Experimental Trial

Activation of the RAGE/STAT3 Pathway in the Dorsal Root Ganglion Contributes to the Persistent Pain Hypersensitivity Induced by Lumbar Disc Herniation

Xin-Sheng Zhang, MD, Xiao Li, MD, Hai-Jie Luo, MD, Zhu-Xi Huang, MD, Cui-cui Liu, MD, Qing Wan, MD, PhD, Shu-Wei Xu, MD, Shao-Ling Wu, MD, PhD, Song-Jian Ke, MD, and Chao Ma, MD, PhD

From: Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Department of Rehabilitation Medicine, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, 510120, China

Address Correspondence:
Chao Ma, MD, PhD
Department of Rehabilitation Medicine
Sun Yat-sen Memorial Hospital
Sun Yat-sen University, 107 Yanjiang West Road
Guangzhou 510120, Guangdong, China
E-mail: ma_chao99@126.com

Disclaimer: Authors Xin-Sheng Zhang, MD, Xiao Li, MD, and Hai-Jie Luo, MD contributed equally to this work. This work was supported by National Natural Science Foundation of China (81171469, 81671088, 81600959, 81601978); Natural Science Foundation of Guangdong Province (2016A030311045); Guangzhou Science and Technology Plan Projects (201607010254); Fundamental Research Funds for the Central Universities (17ykzd20); China Postdoctoral Science Foundation (2016M590833); Grant [2013]163 from Key Laboratory of Malignant Tumor Molecular Mechanism and Translational Medicine of Guangzhou Bureau of Science and Information Technology; Grant KLB09001 from the Key Laboratory of Malignant Tumor Gene Regulation and Target Therapy of Guangdong Higher Education Institutes.

Manuscript received: 09-20-2016
Revised manuscript received: 01-19-2017
Accepted for publication: 01-25-2017

Free full manuscript:
www.painphysicianjournal.com

Background: Clinically, chronic low back pain and sciatica associated with lumbar disc herniation (LDH) is a common musculoskeletal disorder. Due to the unawareness of detailed mechanisms, it is difficult to get an effective therapy.

Objective: The aim of the present study was to identify the role of the RAGE/STAT3 pathway in the dorsal root ganglion (DRG) on the formation and development of persistent pain hypersensitivity induced by LDH.

Study Design: Controlled animal study.

Setting: University laboratory.

Methods: After LDH induced by implantation of autologous nucleus pulposus (NP, harvested from animal tail) on the left L5 nerve root was established, mechanical thresholds and electrophysiological tests were conducted at relevant time points during an observation period of 28 days. Protein levels and localization of RAGE and p-STAT3 were performed by using Western blotting and immunohistochemistry, respectively.

Results: LDH induced persistent pain hypersensitivity, increased excitability of DRG neurons, and upregulated the expression of RAGE and p-STAT3 in the DRG. Consecutive injection of both RAGE antagonist FPS-ZM1 (i.t.) and STAT3 activity inhibitor S3I-201 (i.t.) inhibited the enhanced excitability of DRG neurons and mechanical allodynia induced by NP implantation. Furthermore, local knockdown of STAT3 by intrathecal injection of AAV-Cre-GFP into STAT3flox/flox mice markedly alleviated NP implantation-induced mechanical allodynia in mice. Importantly, the expression of p-STAT3 was colocalized with that of RAGE in the DRG and inhibition of RAGE with FPS-ZM1 prevented NP implantation-induced STAT3 activation.

Limitations: More underlying mechanism(s) of the role of the RAGE/STAT3 pathway on the formation and development of persistent pain hypersensitivity induced by LDH will be needed to be explored in future research.

Conclusion: These findings suggest activation of the RAGE/STAT3 pathway plays a critical role in persistent pain induced by LDH, and this pathway may represent novel therapeutic targets for the treatment of LDH-induced persistent pain.

Key words: Lumbar disc herniation, persistent pain, RAGE, STAT3, DRG

Pain Physician 2017; 20:419-427

Lumbar disc herniation (LDH) is highly associated with low back pain and sciatica, which affects about 80% of the population during their lifetime (1). Despite that previous studies have suggested that various factors, including age, hereditary, and environmental factors, might contribute to LDH, there is no well-established treatment to prevent or minimize discogenic pain. So, understanding the mechanism underlying LDH-induced persistent pain has a high research priority.

It is well known that the receptor for advanced glycation end-products (RAGE) is a complex, multi-ligand signaling system associated with the pathogenesis of cardiovascular disease, diabetes, neurodegeneration, and various cancers (2). Recently, the emerging data show that RAGE plays an important role in the states of sensory nerve hyper-excitability associated with peripheral inflammation or nerve injury. For example, RAGE is localized on peripheral nerves and dorsal root ganglions (DRG), and the expression is enhanced following nerve injury, trauma, or other diseases (3). Meanwhile, specific modulation of RAGE effectively attenuates nociceptive sensitivity associated with chronic inflammatory and neuropathic pain states (4). In addition, activation of neuronal RAGE by high mobility group protein box-1 (HMGB1) (5), which is confirmed to contribute to the neuropathic pain (6), results in an increase of neuronal excitability in DRG and RAGE antibody significant decreases the enhancement of DRG neuronal excitability induced by HMGB1 (5). It has been showen that LDH induced by nucleus pulposus (NP) transplantation markedly increased the excitability of DRG neurons (7). However, whether enhanced RAGE via enhancing the excitability of DRG neurons contributes to LDH-induced persistent pain is unknown.

