Animal Study

Reduction of SIRT1-Mediated Epigenetic Upregulation of Nav1.7 Contributes to Oxaliplatin-Induced Neuropathic Pain

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Background: Clinically, neuropathic pain is a severe side effect of oxaliplatin chemotherapy, which usually leads to dose reduction or cessation of treatment. Due to the unawareness of detailed mechanisms of oxaliplatin-induced neuropathic pain, it is difficult to develop an effective therapy and limits its clinical use.

Objectives: The aim of the present study was to identify the role of sirtuin 1 (SIRT1) reduction in epigenetic regulation of the expression of voltage-gated sodium channels 1.7 (Nav1.7) in the dorsal root ganglion (DRG) during oxaliplatin-induced neuropathic pain.

Study Design: Controlled animal study.

Setting: University laboratory.

Methods: The von Frey test was performed to evaluate pain behavior in rats. Real-time quantitative polymerase chain reaction, western blotting, electrophysiological recording, chromatin immunoprecipitation, and small interfering RNA (siRNA) were used to illustrate the mechanisms.

Results: In the present study, we found that both the activity and expression of SIRT1 were significantly decreased in rat DRG following oxaliplatin treatment. The activator of SIRT1, resveratrol, not only increased the activity and expression of SIRT1, but also attenuated the mechanical allodynia following oxaliplatin treatment. In addition, local knockdown of SIRT1 by intrathecal injection of SIRT1 siRNA caused mechanical allodynia in naive rats. Besides, oxaliplatin treatment enhanced the action potential firing frequency of DRG neurons and the expression of Nav1.7 in DRG and activation of SIRT1 by resveratrol reversed this effect. Furthermore, blocking Nav1.7 by ProTx II (a selective Nav1.7 channel blocker) reversed oxaliplatin-induced mechanical allodynia. In addition, histone H3 hyperacetylation at the Nav1.7 promoter in DRG of rats following oxaliplatin treatment was significantly suppressed by activation of SIRT1 with resveratrol. Moreover, both the expression of Nav1.7 and histone H3 acetylation at the Nav1.7 promoter were upregulated in the DRG by local knockdown of SIRT1 with SIRT1 siRNA in naive rats.

Limitations: More underlying mechanism(s) of SIRT1 reduction after oxaliplatin treatment needs to be explored in future research.

Conclusions: These findings suggest that reduction of SIRT1-mediated epigenetic upregulation of Nav1.7 in the DRG contributes to the development of oxaliplatin-induced neuropathic pain in rats. The intrathecal drug delivery treatment of activating SIRT1 might be a novel therapeutic option for oxaliplatin-induced neuropathic pain.

Key words: SIRT1, neuropathic pain, dorsal root ganglion, NAV1.7 voltage-gated sodium channel, oxaliplatin

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Oxaliplatin, a platinum-based antineoplastic medication drug, is extensively used for the therapy of cancers (1,2), with increased frequency of use in recent years. However, painful neuropathy is a severe adverse effect of oxaliplatin therapy that compromises quality of life and leads to discontinuation of chemotherapy in cancer patients (3). Although various therapeutic attempts have been tested to prevent or alleviate oxaliplatin chemotherapy-induced painful neuropathy, none have been uniformly effective (4,5). Therefore, exploring its underlying mechanisms remains a high research priority.

Sirtuin 1 (SIRT1), a member of the mammalian sirtuin family protein, functions as a conserved nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylase, acting on histones and other protein targets to conduct epigenetic control and modulation of transcriptional silencing and cell survival (6). Growing evidence indicates that SIRT1 plays crucial roles in inflammatory pain, bone cancer pain, and neuropathic pain (7-10). Our previous study (7) also shows that reduction of spinal cord SIRT1 participates in chemotherapeutic bortezomib-induced neuropathic pain via upregulating the expression of the inflammation-associated gene, NALP1 (encodes NACHT leucine-rich-repeat protein 1). In addition, a SIRT1-activating molecule, resveratrol, has been reported to attenuate oxaliplatin-induced mechanical and thermal allodynia via upregulating antioxidant mediators and suppressing pro-inflammatory parameters in the dorsal root ganglion (DRG) (11). Thus, SIRT1 may be a promising therapeutic target for chemotherapy-induced neuropathic pain. However, SIRT1 expression and activity in the DRG of oxaliplatin-induced neuropathic pain rats remain unclear. Moreover, the underlying mechanism of SIRT1 in ameliorating oxaliplatin-induced neuropathic pain remains to be elucidated.

