Carnosic acid attenuates neuropathic pain in rat through the activation of spinal sirtuin1 and down-regulation of p66shc expression

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Abstract
Background: It has been reported that carnosic acid (CA) exhibits a range of biological activities including hepatoprotective, antioxidant and anti-inflammatory. However, the effect of carnosic acid in neuropathic pain remained elusive.

Methods: A neuropathic pain model of chronic constriction injury (CCI) was established in adult male Sprague–Dawley rats. Mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were recorded, and western blot was performed to detect sirtuin1 and p66shc content.

Results: Intrathecal administration of carnosic acid attenuated mechanical allodynia and thermal hyperalgesia in rats following chronic constriction injury. Interestingly, carnosic acid analgesic effect was positively associated with spinal sirtuin1 activation; however, p66shc was inhibited by carnosic acid in the spinal cord. In addition, sirtuin1 inhibitor EX-527 reversed the anti-nociceptive effect of carnosic acid.

Conclusions: Carnosic acid is effective in the treatment of the established CCI-induced pain. It may be possible that spinal sirtuin1 activation by carnosic acid attenuates neuropathic pain through a mechanism involving the down-regulation of p66shc expression.

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1. Introduction

Neuropathic pain is a debilitating pain state, which is often caused by injury to the nervous system arising from bone compression in cancer, diabetes mellitus, infection, autoimmune disease, or physical injury (Baron, 2006). It is characterized by the presence of spontaneous ongoing and evoked pain, with the latter presenting as allodynia (pain elicited by a nonnoxious stimulus) or hyperalgesia (increased pain response to anoxious stimulus) (Finnerup and Jensen, 2006; Finnerup et al., 2007), which are refractory to conventional analgesics. From general population studies, neuropathic pain affects up to 5% of the population (Bouhassira et al., 2008; Torrance et al., 2006). These led us to discovery new approaches to treat chronic pain-related disorders.

Previous studies suggest that neuropathic pain might result from multiple pain-related cellular and molecular alternations in primary afferent or spinal cord after nerve injury (Uchida et al., 2010a,b; Adilakshmi et al., 2012). One of the characteristic alterations in gene modification is abnormal histone acetylation, which has been shown to contribute to the development of neuropathic pain. It has been demonstrated that the impact of abnormal histone acetylation, can be modulated by Sirtuin1 (Sirt1) (Uchida et al., 2013; Kiguchi et al., 2012). Sirt1, a member of the highly conserved nicotinamide adenine dinucleotide–dependent class III histone deacetylases, plays important roles in liver protection, genomic stability, inflammation via deacetylation of its target proteins (Zhu et al., 2013; Hao and Haase, 2010; Michan and Sinclair, 2007). Additionally, in a SOD1 G93A transgenic mouse model of amyotrophic lateral sclerosis, the expression of Sirt1 in lumbar segment of the spinal cord was changed (Lee et al., 2012), indicating that Sirt1 may regulate the pathogenesis of neuropathic pain.

Interestingly, recent works suggest that the regulation by Sirt1...
may involve p66shc. Sirt1 transgenic diabetic mice exhibit decreased expression of p66shc (Chen et al., 2012) and Sirt1-mediated p66shc inhibition is crucial for improving hepatocyte function during rats with liver ischemia/reperfusion injury (Yan et al., 2014). P66shc is an isoform of the mammalian adapter protein ShcA and an increasing number of reports demonstrated that it contributes to physiological and pathophysiological process. Notably, p66shc knockout mice did not have age-dependent increases in pain sensitivity as compared with the corresponding wild-type mice (Berry et al., 2007). However, the role of p66shc in neuropathic pain remains unknown.

