Hydrogen attenuates postoperative pain through Trx1/ASK1/MMP9 signaling pathway

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Abstract

Background: Postoperative pain is a serious clinical problem with a poorly understood mechanism and lack of effective treatment. Considering hydrogen (H 2) could reduce neuroinflammation, we hypothesizes that hydrogen may alleviate postoperative pain and investigate the mechanism.

Methods: Mice were used to establish postoperative pain model via performing a plantar incision surgery. Mechanical allodynia was measured using the Von Frey test. Cell signaling was assayed using gelatin zymography, western blotting, immunohistochemistry and immunofluorescence staining. The animals or BV-2 cells were received with/without ASK1 and Trx1 inhibitor to investigate the effects of H 2 on microglia.

Results: Plantar incision surgery significantly decreased the mechanical threshold, and increased MMP-9 activity and ASK1 phosphorylation in the spinal cord of mice. MMP-9 knockout and ASK1 inhibitor NQDI-1 attenuated postoperative pain. H 2 treatment increased Trx1 expression, decreased ASK1, p38 and JNK phosphorylation, MMP-9 activity, pro-IL-1β maturation and IBA-1 expression in the spinal cord of postoperative pain mice, and ameliorated postoperative pain. In vitro, H 2 increased Trx1 expression and reduced MMP-9 activity induced by LPS in BV-2 cells. Additionally, H 2 also reduced ASK1, p38 and JNK phosphorylation, and IBA-1 expression induced by LPS, which were abolished by Trx1 inhibitor PX12 in BV-2 cells.

Conclusions: For the first time the results confirm that H 2 can work as a therapeutic agent for ameliorating postoperative pain through Trx1/ASK1/MMP9 signaling pathway. MMP-9 and ASK1 may be the targeting molecules to relieve postoperative pain.

Background

Postoperative pain is the most common health concern caused by tissues and nerves lesions after surgical injuries. About 80% of patients experience acute postoperative pain, and more than 20% of patients convert to severe chronic pain, which affects 20–50% of patients' normal life [1, 2]. However, the effective treatments for postoperative pain are still scarce [3–5].

Central sensitization, characterized by increased responsiveness of nociceptive neurons in the central nervous system (CNS) and neuroinflammation mediated by glia (especially microglia), play a crucial role in the pathogenesis of pain [4]. Exposed to various irritating stimuli, microglia could be rapidly activated, and release a large number of inflammatory factors such as IL-1β, IL-6 and TNF-α to aggravate neuroinflammation and further promote central sensitization [6–8]. Numerous studies have shown that microglial activation appears in a variety of pain models, including sciatic nerve ligation (SNL), chronic constriction injury (CCI) and plantar incision (PI) [9–11]. Thus, it is particularly important to find new way to reduce microglial activation and neuroinflammation.

Studies show that matrix metalloproteinases (MMPs) represent a novel mechanism and potential therapeutic target for neuroinflammation and pain [12–14]. The main MMPs involved in the generation and maintenance of pain include MMP-2 and MMP-9. After nerve injury, a rapid and transient rise of MMP-9 in early, activates microglia by the cleavage of IL-1β and p38 activation to induce neuropathic pain, while MMP-2 produces a delayed increase in maintaining neuropathic pain [14, 15]. Further studies show that MMP-9 inhibitor suppresses the activation of astrocyte and microglia, relieving pain, and MMP-9 knockout significantly reduces neuralgia and morphine tolerance [14–16]. Our previous study has shown that paeoniflorin inhibits MMP9/2 and suppresses plantar incision-induced postoperative pain [17]. Thus, inhibition of MMP9 may be a novel approach for the treatment of postoperative pain.

ASK1, a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family, is activated by intracellular oxidative stress, activating JNK MAPK and p38 MAPK to amplify the inflammatory cascade and induce apoptosis, which particularly participates in the induction and maintenance of neuroinflammation, aggravating the central sensitization [18, 19]. Association of Trx with ASK1 through a single Cysteine (C32 or C35) induces ASK1 ubiquitination/degradation leading to inhibition of ASK1-induced apoptosis [20]. In addition, studies have shown that nerve injury causes deeply cell-selective activation of MAPK in the spinal cord and brain. The increase in ERK activity is mainly distributed in neurons, while activation of JNK in astrocytes, and p38 activation in microglia. They are all involved in the development of pain and nerve repair [21–24]. Inhibition of ASK1 activation indirectly reduces the expression and activity of MMP-9 and the subsequent apoptosis ischemic injury [25]. Therefore, targeted inhibition of ASK1 activation may be a very attractive analgesic strategy for the management of postoperative pain.

Hydrogen (H_2) has been proven to be a safe product with fewer adverse effects and effective novel antioxidant, which selectively removes •OH and ONOO- without disturbing other metabolic oxidation reduction [26]. For the ability to penetrate biomembranes rapidly, H_2 passes through the BBB and reaches the nucleus and mitochondria to protect against oxidative damage and reduces cell apoptosis, and plays a protective role in various pathological conditions, especially in brain ischemia and reperfusion injury [26, 27] and inflammation[28, 29]. Thus, we speculated that hydrogen may alleviate postoperative pain, and investigated its potential therapeutic effect and underlying mechanism.

Material And Methods

Animals and ethics statement

Adult male wild type and MMP-9 knockout mice (20 ± 2 g) were approved by Nanjing Medical University animal center. All animals were bred under pathogen-free conditions with controlled temperature (22 ± 2 °C) and a standardized light/dark cycle. They were given ad libitum access to food and water. All animals were allowed to acclimate to these conditions for about one week before inclusion in the experiments.

Surgery

The plantar surgery was performed according to previous study [30]. All surgeries were done under anesthesia induced by isoflurane (2% oxygen gas, 300 ml/min). The plantar aspect of the left hind paw was sterilized with a 10% povidone-iodine solution before and after surgeries, and was placed through a hole in a sterile drape. A 1 cm longitudinal incision extending toward the toes from the proximal edge of the heel was made through skin and fascia of the plantar aspect of the foot. Then elevated and longitudinally incised through, leaving muscle origin and insertion intact, then the skin was apposed with sutures of 5-0 nylon. The incision was checked daily, and any plantar incision with signs of wound infection or dehiscence was excluded from the study.

Drugs and reagents

NQDI-1 was purchased from Selleck Chemical Inc. (Houston, TX). PX12 was purchased from MedChem Express (USA). Antibodies for Thioredoxin (#2429S), p-p38 (Thr180/Tyr182) (#9215S), p-JNK (Thr183/Tyr185) (#9255S) and p-ERK1/2 (Thr202/Tyr204) (#4377S) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies for p-ASK1 (Thr845) (sc-109911) and β-actin (sc-4778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for IBA-1 (ab178847) and MMP-9(ab58803) were purchased from Abcam (Cambridge, MA). Antibody for IL-1β was purchased from R&D systems (USA). Lipopolysaccharide (LPS) and Dimethyl Sulfoxide (DMSO) were purchased from Sigma (St. Louis ,MO, USA). All other chemicals were purchased from Sigma Chemical Co (St. Louis, MO).

