Research Paper

Selective class I histone deacetylase inhibitors suppress persistent spontaneous nociception and thermal hypersensitivity in a rat model of bee venom-induced inflammatory pain

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Abstract: To confirm whether class I histone deacetylase inhibitors (HDACIs) are effective in relief of peripheral inflammatory pain, the effects of two selective inhibitors, MS-275 and MGCD0103, were studied in rats inflamed by subcutaneous (s.c.) injection of bee venom (BV). The BV test is characterized by displaying both persistent spontaneous nociception (PSN) and primary hypersensitivity. Intrathecal (i.t.) pre-treatment of either MS-275 or MGCD0103 with a single dose of 60 nmol/20 μL resulted in profound suppression of both PSN and primary thermal hypersensitivity but without significant influence upon the primary mechanical hypersensitivity and mirror-image thermal hypersensitivity. Moreover, the up-regulation of both HDAC1 and HDAC2 induced by s.c. BV injection was completely suppressed by i.t. pre-treatment of MS-275. The present results provide with another new line of evidence showing involvement of epigenetic regulation of chromatin structure by HDAC1/2-mediated histone hypoacetylation in the BV-induced PSN and thermal hypersensitivity and demonstrate the beneficial effects of class I HDACIs in prevention of peripheral inflammatory pain from occurring.

Key words: bee venom; histone deacetylase; persistent spontaneous nociception; thermal hypersensitivity; mechanical hypersensitivity

选择性I型组蛋白去乙酰化酶抑制剂对蜜蜂毒诱致的大鼠自发痛和热痛敏的抑制作用

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摘 要：为了明确I型组蛋白去乙酰化酶(histone deacetylase, HDAC)抑制剂对外周炎性痛的减轻是否有效，本实验采用对大鼠足底皮下注射蜜蜂毒的方法研究了两种选择性I型HDAC抑制剂MS-275和MGCD0103的镇痛作用。蜜蜂毒模型能够呈现多种疼痛表现型，包括持续性自发痛相关行为，原发性热和机械痛敏，以及镜像热痛敏。鞘内提前给予剂量为60 nmol/20 μL的MS-275和MGCD0103能够显著抑制蜜蜂毒诱导的持续性自发痛和原发性热痛敏，而对原发性机械痛敏和镜像热痛敏无显著影响；而且，由皮下注射蜜蜂毒诱导的HDAC1和HDAC2的高表达通过鞘内提前给予MS-275得到了完全抑制。本研究为证
Epigenetics refers to processes that lead to stable and/or heritable changes in gene transcription without alterations in the DNA sequence usually by DNA methylation, histone modification, and RNA interference [1–4]. Among the epigenetic processes, chromatin remodeling by histone acetylation and deacetylation determined by histone acetyltransferase (HAT) and histone deacetylase (HDAC), respectively, has been well studied because HDAC inhibitors (HDACIs) have been approved as clinical drugs for treatment of cancer [5, 6]. There are four classes of HDAC isoforms including class I (HDAC1, 2, 3, and 8), class II (HDAC4, 5, 6, 7, 9, and 10), class III (sirtuins 1–7) and class IV (HDAC11) [5–7]. The HDACIs can increase acetylation of histone proteins (hyperacetylation) by suppressing HDACs, resulting in increased gene transcriptional activity.

Recently, analgesic effects of HDACIs have also been assessed in animal models of both inflammatory [8–11] and neuropathic pain [12–14]. Using three animal models of neuropathic pain [13], it has been demonstrated that intrathecal (i.t.) pre-administration of MS-275 and MGCD0103, two selective class I HDACIs [15–18], were effective in suppression of both thermal and mechanical pain hypersensitivity. However, the use of HDACIs is likely to be limited to prevention of neuropathic pain but not treatment [13]. In contrast, other two studies showed that pan-HDACIs, sodium butyrate and baicalin, could be effective in improvement of both thermal and mechanical hypersensitivity when delivered after establishment of neuropathic pain [12, 14]. With regard to the effects of HDACIs on the inflammatory pain, results varied inconsistently from different animal models and different drug delivery routes. Class I HDACIs, MS-275 and suberoylanilide hydroamic acid (SAHA) were shown to be effective in suppression of phase II of the formalin-induced nociception only when continuously administered for 5 days via subcutaneous (s.c.) delivery [9, 11]. However, i.t. pre-treatment of MS-275 failed to suppress complete Freund’s adjuvant (CFA)-induced pain hypersensitivity although inhibition of class II HDAC was likely to be effective [8]. Contrarily, intraperitoneal administration of SAHA exacerbated incision-induced mechanical pain hypersensitivity through up-regulation of chemokine CC motif receptor 2 (CXCR2) signaling in the spinal dorsal horn [19]. Moreover, s.c. injection of a pan-HDAC JNJ-26481585 produced mechanical hypersensitivity in naïve rats [20]. Collectively, the analgesic effect of HDACIs is largely consistent in neuropathic pain, but it still remains to be further examined in inflammatory pain.

