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Nrf2/HO-1 signaling pathway participated in the protection of hydrogen sulfide on neuropathic pain in rats

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ABSTRACT

Neuropathic pain is evoked by aberrant sensory processing in the peripheral or central nervous system, which is characterized by persistent pain, tactile allodynia, or hyperalgesia. Neuroinflammation is associated with the initiation and maintenance of persistent pain in both the peripheral and central nervous systems. Hydrogen sulfide plays important regulatory roles in different physiological and pathological conditions. Therefore, we investigated the effect of hydrogen sulfide on allodynia, hyperalgesia and cytokine release in rats with neuropathic pain and the related regulatory mechanism. Neuropathic pain was established by chronic constriction injury (CCI) of the sciatic nerve in rats. Nuclear factor erythroid-2 (NF-E2)-related factor 2 (Nrf2) siRNA, hemin, Sn-protoporphyrin (SnPP)-IX and/or NaHS were administered to rats with neuropathic pain, and the spinal cord was collected to detect the expression of Nrf2, hemeoxygenase-1 (HO-1), nuclear factor-kappa B (NF-κb) and the cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and high mobility group box (HMGB)-1 by Western blot (WB) analysis, reverse transcription polymerase chain reaction (RT-PCR), immunofluorescence or enzyme-linked immunosorbent assay (ELISA). Mechanical allodynia, thermal hyperalgesia and the number of paw lifts were measured at different time points after operation. In the present research, neuropathic pain induced Nrf2 and HO-1 expression in the microglial cells of the spinal cord; Nrf2 and HO-1 were necessary to alleviate the hyperalgesia of CCI-induced rats; NaHS mitigated the hyperalgesia and allodynia induced by the CCI operation; and NaHS mitigated the excessive release of the cytokines TNF-α, IL-1β, IL-6 and HMGB1 via the Nrf2/HO-1 pathway in the microglial cells of the spinal cord. These results indicated that NaHS exhibited antinociceptive and anti-inflammatory effects that were associated with the activation of the Nrf2/HO-1 pathway in the spinal cord of rats with neuropathic pain.

1. Introduction

Neuropathic pain is evoked by primary lesions or the dysfunction of the peripheral or central nervous system associated with various chronic conditions and is characterized by allodynia and hyperalgesia, defined as intense pain in response to a normally painful stimulus [1]. Following peripheral nerve injury, a cascade of symptoms and signs in the primary afferents induces peripheral sensitization, resulting in spontaneous nociceptor activity, decreased threshold and increased response to suprathreshold stimuli [2]. The prevalence of neuropathic pain in the human population ranges from 1 to 17.9% [3], which strongly adds to the burden of medical costs in our society. A large proportion of people with neuropathic pain experience poor physical and mental health, and this condition adversely influences quality of life. The exact molecular mechanisms of neuropathic pain are illusive and insufficiently understood, and the elaboration of the mechanisms underlying neuropathic pain is pivotal for the development of treatment strategies. Inflammatory response is associated with both the peripheral and central nervous systems and is considered to be associated with the pathogenesis of neuropathic pain [4]. It is well known that neuropathic pain is a neuro-immune disorder and that the activation of immune and immune-like glial cells results in the release of both proinflammatory and anti-inflammatory cytokines in the injured nerve [5]. The primary mediators of inflammation during the process of

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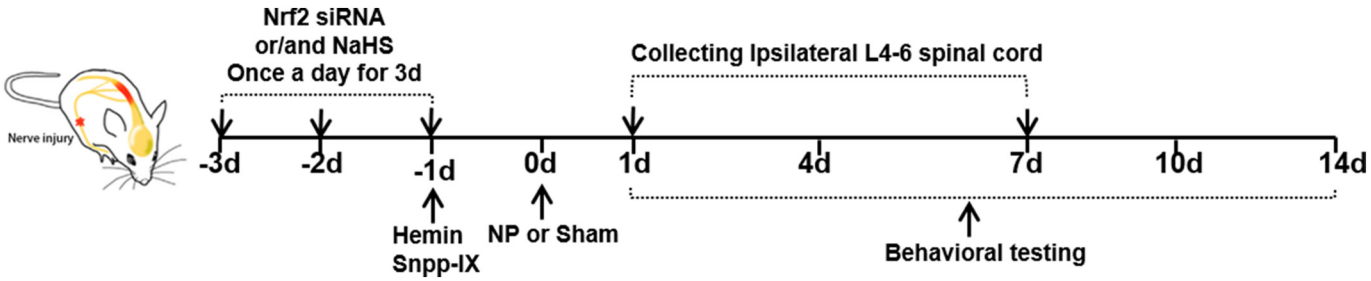

Fig. 1. Experimental design. The rats received the sham operation or the CCI operation to establish the NP model. Spinal cord tissue was collected for the Western blot assay 1 day and/or 7 days after operation. Nrf2 siRNA or/and NaHS were intraperitoneally injected once per day for 3 days before operations. Hemin and SnPP-IX were injected by intraperitoneal injection before operation. The behavior of the rats was tested on days 1, 4, 7, 10, and 14 after operation.

neuropathic pain are spinal microglial cells [6], which contribute to both the initiation and maintenance of neuropathic pain derived from cellular and molecular changes and the generation of behavioral hypersensitivity.

Nrf2, a basic redox-sensitive leucine zipper transcription factor, is the major transcriptional regulator of genes that encode phase II detoxifying/antioxidant enzymes. Under normal circumstances, the nuclear expression of Nrf2 is very low. However, upon exposure to oxidative stress, increasing Nrf2 accumulates in the nucleus, where it binds to antioxidant responsive elements (ARE) via heterodimerization with small Maf proteins and enhances the transcriptional activation of its targets, including HO-1 [7]. HO-1 plays a vital cytoprotective role in various inflammatory diseases [8]. The Nrf2/HO-1 pathway has been reported to play a critical role in inflammatory cytokine expression in LPS-induced macrophages [9]; increases in Nrf2 and HO-1 markedly inhibit the increase in inflammatory cytokines TNF-α, IL-1β and HMGB1 in macrophages stimulated by lipopolysaccharide (LPS), and the HO-1 inhibitor tin protoporphyrin partially reversed the reduction in proinflammatory cytokines. Moreover, J hang et al. [10]. found that monosodium urate (MSU)-induced secretion of IL-1β and activation of the nucleotide-binding oligomerization domain-like receptor pyrin domain containing 3 (NLRP3) inflammasome were inhibited by the knockdown of Nrf2 and via the HO-1 inhibitor zinc (II) protoporphyrin IX (ZnPP-IX), which indicated that MSU-induced gouty inflammation is involved in the Nrf2/HO-1 antioxidant signaling pathway. It is now widely accepted that Nrf2 is the most well-known family member due to its cytoprotective role in the responses to oxidative stress and inflammation [11–14].

For decades, hydrogen sulfide (H_2S) has been known as a colorless, flammable, water-soluble, noxious and toxic gas. H_2S, as well as its two counterparts, nitric oxide (NO) and carbon monoxide (CO), is also a recognized medical gasotransmitter) [15]. An abundance of experimental evidence indicates that H_2S plays important regulatory roles in an array of pathological disturbances, including hypertension, inflammation, heart failure, diabetes, sepsis, cirrhosis, and neurodegenerative disease [16]. Tokuda et al. [17] reported that H_2S exerted a protective effect on the inflammatory response induced by LPS and improved the survival rate in endotoxemic mice. Recently, the physiological roles of this small molecule were elucidated in nervous system diseases [18]. H_2S prevented neuropathic pain behavior via downregulating the excessive release of inflammatory cytokines and activating microglial cells in the spinal cord after peripheral nerve injury [19]. Based on these findings, we designed this study to elucidate the potential mechanism underlying the effect of H_2S on the inhibition of spinal cord neuroinflammation and the specific role of the Nrf2/HO-1 pathway in peripheral nerve injury.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (200–250 g) were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences in Beijing, China and were housed under a 12-h light/dark cycle with food and water provided ad libitum. The experimental protocol conformed to the ethical guidelines of Tianjin Medical University and the local ethics committee. This protocol was in agreement with the Committee on the Ethics of Animal Experiments of Tianjin Medical University, Tianjin, China (permit number: 2018-X3–08).