The procedures of saturated hydrogen-rich saline (HRS) were prepared as previously described [31]. They were prepared by hydrogen gas which was dissolved in normal saline for 12 h under high pressure (0.4 Mpa), and stored at 4 °C in an aluminum bag under normal pressure. In order to make sure the concentration of hydrogen was more than 0.8 ppm, it was prepared freshly every time and detected with gas chromatography ENH-1000 (Trustiex Co, Japan) and Methyene Blue (Seo , Med Gas Res, Japan).

Grouping and treatment

Mice were randomly assigned to the following groups: sham, plantar incision (PI), PI plus HRS (5 ml/kg, i.p.), HRS treatment alone, PI plus NDQI-1(5 μg/10 μl, i.t.) and NDQI-1 treatment alone. Each HRS treatment group, the mice received injections twice daily, and NDQI-1 treatment groups were administered once daily. HRS and NDQI-1 were injected 6 h before plantar incision surgery. Each group consisted of 12 mice.

BV-2 cells were maintained in humidified 5% CO_2 at 37 °C in Dulbecco's modified Eagle's Medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml). 105 cells were seeded onto 6-well plates overnight and divided into groups(n = 4/group) as sham, LPS (1μg/ml), LPS plus H_2

5% CO_2, 20% O_2, 60% H_2, PH-1-A, China, H_2 alone, LPS plus PX12 (8 μM), and LPS plus H_2 and PX12. In LPS plus PX12 treatment groups, PX12 was added 2 h before LPS.

Behavioral analysis

All animals were placed in the testing environment daily for at least 2 days before baseline testing for acclimatization. The first researcher was responsible for numbering the animal groups and drugs as well as data analysis. The second researcher administrated the drugs to the groups according to the numbers, and the third researcher performed the behavioral tests. All staffs involved were blinded. Mechanical sensitivity was detected using von Frey hairs (Woodland Hills, Los Angeles, CA, USA). Animals were placed into boxes with an elevated metal mesh floor for habituation 30 min before testing. A series of Von Frey hairs with logarithmically incrementing stiffness were used to stimulate the plantar surface of each hind paw perpendicularly. Each mouse was tested three times, and the average of the threshold was measured.

Gelatin zymography

Mice were anesthetized and spinal cord segments were rapidly dissected and homogenized in 1% Nonidet P-40 lysis buffer. Then the solubilized proteins were resolved on gels (8% polyacrylamide gels containing 0.1% gelatin). After electrophoresis, each gel was incubated with 50 ml of developing buffer for 48 h (37.5°C) in a shaking bath. Finally, the gels were stained with Coomassie Brilliant Blue (1%, with 10% acetic acid and 10% isopropyl alcohol, diluted with double-distilled H_2O).

Western blotting analysis

The protein concentrations were determined by BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA), and equal amounts of protein per lane were separated by 8-15% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA, USA). After blocked with 5% bovine serum albumin and 5% skim milk for 2 h at room temperature, the membranes were incubated overnight at 4 °C with the primary antibodies and then incubated with horseradish peroxidase(HRP)-conjugated secondary antibodies for 2 h at room temperature The primary antibodies used included Thioredoxin (1:500), p-p38 (1:1000), p-JNK (1:1000), p-ERK1/2 (1:1000), p-ASK1(1:1000), IL-1b (1:500), IBA-1 (1:1000). For loading control, the blots were probed with antibody for β-actin (1:5000). The filters were then developed by enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA, USA) with secondary antibodies Sigma (St. Louis, MO, USA). Finally, the data were acquired with the Molecular Imager (Gel DocTM XR, 170-8170) and analyzed by the associated software Quantity One 4.6.5 (Bio-Rad Laboratories, Hercules, CA).

Immunofluorescence staining

After deep anesthesia, the animal was perfused transcardially with normal saline followed by 4% paraformaldehyde. L4 and/or L5 lumbar segment was dissected out and post-fixed in the same fixative. The embedded blocks were sectioned into 20 μm thickness. Then, sections from each group (six animals in each group) were incubated with rabbit antibodies for IBA-1 (1:100), mouse antibodies for MMP-9 (1:100), the secondary antibody (1:300, Alexa Fluor 488 AffiniPure Donkey Anti-Rabbit/Mouse IgG, #711-545-152/#715-545-150, Jackson ImmunoResearch Laboratories, USA) at room temperature. After washing out three times with PBS, all slides were carried out blindly and then studied under a confocal microscope (Olympus FV1000 confocal system, Olympus, Japan) for morphological details of the staining. Images were randomly coded and the fluorescence intensities were analyzed by Image Pro Plus 6.0 software (Media Cybernetics Inc. Rockville, MD, USA). The average green and red fluorescence intensity of each pixel was normalized to the background intensity in the same image.

Immunohistochemistry staining

The specimens were sectioned into 5 μm thickness, then incubated with first antibodies for Thioredoxin (1:100) and mouse antibodies for MMP-9 (1:100) in 10% donkey serum and 0.3% Triton-X100. After quenching endogenous peroxidase activity, slides were washed in PBS and incubated with the HRP conjugated secondary antibody for 2 h. Diaminobenzidine was used as a chromogen and counterstaining was done with hematoxylin. All immunohistochemistry staining sections were evaluated by 2 independent pathologists. The score for each slide was measured as the cross-product of the value of immunostaining intensity and the value of proportion of positive-staining cells. The immunostaining intensity was divided into four grades: 0, negative; 1, weak; 2, medium; 3, strong. The proportion of positive-staining cells was also divided into four grades: 1, 0-25%; 2, 26-50%; 3, 51-75%; 4, >75%. The score was calculated using the following formula: Total score = Intensity score × Proportion score.

Statistical analysis

SPSS Rel 15 (SPSS Inc., Chicago, IL) was used to conduct all statistical analysis. Continuous variables were presented as means ± SEM. Normally distributed alteration of protein detected expression and changes in behavioral responses were tested with Student's t tests and one-way ANOVA. The differences in latency over time among groups were tested with two-way ANOVA. Bonferroni *post hoc* comparisons was performed between multiple groups. A criterion value of $P < 0.05$ was considered as statistically significant.

Results

MMP-9 participates in the development of plantar incision-induced postoperative pain

To explore the role of MMP-9 in the process of plantar incision-induced postoperative pain, the activity of MMP-9 and the mechanical threshold of mice (WT or MMP-$9^{-/-}$) were measured. As shown in Fig. 1A, compared to sham group, plantar incision surgery significantly decreased the mechanical threshold of mice lasting for 5 days, causing severe postoperative pain. Plantar incision surgery increased the activity of MMP-9 in the spinal cord of mice collected on 5th day after operation (Fig. 1B). Interestingly, the mechanical threshold of MMP-$9^{-/-}$ mice was higher than WT mice after plantar incision surgery. These results suggested that plantar incision surgery could successfully establish postoperative pain model, and MMP-9 play a vital role in the development of postoperative pain.

ASK1-mediated the activation of MMP-9 facilitates the development of postoperative pain

Considering the important role of ASK1 in inflammation and apoptosis [18–20], we further explored the mechanism of ASK1 in postoperative pain. As shown in Fig. 2A, compared to sham group, plantar incision surgery markedly increased the phosphorylation of ASK1. NQDI1 (5 μg/10 μl, i.t./day) was used as ASK1 inhibitor (Fig. 2B) and significantly decreased the activity of MMP-9 induced by plantar incision surgery in the spinal of mice (Fig. 2C). We also measured the mechanical threshold of mice and found that ASK1 inhibition increased the mechanical threshold, attenuating postoperative pain (Fig. 2D). These data provided evidences that ASK1 may be used as a targeting molecule to relieve postoperative pain.

