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Emodin ameliorates diabetic neuropathic pain through inhibiting up-regulation of TRPV1 and pro-inflammatory cytokines in dorsal root ganglions in rats

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Abstract

Background & Objective: Peripheral neuropathy is one of the most common complications of diabetes and leads to sensory symptoms, including diabetic neuropathic pain (DNP). Emodin has potential analgesic effect for the treatment of pain-related diseases. However, the analgesic effect of emodin on DNP and its possible mechanism remains unknown. The aim of the present study is to investigate the effect of emodin on STZ-induced DNP in rats and its potential molecular mechanism. *Methods:* To determine the analgesic effect of emodin on DNP, a mouse model of STZ-induced DNP was established. The pain-related behaviors were evaluated by von Frey test, and hot plate test. The mRNA and protein expression of several TRP channels was detected by qRT-PCR and western blot methods, and the level of inflammatory cytokines was determined by ELISA. *Results:* The mechanical and thermal pain thresholds were significantly decreased in DNP rats. A single injection of emodin treatment significantly reduced DNP. Further results demonstrated that emodin inhibited the up-regulation of *Trpv1* mRNA in the DRG of DNP rats. Our data also indicated that emodin significantly reduced the levels of TNF-α, IL-1β and IL-6 in the DRG of DNP rats.
Conclusions: Emodin ameliorates mechanical allodynia and thermal hyperalgesia in DNP rats by down-regulating the expression of TRPV1 in DRG and the expression of TNF-α, IL-1β and IL-6. Emodin serves as a potent analgesic reagent for treatment and prevention of DNP.

Keywords: Emodin, pain; inflammation, hyperalgesia, inflammatory cytokines; TNF-α

INTRODUCTION

Emodin (1, 3, 8-trihydroxy-6-methylanthraquinone) is a natural anthraquinone derivative, which is the main component of rhubarb and other medicinal materials.[1] A large number of studies have shown that emodin has a variety of biological characteristics.[2,3] Rhubarb, as an important medicinal material for the treatment of many diseases, its active ingredient emodin has become a hot spot of research. Previous reports have shown that emodin has a variety of pharmacological effects, such as antiviral, antibacterial, antiallergic, antidiabetic, immunosuppressive and hepatoprotective activities.[2,3] More importantly, in a large number of clinical practices of Chinese medicine, emodin is often used for the treatment of pain-related diseases.[4,5,6] However, the analgesic effect of emodin on DNP and its possible mechanism remains unknown.

Diabetes mellitus (DM) is a major cause of peripheral neuropathy. The global prevalence of DM in 2011 was approximately 366 million (8.3%), and the prevalence is expected to increase to 552 million (9.9%) by 2030.[7] More than 90% of all cases of DM belong to type 2 diabetes mellitus (T2DM). DM is a metabolic disorder characterized by hyperglycemia.[8] Diabetic peripheral neuropathy is the most common complication of DM, it results in sensory symptoms, including diabetic neuropathic pain (DNP). DNP is also known as painful diabetic peripheral neuropathy.[9] As is widely known, pain is one of the most common diseases seen in the general clinic. DNP seriously affects the quality of life and function of the patients, it also adds to the of the patients' families. Statistics indicate that 10% of diabetic patients suffers from DNP. Growing evidence supports that those with metabolic syndrome, including the prediabetes, may also suffer from neuropathy.[10]

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DNP is characterized by an increased response to painful stimuli (hyperalgesia) and pain in response to a stimulus that does not normally provoke pain (allodynia).[11] DNP is a type of chronic pain whose mechanisms are complex and poorly understood, and effective therapies for DNP remain elusive[12], making DNP as one of the major difficulties in the field of pain research.[13] Also as the treatment effect of DNP remains unsatisfactory[14-16], development of novel analgesia for DNP treatment is warranted.

Transient receptor potential (TRP) channels have been reported to be involved in nociception and thermo-sensation in nervous systems.[17] TRP vanilloid 1 (TRPV1) is activated by multiple mechanisms, such as noxious heat, protons and some pungent chemicals such as capsaicin.[18] TRPV1 is reported to have a crucial role in nociceptive transmission under pathological forms of pain.[19,20] Spinal synaptic plasticity accounts for the transition from acute pain to a chronic pain in which TRPV1 plays a critical role.[21] TRPV1 expression in nociceptors is altered in different models of neuropathy under pathological conditions.[19,20] It has been reported that TRPV1 is also involved in DNP.[22]

In this study, we aim to elucidate the effect of emodin on DNP in rats, and further clarify its potential molecular mechanism of analgesia.