2.2. Neuropathic pain models

According to the method by Bennett and Xie [20], rats with neuropathic pain were produced by chronic constriction injury (CCI). Under sodium pentobarbital anesthesia, after a midline scalp incision was made in the right thigh, the biceps femoris and the gluteus superficialis were separated, and the sciatic nerve was exposed. Two loose ligatures spaced 1 mm apart were then tied around the nerve distal to the sciatic notch using 6–0 poly-glycolic acid synthetic absorbable sutures. Then, brief twitch was elicited in the respective hind limb of the rats. The skin and muscle were sewed using 5–0 interrupted nylon sutures. The same surgery was performed in sham-operated rats, but only the right sciatic nerve was exposed without ligation.

2.3. Experimental design (Fig. 1)

2.3.1. Experiment 1

Male Sprague-Dawley rats were randomly divided into the following six groups: sham group, sham + NaHS group, neuropathic pain group (NP group), neuropathic pain plus low-dose NaHS group (NP + low NaHS group), neuropathic pain plus medium-dose NaHS group (NP + NaHS group), and neuropathic pain plus high-dose NaHS group (NP + high NaHS group). Neuropathic pain was induced by CCI of the sciatic nerve, and different doses of NaHS were injected into the abdominal cavity once per day for 3 days before the operation. A solution of 30 mg/kg NaHS was used to treat the sham + NaHS group, 15 mg/kg NaHS was used to treat the NP + low NaHS group, 30 mg/kg NaHS was treated at the NP + NaHS group, and 60 mg/kg NaHS was used to treat the NP + high NaHS group. Mechanical allodynia, thermal hyperalgesia and the number of paw lifts were measured at 1, 4, 7, 10, and 14 days after operation. According to the above results, we chose the best concentration of NaHS, 30 mg/kg, NaHS to treat the rats with neuropathic pain the next experiment.

2.3.2. Experiment 2

Rats with neuropathic pain were produced by CCI. L4-6 spinal cord segments were collected to detect the protein and mRNA expression of Nrf2 by Western blot analysis, RT-PCR and immunofluorescence assay

1 day and 7 days after operation. In another part of this experiment, Nrf2 siRNA was administered by intrathecal (i.t.) injection once per day for 3 days before the operation. L4–6 spinal cord segments were collected to detect the protein and mRNA expression of Nrf2 and HO-1 by Western blot analysis and RT-PCR 1 day after operation. Mechanical allodynia, thermal hyperalgesia and the number of paw lifts were detected 1, 4, 7, 10, and 14 days after operation.

2.3.3. Experiment 3

Rats with neuropathic pain were produced by CCI. The L4–6 spinal cord segments of rats in the control group and NP group were collected to detect the mRNA expression, protein expression, and activity of HO-1. Rats were separately treated with hemin and Sn-protoporphyrin (SnPP)-IX by intraperitoneally injection 24 h before operation, and L4–6 spinal cord segments were collected to detect the protein expression, mRNA expression and activity of HO-1 one day after operation. Mechanical allodynia, thermal hyperalgesia and the number of paw lifts were detected at 1, 4, 7, 10, and 14 days after operation.

2.3.4. Experiment 4

Rats were divided into the following three groups: NP group, NP + NaHS group and NP + NaHS + siRNA group. L4–6 spinal cord segments were collected to detect Nrf2, NF-κb, HO-1 and IBA1 protein expression 1 day after operation by Western blot analysis.

2.3.5. Experiment 5

Male Sprague-Dawley rats were randomly divided into the following six groups: sham group, NP group, NP + NaHS group, NP + NaHS + siRNA group, NP + NaHS + hemin group, and NP + NaHS + SnPP-IX group. The siRNA, hemin and SnPP-IX treatments were the same as those in Experiments 1, 2 and 3. L4–6 spinal cord segments were collected to detect the cytokines TNF-α, IL-1β, IL-6 and HMGB1 1 day after operation.

2.4. Intrathecal catheterization

After the rats were deeply anesthetized, a polyethylene (PE)-10 i.t. catheter was inserted through an incision in the atlantooccipital membrane, which reached the enlarged lumbar. After i.t. catheterization, the rats were subjected to the neuropathic pain modeling and did not exhibit locomotion deficits or death.

2.5. SiRNA transfection

Nrf2 siRNA oligonucleotides (Nrf2 siRNA) were purchased from GenePharma (Shanghai, China). Rats were transfected via an i.t. injection of Nrf2 siRNA by i.t. catheter once per day for 3 days before the CCI operation. The sequence of the sense strand of the Nrf2 siRNA was 5'-GAGGAUGGGAAACCUUACUTT-3′, and the sequence of the antisense strand was 5'-AGUAAGGUUUCCCAUCCUCTT-3′. To verify the efficiency of inhibition of Nrf2 expression, the dorsal horn of the spinal cord segment L4-L6 was obtained for PT-PCR after 1 week.

2.6. Behavioral analysis

2.6.1. Mechanical allodynia

Mechanical allodynia was detected by von Frey filament (Stoelting, North Coast, USA) stimulation and an up-and-down method. As described in the method of Chaplan et al. [21], the rats were placed in wire mesh dome 5 min before the experiment. The von Frey filament was applied to the midplantar surface of the hind foot until the was foot withdrawn during or after the stimulus, and the withdrawal was automatically detected.

2.6.2. Thermal hyperalgesia

Thermal hyperalgesia was measured with a plantar heat test (Ugo Basile, Italy) as previously described [22]. Briefly, each animal was acclimated 2 days before CCI, and the test was performed in an enclosed, clear Plexiglass box. After 30 min, during which the animals were allowed to freely explore to habituate to the apparatus, the surface of the forepaw was irradiated by an infrared light beam until paw withdrawal latency. The latency was recorded in seconds as the measurement of thermal hyperalgesia. The infrared stimulus application automatically shut off at 30 s, and rats were kept away from the infrared stimulus to avoid tissue damage. Six trials were randomly performed on the right forepaw with at least 2 min between each trial.

2.6.3. Cold sensitivity

According to a previous report [23], the cold sensitivity of the hind paw was detected. The rats were acclimated in the experimental environment, a copper plate that was cooled to 4 °C, for > 10 min. The number of right hind paw withdrawals from the cold surface over the subsequent 20-min period was counted.

2.7. Enzyme-linked immunosorbent assay

Rats were sacrificed 1 day after operation to detect the cytokines TNF-α, IL-1β, IL-6 and HMGB1. Under deep anesthesia, ipsilateral L4–6 spinal cord samples were harvested, dissected on ice, weighed, homogenized in phosphate-buffered saline (PBS) and centrifuged at 15,000 × *g* at 4 °C for 60 min; the supernatant was collected for assays. The cytokines in spinal cord tissue were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (rat TNF-α, IL-6, IL-10, ELISA kits were from R&D Systems, Minneapolis, Minn; HMGB1 ELISA kit was from IBL, Hamburg, Germany) with a microplate reader (CA 94089; Molecular Devices).