Accumulating data shows that SIRT1 has been shown to be involved in regulating ion channel expression of DRG neurons and synaptic plasticity of spinal dorsal horn neurons (12-15). Nav1.7, a voltage-gated sodium channel of peripheral neurons, amplifies generator potentials and modulates neuronal excitability in normal and pathological pain states (16,17). Evidence is accumulating that genetic variants in SCN9A, a gene coding for Nav1.7, were associated with neuropathic pain in oxaliplatin-treated cancer patients (18,19) and Nav1.7 upregulation in the DRG contributes to paclitaxel-induced neuropathic pain in rats and humans (20). In addition, a recent research has found that the DRG from rats with chronic constriction injury (CCI) displayed lower SIRT1, and a SIRT1 activator resveratrol alleviated CCI-evoked neuropathic pain possibly via suppression of Nav1.7 expression (15). Moreover, SIRT1-mediated epigenetic upregulation of mGluR1/5 gene expression in the spinal cord attenuated diabetic neuropathic pain via increasing the acetylation levels of histone H3 at the Grm1/5 promoter (21). However, it is still not known whether SIRT1-mediated epigenetic upregulation of Nav1.7 in the DRG contributes to oxaliplatin-induced neuropathic pain via increasing H3 acetylation levels at the Nav1.7 promoter regions.

Methods

Animals

Male Sprague Dawley rats (weighting 220-250g) were obtained from the Institute of Experimental Animals of Sun Yat-sen University. All rats were housed in a constant-temperature room with 24 ± 1°C and 50% to 60% humidity on a 12-hour light /12-hour dark cycle and fed rodent chow and water freely.

The rats were randomly divided into 8 groups. The oxaliplatin-induced neuropathic pain group comprised 111 rats (paw withdrawal threshold, 18 rats; real-time quantitative polymerase chain reaction [PCR] assay, 36 rats; western blotting assay, 18 rats; SIRT1 activity assay, 30 rats; electrophysiological recording, 3 rats; chromatin immunoprecipitation, 6 rats), which were intraperitoneally (i.p.) injected with oxaliplatin and verified based on paw withdrawal threshold. The vehicle-treated group comprised 66 rats (paw withdrawal threshold, 18 rats; real-time quantitative PCR assay, 18 rats; western blotting assay, 9 rats; SIRT1 activity assay, 12 rats; electrophysiological recording, 3 rats; chromatin immunoprecipitation, 6 rats), which were given the same dose of vehicle as the oxaliplatin-induced neuropathic pain group. The oxaliplatin + resveratrol group consisted of 39 rats (paw withdrawal threshold, 6 rats; real-time quantitative PCR assay, 12 rats; western blotting assay, 6 rats; SIRT1 activity assay, 6 rats; electrophysiological recording, 3 rats; chromatin immunoprecipitation, 6 rats), which were intrathecally injected with resveratrol. Rats in the oxaliplatin + 15 µg ProTx II group consisted of 6 rats (paw withdrawal threshold, 6 rats), which were intrathecally injected with ProTx II. Rats in the oxaliplatin + 60 µg ProTx II group consisted of 6 rats (paw withdrawal threshold, 6 rats). The control group comprised 30 rats (paw withdrawal threshold, 6 rats; real-time quantitative PCR assay, 12 rats; western blotting assay, 6 rats;

chromatin immunoprecipitation, 6 rats). The scramble small interfering RNA (siRNA) group comprised 30 rats (paw withdrawal threshold, 6 rats; real-time quantitative PCR assay, 12 rats; western blotting assay, 6 rats; chromatin immunoprecipitation, 6 rats). The SIRT1 siRNA group comprised 30 rats (paw withdrawal threshold, 6 rats; real-time quantitative PCR assay, 12 rats; western blotting assay, 6 rats; chromatin immunoprecipitation, 6 rats).

All of the experimental protocols in this study were approved by the Institutional Animal Care Committee and carried out in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number and the suffering of rats used. All rats were randomly assigned to different experimental or control groups.

Injection of Drug and Adenovirus-Associated Vector

Oxaliplatin was purchased from MillporeSigma (United States) and dissolved in 5% glucose solution (22). Oxaliplatin was i.p. injected at 4 mg/kg once per day for 5 consecutive days to induce mechanical allodynia. An equivalent volume of 5% glucose solution was i.p. injected in the control rat.