METHOD

Animals

All experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Second Affiliated Hospital of Fujian Medical University. All efforts were made to minimize the number of animals used and alleviate their discomfort. Male Sprague–Dawley rats (180-220 g) were used for all experiments. Rats were housed in individual cages under conditions of a temperature-controlled micro isolator filter cages (23 ± 1°C) under a 12h light-dark cycle, with free access to chow and water. All rats were adapted to the experimental circumstances for a week before experiments.

Diabetic mellitus rat model

Rat was given a single injection (i.p.) of streptozotocin (STZ; 60 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 M ice-cold citrate buffer (pH 4.5). Control group rats were given same volume of saline. Since the increase of blood glucose level is regarded as the key parameter for the successful induction of DM animal model, the glucose level of blood samples from the tail vein was measured with a glucometer (Accu-Chek Active, Roche, Basel, Switzerland) every week. Blood glucose levels higher than 16.7 mmol/L after STZ injection were considered as DM rats.[23] Body weight was measured once a week.

Drug administration and experimental design

Emodin was purchased from Sigma (Sigma, USA) and dissolved in DMSO and diluted to the final working solution in 0.9% saline. For behavioral experiments, the rats were randomly divided into four groups (n = 8): a saline group, a DNP group treated with emodin (10 μg/kg), a DNP group treated with emodin (30 μg/kg) and a DNP group treated with emodin (100 μg/kg). DNP rats were used for a single dose of emodin intraperitoneal (i.p.) injection at 49 days after STZ injection. For repeated dose of emodin administration, emodin (i.p., 100 μg/kg) was injected every two days in rats after STZ injection.

Behavioral tests

Mechano-allodynia (von Frey) test

Mechanical sensitivity was measured using von Frey to assess response to mechanical stimulation by paw withdrawal response to serially increasing filament stiffness. Rats were placed in plastic cages with wire mesh at the bottom, and each rat was allowed to adapt before the test. Von Frey filaments were used on the middle surface of the sole of the left hind paw of each rat, starting from 1g and ranging from 0-50g. The filament touches the hind paw until it bends, while the filament remain perpendicular to the side of the planter of the paw for about 6-8 s.[24] The withdrawal threshold data were determined in grams as paw withdrawal thresholds (PWT).

Thermal pain sensitivity test

For the thermal pain sensitivity test of rats, the hot plate were used to test the paw retraction latency (PWL) of all rats to evaluate the thermal pain threshold. The hot plate consisted of a 25 cm × 25 cm metal plate and a cage with glass. Before the test, each rat was placed in a hot plate device for 10 minutes to allow the rat to adapt without heating. The two hind claws of the rat was allowed to gently touch the surface of the plate without any force applied, and the hot plate

was set at 54°C with a cut-off time of 30 s. The pain threshold was measured by the response time of shaking, retracting or licking the paw of rats, and the threshold data was determined from the average interval of 15 minutes in three experiments.

Cold plate test

Behavioral tests of cold allodynia were performed in rats as previously described.[25] The rats in each experimental group were placed on a glass plate kept at a low temperature (4 ± 1°C), and the incubation period of evacuation was recorded. The stimulation time of each mouse's alternately stimulating paws was 5 minutes, and each paw was tested four times in total.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

According to the manufacturer's instruction manual, Lumbar 4-6 DRG specimens were taken from the ipsilateral lumbar rat of each experimental group, total RNA of each sample was extracted using TRIzol reagent (Invitrogen, USA), and RNA concentration of each sample was quantified by Nanodrop 2000 (Thermo Fisher Scientific, USA) spectrophotometry. Next, 1 μg of each sample was reverse transcribed into cDNA using the PrimerScript^{MT} RT Master Mix kit (Takara Bio, Japan), followed by qPCR experiments. Prepare qPCR reaction system according to SYBR® Premix Ex Taq™ (Takara Bio, Japan) kit instructions. The qPCR reaction system was incubated at 95°C for 3 minutes, and then the target gene fragment was amplified by 40 cycles at 95°C for 10 seconds and 58°C for 10 seconds. The expression level of the gene was calculated by the quantitative cycle (Cq) value according to the formula $2^{-\Delta\Delta Cq}$, and the expression level of the GAPDH gene was used as an internal control. The primer sequences used was as previously reported.[26]

Enzyme-linked immunosorbent assay (ELISA)

The DRG of each experimental group was collected, and the sample homogenate and supernatant were collected rapidly under low temperature. The total protein content of each sample was determined by BCA method. The TNF-α, and IL-6 level in the protein samples was measured by a Parameter™ TNF-α, and IL-6 Immunoassay ELISA kit (R&D Systems, USA) according to the manufacturer's protocol.