Thus we reasoned that Sirt1 inhibiting p66shc after peripheral nerve injury may lead to effective approach to the treatment of neuropathic pain. Carnosic acid (CA) is a major constituent of the labiate herbal plant rosemary and it has been found to exhibit a range of biological activities including hepatoprotective, antiinflammatory, anti-inflammation (Yan et al., 2014; Ibarra et al., 2011). However, little research has been conducted regarding the therapeutic efficacy of CA in neuropathic pain. Here, we explored whether CA, which shared a structural similarity with Sirt1 activators like resveratrol and quercetin (Chung et al., 2010), attenuated neuropathic pain at the spinal level and we aimed to elucidate whether its function was linked to Sirt1 and p66shc. To address these issues, the effect of intrathecal injection of CA on the development of neuropathic pain in rats following chronic constriction injury (CCI) was examined. Changes in the expression of spinal Sirt1 and p66shc relation to neuropathic pain were examined by using Western blot. The effects of CA on the regulation of Sirt1 and p66shc were evaluated. Finally, we characterized the nociceptive behavior of CCI rats using Sirt1 inhibitor EX-527 (6-chloro-2, 3, 4, 9-tetrahydro-1H-carbazole-1-carboxamide), which is more potent and selective than other current Sirt1 inhibitors (Napper et al., 2005), before intrathecal injection of CA to further elucidate the role of Sirt1 in CA analgesic effect.

2. Materials and methods

2.1. Experimental animals

All experimental protocols were performed according to the standards established in the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animals Resources of the National Research Council (United States) and were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University (Wenzhou, China). 162 male Sprague Dawley rats weighing 230–250 g were purchased from SLAC Experimental Animals Company of Shanghai (Shanghai, China) and housed in a temperature-controlled (22 °C) colony room under a 12 h/12 h light/dark cycle regime, with food and water available ad libitum at the Experimental Animal Center of the Wenzhou Medical University. All behavioral testing was performed during the light cycle between 10:00 AM and 2:00 PM.

2.2. Lumbosacral intrathecal surgery

Lumbosacral intrathecal (IT) catheters was inserted into the lumbar enlargement according to the method described previously (Milligan et al., 1999). The lumbar approach was utilized because it avoided pressure on the spinal cord. Briefly, rats were anesthetized with 4% chloral hydrate (400 mg/kg, intraperitoneally [IP]). A rectangular area of the skin above the L2-L6 lumbar vertebral was shaved and sterilized with alcohol. A 2 cm longitudinal skin incision was made. At a point 15 mm caudal to the L5 – L6 lumbar gap, a 20 gage guide cannula was inserted at an angle about 20° into the muscle and was carefully advanced rostrally along the dorsal surface of the L6 vertebra, as the angle of the syringe was reoriented to about 15° until the cannula was entered the subarachnoid space. The correct intrathecal localization was characterised by a sudden slight flick of the tail or a paw retraction. A PE-10 polyethylene catheter, with an outer diameter of 0.5 mm was used. Submerged in water of 60 °C, one end of the catheter was reduced in diameter by stretching it to about 150% of the original length. A 14-cm length of PE-10 catheter with volume of approximately 7 μl was easily inserted through the cannula and advanced about 3 cm beyond the tip of the inserted cannula to reach the level of the lumbar enlargement. The cannula was then removed, leaving the catheter in place. A loop knot was tied in the catheter and slightly tightened. The knot itself was anchored to white collagenous tissue with a sterile suture of 4–0. The catheter was tunneled under the skin, appearing close to the base of the tail. A head 10 cm from the tip was made on the catheter to prevent the catheter from being dislocated. After injecting 10 μl Evans blue dye to ensure the presence of Evans blue dye over cauda equina at the lumbosacral level. The skin incision was then closed with silk sutures. The cannulated rats were allowed to recover from anesthesia and were housed individually. Those rats exhibiting hindlimb motor weakness or other abnormalities were excluded from the experiments. All catheter placements were verified upon completion of behavioral testing by visual inspection. Data were analyzed only from animals in which the catheter tip was verified as being at the lumbosacral spinal level.