The signal transducer and activator of transcription factor 3 (STAT3) is an efficient regulatory protein dedicated to the regulation of gene expression. Studies indicate that STAT3 is intensively involved in the process of cellular growth, survival, development, and differentiation. It is well known that STAT3 is involved in the change of synaptic plasticity in the hippocampus (8). Recent studies further demonstrated that nerve injury induced STAT3 activation in the spinal cord and inhibition of STAT3 significantly attenuated the pain hypersensitivity, which suggested that activation of STAT3 might be involved in neuropathic pain (9,10). However, it remains unknown whether STAT3 participates in LDH-induced enhancement of DRG neuronal excitability and persistent pain. In addition, accumulating evidence that activation of RAGE, as a transmembrane receptor of the immunoglobulin superfamily, via activating NF-κB, MAPK signal transduction molecule initiated inflammatory response and contributes to the diseases of the central nervous system (CNS) (11). Importantly, recent studies showed that RAGE also promoted the phosphorylation of STAT3 and affected cancer cell proliferation (12). However, whether RAGE through activation of STAT3 in DRG neurons contributes to the enhancement of neuronal excitability and persistent pain induced by LDH is still unclear.

Methods

Animals and Surgery

Male Sprague Dawley rats weighing 200–220 g and male C57BL/6 background mice weighing 20-30 g were obtained from the Institute of Experimental Animals of Sun Yat-sen University. STAT3flox/flox mice (ID: 016923) were purchased from the Jackson Laboratory. All animals were housed in separated cages with ad libitum to food and water. The room was kept at 24°C temperature and 50% – 60% humidity, under a 12/12 hour light/dark cycle. All experimental procedures were approved by the Institutional Animal Care Committee of Sun Yat-Sen University and were carried out in accordance with the guidelines of the National Institutes of Health guide for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

For intrathecal injection of the drug, a polyethylene-10 (PE-10) catheter was implanted as described by Liu et al (13) and Kawakami et al (14). In brief, rats were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg); a sterile catheter filled with saline was inserted into the subarachnoid space through the L2-L3 intervertebral space, and gently advanced caudally to the spinal lumbar enlargement level. Then, the catheter was fixed under the skin with paravertebral muscles and sutured at the head of rats. After rats completely recovered from anesthesia, the correct placement of the catheter was confirmed by observing the behavior of dragging or paralysis in bilateral hind limbs following the injection of 2% lidocaine (10 μl). Finally, rats with catheter prolapse, infection, or neurological deficit were excluded from the following experiments. All rats were observed for 3 days before the implantation of NP to L5 DRG.

Surgery for the LDH model was performed as previously described by Wang et al (15) and Anzai et al (16). In brief, rats were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg) and laminecto-

mies were performed in which the left L5 nerve roots and corresponding DRG were exposed. Autologous NP was harvested from the disc level between the second and third coccygeal intervertebral disc of each tail and applied to the left L5 nerve roots just proximal to the corresponding DRG. The right side of the dorsal roots was to be kept intact without surgery in all rats. The surgical procedure in the sham control group was identical to the NP treated group, including exposing nerve roots and harvesting NP from the tail, except for the application of NP. Special care was taken to prevent infection and minimize the influence of inflammation.

Injection of Adenovirus-associated Vector (AAV)

Recombinant adeno-associated virus encoding Cre and GFP marker (AAV8-Cre-GFP) and AAV encoding GFP (AAV8-GFP) were purchased from Beijing Vector Gene Technology Company Ltd. Four μl AAV8-Cre-GFP was intrathecally injected into the subarachnoid space of the L4-L6 spinal cord of STAT3flox/flox mice. Control mice were injected with the same amount of AAV encoding GFP (AAV8-GFP). The implantation of autologous NP on left L5 spinal nerve roots was performed on day 21 after the virus injection.

Behavioral Test

The 50% withdrawal threshold was assessed using von Frey hairs as described previously (15). Briefly, each animal was loosely restrained beneath a plastic box on a metal mesh for at least 15 minutes once daily for 3 separate days and mechanical allodynia in the NP and sham groups were examined one day before surgery. Then, the animals recovered for 7 days, and the test was performed weekly until 4 weeks postoperatively. Mechanical allodynia was assessed by the hindpaw withdrawal threshold in response to probing with a series of von Frey filaments. A nociceptive response was defined as a brisk paw withdrawal or flinching of the paw following von Frey filament application. In the presence of a response, a von Frey filament with the next lower force was applied. In the absence of a response, a von Frey filament with the next greater force was applied. As described previously, the tactile stimulus producing a 50% likelihood of withdrawal was defined by the "up-down" calculating method (15). Each test was repeated for 2 – 3 times at approximately 2 minute intervals, and the average value of von Frey filament force was determined as the force to evoke a withdrawal response. The experimenter who conducted the behavioral test was blinded to all treatments.

Western Blot

Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) at different time points. Left L5 DRG were immediately removed and homogenized on ice in 15 mmol/l Tris containing a cocktail of proteinase inhibitors and phosphatase inhibitors. Protein samples were separated by gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. The blots were placed in the block buffer for one hour at room temperature and incubated with primary antibody against RAGE (1:2000, Abcam, USA), phosphorylated STAT3 (1:1000, Abcam, USA), STAT3 (1:1000, Abcam, USA), or β-tubulin (1:2000, Cell Signaling Technology, USA) overnight at 4°C. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody. ECL (Pierce, USA) was used to detect the immune complex. The band was quantified with computer-assisted imaging analysis system (NIH ImageJ).

Immunohistochemistry

Immunohistochemistry was performed as previously described (13). Briefly, rats were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg) and immediately perfused through the ascending aorta with 4% paraformaldehyde. The left L5 DRG were removed and post-fixed in the same fixative overnight. Cryostat sections (16 μm) were cut and processed for immunohistochemistry with primary antibody for RAGE (1:300, Abcam, USA), phosphorylated STAT3 (1:1000, Abcam, USA), NF200 (1:500, Chemicon, USA), GFAP (1:500, Chemicon, USA), or IB4 (1:500, Chemicon, USA). After incubation overnight at 4°C, the sections were incubated with cy3-conjugated and fluorescein isothiocyanate-conjugated secondary antibodies for one hour at room temperature. The stained sections were then examined with a Leica (Leica, Germany) fluorescence microscope, and images were captured with a Leica DFC350 FX camera.

