral paws. In contrast, the same regimen of morphine (20 μg) or β-endorphin (1 μg) induced a progressive and complete tolerance to anti-allodynia. At the beginning of the 7-day treatments, concurrent injection of the same doses of SM and morphine, or SM and β-endorphin, generated apparently additive anti-allodynia, which was entirely lost at the end of the study.

In addition, consecutive intrathecal injection of SM and morphine, or SM and β-endorphin, on the eighth day produced marked and reversible anti-allodynia in the rats subjected to the bi-daily saline treatments during the initial 7 days. The anti-allodynic effect of SM was kept intact in the rats treated with the 7-day SM. However, it was completely lost in the rats treated with morphine, β-endorphin, SM + morphine, or SM + β-endorphin, for 7 days. The anti-allodynic effect of morphine was maintained in the rats treated with the 7-day SM, but it completely disappeared in the rats multiply treated with morphine or SM + morphine. β-
Endorphin did not produce an anti-allodynic effect in the rats multiply treated with bi-daily β-endorphin or SM + β-endorphin injections (Fig. 7C, D).

In addition, an ELISA assay was applied to naïve neuropathic rats (n = 3) in a separate study to measure the gross spinal β-endorphin level after exogenous β-endorphin injection. At 0.5 h after a single intrathecal injection of β-endorphin (1 μg), the β-endorphin level in the spinal homogenates was 4.7 ± 0.7 ng/mg of protein.

Furthermore, bi-daily intrathecal injections of exenatide (100 ng) over 7 days did not induce tolerance to anti-allodynia, whereas the 7-day morphine (20 mg) induced a progressive and complete tolerance. At the beginning of the 7-day treatments, concurrent injections of the same doses of exenatide and morphine produced apparently additive anti-allodynia, which was completely lost at the end of the experiment (Fig. 7E). Consecutive intrathecal injection of exenatide and morphine produced marked and reversible anti-allodynia in the rats treated with saline during the initial 7 days. The anti-allodynic effect of exenatide was kept intact in the rats subjected to the 7-day treatments with exenatide, but entirely lost in the rats multiply treated with morphine, or exenatide + morphine (Fig. 7F).

4. Discussion

Oral administration of the L. rotata aqueous extract has been shown to markedly reduce formalin-induced tonic hyperalgesia and peripheral nerve injury- and bone cancer cell inoculation-induced mechanical allodynia by 50%–80%, with similar half-effective doses ranging between 130 and 250 mg/kg (Zhu et al., 2014). In this study, we characterized the anti-allodynic effects of SM, the principle effective ingredient of L. rotata, in the rat model of neuropathy. Intrathecal injection of SM did not alter normal nociceptive thresholds in contralateral paws. However, SM was able to dose-dependently and long-lastingly (>4 h) reduce mechanical allodynia in ipsilateral paws of neuropathic rats, with a projected ED50 value of 40 μg and an Emax Value of 49% MPE, similar to that obtained from the L. rotata aqueous extract in neuropathic rats (Zhu et al., 2014). The anti-allodynic effect of SM was shown to be totally prevented by the pretreatment with the orthosteric GLP-1 receptor antagonist exendin(9–39). Repetitive treatments with SM over 7 days did not induce self-tolerance to anti-allodynia, a finding consistent with our previous result that long-term treatment with the oral L. rotata aqueous extract did not induce anti-allodynic tolerance (Zhu et al., 2014). The anti-allodynic effect of SM was shown to be totally prevented by the pretreatment with the orthosteric GLP-1 receptor antagonist exendin(9–39). Repetitive treatments with SM over 7 days did not induce self-tolerance to anti-allodynia, a finding consistent with our previous result that long-term treatment with the oral L. rotata aqueous extract did not induce anti-allodynic tolerance (Zhu et al., 2014). In addition, repetitive treatments with SM over 7 days did not induce cross-tolerance to morphine anti-allodynia and concurrent treatment with SM and morphine was apparently additive in anti-allodynia in the acute setting. However, SM did not prevent morphine anti-allodynic tolerance and was not effective in relieving mechanical allodynia in morphine-tolerant rats. These results suggest that although L. rotata and its effective ingredient SM may not be effective in blocking pain in morphine-tolerant patients, they can be used for the long-term treatment of chronic pain. Indeed, Tong (2002) showed that long-term application of L. rotata was effective in relieving pain in neuropathic patients.
Endorphin is produced from neurons in the pituitary gland and hypothalamus (Fichna et al., 2007), or from microglia in the brain and spinal cord (Fan et al., 2015; Gong et al., 2014b; Sacerdote et al., 1993). Endogenously released β-endorphin can specifically activate the opioid receptors located on post-synaptic neurons and modulate pain transmission and transduction (Bach, 1997; Petraschka et al., 2007). In this study, we demonstrate that spinal microglial β-endorphin expression and subsequent activation of neuronal μ-opioid receptors are the mechanisms responsible for SM anti-allodynia. SM stimulated β-endorphin expression in the spinal cord and primary cultures of microglia (but not neurons or astrocytes) measured in both the β-endorphin levels and β-endorphin precursor POMC gene expression. In addition, SM-induced anti-allodynia and β-endorphin expression were completely inhibited by the specific microglial inhibitor minocycline. More specifically, the β-endorphin antiserum completely prevented SM anti-allodynia. Furthermore, the selective μ-opioid receptor antagonist CTAP also entirely blocked SM anti-allodynia. Neither the κ-opioid receptor antagonist nor-BNI, nor δ-opioid receptor antagonist naltrindole was effective in blocking SM anti-allodynia. Although the peptidic GLP-1 receptor agonist exenatide (with a molecular weight of approximately 4200 Da) and non-peptidic agonist WB4-24 (with a molecular weight of approximately 1000 Da) also exhibited anti-allodynic effects (Fan et al., 2015; Gong et al., 2014b), the small molecule agonist SM (with a molecular weight of approximately 390 Da) offers a better option for treating chronic pain due to its better intestinal absorption and penetration of the central nervous system. The discovery of