2.3. Chronic constriction injury model

One or two days after days after the catheter implantation, rats received either chronic constriction injury of the sciatic nerve or sham surgery. CCI model as one of neuropathic pain models was prepared based on previous description (Bennett and Xie, 1988). Rats were anesthetized with 4% chloral hydrate (400 mg/kg, intraperitoneally [IP]) during surgical procedures. The right sciatic nerve was exposed at mid-thigh level. Around the dissected nerve, four ligature knots (4–0 chronic gut) were performed loosely. Intervals among ligature knots were about 1 mm. The tension of the constriction was guided by the occurrence of a short flick of the ipsilateral hind limb. Skin wound was closed with 4–0 sterile silk. For sham operation, the sciatic nerve was exposed but not manipulated. The rectal temperature of all the rats was maintained by constant temperature blanket during surgical procedures until the rats recovered from anesthesia.

2.4. Drugs injections

Injection of drugs was conducted in restrained animals placed in a pile of soft towels during drug administration. The animals were kept horizontal during injections. After an initial more than 30 s acclimation period, the sealed tip was cut and the drugs were microinjected intrathecally with a 10-μl microsyringe in a volume of 10 μl followed by a DMSO flush. The drug was injected about a 1 min period into the subarachnoid space. The needle of microsyringe was left in place for further 15 s before withdrawal. The time taken for the total procedure was about 3 min. Carnosic acid and EX-527 were purchased from Sigma (St. Louis, MO) and were dissolved in 10 μl 10% DMSO (100% DMSO diluted in PBS). The doses of carnosic acid and EX-527 were selected according to previous reports (Peck et al., 2010; Satoh et al., 2008; Shao et al., 2014) and our preliminary experiments.

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2.5. Assessment of mechanical withdrawal threshold (MWT)

The 2390 Electronic von Frey Anesthesiometer (IITC Life Science, USA) was used to test the MWT to evaluate mechanical allodynia. Rats were placed individually into wire mesh-bottom cages (22 × 22 × 22 cm) allowed to acclimatize for 30 min. The probe was positioned below the plantar surface of the paw with Von Frey filaments at range of 0.1–90 g, increasing force until the rat twitches its paw. The maximum force was recorded at the time of paw withdrawal. Each paw was tested alternately in 5 min intervals and each rat was at least tested four times over the course of the experiment. The average value attained by this method was expressed as the MWT.

2.6. Assessment of thermal withdrawal latency (TWL)

The TWL was applied to estimate the thermal hyperalgesia by the 336 Plantar Test Apparatus (IITC Life Science, USA). Rats were placed in a transparent, square, bottomless acrylic box (20 × 22 × 20 cm). After an initial 15 min acclimation period, the infrared source placed under a glass plate is positioned by the operator directly beneath the plantar surface of the hind paw. Withdrawal of the paw, indicating sensation of pain in the rat, caused the infrared source to switch to the off position and the reaction time counter to stop. The hind paw was tested alternately at 5 min intervals. A 25-s cutoff was used to prevent tissue damage in the absence of a response. Each rat was tested five times and the average value expressed as the TWL. The average of the last 3 trials was then determined (Hargreaves et al., 1988).