Culture of DRG Neurons and Electrophysiological Recordings

DRG neurons were dissociated using enzyme digestion as previously described with minor modifications (17). In brief, left L5 DRG were excised, freed from their connective tissue sheaths, and broken into pieces with a pair of sclerotic scissors in DMEM/F12 medium (GIBCO, USA) under low temperature (in a mixture of ice and water). After enzymatic and mechanical dissociation, DRG neurons were seeded on cover lips coated with Poly-L-Lysine (Sigma-Aldrich, USA) in a humidified atmosphere (5% CO2, 37°C) and then used for patch-clamp investigation 4 hours after plating.

Whole-cell patch-clamp recordings from DRG neurons were performed using an EPC-10 amplifier and the PULSE program (HEKA Electronics, Lambrecht, Germany) as previously described (18). Patch pipettes with one to 3 MΩ resistance were fabricated from borosilicate glass capillaries (Sutter Instruments, Novato, CA) using a Sutter P-87 puller (Sutter Instruments, Novato, CA). The action potentials of DRG neurons were recorded in current clamp mode. Action potentials were elicited by a constant current 500 pA (one second). The external solution contained (in mM): 140 NaCl, 3 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES, pH adjusted to 7.3 with NaOH. The pipette solution contained (in mM): 140 KCl, 0.5 EGTA, 5 MgATP, and 5 HEPES, pH 7.3 with KOH.

Statistical Analysis

All data were expressed as means ± SEM, and analyzed with SPSS 13.0 (SPSS, USA). Western blot and electrophysiological data were analyzed by 2-way ANOVA followed by Tukey post hoc test. For behavioral test, one-way or 2-way ANOVA with repeated-measures followed by Tukey post hoc test was carried out. The criterion for statistical significance was $P < 0.05$. While no power analysis was performed, the sample size was determined according to our and peers' previous publications in painful behavior and pertinent molecular studies.

Results

LDH by Implantation of Autologous NP Induced Mechanical Allodynia and Enhanced the Excitability of DRG Neurons

Consistent with previous studies (15,19), rats exhibited significant and prolonged mechanical allodynia after NP implantation (Fig. 1A) on the ipsilateral side. Compared to the values of the sham group (19.25 ± 2.47) and the pre-treatment baseline (19.88 ± 2.59), the 50% withdrawal threshold on the ipsilateral side significantly decreased to 4.15 ± 1.62, 3.60 ± 1.36, 4.98 ± 1.27, and 5.73 ± 1.45 on days 7, 14, 21, and 28 after NP implantation, respectively (Fig. 1A). To define whether LDH induced by NP implantation enhanced the DRG neurons excitability, which contributed to the persistent pain, we observed the number of action potentials in the separated left L5 DRG neurons of rats. Interestingly, electrophysiological results showed that the number of action potentials of DRG neurons was significantly increased on day 7, 14, and 28 following NP implantation (Fig. 1B). These results implied that LDH might mediate mechanical allodynia via enhancing excitability of DRG neurons.

Upregulated RAGE in DRG Neurons Contributes to the Enhanced Excitability of DRG Neurons and Mechanical Allodynia Induced by LDH

Next, we examined the expression of RAGE in DRG of rats by western blot analysis. The results showed that the expression of RAGE was significantly upregulated on day 7 and maintained to the end of the experiment (day 28) following LDH induced by implantation of autologous NP (Fig. 2A). Immunohistochemistry results further confirmed that the expression of RAGE in LDH rats on day 14 was significantly increased compared with the sham group (Fig. 2B). The time course of upregulated RAGE is consistent with that of mechani-

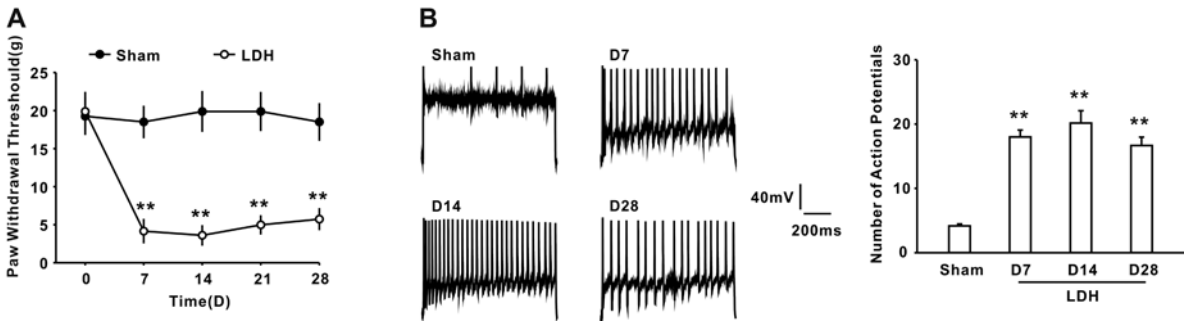

Fig. 1. *LDH induced mechanical allodynia and enhanced the excitability of DRG neurons. (A) The paw withdrawal threshold of rats was significantly decreased following NP implantation (** $P < 0.01$ versus the sham group, $n = 12$ in each group). (B) The number of action potentials in DRG neurons were increased after NP implantation (** $P < 0.01$ versus the sham group, $n = 36$ in each group).*