Data and statistical analysis

Experimental data are expressed as the mean ± standard error of the mean. Independent sample t test or one-way analysis of variance was used to analyze the quantitative data between the experimental groups. $P < 0.05$ is a significant difference. All data were processed in GraphPad Prism 5 software (La Jolla, USA).

RESULTS

Establishment of DNP model in rats

To establish STZ-induced DM rats, a dose of STZ (STZ; 60 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.1 M ice-cold citrate buffer (pH 4.5) was injected (i.p.). The blood glucose levels and body weights were monitored every week to confirm the establishment of DM rats. As show in Figure 1A, the blood glucose level was dramatically increased and reached to 21.5 **±** 0.70 mmol/L (>16.7 mmol/L) 1 week after STZ administration and was sustained for at least 7 weeks ($P < 0.01$, n = 8, repeated ANOVA). On the other hand, the body weight of STZ-injected rats was significantly decreased (Figure 1B, $P < 0.05$ or $P < 0.01$, n = 8, repeated ANOVA). These results demonstrate that STZ-induced DNP model rats has been successfully established.

After the onset of DM, mechanical thresholds in response to mechanical stimuli and paw withdrawal latency in response to noxious heat were examined. The STZ-induced DM rats began to show obvious mechanical sensitivity 2 weeks and continued until 7 weeks after STZ injection (Figure 1C, $P < 0.01$, n = 8, repeated ANOVA). On the other hand, the paw withdrawal latency in response to noxious heat stimulation was also dramatically decreased 3 weeks after STZ administration (Figure 1D, $P < 0.01$, n = 8, repeated ANOVA), indicating a significant mechanical allodynia and thermal hyperalgesia have occurred in DM rats and lasted until 7 weeks. Therefore, DM rats were used 7 weeks after STZ injection in the following experiment. In addition, we also examined the mRNA expression levels of *Trpa1*, *Trpv1*, *Trpv2*, *Trpv4*, and *Trpm8* in DRG. The results reveal that the expression of *Trpv1* and *Trpv4* are significantly increased in the DRG of DM rats 7 weeks after STZ injection (Figure 1E, $P < 0.01$, n = 8, student's *t* test).

Emodin dose-dependently reverses STZ- induced allodynia and hyperalgesia in rats

To evaluate the effect of emodin on DNP rats,

the STZ-induced DNP rats were used to examine the analgesic effect of emodin. Emodin (10, 30, 100 μM) was administrated (i.p.) to DNP rats 7 weeks after STZ injection. The results of mechanical test showed that compared with saline group without emodin injection, intraperitoneal (i.p.) injection of emodin significantly alleviated STZ-induced mechanical threshold in a dose-dependent manner, and the mechanical threshold increased by more than 350% when emodin was injected at 100 μM (Figure 2A, $P < 0.05$ or $P < 0.01$, n = 8, repeated ANOVA). In DNP rats with mechanical allodynia, the potent analgesic dose of emodin was 100 μg/kg. The increased mechanical threshold reached its peak at 15 min, lasted for at least 45 min and disappeared 60 min after emodin injection (Figure 2A). Similarly, in the hot plate test, the effect of 100 μM emodin group was significantly higher than that of saline