2.7. Western blot

The animals were euthanized by overdose chloral hydrate after the supposed survival time. Fresh tissue samples taken from the L4 and L5 dorsal spinal cord were removed as quickly as possible. The L4-5 spinal cord segment was dissected according to the termination of the L4 and L5 dorsal roots and the dorsal horn of the right half of L4 and L5 (ipsilateral to the CCI side) was isolated. After dissection, all tissues were rapidly frozen in liquid nitrogen and stored −80 °C until further processing. Tissue samples were homogenized in lysis buffer (12.5 μl/mg tissue) containing a cocktail of protease inhibitors and phenylmethylsulfonyl fluoride (Sigma–Aldrich Co. LLC). The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant was collected for protein concentration determination by using Bradford method. To detect Sirt1, p66shc, Protein bands on Western blots were visualized by ECL Plus detection system (Amersham Biosciences, Buckinghamshire, UK), rabbit anti-p66shc (1:2000; Abcam, UK) or mouse anti-Sirt1 (1:1000; Abcam, England, MA). The filters were blocked with 5% nonfat milk and incubated overnight at 4 °C with a mouse anti-Sirt1 (1:1000; Abcam, Cambridge, UK), rabbit anti-p66shc (1:2000; Abcam, UK) or mouse anti-β-actin (1:5000; Bioworld, USA) primary antibody. The blots were washed with Tris-buffered saline with Tween (×3, 10 min each) and incubated for 2 h at room temperature with goat anti-mouse or goat anti-rabbit secondary antibody (1:1000 in 5% milk TBS-T). Protein bands on Western blots were visualized by ECL Plus according to the manufacturer's instructions and were exposed onto X-Omat film. The expression level of proteins was calculated as the average of the densities in each band area from different rats. The expression of proteins was evaluated relative to β-actin. The density of each band was quantified by using ImageJ analysis software.

2.8. Experimental protocol

Experiment Set I: The first set of the experiments was undertaken to determine examining the effect of carnosic acid (CA) on CCI-induced mechanical allodynia and thermal hyperalgesia. Rats were intrathecally injected with carnosic acid (25 [CA1, 100 [CA2]/10 μl) or DMSO once a day from 3 day after CCI to 6 day after CCI. The behavioral measurements were carried out 1 day before, and 3, 7, 9 days after CCI. Forty-two rats were randomly assigned to six groups (n = 7): sham group, sham + CA1 group, CCI + CA1 group, CCI + CA2 group, CCI + DMSO group.

Experiment Set II: The second set of the experiments was undertaken to determine the effect of CCI operation on the expression of Sirt1 and p66shc in L4-L5 spinal cord. After performing baseline behavioral assessments, rats underwent CCI operation (day 0). On day 1, 5, 9 and 14 after CCI surgery, rats were killed, and the L4-5 spinal cord was removed for Western blot analysis. Sham rats were killed on day 14. To assess the development of allodynia and hyperalgesia, the behaviors of the rats were tested immediately before they were killed. Rats were randomly assigned to five groups (n = 5): sham group, CCI day 1 group, CCI day 5 group, CCI day 9 group, CCI day 14 group.

Experiment Set III: The third set of the experiments was to assess the effect of carnosic acid on the expression of Sirt1 and p66shc in L4-L5 spinal cord of CCI rats. Rats were intrathecally injected with carnosic acid (25 [CA1, 100 [CA2]/10 μl) or DMSO once a day from 3 day after CCI to 6 day after CCI. Thirty rats were randomly assigned to five groups (n = 6): sham group, CCI group, CCI + CA1 group, CCI + CA2 group, CCI + DMSO group. Rats were euthanized for molecular detection at day 7 after CCI or sham surgery.

Experiment Set IV: The fourth set of the experiments was to examine the role of EX-527 on nociceptive-induced allgesia. EX-527 (3 μg/10 μl), a selective Sirt1 inhibitor, was administrated intrathecally 1 h before CA administration once a day from 3 day after CCI to 6 day after CCI. Behavioral experiments were performed 7, 9 days after CCI operation. Thirty-five rats were randomly assigned to five groups (n = 7): sham + DMSO + DMSO group, CCI + DMSO + DMSO group, CCI + DMSO + EX-527 group, CCI + CA2 + DMSO group, CCI + CA2 + EX-527 group.

Experiment Set V: The fifth set of the experiments was to examine the effect of EX-527 on nociceptive acid attenuating CCI-induced elevation of p66shc after CCI surgery. EX-527 (3 μg/10 μl) was administered intrathecally 1 h before CA administration once a day from 3 day after CCI to 6 day after CCI. Thirty rats were randomly assigned to five groups (n = 6): sham + DMSO + DMSO group, CCI + DMSO + DMSO group, CCI + CA2 + DMSO group, CCI + DMSO + EX-527 group, CCI + CA2 + EX-527 group. Rats were euthanized for molecular detection at day 7 after CCI or sham surgery.