Hydrogen alleviates postoperative pain via decreasing ASK1 phosphorylation and MMP-9 activity in mice

Based on the advantages of hydrogen in the rapid membrane translocation to inhibit inflammasome activation which promotes the maturation of pro-IL-1β and induces inflammation [9, 26], we explored the effects of hydrogen on ASK1-mediated signaling pathway and IL-1β expression. As shown in Fig. 3A, compared to sham group, plantar incision surgery significantly increased the phosphorylation of ASK1 and the downstream targets p38 and JNK, which were reduced by hydrogen-rich saline. We also measured the activity of MMP-9 and the expression of IL-1β and pro-IL-1β. As shown in Fig. 3B and 3C, plantar incision surgery increased the activity of MMP-9 and the maturation of pro-IL-1β, which were also decreased by hydrogen-rich saline. To further explore the effects of hydrogen on postoperative pain, we measured the mechanical threshold of mice and found that hydrogen-rich saline could significantly attenuate mechanical allodynia (Fig. 3D). These data suggested that hydrogen may work as a potential therapeutic agent to ameliorate postoperative pain.

Hydrogen suppresses the activation of microglia induced by plantar incision surgery in the spinal cord of mice

Activation of microglia in the spinal cord could release a large number of pro-inflammatory factors, such as MMP-9 and IL-1β, resulting in central sensitization and neuropathic pain [8, 14]. Thus, we measured

the expression of IBA-1 (microglia marker) in the spinal cord by immunofluorescence staining analysis and western blot to investigate the effects of hydrogen on microglia activation. As shown in Fig. 4A and 4B, compared to sham group, plantar incision surgery significantly increased the activation of microglia in the spinal cord of mice, which was reduced by hydrogen-rich saline. Western blot results showed that plantar incision surgery increased the activation of microglia in the spinal cord of mice, which was also decreased by hydrogen-rich saline (Fig. 4C). Moreover, further studies found that plantar incision-induced MMP-9 activation was primarily co-labeled with microglia, and hydrogen-rich saline could remarkably decrease MMP-9 activity (Fig. 4A, B). These data suggested that hydrogen could reduce the plantar incision-induced the activation of microglia and MMP-9 in the spinal cord.

Hydrogen increases the expression of thioredoxin in the spinal cord of mice

To further elucidate the early molecular mechanisms of postoperative pain, we measured the related proteins level on 24 hour after plantar incision surgery. Since thioredoxin (Trx) promoting ubiquitination and degradation of ASK1 inhibit ASK1-mediated apoptosis in a redox activity-independent manner [20]. The expression of Trx1 was measured and we found that hydrogen-rich saline could significantly increase the expression of Trx1 both at 24 h (Fig. 5A) and on 5th day (Fig. 5C), and decrease the activity of MMP-9 at 24 h (Fig. 5B). The results of immunohistochemistry also showed that hydrogen-rich saline could increase the Trx1 expression induced by plantar incision surgery (Fig. 5D). These data suggested that Trx1 may be involved in the protection of hydrogen against postoperative pain.

Hydrogen attenuates microglial activation via regulating Trx1/ASK1/MMP9 signaling pathway in vitro

To further confirm the mechanism that hydrogen inhibits the activation of microglia, BV-2 cell was used for in vitro experiments. As shown in Fig. 6, LPS established cellular inflammation model. Compared to LPS group, hydrogen gas significantly increased the expression of Trx1 (Fig. 6A) and decreased the activity of MMP-9 (Fig. 6B). We also found that hydrogen gas could decrease the phosphorylation of ASK1, p38 and JNK compared to LPS group, which were abolished by the compound PX12 (the inhibitor of Trx1). We further measured the NF-κB translocation from the cytosol to the nucleus and the expression of microglia marker IBA-1, as shown in Fig. 6D-F, hydrogen gas significantly decrease p65 translocation and the expression of IBA-1 induced by LPS, which were abolished by Trx1 inhibitor PX12. These data suggested that Hydrogen could reduce the activation of microglia via regulating Trx1/ASK1/MMP9 signaling pathway in vitro.

Discussion

In this study, the main findings include the following: (1) MMP-9 knockout delayed and relieved the development of postoperative pain induced by plantar incision surgery. (2) ASK1 inhibitor depressed MMP-9 activity and attenuated postoperative pain. (3) H_2 up-regulated Trx1 expression and inhibited ASK1/MMP-9 to ameliorate postoperative pain. (4) H_2 decreased MMP-9 activity via Trx1/ASK1 signaling pathway and reduced microglial activation in BV-2 cells.

MMPs are implicated in the pathogenesis of neuropathic pain. The mechanism of MMPs activation involves modifying the cysteine residues (such as S-nitrosylation alkylation and oxidization) and dissociating the cysteine residue from the zinc-binding site of MMPs [32, 33]. Since the production of proinflammatory factors and cytokines such as TNF-α, IL-1β, ROS etc., microglial activation are increasingly implicated. Further oxidation resulted in a stable posttranslational modification with pathological activity, activated MMP-9 and induced apoptosis [7, 34]. Our previous study demonstrated that plantar incision could increase MMP-9 activity, activate microglia and promote the development of postoperative pain [17]. In this study, we further demonstrated the importance of MMP-9 in the development of postoperative pain using MMP-9 knockout mice (Fig. 1C). Moreover, MMP-9 co-localized with microglia IBA-1 and aggravated the IL-1β cleavage (Fig. 4). We also found ASK1 inhibition could ameliorate pain and reduced MMP-9 activity (Fig. 2), suggesting that MMP-9 and its upstream ASK1 are associated with a facilitation in postoperative pain. Regulation of ASK1/MMP9 in microglia may receive considerable attention as a potential therapeutic target for ameliorating postoperative pain.

Hydrogen-rich saline and H_2 gas have been confirmed have effects on alleviating the hyperpathia and activating autophagy in neuropathic pain model [9, 10]. In this study, for the first time hydrogen-rich saline was used for treating postoperative pain. In accordance with the effects of H_2 on p-ASK1, p-p38 and p-JNK (Fig. 3A), H_2 decreased the activity of MMP-9 (Fig. 3B) and attenuates incision-induced postoperative pain effectively (Fig. 3D). But how does H_2 regulate the phosphorylation of ASK1, p38 and JNK? Trx1 is an endogenous 12 kDa multifunctional protein with two redox active half-cysteine residues -Cys-Gly-Pro-Cys- [35]. It has been identified in all living cells and related to cell proliferation and apoptosis, which is responsible for protecting cells from oxidative stress by scavenging ROS [35, 36]. Experimental results show that intravenous administration of recombinant human thioredoxin and overexpression of Trx1 in transgenic mice confer resistance to ROS-induced cells death, finally decrease the brain damage in cerebral ischemia model [37, 38]. Trx1 overexpression extends antioxidant protection, attenuates mitochondrial damage and prolongs survival during sepsis[39]. Furthermore, accompany with the decrease of Trx1 level, NLRP3 expression increase promoting inflammation in injured tissue[40]. Since Trx1 exerts its role through interacting with its binding proteins ASK1 and inhibits the activation of ASK1 [20], and our data revealed that H_2 could increase the expression of Trx1 (Fig. 5A and 5C), indicating that Trx1 is an endogenous neuroprotective protein in H_2 treatment against postopreative pain. To further investigate the protection effects of H_2, we collected and analyzed BV-2 cells after H_2 treatment. We found H_2 treatment time-dependently increased the expression of Trx1 and decreased the phosphorylation of ASK1, p38 and JNK, and decreased MMP-9 activity, which were abolished by the Trx1 inhibitor PX12 in BV-2 cell. These data demonstrated that H_2 could attenuate postoperative pain via regulating Trx1/ASK1/MMP-9 signaling pathway, enriching the mechanism of H_2 therapy.