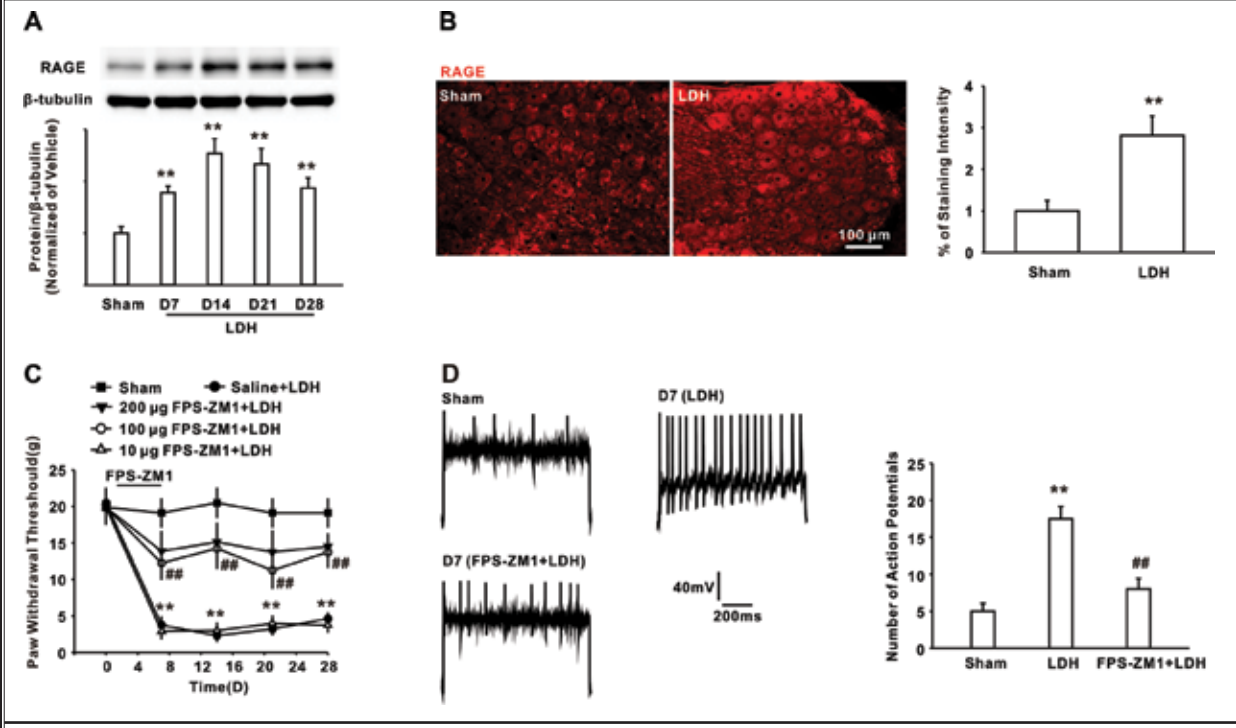

*Fig. 2. Upregulation of RAGE in DRG neurons plays an important role in the mechanical allodynia and enhanced excitability of sensory neurons induced by LDH. (A) Representative blots and histogram showed the upregulation of RAGE in DRG induced by LDH (** $P < 0.01$ versus the sham group, $n = 6$ in each group). (B) Immunohistochemistry results indicated that the expression of RAGE in DRG on day 14 after LDH was significantly increased (** $P < 0.01$ versus the sham group, $n = 5$ in each group). (C) Intrathecal injection of RAGE antagonist FPS-ZM1 at dose of 100μg/10μl or 200 μg/10 μl, but not 10μg/10μl, obviously attenuated the mechanical allodynia caused by NP implantation (** $P < 0.01$ versus the sham group, ## $P < 0.01$ versus the corresponding LDH group, $n = 12$ in each group). (D) Blockage of RAGE with FPS-ZM1 at dose of 100μg/10μl (i.t.) significantly decreased the enhanced number of action potentials on day 7 following NP implantation (** $P < 0.01$ versus the sham group, ## $P < 0.01$ versus the corresponding LDH group, $n = 25$ in each group).*

cal allodynia. To define the role of RAGE in persistent pain induced by LDH, RAGE antagonist FPS-ZM1 (i.t.) was daily intrathecally administrated for consecutive 7 days. The results demonstrated that RAGE antagonist at doses of 100 μg/10 μl or 200 μg/10 μl, but not 10 μg/10μl, significantly inhibited LDH-induced mechanical allodynia (Fig. 2C). Meanwhile, FPS-ZM1 treatment at a dose of 100 μg/10 μl (i.t.) also markedly reduced the enhancement of action potentials in DRG neurons on day 7 following LDH induced by NP implantation (Fig. 2D). These results suggested that LDH upregulated RAGE via enhancing the DRG neurons excitability contributed to the persistent pain.

Activation of STAT3 Mediated Mechanical Allodynia Induced by LDH

Recent studies indicated that STAT3 activation played a critical role in the neuropathic pain (20). In this study, the western blot results revealed that the phosphorylation of STAT3 in DRG was significantly increased on day 7 and persisted to the end of the experiment (day 28) after NP implantation in rats (Fig. 3A). Immunohistochemistry results also showed that the phosphorylated STAT3 was upregulated in DRG on day 14 after NP implantation in rats (Fig. 3B), and the phosphorylated STAT3 mainly was expressed in IB4 and NF200 positive cells, but not GFAP positive cells (Fig. 3B). Importantly, consecutive intrathecal injection of STAT3 activity inhibitor S3I-201 at doses of 100 μg/10 μl or 200 μg/10 μl, but not 25 μg/10 μl for 7 days, significantly ameliorated the mechanical allodynia induced by NP implantation in rats (Fig. 3C). Meanwhile, S3I-201 treatment at a dose of 100 μg/10 μl (i.t.) also markedly decreased the enhanced number of action potentials on day 7 following NP implantation (Fig. 3D). To further define the role of STAT3 in DRG in persistent pain induced by LDH, the