To further confirm the effects of class I HDACIs on peripheral inflammatory pain, bee venom (BV) model was used in the current study due to the following advantages [21, 22]: (1) a naturally occurring chemical tissue injury-induced inflammatory pain; (2) multiple phenotypes of pain in one animal displaying persistent spontaneous nociception (PSN) similar to the formalin test, primary thermal and mechanical pain hypersensitivity, as well as mirror-image thermal hypersensitivity; (3) the use of the BV-induced multiple phenotypes of pain can utmost avoid inter-individual and inter-experimental differences.

1 MATERIALS AND METHODS

The experiments were carried out on male Sprague-Dawley albino rats (weighing 180–220 g) provided by Laboratory Animal Center of the Fourth Military Medical University (FMMU), Xi’an, China. The animals were housed one per cage with access to water and food ad libitum and maintained on a 12 h light/dark cycle (with the lights on between 8:00 a.m. and 8:00 p.m.) at room temperature (22–26 °C). All experimental procedures were approved by the Institutional Animal Care and Use Committee of FMMU and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and followed ethical guidelines of the International Association for the Study of Pain (IASP) for pain research in conscious animals [23]. Chronic i.t. catheterization was performed as described in our previous report [21]. Animals were recovered for 3–4 days and only those without motor
disturbance and other neurological deficits were included for further experiments.

A volume of 0.05 mL saline containing 0.2 mg lyophilized whole venom (from *Apis millifera* kindly provided by Floret Ltd. and its partner company New Techniques Laboratory Ltd., Tbilisi, Georgia) was used during the whole experiment. Subcutaneous BV injection was administered into the posterior plantar surface of one hindpaw of rats as reported previously [21, 22]. Selective class I HDACIs, MS-275 (N-(2-aminophenyl)-4-[N-(pyridin-3-yl-methoxycarbonyl)amino-methyl]benzamide) and MGCD0103 (N-(2-aminophenyl)-4-[N-(pyridin-3-yl-pyrimidin-2-ylamino)-methyl]-benzamide), were purchased from Cayman Chemical and Selleck Chemicals, separately. Both MS-275 and MGCD0103 were dissolved in 30% dimethylsulfoxide (DMSO) with saline solution and stored at −20 °C before its application. DMSO was used as vehicle control. Both MS-275 and MGCD0103 were administered through i.t. route at 60 nmol which has been proved to be effective in improvement of neuropathic pain [31]. All vehicle or drugs were administered 30 min before s.c. BV injection.

At the beginning of all behavioral testing, rats were randomized into groups, and all of the behaviors were tested blindly. PSN was quantified by counting the number of paw flinches during every 5-min interval for 1 h following s.c. BV injection [22]. To assess thermal sensitivity, rats were placed in a plastic chamber on the surface of a 2 mm thick glass plate and the sensitivity to heat stimuli by a TC-1 radiant heat stimulator (Xi’an Bobang Technologies of Chemical Industry Co. Ltd., China; 10 V) was measured. The heat stimuli were applied to both the injection site and the corresponding area of the contralateral paw. The latency was determined as the duration from the beginning of heat stimuli to the occurrence of a marked paw withdrawal reflex. Five stimuli were repeated for each site and the latter three or four values were averaged as mean paw withdrawal thermal latency (PWTL, s) [22]. The inter-stimulus interval for each heat test was more than 15 min at the same region and 10 min at the different paws. For evaluating mechanical sensitivity, mechanical stimuli were applied by using ascending graded individual von Frey monofilaments with bending forces of 4.9, 9.8, 19.6, 39.2, 58.8, 78.4, 98.0, 117.6, 137.2, 156.8, 176.4, 196.0, 245.0, 343.0, 441.0, and 588.0 mN. A single von Frey filament was applied 10 times (once every several seconds) to each testing site of bilateral hindpaws. A bending force being able to evoke an approximate 50% occurrence of paw withdrawal reflex was expressed as the paw withdrawal mechanical threshold (PWMT, mN) [22].