2.8. Western blot analyses

L4-6 spinal cord segments were collected for the detection of Nrf2, HO-1, NF-κb and IBA1 by Western blotting. The samples were homogenized in a six-volume lysis buffer and then centrifuged at 15,000 × *g* at 4 °C for 20 min. The supernatant was kept at −80 °C and used for further analysis. The samples were electrophoresed on a 12% SDS polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) filters (Millipore, USA) in a wet-transfer apparatus. After blocking in 5% BSA for 1 h, the filters were incubated overnight at 4 °C with primary antibody (diluted 1:1000 for Nrf2, diluted 1:2000 for HO-1, diluted 1:1000 for NF-κb, diluted 1:1000 for and IBA1, and diluted 1:2000 for β-actin; Abcam, USA). After washing with PBS (0.01 M PBS mixed with 0.1% Tween-20), the filters were incubated with TBST (5 min each), and the immunoblots were incubated with horseradish peroxidase-conjugated IgG (1:5000 dilution in blocking buffer, Abcam, USA) for 1 h at room temperature. After washing with PBS again, the protein bands were measured with enhanced chemiluminescence (ECL) reagents and then visualized and photographed using a quantitative Gel Quantity One system (Bio-Rad, Tokyo, Japan).

2.9. Reverse transcription polymerase chain reaction(RT-PCR)

L4-6 spinal cord segments were harvested for the detection of Nrf2 and HO-1 mRNA 1 day and 7 days after CCI by RT-PCR. Total RNA extraction was performed by the RNA TRIzol isolation reagent kit (Invitrogen, USA). TRIzol reagent (Takara Co., Ltd., Japan) was used for total RNA extraction, and the RNA quality was tested by NanoDrop 1000 (Thermo Scientific, USA). Then, 5.0 μg total RNA was used for cDNA synthesis by using a third strand cDNA synthesis kit (Invitrogen, CA, USA). Quantitative PCR was performed under the following conditions with 2× Power SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM forward and reverse primers: 5 min at 95 °C and 40 cycles of 15 s at 95 °C and 60 °C for 1 min. All primers were synthesized by Invitrogen (CA, USA), and the primer sequences are

Table 1
The primers of the genes.

Target gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Nrf2	GAATAAAGTTGCCGCTCAGAA	AAGGTTTCCCATCCTCATCAC
HO-1	TGCTCGCATGAACACTCTGGAGAT	ATGGCATAAATTCCCACTGCCACG
GAPDH	TGGAGGTGCTGGAAGAGTT	TCACGCCACAGCTTTCCA

listed in Table 1.

2.10. Immunofluorescence

One day or 7 days after operation, rats were perfused with PBS, and then L4-L6 lumbar spinal cords were collected, placed in 30% (*w/v*) sucrose, frozen, cut into sections and mounted on positively charged microscope slides. The sections were blocked for 1 h in goat serum in 0.3% Triton X-100 in PBS and incubated with the primary antibody (Nrf2 diluted 1:200, HO diluted 1:1:200, IBA1 diluted 1:500; Abcam, Cambridge, UK) in blocking solution overnight at 4 °C. After washing, the cells were incubated with the secondary antibody (1:1000 dilution, DyLight 488-labeled IgG or DyLight 594 AffiniPure IgG, EarthOx, USA) for 2 h at room temperature. After immunostaining, the nuclei of the cells were stained with 1 μg/ml DAPI, and the sections were imaged using a fluorescence microscope (Olympus, Japan).

2.11. HO-1 activity

L4-6 spinal cord tissues were harvested for HO-1 activity determination by using an HO-1 activity kit according to the manufacturer's instructions (GenMed Scientifics Inc., Genmed Scientifics, Arlington, MA).

2.12. Statistical analysis

Statistical analysis was performed by using SPSS 18.0 statistical software. The data are expressed as the mean ± SD. Significant differences between groups were analyzed using one-way ANOVA with post hoc Dunnett's or LSD's test, and a $P < 0.05$ was considered statistically significant.

3. Results

3.1. The effect of different doses of NaHS on hyperalgesia in neuropathic pain induced by CCI

In the present study, we measured pain thresholds and performed behavioral observations from 1 day to 14 days after operation. The withdrawal latency, withdrawal threshold and number of paw lifts of the NP group were lower than those of the sham group from 1 day to 14 days after operation (Fig. 2A, B and C; $P < 0.05$). NaHS improved the withdrawal threshold, withdrawal latency and number of paw lifts of the NP+ low NaHS group, NP + NaHS group and NP + high NaHS group compared with those of the NP group. Among the NP + NaHS groups, hyperalgesia was improved more in the NP+ low NaHS group than in the NP+ high NaHS group, but there was no statistical significance between the NP+ low NaHS group and the NP + NaHS group or between the NP + NaHS group and the NP+ high NaHS group. These results suggested that 30 mg/kg NaHS was a more beneficial treatment for CCI-induced rats.

3.2. Nrf2 expression in the microglial cells of the spinal cord of neuropathic pain rats

The Nrf2 pathway plays an important role in chronic neuropathic pain [24]. Neuropathic pain induced the mRNA expression, nuclear protein expression and total protein expression of Nrf2 from 1 day to 7 days after operation (Fig. 3A, B, C and D; $P < 0.05$), and the expression levels at 1 day were greater than those at 7 days. From the immunofluorescence results, we found that Nrf2 expression had increased colocalization with IBA1 in the NP group compared with the sham group (Fig. 3E) from 1 day to 7 days after operation, which indicated that Nrf2 expression occurred in the microglial cells of CCI-induced rats.

3.3. Nrf2 is necessary for regulating HO-1 expression and hyperalgesia in CCI-induced rats

To discuss the effect of Nrf2 on HO-1 expression and hyperalgesia in CCI-induced rats, Nrf2 siRNA was used to inhibit Nrf2 expression. As shown in Fig. 3, siRNA treatment markedly inhibited the nuclear (Fig. 4A and B, $P < 0.05$) and total protein expression (Fig. 4A and C, $P < 0.05$) and the mRNA expression (Fig. 4E, $P < 0.05$) of Nrf2 and further diminished the protein (Fig. 4A and D, $P < 0.05$) and mRNA expression of HO-1 (Fig. 4F, $P < 0.05$) 1 day after the CCI operation in the NP + siRNA group compared with the NP group.

Withdrawal threshold, withdrawal latency and the number of paw lifts are widely used as behavioral indexes of neuropathic pain. Compared with the sham group, the group that received the CCI operation (NP group) had decreases in withdrawal threshold (Fig. 5A, $P < 0.05$), withdrawal latency (Fig. 5B, $P < 0.05$) and the number of paw lifts (Fig. 5C, $P < 0.05$) from 1 day to 14 days after operation. The inhibition of Nrf2 by siRNA further exacerbated the deterioration of the withdrawal threshold (Fig. 5A, $P < 0.05$), withdrawal latency (Fig. 5B, $P < 0.05$) and number of paw lifts (Fig. 5C, $P < 0.05$) in the NP + siRNA group, which indicated that the inhibition of Nrf2 worsened the hyperalgesia induced by CCI in rats.