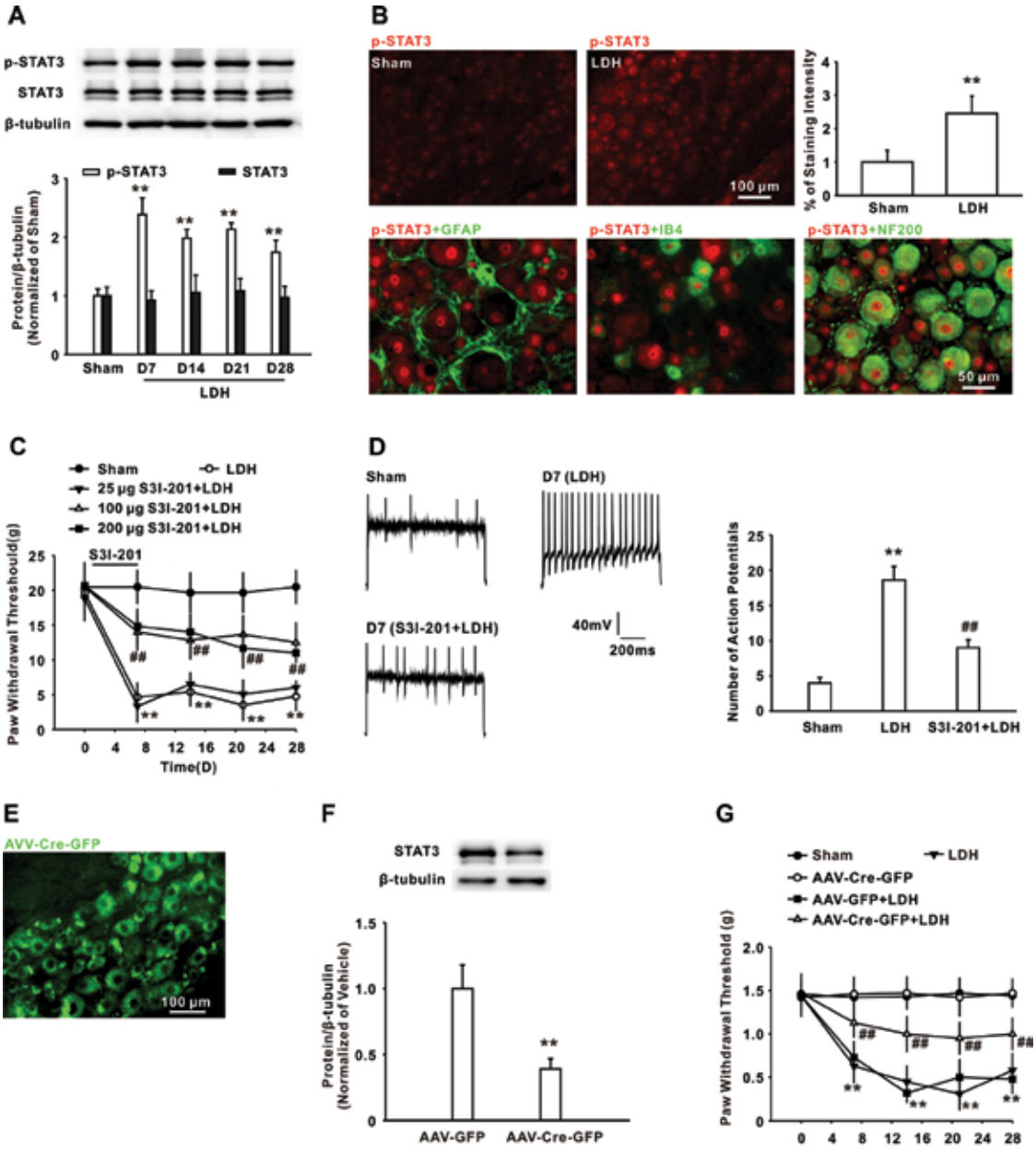

Fig. 3. *Activation of STAT3 contributed to mechanical allodynia induced by LDH. (A) NP implantation significantly upregulated the expression of p-STAT3, but not the expression of STAT3 in DRG of rats (** P < 0.01 versus the sham group, n = 6 in each group). (B) The expression of p-STAT3 were markedly increased in DRG on day 14 after LDH (** P < 0.01 versus the sham group, n = 5 in each group) and colocalized with IB4 positive cells and NF200 positive cells, but not GFAP positive cells in DRG of LDH rats (n = 6 in each group). (C) Intrathecal injection of STAT3 activity inhibitor S3I-201 at dose of 100μg/10μl or 200μg/10μl, but not 25μg/10μl, significantly attenuated the mechanical allodynia induced by LDH in rats (** P < 0.01 versus the sham group, ## P < 0.01 versus the corresponding LDH group, n = 12 in each group). (D) Inhibition of the activity of STAT3 with S3I-201 at dose of 100 μg/10 μl (i.t.) markedly decreased the enhanced number of action potentials on day 7 following NP implantation (** P < 0.01 versus the sham group, ## P < 0.01 versus the corresponding LDH group, n = 25 in each group). (E) A marked green fluoresces in DRG of mice was observed on days 21 after AAV-Cre-GFP injection (n = 6 in each group). (F) STAT3 expressions in DRG were significantly reduced on day 21 after AAV-Cre-GFP injected STAT3flox/flox mice (** P < 0.01 versus corresponding AAV-GFP group; n = 6 in each group). (G) The mechanical allodynia was greatly attenuated in AAV-Cre-GFP-injected STAT3flox/flox mice compared to that of AAV-GFP-injected STAT3flox/flox mice following NP implantation (** P < 0.01 versus the sham group, ##P < 0.01 versus the corresponding AAV-GFP +LDH group, n = 12 in each group).*