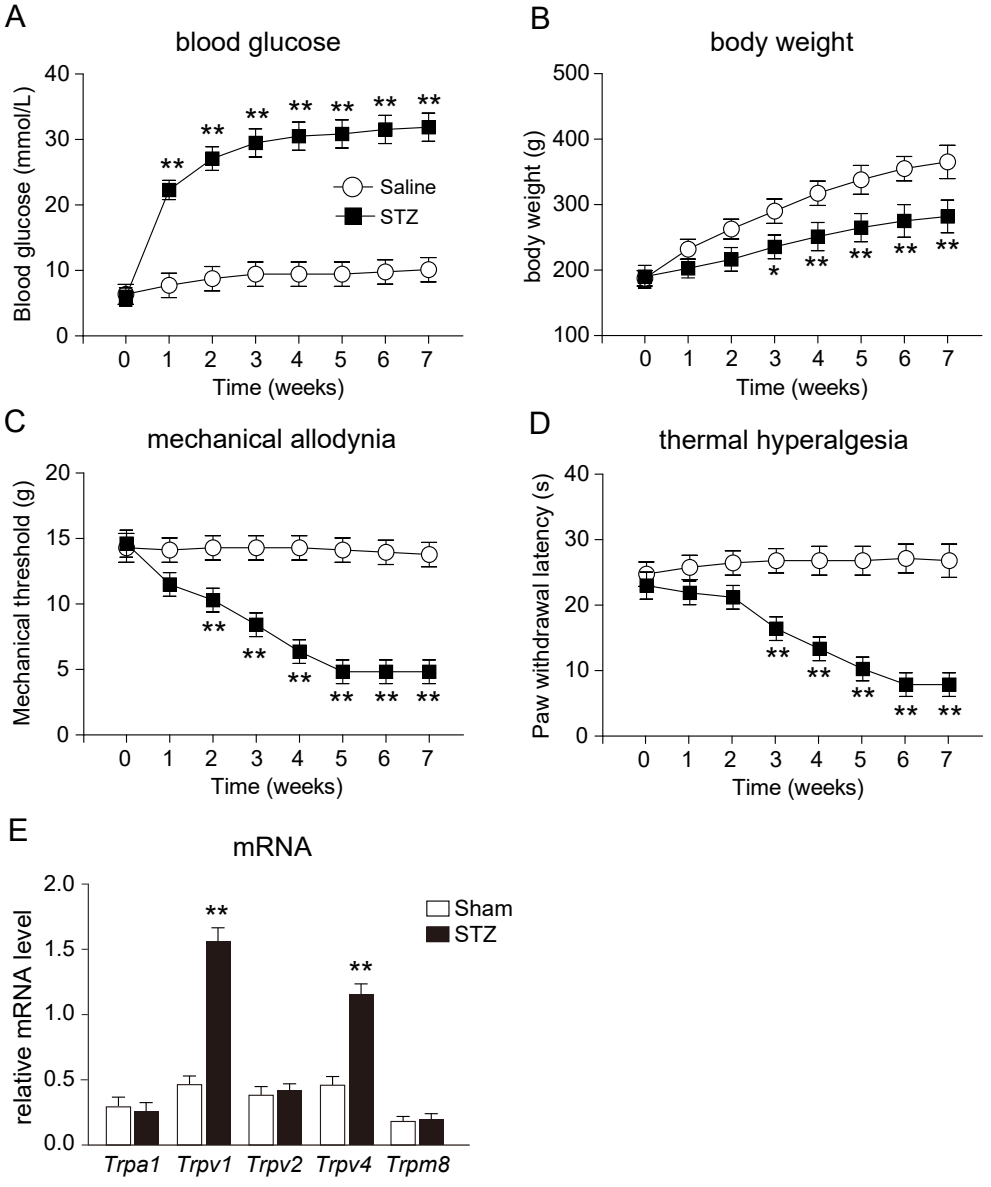

Figure 1. Parameter changes of streptozotocin-induced diabetes mellitus rats.
A and **B** The blood glucose levels (A) and body weight changes (B) in streptozotocin (STZ)-induced diabetes mellitus (DM) rats. **C** and **D** Behavioral tests of mechanical threshold (C) and paw withdrawal latency in response to thermal stimulation (D) in STZ-induced DM rats. Mean ± SEM, n = 8; * $P < 0.05$ and ** $P < 0.01$. Repeated measures ANOVA followed by LSD post hoc test.