H_2 has been proven to be a safe product with fewer adverse effects. Compared to Vitamin E and superoxide dismutase, H_2 is a selective antioxidant in reducing cytotoxic oxygen radicals [26]. Inhalation of hydrogen gas, drinking hydrogen water, injection of hydrogen saline and direct incorporation of molecular hydrogen by diffusion including eye drops, bath and cosmetics are the main methods to ingest

or consume H_2 [27]. After oxygen glucose deprivation and reperfusion, H_2 increase neuron survival and vitality to protect neurons against oxidative stress-induced cell death in vitro [26, 31]. Inhaling of H_2 gas suppresses not only the initial brain injury, but also its progressive damage [26]. Moreover, hydrogen has beneficial effects involve promotion of microglia M2 polarization and inflammation reduction [28, 31]. In clinical trials, hydrogen-rich water can restore an optimal oxidative balance and reduce gastroesophageal reflux disease symptoms rapidly [41], and there are also studies on hydrogen-rich water in Parkinson's disease [42]. Therefore, H_2 treatment for postoperative pain is easier to apply clinical transformation from basic items.

Conclusions

In summary, the present results demonstrated that H_2 attenuates plantar incision-induced postoperative pain via inhibiting ASK1/JNK/p38/MMP9 signaling pathway and microglia activation, which may be related to Trx1 (Fig. 7). We suggested that H_2 may be a potential drug candidate for postoperative pain treatment.

Abbreviations

MMPs: matrix metalloproteases; ASK1: apoptosis signal-regulated kinase 1; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor alpha; ERK: extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase; Trx1: Thioredoxin; •OH: hydroxyl radical; ONOO-: peroxynitrite; HRS: hydrogen-rich saline; H2: hydrogen gas; LPS: Lipopolysaccharide; DMSO: Dimethyl Sulfoxide; IBA-1: anti-ionized calcium-binding adaptor protein 1; NLRP3: Nod-like receptor family pyrin domain containing protein 3.

Declarations

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Availability of data and materials

The datasets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL, QL, and LH designed and performed the experiments, analyzed the results, and drafted the manuscript. PT, YS and JL carried out the behavioral measures, gelatin zymography and immunofluorescence. QL and LZ carried out the western blotting analysis. WL, MD, PY and LH conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethical Approval and Consent to participate

All procedures were performed in accordance with the regulations of the ethics committee of the International Association for the Study of Pain and the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China). All animal experiments were designed to minimize suffering and the number of animals used.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Figures

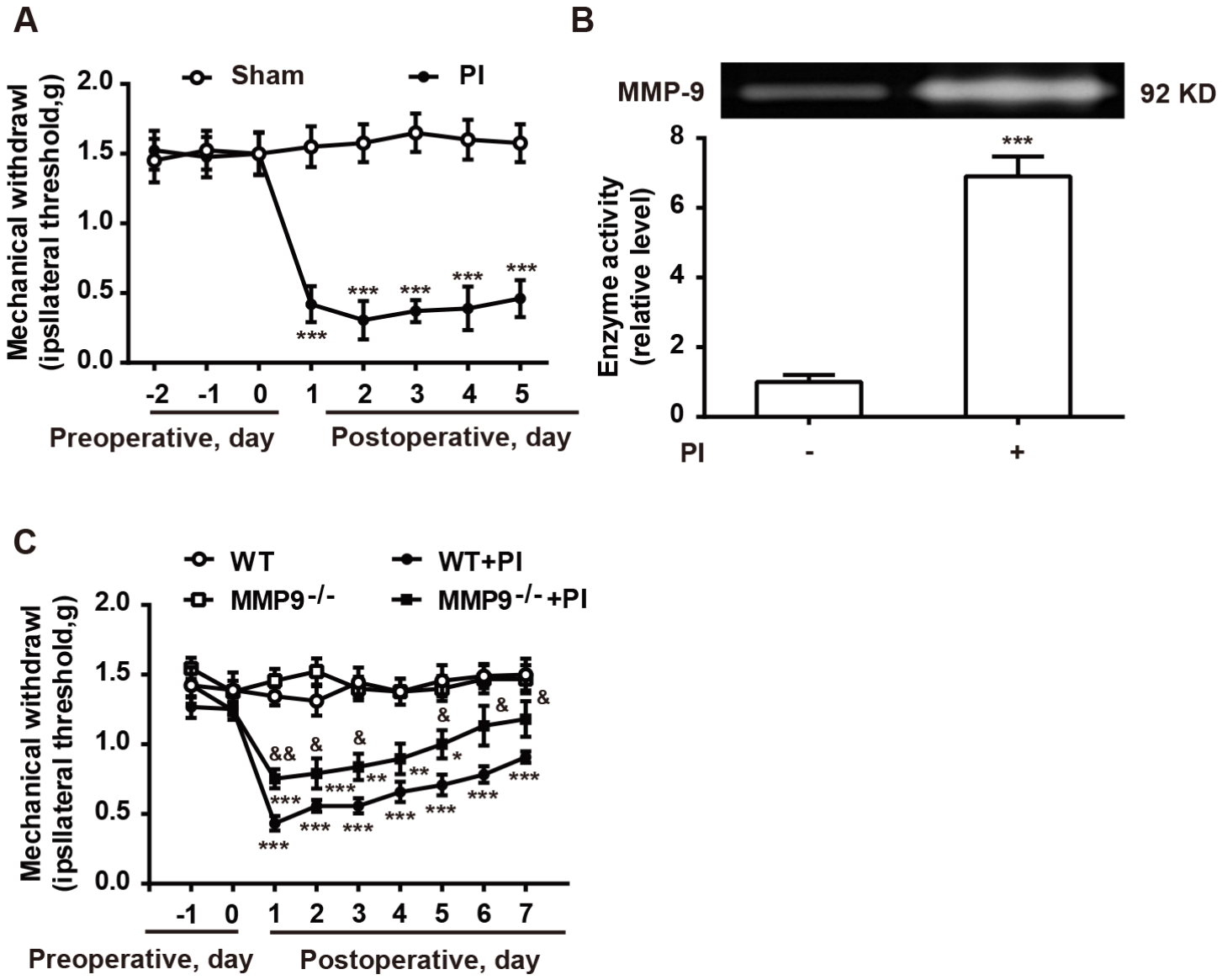

Figure 1

MMP-9 plays a crucial role in the development of plantar incision-induced postoperative pain. A. Mechanical thresholds of mice received plantar incision surgery (n = 12). B. The activity of MMP-9 was measured by zymography after surgery in the spinal cord of mice (n = 6). The lumbar spines (L1–L6) were collected and analyzed 5 days after surgery. C. Mechanical thresholds of wild-type mice or MMP-9 KO mice that received plantar incision surgery (n = 6). PI = plantar incision. A significant difference was revealed following Student's t tests (*P < 0.05, **P < 0.01, ***P < 0.001 vs. sham; &P < 0.05, &&P < 0.01 vs. plantar incision group).