β-Endorphin is produced from neurons in the pituitary gland and hypothalamus (Fichna et al., 2007), or from microglia in the brain and spinal cord (Fan et al., 2015; Gong et al., 2014b; Sacerdote et al., 1993). Endogenously released β-endorphin can specifically activate the opioid receptors located on post-synaptic neurons and modulate pain transmission and transduction (Bach, 1997; Petraschka et al., 2007). In this study, we demonstrate that spinal microglial β-endorphin expression and subsequent activation of neuronal μ-opioid receptors are the mechanisms responsible for SM anti-allodynia. SM stimulated β-endorphin expression in the spinal cord and primary cultures of microglia (but not neurons or astrocytes) measured in both the β-endorphin levels and β-endorphin precursor POMC gene expression. In addition, SM-induced anti-allodynia and β-endorphin expression were completely inhibited by the specific microglial inhibitor minocycline. More specifically, the β-endorphin antiserum completely prevented SM anti-allodynia. Furthermore, the selective μ-opioid receptor antagonist CTAP also entirely blocked SM anti-allodynia. Neither the κ-opioid receptor antagonist nor-BNI, nor δ-opioid receptor antagonist naltrindole was effective in blocking SM anti-allodynia. Although the peptidic GLP-1 receptor agonist exenatide (with a molecular weight of approximately 4200 Da) and non-peptidic agonist WB4-24 (with a molecular weight of approximately 1000 Da) also exhibited anti-allodynic effects (Fan et al., 2015; Gong et al., 2014b), the small molecule agonist SM (with a molecular weight of approximately 390 Da) offers a better option for treating chronic pain due to its better intestinal absorption and penetration of the central nervous system. The discovery of
structurally diverse small molecule orthosteric and allosteric agonists has been a rapidly-developing but challenging research area in recent years (Wang et al., 2010; Willard et al., 2012). Our data provides a novel structure for discovering and developing small molecule GLP-1 receptor agonists originated from natural herbs.