2.9. Statistical analysis

All data are presented as mean ± SD. Statistical analyses were evaluated using SPSS v16.0 statistical software package (SPSS, Chicago, IL). The data from both thermal hyperalgesia and mechanical allodynia tests were analyzed by using a two-way repeated-measures ANOVA followed by LSD post hoc tests. Western blot data between the groups were analyzed by one-way analysis of variance. For all tests, P values less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of CA on the development of neuropathic pain behaviors

To examine whether carnosic acid (CA) is effective in treating neuropathic pain, we intrathecally injected carnosic acid to rats
with CCI-induced neuropathic pain. The behavioral measurements were carried out 1 day before, and 3, 7, 9 days after CCI. We observed that the rats developed mechanical allodynia and thermal hyperalgesia on postoperative day 3 and continued on postoperative day 9 in CCI rats (Fig. 1A and B). Intrathecal administration of 100 μg CA once daily from 3 days after CCI to 6 days after CCI, but not 25 μg, significantly suppressed the decrease of MWT (Fig. 1A) and TWL (Fig. 1B) on the ipsilateral side from day 7 to day 9 after CCI as compared with DMSO-treated CCI rats. However, CA did not significantly alter withdrawal thresholds on contralateral paws. We also examined the effects of 100 μg CA on paw withdrawal latencies in sham-operation rats and found no significant differences in latency times between sham-operation rats and CA-treated sham-operation rats. These results suggested that abundant CA administration intrathecally attenuated the development of CCI-induced neuropathic pain.

3.2. Changes in spinal Sirt1 and p66shc expression after CCI

To assess whether CCI induced significant changes in the expression of spinal Sirt1 and p66shc, rats were immediately euthanized after completion of the behavior test for protein detection by Western blot. CCI surgery induced an increase in mechanical allodynia and thermal hyperalgesia at 1, 5, 9 and 14 days after CCI, as compared with sham group (data not shown). A significant decrease in Sirt1 protein in the ipsilateral spinal cord was observed at 1, 5, 9 and 14 days after CCI in contrast to sham-operated rats (Fig. 2A and B). However, an initial increase in p66shc expression was accompanied with a decreased Sirt1 expression in CCI rats (Fig. 2A and C). The expression levels of Sirt1 (Fig. 2D and E) and p66shc (Fig. 2D and F) protein in the contralateral side spinal cord did not significantly alter.

3.3. Effects of CA on spinal Sirt1 and p66shc expression

Based on the finding of 100 μg carnosic acid has an anti-nociceptive effect in CCI rats, we further investigated whether anti-nociceptive effect of CA was associated with Sirt1 activation and p66shc inhibition. As shown in Fig. 4, after CCI, the spinal cord exhibited a decrease in Sirt1 (Fig. 3A and B) with an immediate increased P66shc levels (Fig. 3A and C). Nevertheless, 100 μg CA administration markedly up-regulated Sirt1 (Fig. 3A and B) and consequentially reversed the increased p66shc expression in CCI rats (Fig. 3A and C). The expression levels of Sirt1 (Fig. 3D and E) and p66shc (Fig. 3D and F) protein in the contralateral spinal cord did not significantly alter. We also found that no significant effect of 25 μg carnosic acid on Sirt1 and p66shc within the spinal cord ipsilateral to CCI as compared with the CCI + DMSO group in the Western analysis (Fig. 4).