For Western blotting, after the behavioral experiment, all rats were immediately sacrificed by decapitation, and the lumbar spinal cord enlargement were quickly removed by injecting saline into the spine and stored at −70 °C until used for Western blotting. The tissue was homogenized in an ice-cold mixture of RIPA lysis buffer and protease inhibitors and then the homogenates were centrifuged at 12 000 g for 10 min at 4 °C. The total sample protein concentration was measured by using a BCA protein assay kit (Thermo, IL, USA). The aliquots of proteins (60 μg) were heated for 10 min at 98 °C and then stored at −70 °C until use. Samples were fractionated on a 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to a PVDF membrane (Immobilon, Millipore). The membranes were blocked with 5% skimmed milk solution for 2 h at room temperature and incubated overnight at 4 °C with mouse anti-HDAC1 (1:800, Santa Cruz Biotechnology, USA) and anti-HDAC2 (1:500, Sigma, USA) as primary antibodies. Mouse anti-β-actin antibody (1:2 000, Sigma, USA) was used for normalization. The membranes were visualized with enhanced chemical luminescence reagents (ECL) and photographed by a gel image analysis system (Alpha Innotech Corp., USA). The intensities were measured with AlphaImager Software and relative intensities of target bands were analyzed and normalized to β-actin intensity. All Western blot samples were performed at least three times from at least three rats.

All results were presented as mean ± SEM. Two-way repeated ANOVA was used to analyze group differences in mean time courses of PSN. One-way ANOVA (Fisher’s PLSD test) was applied to compare differences in averaged mean numbers of flinching reflex for 1 h in the pre-treatment paradigm, the primary thermal or mechanical hypersensitivity measurements as well as mirror-image thermal hypersensitivity assays. A statistical difference was accepted as significant if *P* < 0.05.

2 RESULTS

As shown in Fig. 1A and B, i.t. pre-treatment with either MS-275 or MGCD0103 (60 nmol in 20 μL
DMSO for both) resulted in a profound suppression of the paw flinching reflex compared with the control. The total number of paw flinches averaged from 1 h time course for each drug was shown, respectively, in Fig. 1C and D. The inhibitory rate of MS-275 and MGCD0103 was 48.89% (n = 13, P < 0.001) and 42.35% (n = 10, P < 0.001), respectively.

Intrathecal administration of MS-275 or MGCD0103 into naïve animals produced no significant changes in basal pain sensitivity (data not shown). Nevertheless, in the BV-inflamed rats, i.t. pre-treatment with MS-275 prevented the occurrence of primary thermal hypersensitivity with the inhibitory rate at 121.01% relative to the control (n = 13, P < 0.001) (Fig. 2A). Pre-treatment with MGCD0103 also completely blocked the induction of primary thermal hypersensitivity (Fig. 2B); and the thermal latency in the injection site was increased by 200.62% relative to the DMSO control (n = 10, P < 0.001). Fig. 2C and D illustrated the effects of i.t. application of MS-275 or MGCD0103 on the BV-produced mechanical hypersensitivity. Pre-treatment with either MS-275 or MGCD0103 did not cause any significant influence upon the primary mechanical hypersensitivity. Neither of the two drugs produced any anti-hyperalgesic effects on the mirror-image thermal hypersensitivity (Fig. 2A and B). Since s.c. injection of BV did not produce mechanical hyperalgesia in the non-inflamed hindpaw as previously reported [21, 22], no changes in PWMT were observed following i.t. pre-treatment of the two drugs either (Fig. 2C and D).
To examine whether the anti-nociceptive and anti-hyperalgesic effects of class I HDACIs are caused by inhibiting HDAC activities in the spinal cord, the expression levels of both HDAC1 and HDAC2 were studied by Western blotting. It was found that s.c. injection of BV significantly upregulated the HDAC1 and HDAC2 expression in the rat spinal cord (Fig. 3). However, the up-regulation of both HDAC1 and HDAC2 in the spinal cord was reversed to the saline control level following pre-treatment of MS-275 (Fig. 3).