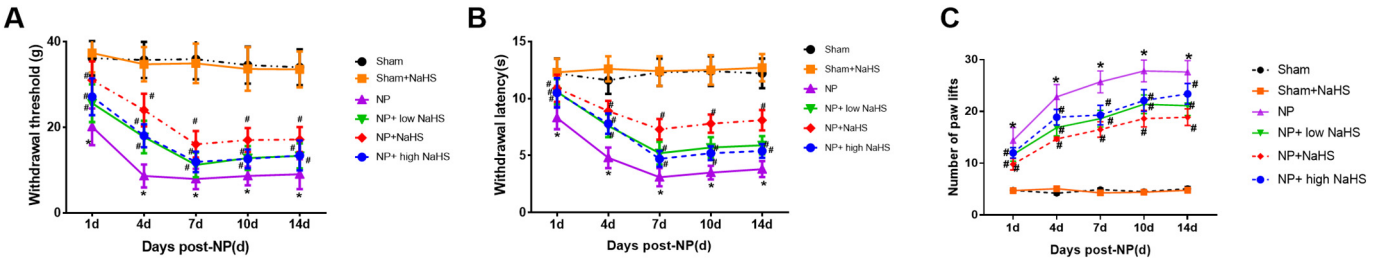

Fig. 2. The effect of different doses of NaHS on hyperalgesia in rats with neuropathic pain. The doses of 15 mg/kg, 30 mg/kg and 60 mg/kg NaHS were injected into the abdominal cavity once per day for 3 days before operations. Mechanical allodynia (A), thermal hyperalgesia (B) and cold sensitivity (C) of rats were tested on days 1, 4, 7, 10, and 14 after operation. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs the sham group. $^{\#}P < 0.05$ vs the NP group.

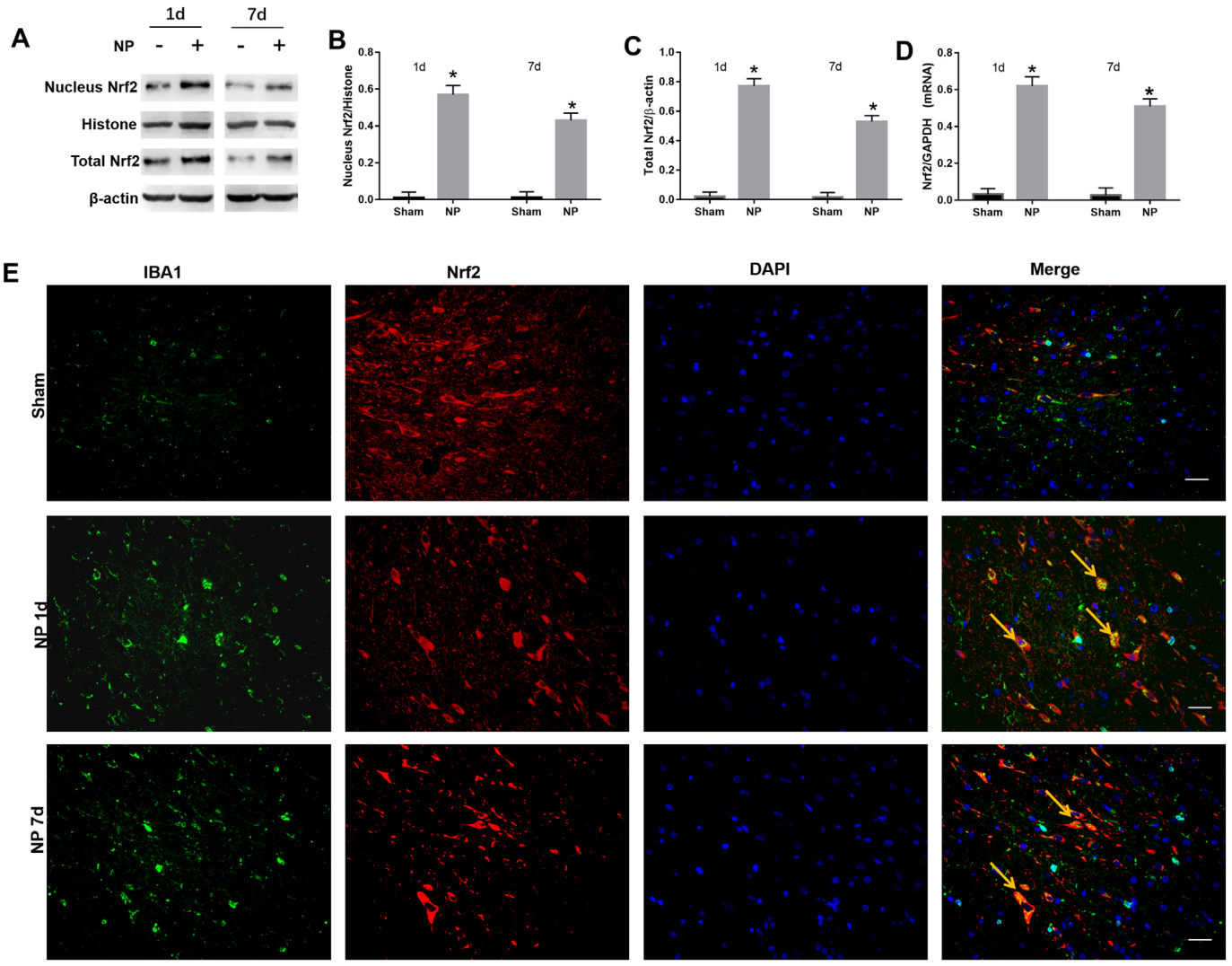

Fig. 3. Nrf2 expression in microglial cells of rats with neuropathic pain. Neuropathic pain was established by the CCI operation. L4–6 spinal cord samples were collected to detect nuclear and total Nrf2 protein and mRNA expression by Western blot analysis (A, B and C), RT-PCR (D) and immunofluorescence (E, bars = 50 μm) 1 day and 7 days after operation. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs the sham group.

3.4. Neuropathic pain induced HO-1 expression in the spinal cord of CCI rats

It has been reported that HO-1 is the main target gene of the Nrf2-ARE pathway in cells. We found that compared with the sham operation, the CCI operation stimulated increases in the protein expression (Fig. 6A and B, $P < 0.05$), mRNA expression (Fig. 6C, $P < 0.05$), and activity of HO-1 (Fig. 6D, $P < 0.05$) 1 day after operation. Additionally, CCI operation induced the colocation of HO-1 and IBA1 in the spinal cord of CCI-induced rats (Fig. 6E), which showed that HO-1 expression was increased in the microglial cells of the spinal cord in CCI-induced rats.

3.5. The effect of hemin and SnPP-IX on HO-1 and hyperalgesia in rats with neuropathic pain

To investigate the effect of HO-1 on CCI-induced rats 1 day after operation, we used hemin and SnPP-IX to activate and inhibit HO-1 expression in rats, respectively. Compared with the sham group, the NP groups treated with hemin (the NP + hemin group) and SnPP-IX (the NP + SnPP-IX group) had markedly induced and inhibited, respectively, HO-1 protein expression (Fig. 7A and B, $P < 0.05$), mRNA expression (Fig. 7C, $P < 0.05$) and activity (Fig. 7D, $P < 0.05$). In the subsequent experiments, we discussed the effect of HO-1 on hyperalgesia. Withdrawal threshold, withdrawal latency and the number of paw lifts were detected from 1 day to 14 days after operation. The results showed that hemin elevated the withdrawal threshold (Fig. 7E, $P < 0.05$) and withdrawal latency (Fig. 7F, $P < 0.05$) and mitigated the number of paw lifts (Fig. 7G, $P < 0.05$) in the NP + hemin group compared with the NP group, while SnPP-IX aggravated the withdrawal threshold (Fig. 7E, $P < 0.05$), withdrawal latency (Fig. 7F, $P < 0.05$) and number of paw lifts (Fig. 7G, $P < 0.05$) in the NP + SnPP-IX group compared with the NP group. These data suggested that HO-1 is necessary to reduce the hyperalgesia in neuropathic pain rats.