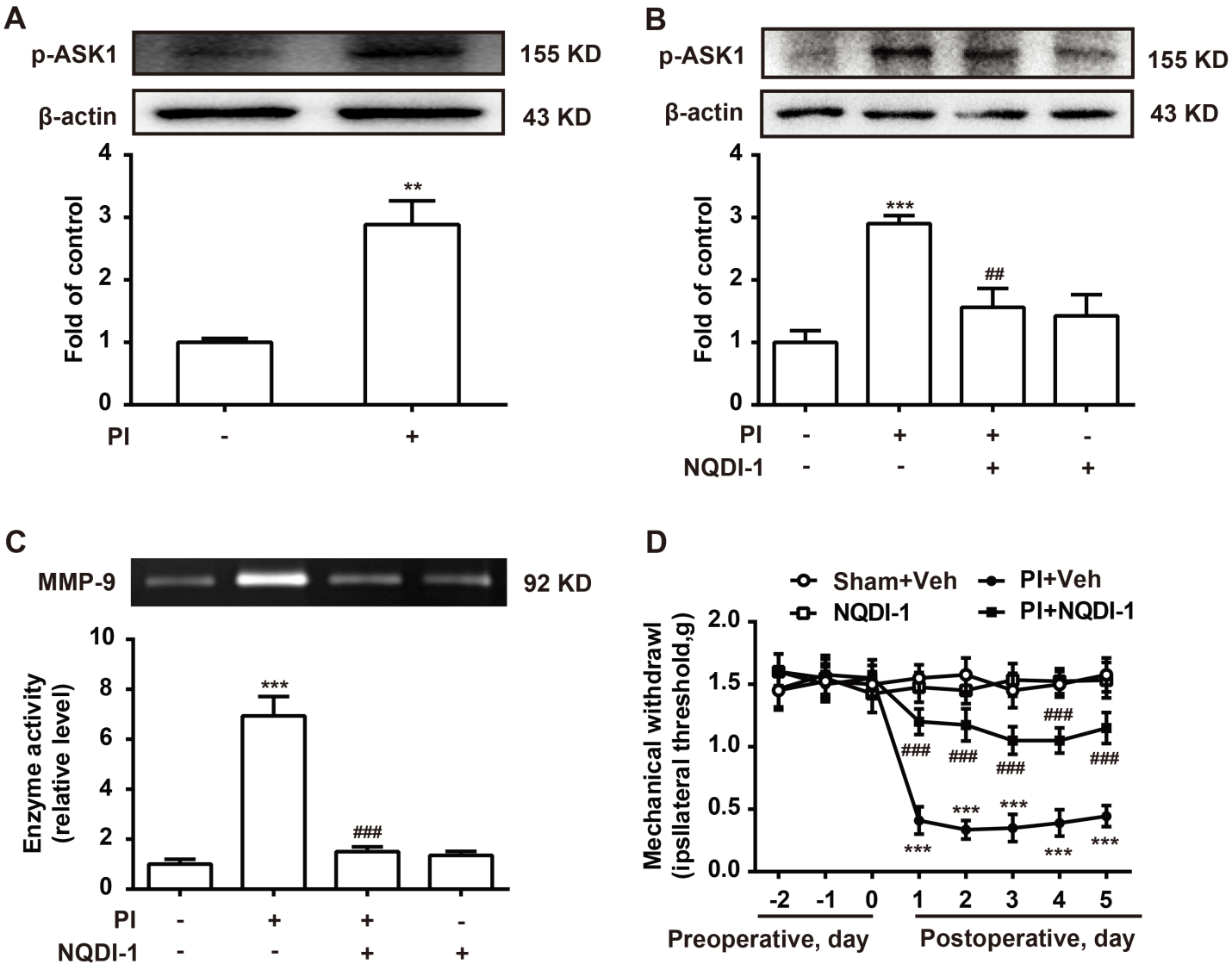

Figure 2

ASK1 inhibition decreased the activity of MMP-9 and attenuated postoperative pain. A. The expression of ASK1 phosphorylation was measured after surgery in the spinal cord of mice (n = 6). The expression of p-ASK1 (B) and MMP-9 activity (D) induced by PI in the spinal cord of mice under the treatment of the ASK1 inhibitor NQDI-1 (5 μg/10 μl, i.t./day) (n = 6). The lumbar spines (L1–L6) were collected and analyzed 5 days after surgery. D. Intrathecal injection the inhibitor NQDI-1 changed mechanical threshold in plantar incision surgery (n = 12). PI = plantar incision; Veh = vehicle. A significant difference was revealed following one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001 vs. sham; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. plantar incision group; Bonferroni post hoc tests).