3 DISCUSSION

The major gain of the present study was to further confirm that i.t. pre-treatment of two selective class I HDACIs (MS-275 and MGCD0103) could prevent peripheral inflammatory pain from occurring in a rat BV model. However, the two drugs were only effective to block the development of PSN and primary thermal hypersensitivity, but not effective in prevention of primary mechanical and mirror-image thermal hypersensitivity. We also confirmed that the analgesic effects of class I HDACIs were caused by inhibition of HDAC1 and HDAC2 expression that were up-regulated in the spinal cord induced by s.c. BV injection. Because MS-275 can preferentially inhibit HDAC1 and does not inhibit HDAC8, while MGCD0103 is HDAC1 and HDAC2 selective $^{16-18}$, it can be concluded that hyperactivity of HDAC1 and HDAC2 is involved in development of the BV-induced PSN and primary thermal hypersensitivity.
hyperalgesia via hypoacetylation of histone proteins.

Pharmacologically, HDACIs can induce histone hyperacetylation by inhibiting the removal of acetyl groups from histone proteins by HDACs, resulting in loosening of the condensed chromatin and increase in transcriptional activities of the silenced genes \[5-7\]. As a line of supporting evidence, Bai et al. (2010) examined the expression level of acetylated H3 lysine 9/18 residues (H3K9/18ac) and found that i.t. pre-treatment of MS-275 could result in up-regulation of H3K9/18ac in neurons mainly in the superficial layers of the spinal dorsal horn in naïve mice \[8\]. The spinal up-regulation of H3K9ac was also shown to be in a dose-dependent manner following i.t. treatment of MS-275 (7.5–60 nmol/day) or MGCD0103 (30–60 nmol/day) in naïve rats \[13\]. However, in contrast to our current result, in the CFA model of inflammatory pain, significant up-regulation of HDACs was only seen for class II (HDAC4, 5, 7, 9), but not for class I (HDAC1, 2, 3) \[8\]. In the same study, the authors found that pan-HDACIs were effective to prevent the CFA-induced pain hyper-sensitivity, whereas MS-275 was not effective \[8\]. Because the dose of MS-275 (0.5 μg in 5 μL, i.t.) used in mice was effective to up-regulate global acetylation in the spinal cord \[8\], the lack of analgesic effect of MS-275 seen in the CFA model was not likely to be caused by shortage of dose but instead by ‘off-target effect’. Taken our current result together, it is suggested that different isoforms of HDACs would be involved in different processes of inflammatory pain caused by different insults. Thus, the therapeutic use of HDACIs in treatment of clinical pain warrants being further studied by different animal models. Moreover, whether any isoform of class II HDACs is involved in development of the BV-induced primary mechanical or mirror-image thermal hypersensitivity also warrants being further studied.

The underlying downstream molecular and cellular mechanism of HDACIs-produced analgesia is largely unknown although several signaling targets have been suggested \[1-4\]. At the spinal level, it has been demonstrated that HDACIs produce analgesia by up-regula-
tion of type 2 metabotropic glutamate receptor (mGluR2) via acetylation-promoted activation of NF-κB transcription factor, p65/ReLA \[9, 24\]. Activation of mGluR2 has been shown to inhibit pre-synaptic neurotransmitter release to the spinal dorsal horn, resulting in improvement of both inflammatory and neuropathic pain \[9, 24\]. At the supraspinal level, HDACIs-produced histone hyperacetylation has been shown to promote Gad2 transcription and restore GABAergic synaptic function, enhancing descending pain-modulatory system and producing analgesia in both inflammatory and neuropathic pain \[25\]. At the peripheral level, the results of the HDACIs appear to be complicated and in conflict with each other. Using pan-HDACIs, it was found that valproic acid produced anti-inflammatory and anti-nociceptive actions by down-regulation of TNF-α related pathway possibly via hyperacetylation of histone proteins \[10\]. Subcutaneous injection of the pan-HDACI JNJ-26481585 resulted in mechanical pain hypersensitivity via up-regulation of both NF-κB pathway and interleukin-1β in the spinal dorsal horn \[20\]. Systemic administration of SAHA, a class I HDACI, was shown to exacerbate incisional mechanical, but not thermal hypersensitivity possibly via up-regulation of CXCR2 in the spinal cord dorsal horn \[19\]. So far, the above discrepancies in the effects of HDACIs against pathological pain are not clear and require to be further studied in pre-clinical research. More selective inhibitors targeting different isoform of different classes of HDACs are required for assessment of efficacy and safety of the HDACIs in use for treatment of clinical chronic pain.

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