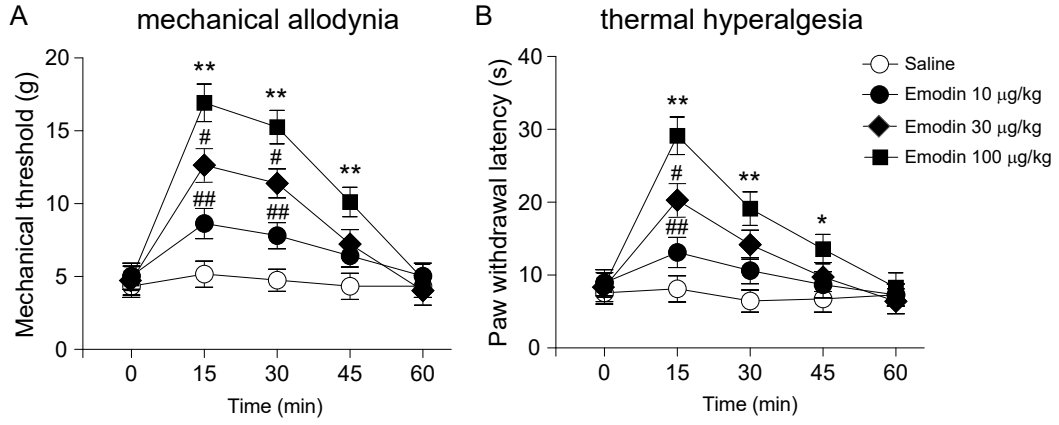

Figure 2. The effect of a single injection of emodin on mechanical allodynia and thermal hyperalgesia in STZ-induced diabetic neuropathic pain rats.

A and **B** Mechanical thresholds (A) and paw withdrawal latencies (B) in STZ-induced diabetic neuropathic pain rats treated with a single injection of emodin administration (i.p., 10, 30 or 100 μg/kg). Mean ± SEM, n = 8; * $P < 0.05$, ** $P < 0.01$ vs. STZ-induced DNP rats group; # $P < 0.05$, ## $P < 0.01$ vs. STZ-induced DNP rats group. Repeated measures ANOVA followed by LSD post hoc test.

group without emodin treatment by about 270% (Figure 2B, $P < 0.05$ or $P < 0.01$, n = 8, repeated ANOVA). The analgesic effect of emodin (100 μg/kg, i.p.) reached its maximum effect at 15 min, lasted for 45 min, although it decreased quickly 15 min after injection. These results suggest that emodin has a transient, dose-dependent analgesic effect in STZ-induced DNP rats.

Emodin effectively prevents the progress of STZ-induced mechanical allodynia and thermal hyperalgesia in rats

In the previous results, we found that emodin can effectively alleviate STZ-induced mechanical allodynia and thermal hyperalgesia in rats. In order to further evaluate the role of emodin in the occurrence and progress of DNP, a repeat dose of emodin was injected in the rats after STZ injection. Different dose of emodin was administrated (i.p.) every two days in STZ-induced DM rats. As shown in Figure 3, the mechanical thresholds in STZ+emodin (100 μg/kg) group were not decreased as much as in STZ

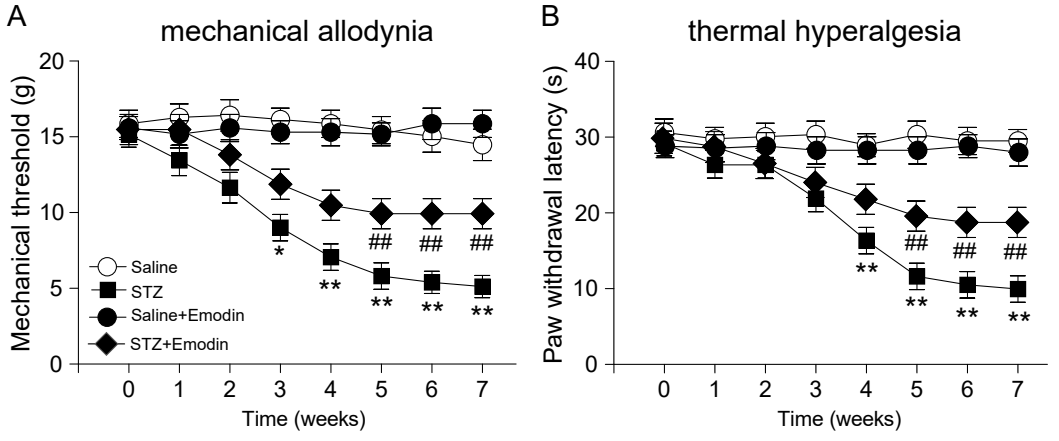

Figure 3. The effect of repeated injection of emodin on the progress of STZ-induced mechanical allodynia and thermal hyperalgesia.