The specific role of microglial cells in the pathogenesis of neuropathic pain has been demonstrated over the last decade. Spinal microglia are activated and upregulated following peripheral nerve injury and play an important role in induction of neuropathic pain (Gosselin et al., 2010; Mei et al., 2011; Tsuda et al., 2004; Talbot et al., 2010). The activated microglia evoke the expression of the pro-inflammatory factors such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (Chauvet et al., 2001; Taves et al., 2013). On the other hand, recent efforts have revealed that microglia also have a “protective” state that activates the anti-inflammatory cascades or tissue repair in addition to their “destructive” inflammatory state (Taves et al., 2013). We recently showed that the activation of GLP-1 receptors by exenatide and WB4-24 stimulated spinal microglia to express β-endorphin and produce anti-hypersensitivity in a variety of animal models of pain hypersensitivity (Fan et al., 2015; Gong et al., 2014b). Our current data provide additional evidence that the small molecule agonist of herbal origin stimulates β-endorphin expression in spinal microglia and suggests that endogenous β-endorphin is one of the transmitters for microglia to produce analgesia. Furthermore, although we did not test the effects of SM on the expression of pro-inflammatory cytokines in the current study, our previous study showed that WB4-24 did not affect the microglial expression of IL-1β, IL-6, and TNF-α induced by lipopolysaccharides (LPS) in vitro and the spinal expression of these pro-inflammatory cytokines induced by complete Freund’s adjuvant (CFA) in vivo (Fan et al., 2015). It is important to note that activation of microglia may not be required for SM to induce β-endorphin expression, as SM significantly stimulated β-endorphin expression by nearly the same degree in both contralateral and ipsilateral sides of the spinal cord from neuropathic rats, and as SM induced β-endorphin expression in the primary culture of microglia in the “resting” condition without incubation with LPS. This is supported by the fact that WB4-24 stimulated primary microglia to express β-endorphin regardless of whether or not LPS was applied (Fan et al., 2015). Furthermore, exenatide and WB4-24 stimulated spinal β-endorphin expression by similar degrees in sham, neuropathic, and CFA-challenged rats (Fan et al., 2015; Gong et al., 2014b).

Our recent studies (Fan et al., 2015; Gong et al., 2014b), along with the present study, show that exenatide, WB4-24, and SM stimulate β-endorphin expression in spinal microglia in a minocycline-sensitive manner. Minocycline is a specific microglial inhibitor (Ledeboer et al., 2005; Taves et al., 2013; Tsuda et al., 2004) and blocks the nuclear translocation of NF-kB to the nuclear promoter through MAPK activation (Kobayashi et al., 2013; Nikodemova et al., 2006). The anti-nociceptive role of minocycline in neuropathic pain is complex when different treatment regimens are in consideration, including single treatment vs. multiple treatments, prevention vs. reversion, and administration routes. It is generally accepted that treatment with minocycline may not be anti-nociceptive in established neuropathy, but its preemptive treatment prevented the induction and/or early development of neuropathic pain (Ledeboer et al., 2005; Padi and Kulkarni, 2008; Raghavendra et al., 2003; Lin et al., 2007; Zhang et al., 2012). It was reported that a single intrathecal minocycline injection reduced spinal nerve injury-induced mechanical allodynia by approximately 10% on the 7th, although not on the 10th or 21st day after surgery (Mei et al., 2011). Our current and previous results showed that the single intrathecal injection of minocycline was not anti-alloedyic in neuropathic pain rats 1–2 weeks after peripheral nerve injury (Gong et al., 2014b), but it totally inhibited spinal SM-induced anti-allodynia and β-endorphin expression. Many studies have found that minocycline inhibited p38 MAPK activation (Hua et al., 2005; Mei et al., 2011; Tikka and Koistinaho, 2001; Won et al., 2012). Whereas some have reported that minocycline blocked ERK1/2 (Cho et al., 2012; Nikodemova et al., 2006) and JNK MAPK phosphorylation as well (Nikodemova et al., 2006; Song et al., 2008). Our results demonstrated that SM and exenatide specifically activated spinal p38 MAPK phosphorylation without affecting the phospho-ERK1/2 or phospho-JNK signaling in neuropathic rats in which the spinal p38 MAPK phosphorylation had already been increased secondary to peripheral nerve injury (Gosselin et al., 2010; Mei et al., 2011; Tsuda et al., 2004). Furthermore, the p38 MAPK activation inhibitor SB203580 completely blocked SM-induced microglial β-endorphin expression and subsequent anti-allodynia in neuropathic rats but the ERK1/2 activation inhibitor U0126 and JNK inhibitor SP600125 had no such blockade effects. All of these results illustrate that SM-induced β-endorphin expression in microglia occurs through specific activation of the p38 MAPK signaling. It seems contradictory to associate p38 MAPK signaling with GLP-1 induced β-endorphin expression rather than with pro-inflammatory cytokine expression, as it is known that the phosphorylation of p38 MAPK stimulates the expression of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α (Chauvet et al., 2001; Taves et al., 2013). However, there are four isoforms of p38, i.e., α, β, γ, and δ. The α and β isoforms are the major forms in the mature nervous system and the targets of most p38 MAPK inhibitor drugs (Ji and Suter, 2007; Kumar et al., 2003; Svensson et al., 2005). It was recently shown that the signaling of p38α/p38β but not p38γ/p38δ MAPK was responsible for the expression of p38 MAPK-induced cytokines (Bachstetter et al., 2011; Tang et al., 2006; Xing et al., 2013). Further studies are warranted to demonstrate which specific isoform of p38 MAPK is involved in GLP-1 induced β-endorphin expression.