3.6. NaHS increased HO-1 protein expression and reduced NF-κb and microglial activation via Nrf2 in neuropathic pain

An essential role of Nrf2-mediated HO-1 upregulation was reported to be in the protection against excessive inflammatory responses [25]. In the above results, we found that 30 mg/kg NaHS has a preferable protective effect on mechanical allodynia and thermal hyperalgesia in CCI-induced rats. Therefore, in the present study, we chose 30 mg/kg NaHS as the dose to treat rats that underwent operation. Compared with the NP group, the NP + NaHS group had increased Nrf2 and HO-1 expression and inhibited NF-κb and IBA1 expression in the spinal cord

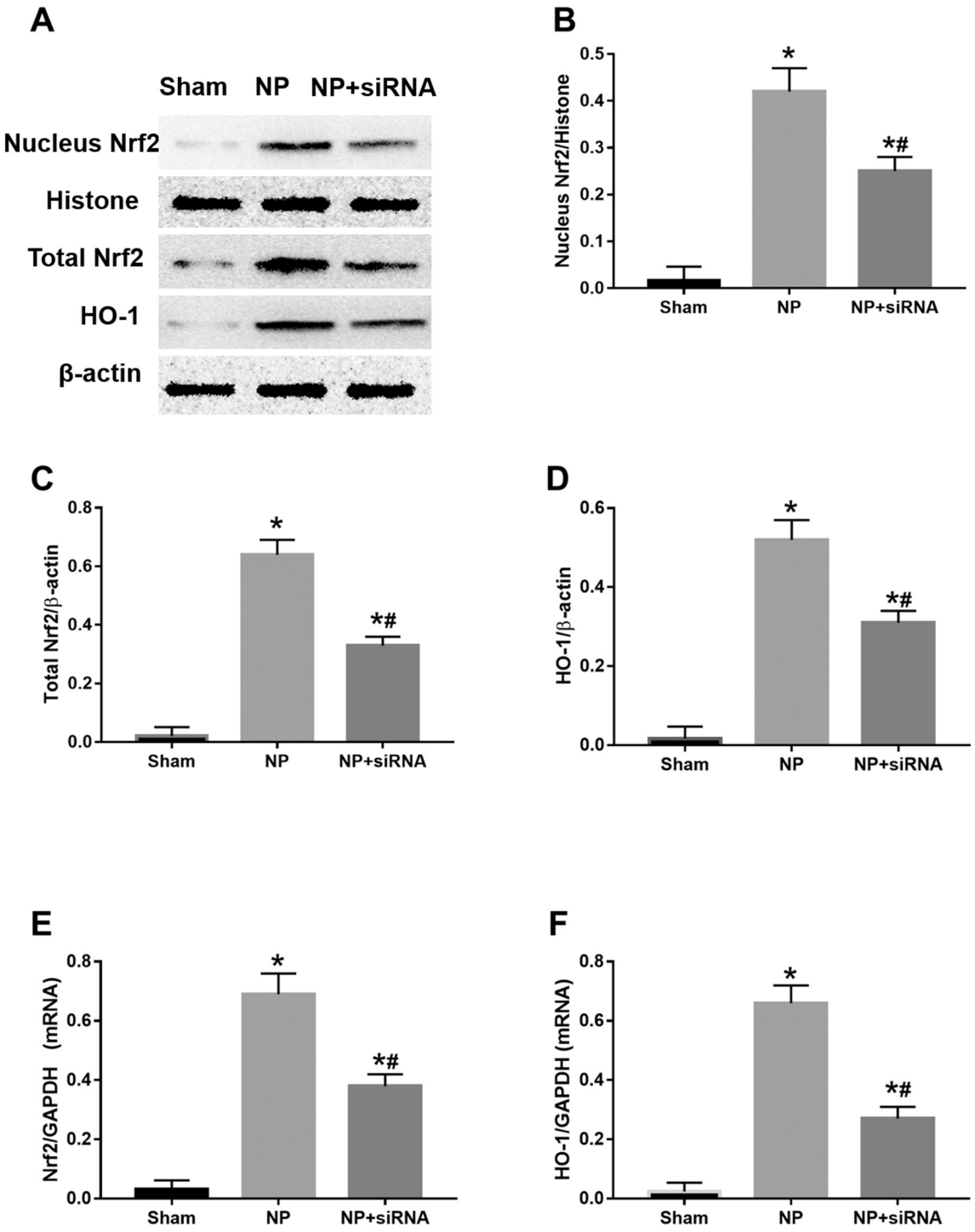

Fig. 4. The absence of Nrf2 reduced the expression of Nrf2 and its downstream effector HO-1 in rats with neuropathic pain. Nrf2 siRNA was administered by i.t. injection before the operation, and neuropathic pain was established by the CCI operation. L4–6 spinal cord samples were collected to detect the nuclear and total protein expression of Nrf2 and HO-1 by Western blot analysis (A, B, C and D) and the mRNA expression of Nrf2 and HO-1 by RT-PCR (E and F) 1 day after operation. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs the sham group. $^{\#}P < 0.05$ vs the NP group.

of CCI-induced rats (Fig. 8A, B, C, D and E; $P < 0.05$). Nrf2 siRNA reversed the NaHS-induced promotion of Nrf2 and HO-1 and inhibition of NF-κb and IBA1 expression in the NP + NaHS + siRNA group (Fig. 8A, B, C, D and E; $P < 0.05$). These results indicated that NaHS increased HO-1 protein expression and reduced NF-κb and microglial activation via Nrf2 in CCI-induced rats.

3.7. NaHS alleviated cytokine release via the Nrf2/HO-1 pathway in neuropathic pain

Compared with the sham operation, the CCI operation induced the excessive release of the inflammatory cytokines TNF-α, IL-1β, IL-6 and HMGB1 (Fig. 9A, B, C and D; $P < 0.05$). In the present study, we investigated the effect of Nrf2 and HO-1 on cytokines in NaHS-treated rats after the CCI operation. siRNA, hemin and SnPP-IX were administered to the rats that underwent the CCI operation. NaHS inhibited the

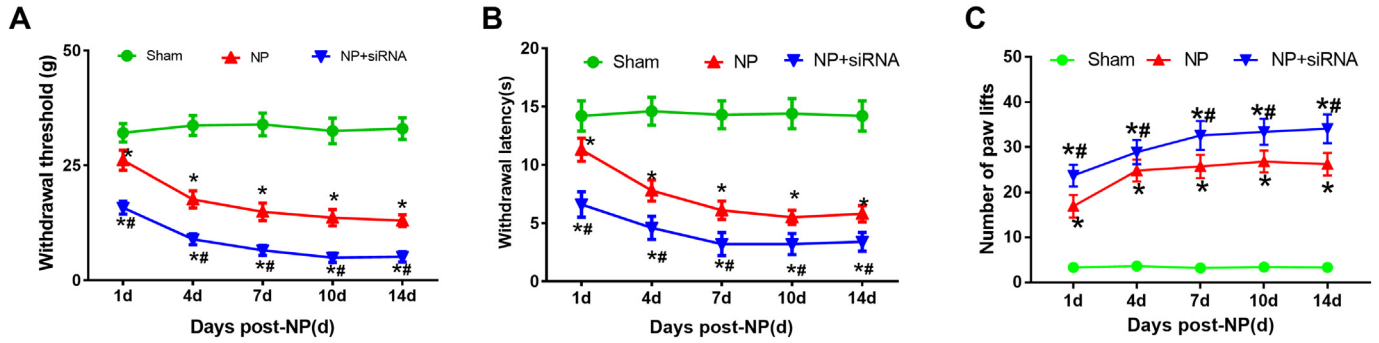

Fig. 5. The absence of Nrf2 aggravated the hyperalgesia in rats with neuropathic pain. Nrf2 siRNA was administered by i.t. injection before the operation, and neuropathic pain was established by the CCI operation. The withdrawal threshold (A), withdrawal latency (B) and number of paw lifts (C) of rats was tested on days 1, 4, 7, 10, and 14 after operation. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs the sham group. $^{\#}P < 0.05$ vs the NP group.