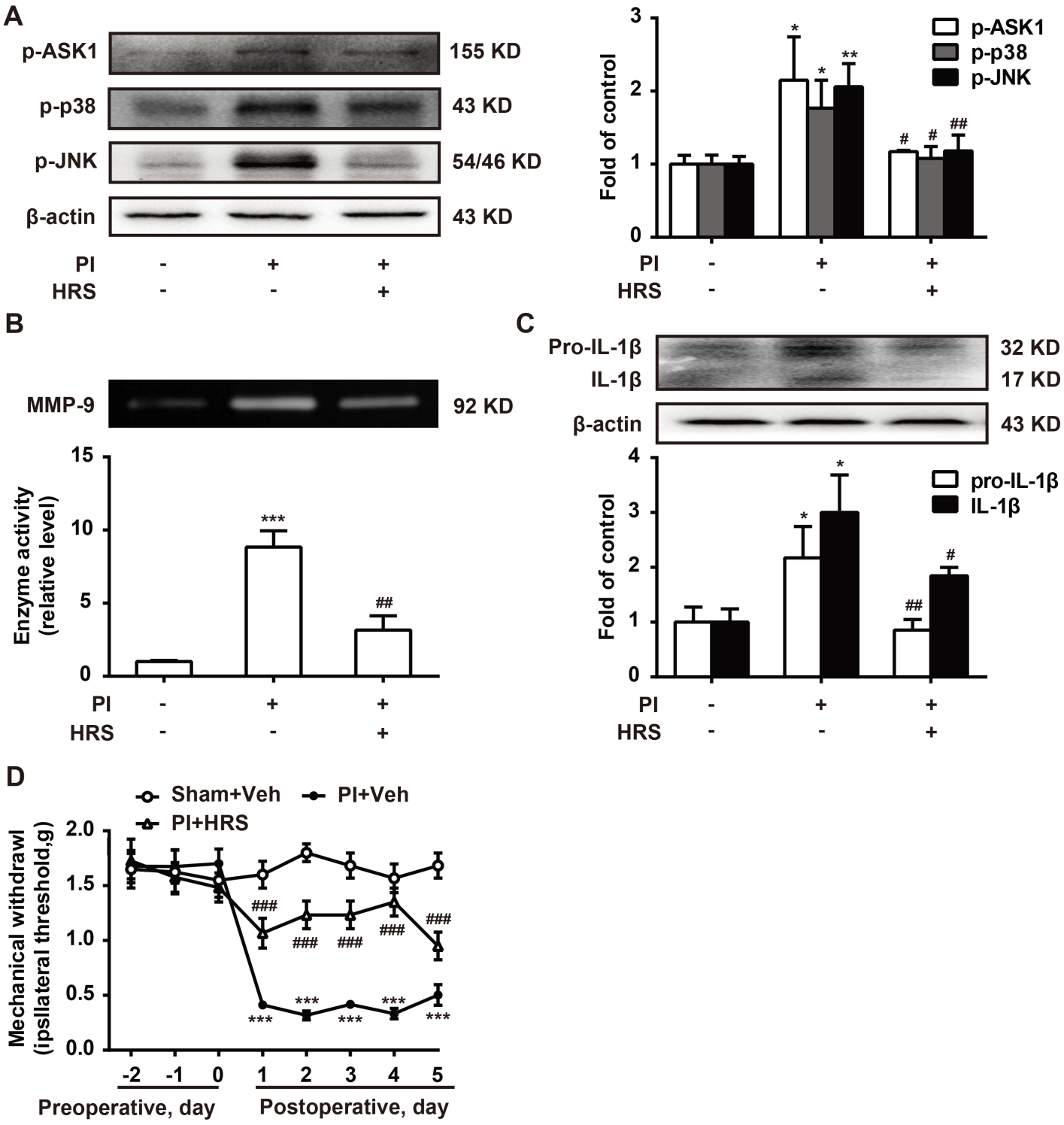

Figure 3

Hydrogen decreased MMP-9 activity and ASK1 phosphorylation to attenuate postoperative pain in mice. With hydrogen-rich saline (5 ml/kg, i.p., twice/day) injecting by intraperitoneal, he expression of p-ASK1, p-p38, p-JNK (A), IL-1β (C) and MMP-9 activity were measured by western blot and zymography in the spinal cord. D. Mechanical allodynia after plantar incision (PI) surgery was measured by using the Von Frey test (n = 12).The lumbar spines (L1–L6) were collected and analyzed 5 days after surgery. HRS = hydrogen-rich saline; PI = plantar incision; Veh = vehicle. A significant difference was revealed following

one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001 vs. sham; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. plantar incision group; Bonferroni post hoc tests).

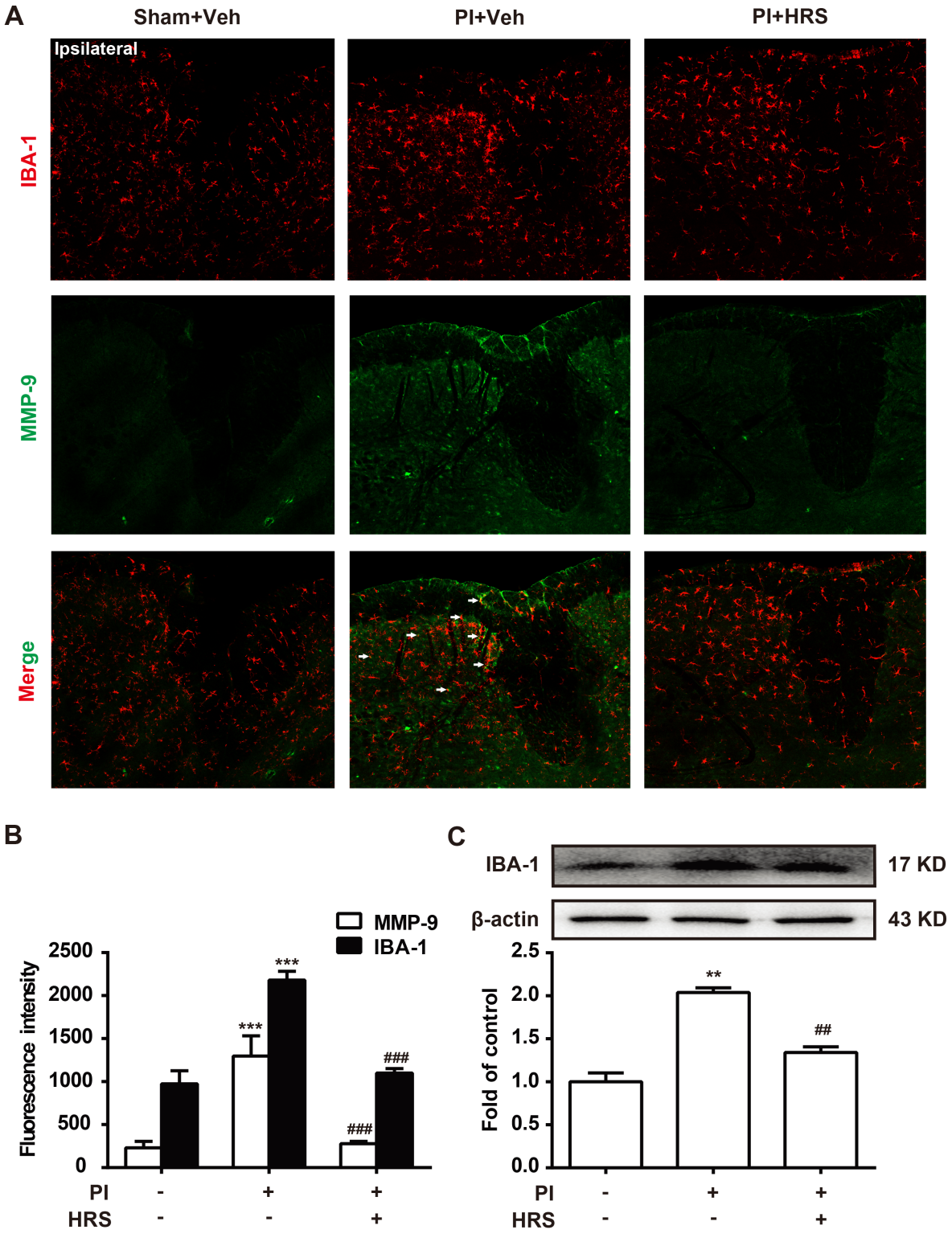

Figure 4

Hydrogen decreased the activation of microglia induced by plantar incision surgery in the spinal cord of mice. A. Confocal images of MMP-9 (green)and its co-localization with microglia (IBA-1, red)in the spinal cord after administering HRS induced by incision surgery. Magnification: 100×. (n = 6). The white arrows

indicate IBA-1 co-localization with MMP-9. B. Immuofluorescence staining analysis after the treatment of HRS. C. HRS has effect on plantar incision induced IBA-1 expression in the spinal cord (n = 6).The lumbar spines (L1–L6) were collected and analyzed 5 days after surgery. HRS = hydrogen-rich saline; PI = plantar incision; Veh = vehicle. A significant difference was revealed following one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001 vs. sham; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. plantar incision group; Bonferroni post hoc tests).