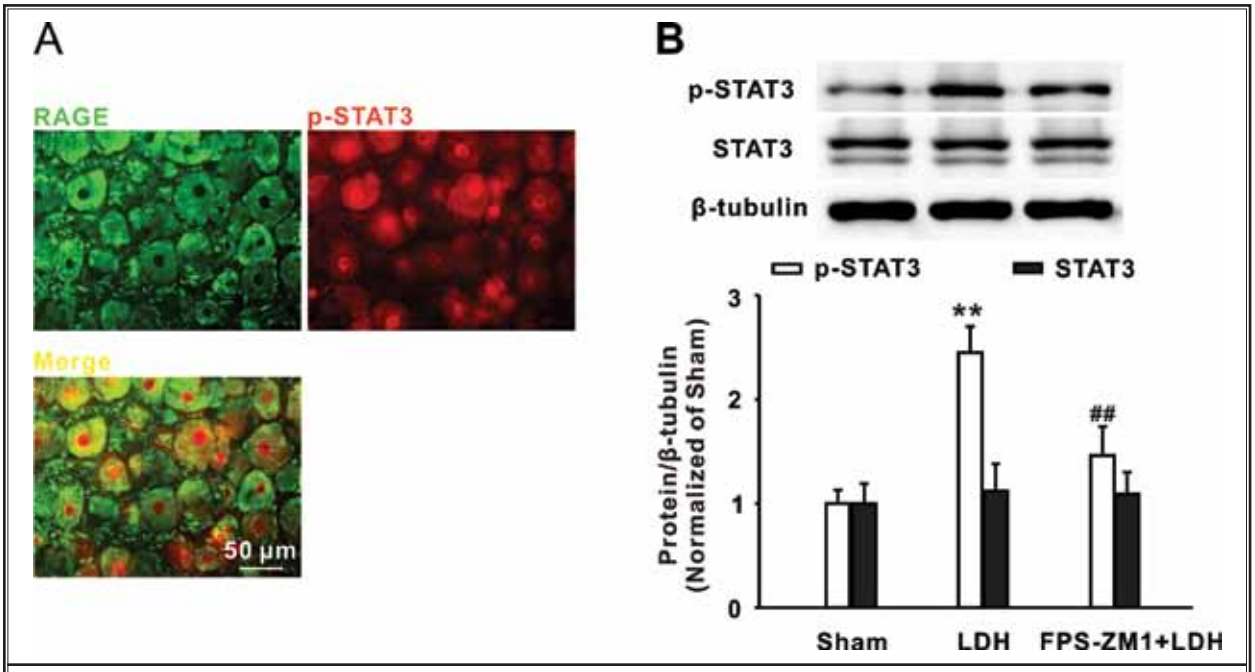

Fig. 4. *Blockage of RAGE inhibited the activation of STAT3 in DRG induced by NP implantation. (A) The expressions of RAGE were colocalized with p-STAT3 positive cells in DRG of rats (n = 6 in each group). (B) Blockage of RAGE with FPS-ZM1 at dose of 100 μg/10 μl (i.t.) significantly inhibited the activation of STAT3 in DRG of rats induced by NP implantation on day 28 (**P < 0.01 versus the sham group, ## P < 0.01 versus the LDH group, n = 6 in each group).*

recombinant adeno-associated virus encoding Cre and GFP (AAV-Cre-GFP) was intrathecally injected into the subarachnoid space of L4-L6 spinal cord of STAT3flox/flox mice. Control mice were injected with AAV encoding GFP (AAV-GFP). Twenty-one days after virus injection, marked green fluoresces in the DRG suggested a high efficiency of transfection (Fig. 3E). Western blot analysis indicated that the expression of STAT3 in DRG was significantly reduced on day 21 after AAV-Cre-GFP injection in STAT3flox/flox mice (Fig. 3F). Importantly, the mechanical allodynia was greatly inhibited in AAV-Cre-GFP injected STAT3flox/flox mice compared to that of AAV-GFP-injected STAT3flox/flox mice following LDH induced by NP implantation (Fig. 3G).

Blockage of RAGE Reduced STAT3 Activation Induced by Implantation of Autologous NP

Due to the consistent time course between the upregulated RAGE and p-STAT3, we further observed whether the upregulation of RAGE mediated STAT3 activation following LDH induced by NP implantation. Immunohistochemistry results showed that RAGE-positive cells were colocalized with p-STAT3-positive cells (Fig. 4A). Furthermore, consecutive administration of RAGE antagonist FPS-ZM1 (100 μg/10 μl, i.t.) significantly inhibited the STAT3 phosphorylation of DRG on day 21 induced by NP implantation (Fig. 4B). These results suggested that the upregulation of RAGE may activate STAT3 in DRG, thus contributing to the persistent pain induced by LDH.

Discussion

A number of studies show that implantation of autologous NP in animals, which mimicked the LDH, induced significant and prolonged mechanical allodynia (15,19). Here, we confirmed the previous study that 7 days after LDH induced by implantation of autologous NP, rats exhibited significant mechanical allodynia. We further found that LDH also significantly enhanced the excitability of DRG neurons, which is consistent with the finding in a rat model of LDH-induced pain hypersensitivity (7). It is well known that the peripheral sensitization involves the increased excitability of primary afferent nociceptors such as DRG neurons, which mainly convey peripheral stimuli into the action potentials, and thus propagate to the CNS (7,21). All these suggested that the enhancement of neuronal excitability of DRG might contribute to pe-

ripheral sensitization and persistent pain induced by LDH.