3.4. EX-527 reverses the anti-nociceptive effects of CA

To further assess whether Sirt1 activation plays a key role in carnosic acid (CA) anti-nociceptive effect, we tested the effect of Sirt1 inhibitor EX-527 on the anti-nociceptive effect of CA. As shown in Fig. 5, intrathecal treatment with 3 μg 1 h before CA administration once daily for 4 days significantly reversed the anti-nociceptive effect of 100 μg CA on the ipsilateral side as compared with the DMSO-treated CA-CCI group (Fig. 5A and B). CA did not significantly alter withdrawal thresholds on contralateral paws. CCI rats treated with EX-527 did not show reliable exacerbation of mechanical allodynia or thermal hyperalgesia as compared with CCI rats treated with DMSO (Fig. 5A and B). In addition, intrathecal treatment with EX-527 effectively blocked the effect of carnosic acid attenuating CCI-induced elevation of p66shc after CCI surgery (Fig. 6).

4. Discussion

Neuropathic pain is a chronic and persistent pain that is difficult to treat with conventional analgesics. Laboratory studies and clinical studies have reported relatively poor efficacy for morphine in neuropathic pain (Arnér and Arnér, 1985; Jadad et al., 1992; Bian et al., 1995). However, some other pharmacological interventions have been reported to exert beneficial effects on neuropathic pain. Previous investigation reported that resveratrol produces an anti-nociceptive effect in neuropathic model (Bermudez-Ocana et al., 2006). Here, we observed that repeated IT infusions of 100 μg CA, which shares a structural similarity with resveratrol was also able to produce analgesic effects in CCI rats as demonstrated in Fig. 1. The results revealed that 100 μg CA markedly blocked peripheral nerve injury induced mechanical allodynia and thermal hyperalgesia on the ipsilateral hind paw. In contrast, 25 μg CA was not effective in the established nociceptive pain responses.

Obviously, persistent painful state caused by partial sciatic nerve ligation could drive nerve cells change in gene expression (Adilakshmi et al., 2012). This also was supported by our present data that Sirt1 expression in the ipsilateral spinal cord was down-

![Fig. 1. Intrathecal injection of carnosic acid (CA) inhibited the established CCI-induced mechanical and thermal sensitivity in the ipsilateral hind paw. Intrathecal administration of 100 μg carnosic acid (CA2) once daily for 4 days beginning on postoperative day 3, but not 25 μg carnosic acid (CA1) produced inhibition of mechanical allodynia (A) and thermal hyperalgesia (B) on the ipsilateral side when rats were examined on postoperative days 7 and 9. An arrow indicates beginning of CCI surgery. n = 7 in each group for behavioral test. *P < 0.05 versus the sham group; **P < 0.05 versus the CCI + DMSO group.

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regulated by neuropathic pain as shown in Fig. 2A, but the SirT1 levels in the contralateral spinal cord remained unchanged between sham and CCI rats (Fig. 2D). Several lines of evidence support that activating SirT1 plays a critical role in mediating injury in various experimental models. It has been shown that SirT1 attenuates nerve injury characterized by axonopathy and neurodegeneration (Araki et al., 2004). SirT1 has been implicated in molecular pathways associated with pain, and a most recent study indicated that SirT1 activation plays a role in ameliorating the development of neuropathic pain in vivo conditions (Shao et al., 2014). Consistent with this observation, we found that IT administration of 100ug CA markedly increased SirT1 expression within the spinal cord ipsilateral to CCI and this increase was consistent with the behavioral change as shown in Fig. 3A. In contrast to changes in protein levels in the ipsilateral spinal cord, Fig. 3D and E shows that 100ug CA did not change contralateral SirT1 expression, which was also consistent with the behavioral change. This assumption is further supported by the fact that the anti-nociceptive effect of CA was reversed by SirT1 inhibitor EX-527 as shown in Fig. 5. However, 25 μg CA could not change ipsilateral SirT1 expression level as compared with the CCI + DMSO group in the Western analysis as demonstrated in Fig. 4. The CA-induced analgesic effects in CCI rats was coupled with increased SirT1 expression and activation, indicating that the effects of CA on

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neuropathic pain behaviors in CCI rats, should be considered to involve the regulation of Sirt1.