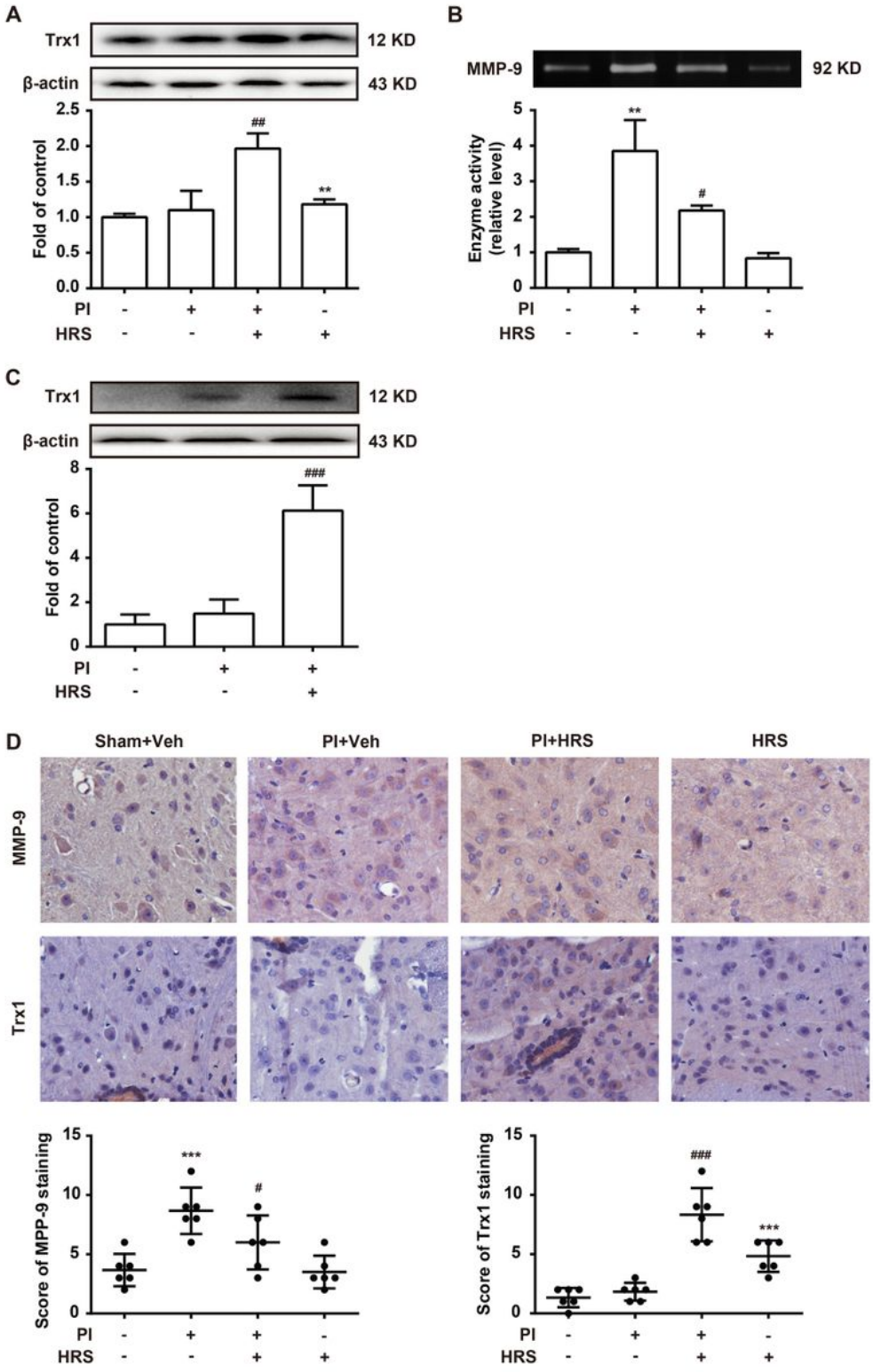

Figure 5

Hydrogen increased the expression of Trx1 in the spinal cord of mice. The expression of Trx1 was measured by western blot at 24h h (A) and 5 d (C) after plantar incision surgery in the spinal cord of mice (n = 6). The lumbar spines (L1–L6) were collected and analyzed 5 days after surgery. B. HRS decreased the MMP-9 activity in the spinal cord of mice after operating 24 h. (n = 6). D. Representative images of immunohistochemistry and IHC score values in HRS treatment groups at 24 h. Magnification: 400× (n = 6).The lumbar spines (L1–L6) were collected and analyzed 1 days after surgery. HRS = hydrogen-rich saline; PI = plantar incision; Veh = vehicle. A significant difference was revealed following one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001 vs. sham; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. plantar incision group; Bonferroni post hoc tests).