Accumulated evidence revealed that RAGE, the receptor for advanced glycation end products, plays a pivotal role in the progression of neuroinflammation, neurodegeneration, and various neurodegenerative conditions such as diabetic neuropathy and familial amyloid polyneuropathy (22,23). In the present study, we further found that implantation of autologous NP significantly increased the RAGE expression in DRG, and the time course of upregulated RAGE is consistent with mechanical allodynia. In addition, intrathecal application of RAGE antagonist FPS-ZM1 markedly attenuated the mechanical allodynia induced by LDH. Importantly, inhibition of RAGE by using FPS-ZM1 significantly reduced the enhanced excitability of DRG neurons following implantation of autologous NP. Studies showed that RAGE activation can trigger several signal pathways such as NF-κB and JNK pathways, thereby inducing an increase of proinflammatory molecules (22,23). These proinflammatory molecules such as TNF-α and IL-1β play a critical role in the hyperalgesia and mechanical allodynia via increasing the sensory neurons excitability (24). Altogether, the present results indicated that upregulation of RAGE via enhancing DRG neurons excitability contributed to LDH-induced persistent pain, and was supported by the study that activation of neuronal RAGE was involved in the increased neuronal excitability and neuropathic pain induced by nerve injury (5). It is known that FPS-ZM1 is not toxic to mice and can easily cross the blood-brain barrier (25). FPS-ZM1 might be a novel therapeutic agent to treat LDH patients.

A recent study showed that STAT3, as a transcription factor, was involved in the induction and maintenance of neuropathic pain following nerve injury. For example, a marked activation of STAT3 in spinal astrocytes was observed following spinal nerve injury (26), and inhibition of STAT3 significantly attenuated the nerve injury-induced neuropathic pain (9,20). Our results also showed that LDH significantly increased the phosphorylated STAT3 expression in DRG, and the upregulated p-STAT3 was only co-localized with the IB4 or NF200-positive cells. Although the present results were supported by many studies that p-STAT3 was expressed on the neurons in the brain (27,28), some studies also showed that activated STAT3 can be expressed on the astrocytes or microglia (20,26). We also found that STAT3 inhibitor S3I-201 significantly attenuated the mechanical allodynia and the enhancement of DRG neurons excitability induced by LDH. In addition, the local deficiency of STAT3 by intrathecal injection of AAV-Cre-GFP into the STAT3flox/flox mice significantly ameliorated the STAT3 expression and mechanical allodynia in the mice with LDH. These results indicated that activation of STAT3 in DRG neurons might be also implicated in LDH-induced mechanical allodynia. Importantly, we found that p-STAT3-positive cells were co-localized with the RAGE-positive cells. Furthermore, intrathecal injection of RAGE antagonist FPS-ZM1 significantly prevented the STAT3 activation on day 7 following implantation of autologous NP. Although studies showed that many molecules such as hormones, growth factors, or cytokines via acting on their respective receptor can activate the STAT3 (29), the present study showed that RAGE was an upstream molecule for STAT3 activation in persistent pain induced by LDH, and this result was consistent with the study that RAGE activated STAT3 in pancreatic ductal adenocarcinoma (12). However, the mechanisms underlying p-STAT3-mediated persistent pain in the LDH need further investigation.

Conclusion

Taken together, these data indicated that activation of RAGE/STAT3 pathway plays a critical role in the persistent pain induced by implantation of autologous NP in a rat model of LDH, and this pathway may represent novel therapeutic targets for the treatment of LDH-induced persistent pain.

Author contributions

XSZ, XL, HJL, SLW and CM conceived of the project, designed the experiments. XSZ, HJL, ZXH, CCL, SJK and SWX carried out all experiments. XSZ, XL, CCL and QW analyzed the data and prepared the figures. SLW and CM supervised the overall experiment. SLW and CM revised the manuscript. All authors have read and approved the final manuscript.

Conflicts of Interest

The authors declared no conflict of interests with respect to the research, authorship, and/or publication of this article.