Although morphine and other opioid analgesics are widely prescribed as powerful painkillers for moderate and severe pain, the induction of analgesic tolerance and hyperalgesia after chronic treatment significantly limits their clinical use (Waldhoer et al., 2004). The inability of SM and the L. rotata aqueous extract to induce anti-nociceptive tolerance lends both a definite advantage over morphine and other opioid narcotics. However, it seems unlikely for them to stimulate β-endorphin expression to produce anti-nociception while not inducing anti-nociceptive tolerance because it is known that chronic treatment with β-endorphin induces anti-nociceptive tolerance in addition to the tolerance to hypothermia and catalepsy (Bhargava, 1981a; Fichna et al., 2007). In order to elucidate the confusion, we studied the interactions between SM and exogenous morphine or β-endorphin and measured the spinal level of β-endorphin after its intrathecal injection. As expected, multiple bi-daily intrathecal injections of morphine and β-endorphin over 7 days produced progressive and complete self-tolerance to anti-allodynia and cross-tolerance to SM anti-allodynia. In contrast, SM induced neither self-tolerance to anti-allodynia nor cross-tolerance to morphine. Furthermore, the peptidic agonist exenatide did not induce either anti-nociceptive tolerance or cross-tolerance to morphine. Morphine, in contrast, induced anti-nociceptive-tolerance and cross-tolerance to exenatide. These findings are also supported by the observation that the 7-day treatment with geniposide, which is a SM-structurally related iridoid glycoside, failed to induce anti-nociceptive tolerance in the rat formalin test (Gong et al., 2014a). All of our results indicate that the agonism of the GLP-1 receptors with SM (and L. rotata), geniposide, and exenatide produce anti-hypersensitivity without inducing tolerance to anti-nociception.

The mechanism underlying the above discrepancy is not
completely understood. Sufficiently high concentrations and durations are required for morphine and β-endorphin to induce antinociceptive tolerance through the mechanisms involved in μ-opioid receptor endocytosis, desensitization of the G-protein coupling, and decrease in the μ-opioid receptor expression (Bhargava and Gulati, 1990; Huang et al., 2012; Martini and Whistler, 2007). The present and the previous studies (Gong et al., 2014b) demonstrated that SM- and exenatide-stimulated β-endorphin expression at a level of approximately 20 pg/mg protein in the spinal cord, which is in sharp contrast to that (approximately 4.7 ng/mg protein) obtained after intrathecal exogenous β-endorphin injection at a dose (1 μg) to induce anti-nociceptive tolerance. Thus, SM- and exenatide-expressed β-endorphin level may not be high enough to induce tolerance to anti-nociception, although it is sufficient to mediate anti-hypersensitivity in central sensitization-involved neuropathic pain states, such as neuropathic pain. Nevertheless, the discrepancy of the induction anti-nociceptive tolerance between SM and exogenous β-endorphin may introduce a new strategy for the discovery and development of new analogs for the long-term treatment of chronic pain. Through stimulation of the endogenous β-endorphin expression, the indirect agonists may be superior to the direct agonists (such as morphine) of neuronal μ-opioid receptors, when analgesic tolerance is in consideration.

Author disclosure statement

The authors declare no conflict of interest.

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