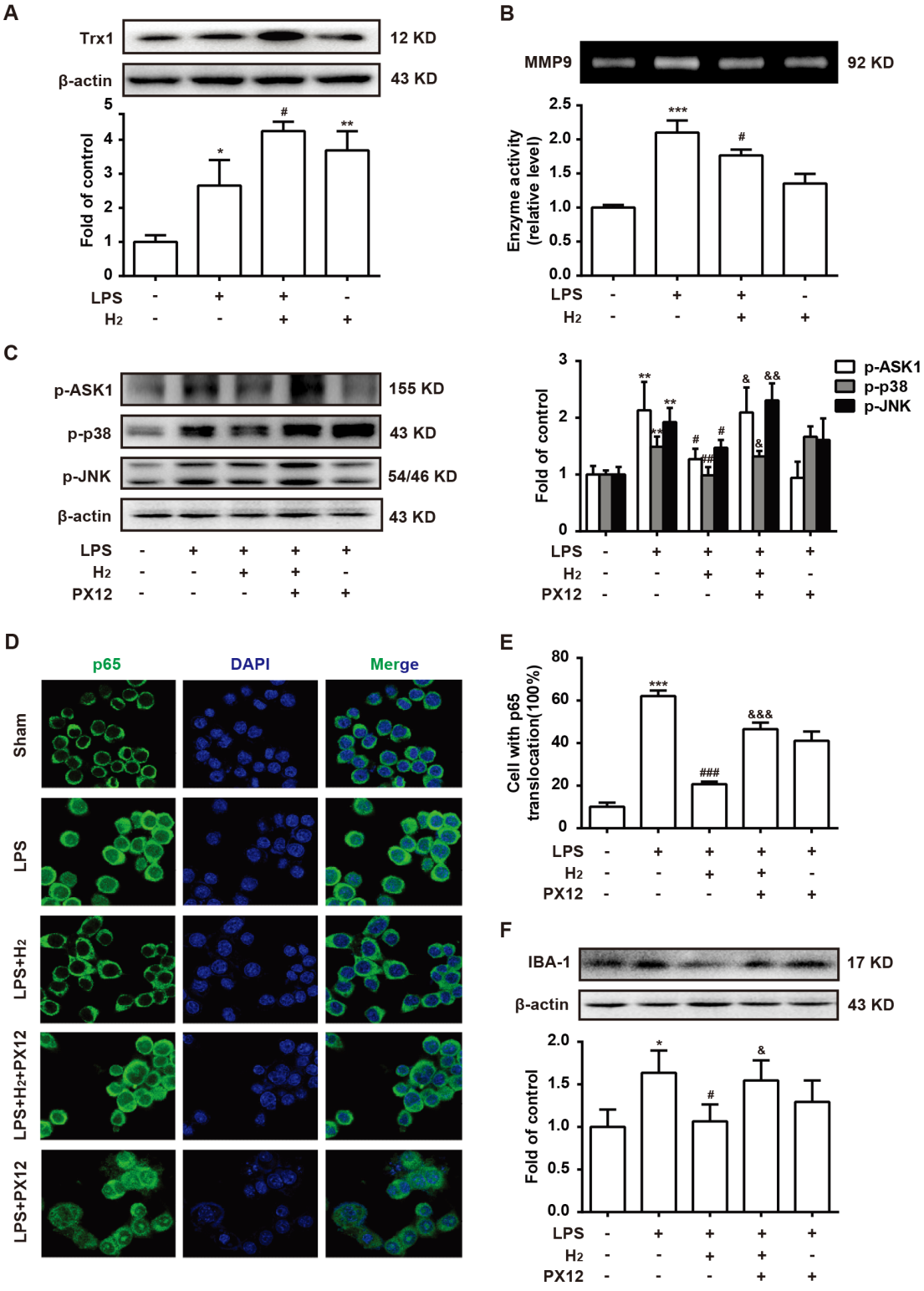

Figure 6

Hydrogen acted on the expression of Trx1/ASK1/MMP9 pathway proteins in vitro. A. BV-2 cells were cultured under 60% H2 after inducing by LPS(1μg/ml) in the expression of Trx1 (n = 4). B. H2 decreased the expression of p-ASK1, p-p38, p-JNK and MMP-9 induced by LPS BV-2 cells. (n = 4). C, F. Effects of Trx1 inhibitor PX12 (8 μM) on Trx1/ASK1/MMP9 pathway and IBA-1 in BV-2 cells after LPS stimulation (n = 4). D, E. Representative images show the NF-kB translocation from the cytosol to the nucleus after

different treatments in BV-2 cells. Magnification: 400× (n=4). Cells were collected and analyzed 24 h after H2 treatment. A significant difference was revealed following one-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001 vs. sham; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. LPS group; &P < 0.05, &&P < 0.01, &&&P < 0.001 vs. LPS plus H2 group; Bonferroni post hoc tests).

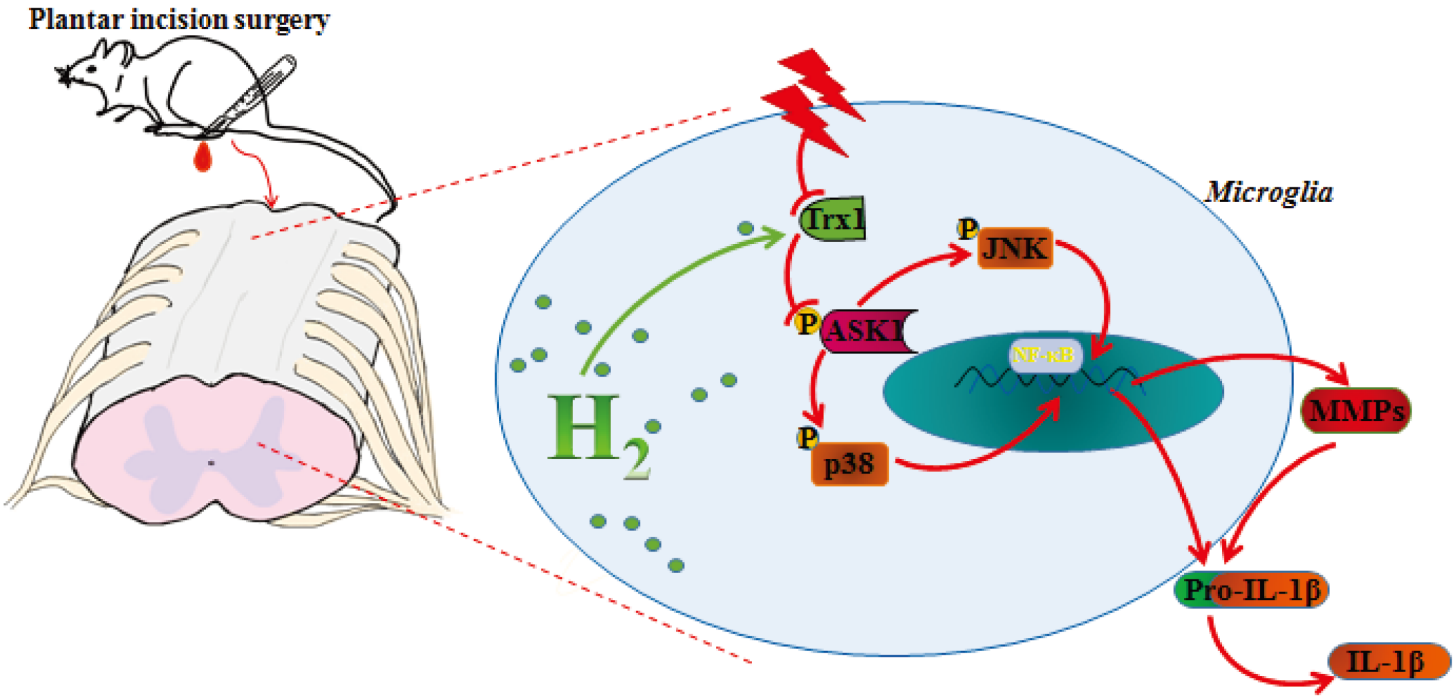

Figure 7

H2 protects against plantar incision-induced postoperative pain by up-regulating Trx1/ASK1/MMP-9 signaling pathway. Plantar incision surgery increases ASK1/JNK/p38 phosphorylation mediated microglia activation, enhances MMP-9 activity and IL-1β cleavage and subsequently induces neuroinflammation, which contributes to the progression of postoperative pain in mice. Inhaling of hydrogen gas and administering of hydrogen-rich saline increasing Trx1 expression promote the amelioration of postoperative pain. R C, DB G, OA dL-C, JM R, S B, T B, T C, CL C, EH C, E D, et al: Management of Postoperative Pain: A Clinical Practice Guideline From the American Pain Society, the American Society of Regional Anesthesia and Pain Medicine, and the American Society of Anesthesiologists' Committee on Regional Anesthesia, Executive Committee, and Administrative Council. The journal of pain : official journal of the American Pain Society 2016, 17:131-157. P G, KR A, PS M: Transition from acute to chronic pain after surgery. Lancet (London, England) 2019, 393:1537-1546. CL W, SN R: Treatment of acute postoperative pain. Lancet (London, England) 2011, 377:2215-2225. AI B, DM B, G S, D J: Cellular and molecular mechanisms of pain. Cell 2009, 139:267-284. LA C, F B, TG H: Perioperative opioid analgesia-when is enough too much? A review of opioid-induced tolerance and hyperalgesia. Lancet (London, England) 2019, 393:1558-1568. SP G, JT W, JR L, J K: Dealing with Danger in the CNS: The Response of the Immune System to Injury. Neuron 2015, 87:47-62. Y K, L Z, JK C, RR J: Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-

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