A and **B** The effect of emodin on STZ-induced mechanical allodynia (A) and thermal hyperalgesia (B) by repeated injection of emodin. Emodin was consecutively injected (i.p., 100 μg/kg every two days) during the induction of DNP. Paw withdrawal threshold in the von Frey test and paw licking latency in the hot plate test were measured before and post the injection of STZ. Mean ± SEM, n = 8, * $P < 0.05$, ** $P < 0.01$ vs. saline group. # $P < 0.05$, ## $P < 0.01$ vs. STZ group. Repeated measures ANOVA followed by LSD post hoc test.

group (Fig. 3A, $P < 0.05$ or $P < 0.01$, n = 8, repeated ANOVA). Moreover, the paw withdrawal latency in response to thermal stimulation were significantly increased in STZ+emodin (100 μg/kg) group, compared to STZ group (Figure 3B, $P < 0.05$ or $P < 0.01$, n = 8, repeated ANOVA). These results suggest that emodin may prevent the progress of STZ-induced mechanical allodynia and thermal hyperalgesia in rats.

Emodin inhibits the up-regulation of TRPV1 expression in DRG of DNP rats

TRPV1 and TRPV4 are pain related ion channels. In order to determine whether the effect of emodin is related to the regulation of TRPV1 and TRPV4 expression, we measured the expression of *Trpv1* and *Trpv4* mRNA in DRG of DNP rats after emodin injection. The expression level of *Trpv1* and *Trpv4* mRNA in DRG of rats were significantly up-regulated in a time-dependent manner after injection of STZ. Moreover, the up-regulation of *Trpv1* mRNA was significantly prevented by emodin. (Figure 4A, $P < 0.05$ or $P < 0.01$, n = 8, one-way ANOVA). This inhibitory effect of emodin was not observed in the mRNA expression of *Trpv4* (Figure 4B, $P < 0.01$, n = 8, one-way ANOVA). Moreover, the up-regulation of TRPV1 protein expression was suppressed