References

1. Andersson GB. Epidemiological features of chronic low-back pain. *Lancet* 1999; 354:581-585.
2. Sparvero LJ, Asafu-Adjei D, Kang R, Tang D, Amin N, Im J, Rutledge R, Lin B, Amoscato AA, Zeh HJ, Lotze MT. RAGE (receptor for advanced glycation end-products), RAGE ligands, and their role in cancer and inflammation. *J Transl Med* 2009; 7:17.
3. Li X, Yang H, Ouyang Q, Liu F, Li J, Xiang Z, Yuan H. Enhanced RAGE expression in the dorsal root ganglion may contribute to neuropathic pain induced by spinal nerve ligation in rats. *Pain Med* 2016; 17:803-812.
4. Brederson JD, Strakhova M, Mills C, Barlow E, Meyer A, Nimmrich V, Leddy M, Simler G, Schmidt M, Jarvis M, Lacy S. A monoclonal antibody against the receptor for advanced glycation end products attenuates inflammatory and neuropathic pain in the mouse. *Eur J Pain* 2016; 20:607-614.
5. Allette YM, Due MR, Wilson SM, Feldman P, Ripsch MS, Khanna R, White FA. Identification of a functional interaction of HMGB1 with receptor for advanced glycation end-products in a model of neuropathic pain. *Brain Behav Immun* 2014; 42:169-177.
6. Feldman P, Due MR, Ripsch MS, Khanna R, White FA. The persistent release of HMGB1 contributes to tactile hyperalgesia in a rodent model of neuropathic pain. *J Neuroinflammation* 2012; 9:180.
7. Yan J, Zou K, Liu X, Hu S, Wang Q, Miao X, Zhu HY, Zhou Y, Xu GY. Hyperexcitability and sensitization of sodium channels of dorsal root ganglion neurons in a rat model of lumber disc herniation. *Eur Spine J* 2016; 25:177-185.
8. Nicolas CS, Peineau S, Amici M, Csaba Z, Fafouri A, Javalet C, Collett VJ, Hildebrandt L, Seaton G, Choi SL, Sim SE, Bradley C, Lee K, Zhuo M, Kaang BK, Gressens P, Dournaud P, Fitzjohn SM, Bortolotto ZA, Cho K, Collingridge GL. The Jak/STAT pathway is involved in synaptic plasticity. *Neuron* 2012; 73:374-390.
9. Xue ZJ, Shen L, Wang ZY, Hui SY, Huang YG, Ma C. STAT3 inhibitor WP1066 as a novel therapeutic agent for bCCI neuropathic pain rats. *Brain Res* 2014; 1583:79-88.
10. Liu S, Mi WL, Li Q, Zhang MT, Han P, Hu S, Mao-Ying QL, Wang YQ. Spinal IL-33/ST2 signaling contributes to neuropathic pain via neuronal CaMKII-CREB and astroglial JAK2-STAT3 cascades in mice. *Anesthesiology* 2015; 123:1154-1169.
11. Xie J, Mendez JD, Mendez-Valenzuela V, Aguilar-Hernandez MM. Cellular signalling of the receptor for advanced glycation end products (RAGE). *Cell Signal* 2013; 25:2185-2197.
12. Kang R, Tang D, Lotze MT, Zeh HJ, 3rd. AGER/RAGE-mediated autophagy promotes pancreatic tumorigenesis and bioenergetics through the IL6-pSTAT3 pathway. *Autophagy* 2012; 8:989-991.
13. Liu C, Luan S, OuYang H, Huang Z, Wu S, Ma C, Wei J, Xin W. Upregulation of CCL2 via ATF3/c-Jun interaction mediated the Bortezomib-induced peripheral neuropathy. *Brain Behav Immun* 2016; 53:96-104.
14. Kawakami M, Tamaki T, Hayashi N, Hashizume H, Nishi H. Possible mechanism of painful radiculopathy in lumbar disc herniation. *Clin Orthop Relat Res* 1998; 351:241-251.
15. Wang Q, Zhu H, Zou K, Yuan B, Zhou YL, Jiang X, Yan J, Xu GY. Sensitization of P2X3 receptors by cystathionine beta-synthetase mediates persistent pain hypersensitivity in a rat model of lumbar disc herniation. *Mol Pain* 2015; 11:15.
16. Anzai H, Hamba M, Onda A, Konno S, Kikuchi S. Epidural application of nucleus pulposus enhances nociresponses of rat dorsal horn neurons. *Spine (Phila Pa 1976)* 2002; 27:E50-E55.
17. Huang ZJ, Song XJ. Differing alterations of sodium currents in small dorsal root ganglion neurons after ganglion compression and peripheral nerve injury. *Mol Pain* 2008; 4:20.
18. Chen X, Pang RP, Shen KF, Zimmermann M, Xin WJ, Li YY, Liu XG. TNF-alpha enhances the currents of voltage gated sodium channels in uninjured dorsal root ganglion neurons following motor nerve injury. *Exp Neurol* 2011; 227:279-286.
19. Kim SJ, Park SM, Cho YW, Jung YJ, Lee DG, Jang SH, Park HW, Hwang SJ, Ahn SH. Changes in expression of mRNA for interleukin-8 and effects of interleukin-8 receptor inhibitor in the spinal dorsal horn in a rat model of lumbar disc herniation. *Spine (Phila Pa 1976)* 2011; 36:2139-2146.
20. Dominguez E, Rivat C, Pommier B, Mauborgne A, Pohl M. JAK/STAT3 pathway is activated in spinal cord microglia after peripheral nerve injury and contributes to neuropathic pain development in rat. *J Neurochem* 2008; 107:50-60.
21. Wang W, Gu J, Li YQ, Tao YX. Are voltage-gated sodium channels on the dorsal root ganglion involved in the development of neuropathic pain? *Mol Pain* 2011; 7:16.
22. Ray R, Juranek JK, Rai V. RAGE axis in neuroinflammation, neurodegeneration and its emerging role in the pathogenesis of amyotrophic lateral sclerosis. *Neurosci Biobehav Rev* 2016; 62:48-55.
23. Pietkiewicz J, Seweryn E, Bartys A, Gamian A. Receptors for advanced glycation end products and their physiological and clinical significance. *Postepy Hig Med Dosw (Online)* 2008; 62:511-523.
24. Schafers M, Sorkin L. Effect of cytokines on neuronal excitability. *Neurosci Lett* 2008; 437:188-193.
25. Deane R, Singh I, Sagare AP, Bell RD, Ross NT, LaRue B, Love R, Perry S, Paquette N, Deane RJ, Thiyagarajan M, Zarcone T, Fritz G, Friedman AE, Miller BL, Zlokovic BV. A multimodal RAGE-specific inhibitor reduces amyloid beta-mediated brain disorder in a mouse model of Alzheimer disease. *J Clin Invest* 2012; 122:1377-1392.
26. Tsuda M, Kohro Y, Yano T, Tsujikawa T, Kitano J, Tozaki-Saitoh H, Koyanagi S, Ohdo S, Ji RR, Salter MW, Inoue K. JAK-STAT3 pathway regulates spinal astrocyte proliferation and neuropathic pain maintenance in rats. *Brain* 2011; 134:1127-1139.
27. Ernst MB, Wunderlich CM, Hess S, Paehler M, Mesaros A, Koralov SB, Kleinridders A, Husch A, Munzberg H, Hampel B, Alber J, Kloppenburg P, Bruning JC, Wunderlich FT. Enhanced Stat3 activation in POMC neurons provokes negative feedback inhibition of leptin and insulin signaling in obesity. *J Neurosci* 2009; 29:11582-11593.
28. Sahu M, Sahu A. Leptin receptor expressing neurons express phosphodiesterase-3B (PDE3B) and leptin induces STAT3 activation in PDE3B neurons in the mouse hypothalamus. *Peptides* 2015; 73:35-42.
29. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. *Eur J Cancer* 2005; 41:2502-2512.