cytokines TNF-α, IL-1β, IL-6 and HMGB1 in the NP + NaHS group compared with the NP group (Fig. 9A, B, C and D; $P < 0.05$). The blockade of Nrf2 by siRNA or HO-1 by SnPP-IX partly reversed the NaHS-induced inhibition of the excessive release of the cytokines TNF-α, IL-1β, IL-6 and HMGB-1 in the NP + NaHS + siRNA group and in the NP + NaHS + SnPP-IX group compared with the NP + NaHS group (Fig. 9A, B, C and D; $P < 0.05$).

4. Discussion

CCI-induced neuropathic pain is caused by aberrant sensory processing in the peripheral nervous system, which is characterized by increased responsiveness to pain stimuli (hyperalgesia) [26], and pain perceived in response to normally non-noxious stimuli (allodynia). Efficacious therapy remains challenging and often insufficient due to the unresolved molecular mechanisms underlying the process of neuropathic pain. In the present study, we investigated the effect of NaHS on hyperalgesia, allodynia, and neuroinflammatory response and the role of the Nrf2/HO-1 pathway in neuropathic pain. We found that 1) NaHS mitigated the hyperalgesia and allodynia induced by CCI; 2) CCI operation induced Nrf2 and HO-1 expression in microglial cells of the spinal cord; 3) Nrf2 and HO-1 are necessary to alleviate the hyperalgesia of CCI-induced rats; and 4) NaHS mitigated the inflammatory response via the Nrf2/HO-1 pathway in the microglial cells of the spinal cord.

NaHS, used to deliver H_2S, is a common donor in experimental research. It has been reported that H_2S plays important regulatory roles in the pathogenetic processes of inflammation, heart failure, sepsis, and neurodegenerative disease [16]. Inhaled H_2S attenuated the excessive release of inflammatory cytokine IL-6 and chemokine (C−C motif) ligand (CCL)2, neuronal damage and microglial activation after peripheral nerve injury, indicating that H_2S confers protective effects on

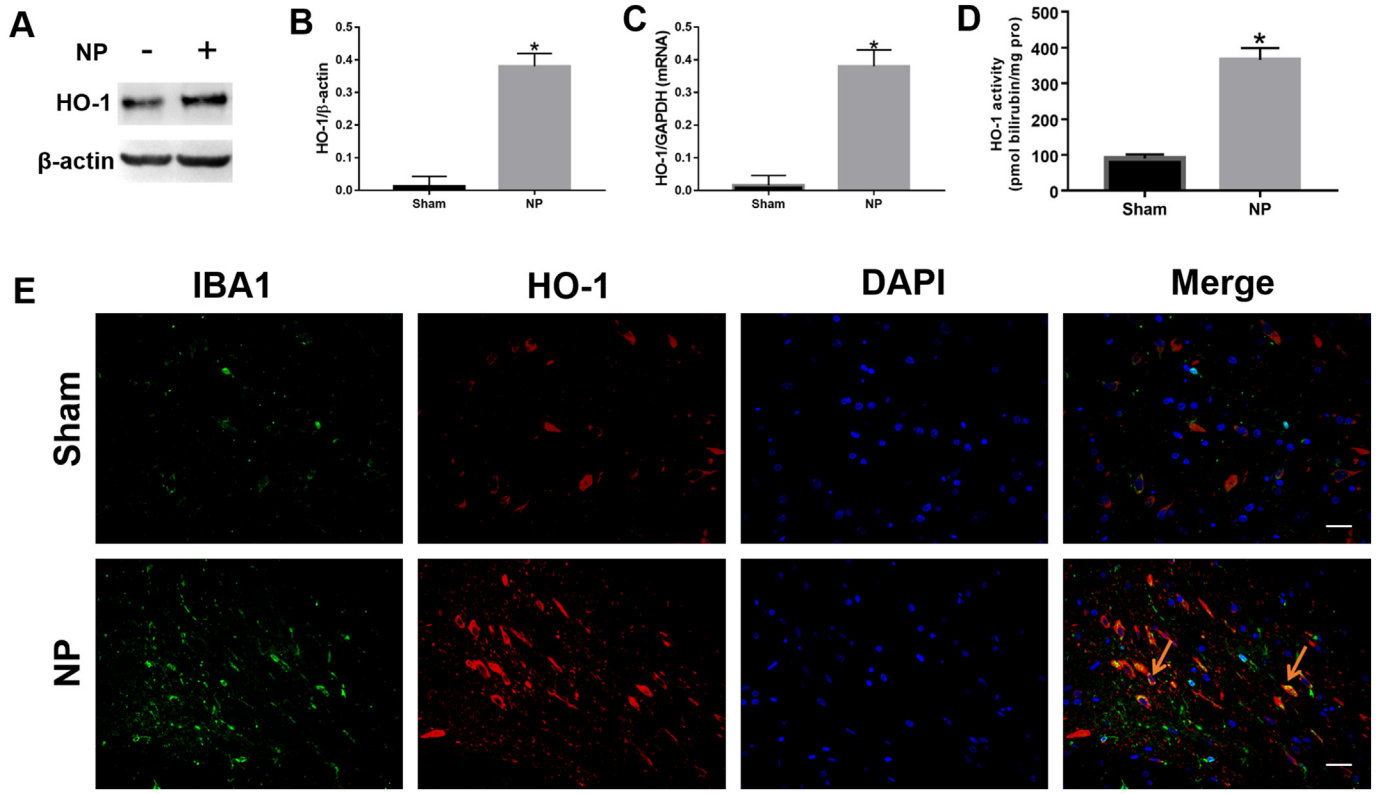

Fig. 6. HO-1 expression and activity in microglial cells in neuropathic pain rats. Neuropathic pain was established by the CCI operation. L4–6 spinal cord samples were collected for the detection of HO-1 protein expression (A and B; E, bars = 50 μm), mRNA expression (C) and activity by Western blot analysis, RT-PCR and immunofluorescence 1 day after operation. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs the sham group.