The upregulation of p66shc has been reported to be involved in various pathophysiological processes, including heart I/R, diabetes, and glomerulopathy (Carpi, 2009; Francia et al., 2009; Menini et al., 2007). It has been demonstrated that deletion of the p66shc gene in mice results in extending life span and in an increase in age-dependent pain threshold (Berry et al., 2007). In contrast to other physiological and pathophysiological processes, there is only little information available about the p66shc that is involved in neuropathic pain. A new finding of the present study is that the expression of p66shc in the ipsilateral spinal cord was upregulated dramatically after CCI surgery as shown in Fig. 2A, although the p66shc in the contralateral spinal cord remained unchanged between sham and CCI rats (Fig. 2D). It should be pointed out that p66Shc is ubiquitously expressed in mammals and was recognized as proinflammatory mediator (Migliaccio et al., 1999). Notably, animal models of pain studies have revealed that several neuropathic pain states, including neuropathy after peripheral nerve injury, are in themselves associated with several pro-inflammatory molecules, which might contribute to the pain processing (Abbadie et al., 2009; Saika et al., 2012). Hence, the effects of altered expression of spinal p66shc on neuropathic pain may be mediated via modulating pro-inflammatory cytokines. Another important finding of this study is that the p66shc upregulation indeed was reduced significantly in CCI rats receiving the 4 d treatment with carnosic acid. Shown in Fig. 4. CCI-induced down-regulation of Sirt1 and up-regulation of p66shc. Expression was not attenuated by 25µg carnosic acid (CA). Intrathecal administration of 25 µg CA once daily for 4 days beginning on 3 day after CCI did not block the CCI-induced down-regulation of Sirt1 and up-regulation of p66shc. (A), Western blot bands from representative CCI and sham rats with ipsilateral spinal protein taken on postoperative day 7. (B), Quantification of the Sirt1 levels in the ipsilateral spinal cord based on Western blot analysis. (C), Quantification of the p66shc levels in the ipsilateral spinal cord based on Western blot analysis.

Fig. 5. EX-527 inhibits the anti-nociceptive effect of carnosic acid (CA). Intrathecal injection of EX-527 3 mg 1 h before 100 µg CA (CA2) administration once daily for 4 days effectively reverses the effect of CA inhibiting the development of mechanical allodynia (A) and thermal hyperalgesia (B) produced by CCI surgery. An arrow indicates the day of CCI surgery. n = 7 in each group for behavioral test. *P < 0.05 versus the sham + DMSO + DMSO group; #P < 0.05 versus the CCI + DMSO + DMSO group; $P < 0.05 versus the CCI + CA2 + DMSO group.
Carnosic acid has been reported to possess hepatoprotective, antioxidant, anti-inflammatory and neuroprotective activities (Van et al., 2014; Ibarra et al., 2011; Satoh et al., 2008). It was found in this study post-treatment of carnosic acid could inhibit the established CCI-induced pain, whether carnosic acid could inhibit the onset of CCI-induced thermal hyperalgesia and mechanical alldynia rats needs further study. Furthermore, CA's analgesic effect was accompanied by the up-regulation of Sirt1 and the down-regulation of p66shc. The results suggest that CA might be an anti-nociceptive and antihyperalgesic agent effective in the management of chronic pain at the animal level. However, accumulating evidence from studies using animal models of neuropathic pain indicates that neuropathic pain result from multiple cellular and molecular alternations in the dorsal horn after nerve injury, it could not be excluded that CA might attenuate neuropathic pain through other pathways. Our ongoing study will focus on extending other mechanisms of the analgesic effect of CA. Additionally, our data showed that post-treatment of CA could inhibit the established CCI-induced pain more than 3 days, indicating that it will be a necessity of continual intrathecal injections of CA to exert a long-lasting antihyperalgesic effect.

In conclusion, the experimental data from the present research have presented evidence that Sirt1 activation and p66shc suppression by CA improved pain threshold in CCI rats. It may be possible that spinal Sirt1 activation could mediate the anti-nociceptive effect of CA through a mechanism involving the down-regulation of p66shc expression.

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References

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References

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