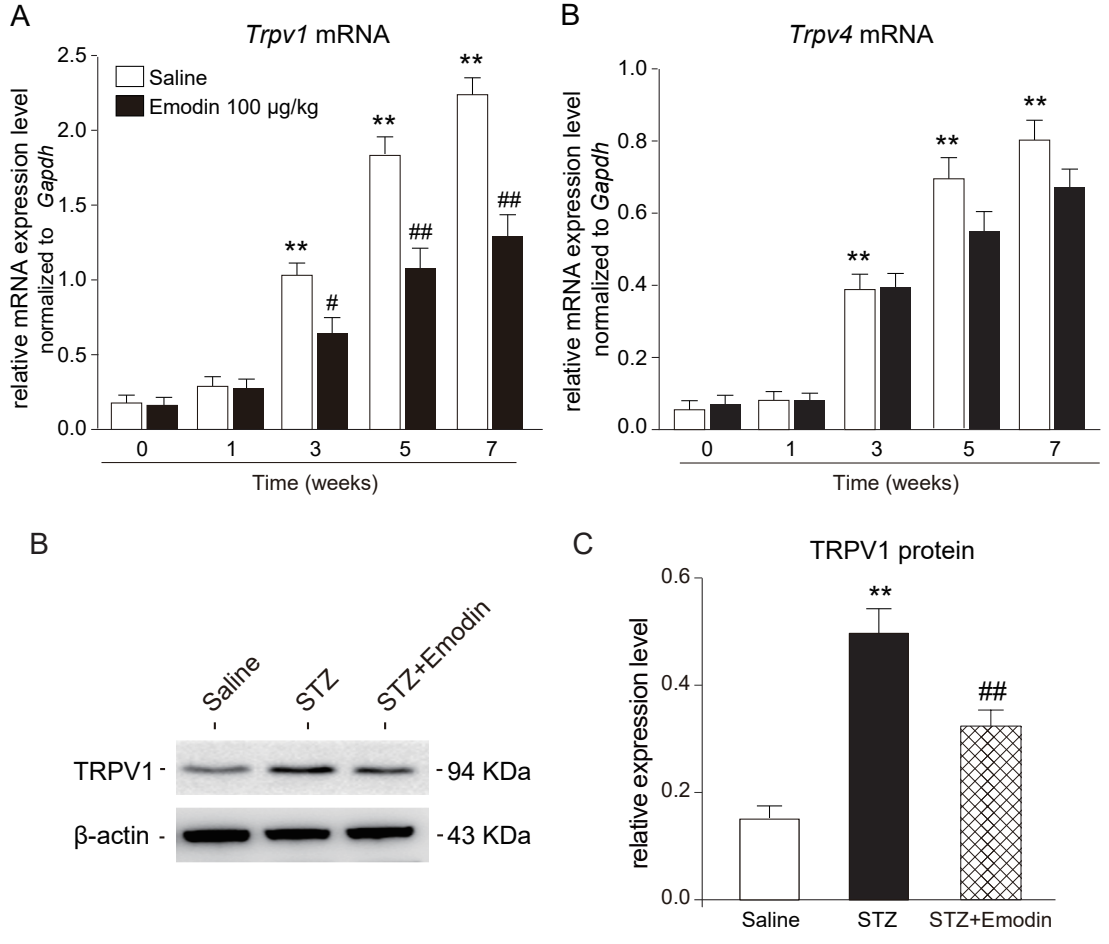

Figure 4. mRNA and protein expression of TRPV1 and TRPV4 in DRG of DNP rats after consecutively emodin treatment.

A and **B** *Trpv1* (A) and *Trpv4* (B) mRNA expression levels normalized to the mRNA expression of Gapdh in DRG of DNP rats after consecutively emodin administration for 49 days. **C** Representative image of western blot result of TRPV1 and GAPDH from DRG of STZ-induced DNP rats consecutively treated with different dose of emodin (i.p., 100 μg/kg). **D** Comparison of TRPV1 protein levels in DRG of STZ-induced DNP rats consecutively treated with different dose of emodin. Mean ± SEM, n = 8, ** $P < 0.01$ vs. sham group, # $P < 0.05$, ## $P < 0.01$ vs. STZ group. One-way ANOVA followed by 2-tailed *t*-test with Bonferroni correction.

by emodin as well (Figure 4C and D, $P < 0.01$, n = 6, one-way ANOVA). These results suggest that emodin may relieve neuropathic pain by preventing TRPV1 up-regulation in DRG of STZ-induced DNP rats.

Emodin down-regulates the levels of TNF-a and IL-6 in DNP rats as well

In order to further clarify the mechanism of emodin in relieving and preventing STZ induced mechanical allodynia and thermal hyperalgesia, we measured the levels of TNF-α and IL-6 in plasma of each rat by ELISA method. As shown in Figure 5, the levels of TNF-α and IL-6 were significantly increased in STZ group. On the other hand, emodin significantly reduced the levels of TNF-α and IL-6 (Figure 5A and B, $P < 0.05$ or $P < 0.01$, n = 8, one-way ANOVA) in the DRG of DNP rats 7 weeks after STZ injection.

DISCUSSION

DNP is one of the most prevalent complications of DM and takes a heavy toll on physical health and well-being in DM patients. DNP is a common neurological complication in diabetic patients, but the current treatment is still far from satisfactory. DNP leads to spontaneous pain, hyperalgesia, and allodynia as well as other atypical paresthesia.[27] The present study demonstrated that emodin prevents DNP and reduces mechanical allodynia and thermal hyperalgesia in STZ-induced DNP rat. Repeated administration of emodin suppresses the up-regulation of TRPV1 and the release of pro-inflammatory cytokines, TNFα and IL6, in DRG induced by STZ. Our results demonstrated the potential analgesic effect of emodin in DNP.

It has already been reported that emodin has potential analgesic effect for the treatment of pain-related diseases.[4,5] The present study demonstrated that the mechanical withdrawal threshold in response to mechanical stimulation and paw withdrawal latency in response to heat stimulation were significantly elevated in DNP rats treated with emodin. Moreover, repeated administration of emodin prevents the progress of STZ-induced DNP. These data suggested that emodin treatment decreases mechanical allodynia and thermal hyperalgesia in STZ-induced DM rats. Together with the previous reports, the present study reveals the potential analgesic effect of emodin in DNP.