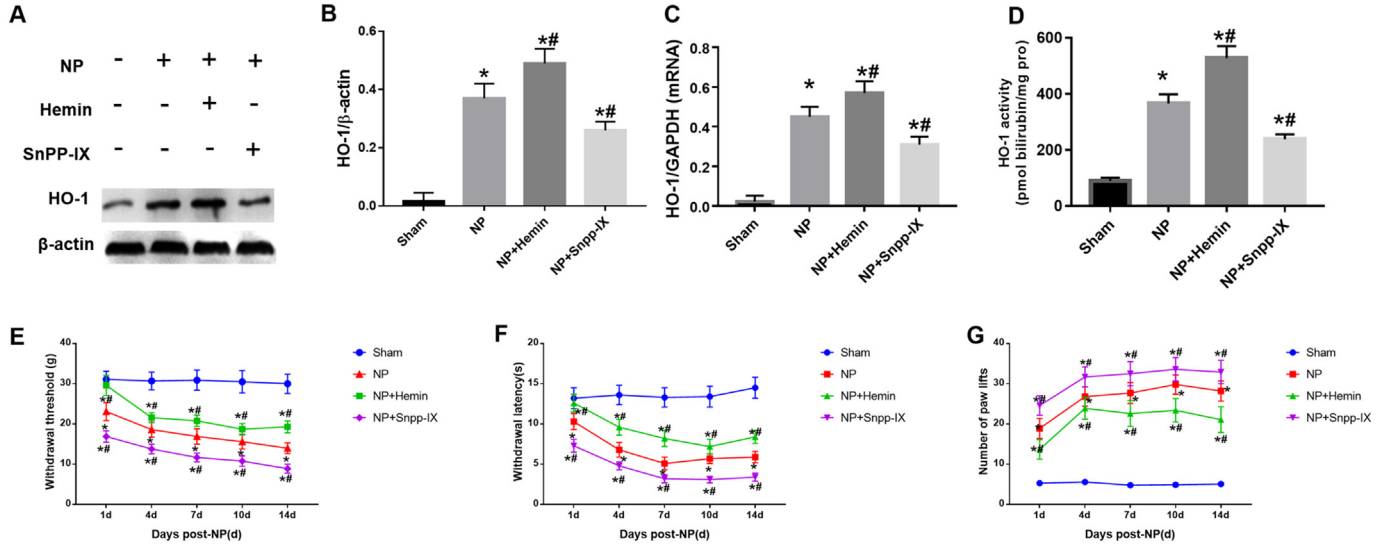

Fig. 7. The effect of hemin and SnPP-IX on HO-1 expression and activity and hyperalgesia in rats with neuropathic pain. Neuropathic pain was established by the CCI operation. Hemin and SnPP-IX were injected by intraperitoneal injection before the operation. L4–6 spinal cord samples were collected for the detection of HO-1 protein expression (A and B), mRNA expression (C) and activity (D) by Western blot analysis and RT-PCR 1 days after operation. The mechanical allodynia (E), thermal hyperalgesia (F) and cold sensitivity (G) of rats were tested on days 1, 4, 7, 10, and 14 after operation. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs the sham group. $^{\#}P < 0.05$ vs the NP group.

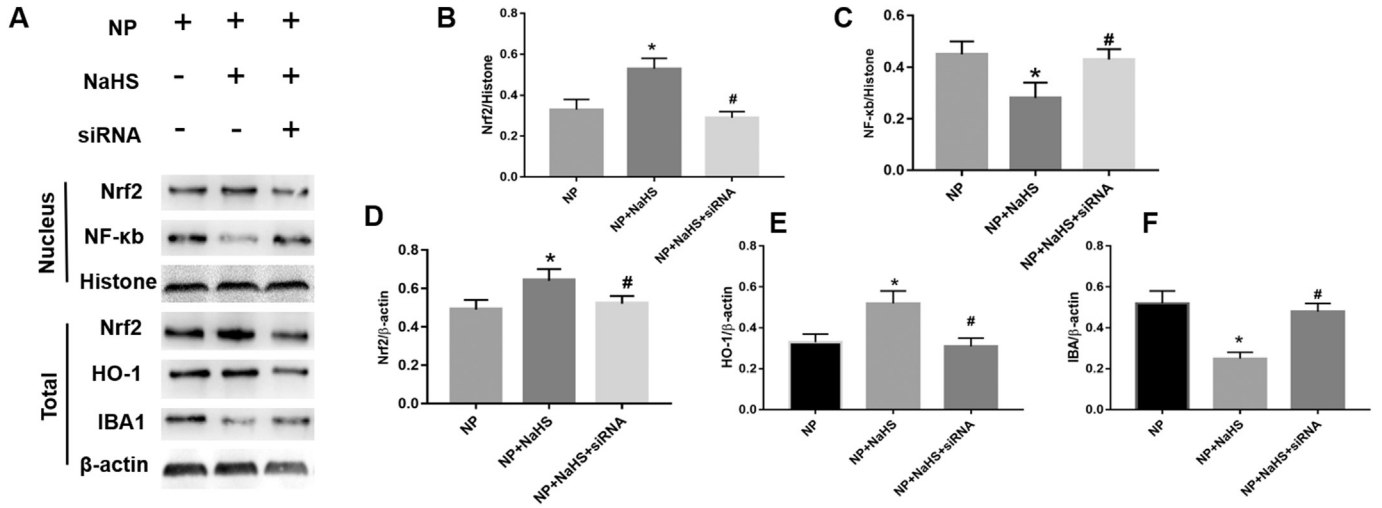

Fig. 8. NaHS increased HO-1 protein expression and reduced NF-κb and microglial activation via Nrf2 in neuropathic pain. Nrf2 siRNA were treated by i.t. injection before the operation, and neuropathic pain was established by the CCI operation. NaHS (30 mg/kg) was injected into the abdominal cavity once per day for 3 days before operations. L4–6 spinal cord samples were collected to detect nuclear (A and B) and total Nrf2 (A and D), nuclear NF-κb (A and C), total HO-1 (A and E) and total IBA1 protein expression (A and F) by Western blot analysis 1 day after operation. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs the NP group. $^{\#}P < 0.05$ vs the NP + NaHS group.

neuropathic pain possibly via inhibiting the inflammatory response and microglial activation [19]. Because H_2S is known as a noxious and toxic gas and the status of H_2S gas is not easy to control, we chose NaHS to deliver H_2S in our research. We used three different concentrations of NaHS to treat rats, and 30 mg/kg NaHS exhibited a more preferable protective effect on hyperalgesia and allodynia than the low and high concentrations of NaHS. Therefore, we chose 30 mg/kg NaHS for further study.

Many findings have also linked the activation of the Nrf2 system to the inflammatory response [27]. Under cellular stress, Nrf2 is released from KEAP1, translocates to the nucleus and initiates the transcription of cytoprotective genes. Nrf2 not only plays a crucial regulatory role in the endogenous defense against various cellular stresses but also is recognized as an important regulator of inflammation in endothelial function [28] and brain injury [27]. The Nrf2 system is also widely expressed in the CNS and regulated in response to both acute cerebral insults and in neurodegenerative disease [29–31]. Therefore, we measured the nuclear and total expression of Nrf2 and the expression of the downstream protein HO-1. We found that CCI operation induced the nuclear and total expression of Nrf2 and the expression of HO-1, and immunofluorescence supported the hypothesis that neuropathic pain increased Nrf2 and HO-1 expression in the microglial cells of the spinal cord of rats with neuropathic pain. The progressive decline in Nrf2 expression was accompanied by a steady reduction in the expression of its downstream protein product, HO-1.

A large amount of literature indicates that enhanced spinal neuroimmune and neuroinflammatory activities initiate and maintain neuropathic pain after primary nerve injury. Damage to the peripheral nerve can lead to an aberrant immune response that results in excessive neuroinflammation of the peripheral or central nervous system. A

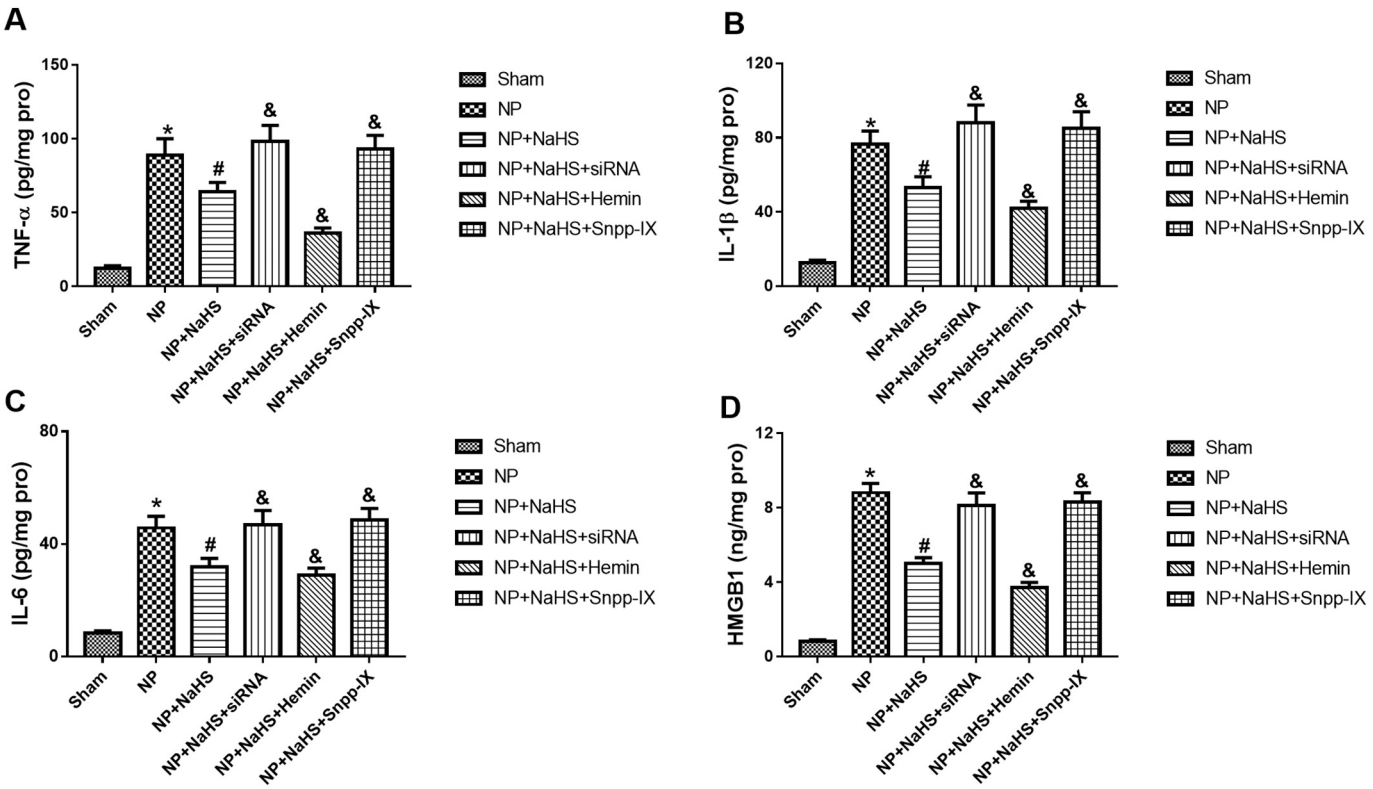

Fig. 9. NaHS alleviated cytokine release via the Nrf2/HO-1 pathway in neuropathic pain. Nrf2 siRNA and/or 30 mg/kg NaHS were administered by i.t. or intraperitoneal (i.p.) injection once per day for 3 days before surgery, and neuropathic pain was established by the CCI operation. L4–6 spinal cord samples were collected to determine the cytokines TNF-α, IL-1β, IL-6 and HMGB1 by ELISA 1 day after operation. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs the sham group. $^{\#}P < 0.05$ vs the NP group. $^{\&}P < 0.05$ vs the NP + NaHS group.

currently unspecified signal from damaged axons induces the activation of the extracellular signal-related kinase (ERK) and mitogen-activated protein kinase (MAP) kinase signaling pathway in Schwann cells, which is one of the main processes that induces the excessive expression of inflammatory mediators and recruits immune cells to the damaged nerve [32]. Once immune cells, including mast cells and macrophages, are activated, NF-κb expression is further activated, and inflammatory mediators, including cytokines (e.g., IL-1β, TNF-α), and chemokines (e.g., monocyte chemoattractant protein (MCP)-1 and fractalkine) are released, which mediates the recruitment of circulating immune cells. Thus, tissue injury, immune cell activation and inflammatory mediator release form a cycle that further induces an excessive inflammatory response and causes serious damage to the body [4]. Proinflammatory cytokines can modulate neuronal excitability and synaptic function, for instance, in the somatosensory system, leading to peripheral and central sensitization. IL-6 is a pleiotropic cytokine with a diverse range of actions, including the modulation of the peripheral and central nervous systems; peripheral nerve injury can induce the synthesis of IL-6 in dorsal root ganglion (DRG) [33], and treatment with an IL-6-neutralizing antibody significantly alleviated allodynia in neuropathic pain [34]. HMGB-1 is a potent inflammatory cytokine. An abnormally inflammatory immune response can induce HMGB1 release in various diseases [35]. The HMGB-1 level was increased in peripheral neurons in response to nerve injury, which contributed to the development of pain hypersensitivity [36]. In our research, we also obtained consistent results; we found that neuropathic pain induced NF-κb activation and the release of the proinflammatory cytokines TNF-α, IL-1β, IL-6 and HMGB-1 1 day after operation. In addition, NaHS has an anti-inflammatory effect on neuropathic pain, and NaHS reduced NF-κb expression and the inflammatory cytokines TNF-α, IL-1β, IL-6 and HMGB1 in the spinal cord after peripheral nerve injury.

To further investigate the effect of Nrf2 on the NaHS-mediated alleviation of hyperalgesia and allodynia in CCI-induced rats, we used siRNA to inhibit Nrf2 expression. In our results, the absence of Nrf2 aggravated hyperalgesia and allodynia in neuropathic pain, and NaHS did not exhibit an alleviating effect on hyperalgesia and allodynia after Nrf2 siRNA treatment in rats with CCI-induced neuropathic pain compared with sham rats. Nrf2 not only regulated hyperalgesia and allodynia but also inhibited the inflammatory response in our research. These results indicated that Nrf2 plays a critical role in the NaHS-mediated improvements in hyperalgesia, allodynia and inflammatory response in neuropathic pain. Interestingly, Nrf2 is primarily involved in the regulation of neuroinflammation via the expression of HO-1 [37,38], and the HO-1/CO signaling pathway was associated with the anti-inflammatory effect of IL-10 and antinociceptive cytokines [38–40]. In the present study, we investigated the effect of HO-1 on cytokines, hyperalgesia and allodynia when NaHS was used to treat rats with neuropathic pain. In our data, the HO-1 inducer, hemin, exhibited synergistic action with NaHS on the anti-inflammatory effect in neuropathic pain. However, the systemic administration of the HO-1 inhibitor SnPP-IX partly reversed the effect of NaHS on anti-inflammation. The data from previous studies and the present study clearly suggest that NaHS acts as an anti-inflammatory agent via the activation of Nrf2/HO-1 in neuropathic pain.

In summary, on the basis of the discussion above, NaHS administration relieves mechanical allodynia, thermal hyperalgesia and excessive cytokines in neuropathic pain and promotes an increase in Nrf2 and HO-1. NaHS produced pronounced anti-inflammatory effect via the activation of the Nrf2/HO-1 signaling pathway.

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