TRPV1 and TRPV4 channels have been widely demonstrated to be expressed in peripheral sensory neurons and are closely related to the development of hyperalgesia.[28,29] Studies have shown that TRPV1 and TRPV4 can act as sensory sensors for nociceptive mechanical stimulation, or play an important role in mechanical hyperalgesia.[30] Similarly, it has been reported that STZ induces the up-regulation of TRPV1 and TRPV4.[29,31] Moreover, nanoparticle-encapsulated emodin reduces DNP probably via P2X3 in DRG.[6] It has also been reported that emodin down-regulates

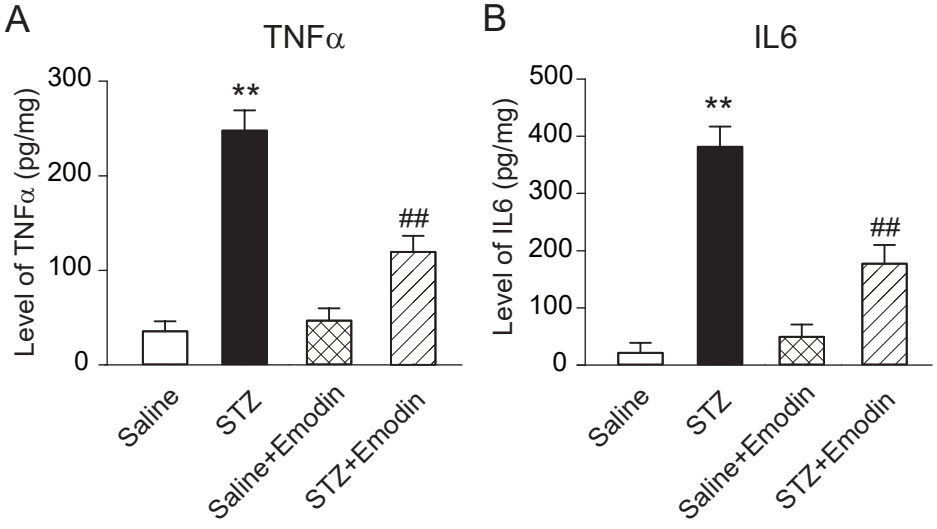

Figure 5. Effect of emodin on the expression of TNF-α, and IL6 in DRG of DNP rats.
A and **B** The level of TNF-α; (A) and IL6 (B) in DRG. All data are shown as the mean ± SD, n = 8, ** $P < 0.01$ vs. saline group, ## $P < 0.01$ vs. STZ group. One-way ANOVA followed by 2-tailed *t*-test with Bonferroni correction.

the expression of TRPV1 mRNA and its function in DRG neurons *in vitro*.[32] In the present study, our results demonstrated that TRPV1 and TRPV4 expression levels were significantly up-regulated in DRG of STZ-induced DNP rats. However, emodin significantly suppresses the up-regulation of TRPV1, but not TRPV4. These results indicate that the analgesic effect of emodin in STZ-induced DNP involves a TRPV1 mechanism.

Inflammatory process is a common pathophysiological process, which is the body's stress response to external physical or chemical factors, and usually involves the accumulation of pro-inflammatory cytokines such as TNF-α and IL-6.[33-35] Numerous studies have shown that pain is often produced in the spinal cord, anterior cingulate cortex, and DRG with the elevated levels of TNF-α and IL-6.[36,37] Inflammatory cytokines such as TNF-α and IL-6 are considered as important biomolecules involved in the development of pain.[38] Previous studies have shown that emodin has a bacteriostatic and anti-inflammatory effect.[2] Therefore, in this study, we examined the level of TNF-α and IL-6 expression levels in DRG. Consistent with the previous data, the expression levels of TNF-α and IL-6 are significantly increased in DRG of STZ-induced DNP rats. Our data indicate that emodin alleviates mechanical allodynia and thermal hyperalgesia in DNP rats share an anti-proinflammatory cytokines mechanism as well.

The present study has some limitations. It is known that STZ-induced diabetic rats show certain structural changes of the peripheral nerves, such as axonal atrophy, axonal disjunction, and fiber demyelination.[39] However, the effects of emodin on pathologic structural changes during STZ-induced DNP were not examined in the present study. This can be explored in future work which exploits the effect of emodin on the peripheral nerve structure in DNP.

In conclusion, our study established a novel analgesic role of emodin as an analgesic agent for DNP. The analgesic role of emodin in DNP might involve TRPV1 and pro-inflammatory cytokines mechanisms. Our data suggested that emodin plays an important analgesic role in DNP and might serve as a potential compound in clinical treatment and prevention of DNP.

DISCLOSURE

Financial support: None

Conflicts of interest: None

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