The siRNA was purchased from Ribobio (Guangzhou Ribobio Co, Ltd, China). The saline was used for siRNA delivery. SIRT1 siRNA (50 μg/15 μl), scrambled siRNA (50 μg/15 μl), ProTx II (15 μg/20 μl, 60 μg/20 μl; Tocris Bioscience, United States), resveratrol (250 μg/10 μl; MillporeSigma, United States), or vehicle saline was intrathecally injected 30 minutes prior to oxaliplatin treatment and maintained for consecutive 7 days. The ProTx II dose was chosen on the basis of previously published studies (20,39).

Intrathecal Injection

Lumbar subarachnoid catheterization for intrathecal drug administration was performed according to a previously described method (7,23). In brief, laminectomy of the L5 vertebra was performed under anesthesia. After the dura was probed with an 8-G needle, a PE-10 catheter was inserted into the subarachnoid space through L5/L6 intervertebral space, and the tip of the catheter was located at the L5 spinal segmental level. The rat was allowed to recover from surgery for at least 5 days prior to subsequent drug injection. Any rats exhibiting hind limb paralysis or paresis after surgery were excluded from the study.

Behavioral Assessment

The von Frey test was used to assess mechanical allodynia in each rat (24). Briefly, rats were placed in the testing environment for 15 minutes daily for 3 consecutive days. Next, the rats were tested using von Frey filaments with a specific bending force to the midplantar surface of the hind paw. A brisk paw withdrawal or paw flinching after filament application was defined as a nociceptive response. Each test was performed for 3 times at 2 minutes rest. The 50% paw withdrawal threshold was calculated according to a previously validated up-down procedure. The experimenter blinded to all treatments conducted the behavioral test.

Electrophysiological Recordings

DRG neurons acutely dissociated by enzyme digestion and mechanical trituration were seeded on cover slips coated with Poly-L-Lysine (MillporeSigma, United States) according to a previously described method (25). Patch-clamp recordings from neurons were performed using an EPC-10 amplifier and the PULSE program (HEKA, United States). Series resistance compensation (80% to 90%) and linear leak subtraction were applied to minimize voltage errors. All recordings were performed on small and medium diameter (20-35μm) neurons (26). The size of neurons was calculated from membrane capacitance, which was read out from the amplifier using PULSE software (HEKA, United States).

Assay of SIRT1 Activity

The activity of SIRT1 was determined by using the SIRT1 fluorometric kit (Abcam, United Kingdom). Briefly, after the protein was extracted in L4 and L5 DRGs, nuclear extract was purified by immunoprecipitation with the antibody (rabbit anti-SIRT1 antibody, Elabscience Biotechnology, China) and Protein A Agarose Beads (Cell Signaling Technology Inc, United States). The reaction mixture was added and the NAD+-dependent deacetylase activity was tested. Fluorescence intensity was read for 60 minutes at 2 minute intervals with excitation wave length at 355 nm and emission wave length at 460 nm using a fluorometric microplate reader (Thermo Scientific, United States).

RNA Extraction and Real-Time Quantitative PCR

Total RNA was harvested from L4 and L5 DRG tissues of rats with Trizol Reagent (Invitrogen, United States). Evo M-MLV Reverse Transcriptase Premix (AG, China) was used to perform the reverse transcription

reactions. The synthesized complementary DNA from total RNA was evaluated by specific primers using SYBR Green Premix qPCR Kit (AG, China). The calculation method of relative expression ratio of messenger RNA (mRNA) was the $2^{-\Delta\Delta CT}$ method (27). Sequences of the primers used for PCR were shown in Table 1.

Western Blotting

Western blotting was performed as described previously (28). Briefly, L4 and L5 DRG tissues of rats were removed and homogenized in lysis buffer. Next, protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were preincubated with a blocking buffer for 1 hour at room temperature and then probed with primary antibodies against SIRT1 (1:2000, Abcam, United Kingdom), Nav1.7 (1:2000, Alomone Labs, Israel) or glyceraldehyde 3-phosphate dehydrogenase (1:1000, Santa Cruz, United States) at 4°C overnight. Next, membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies for one hour at room temperature. Protein bands were visualized in the membranes by chemiluminescent HRP substrate. Expression of protein of interest was normalized to the total protein loaded for each sample, as quantified by computer-assisted imaging analysis system (NIH ImageJ, United States).

Chromatin Immunoprecipitation Assays

Chromatin Immunoprecipitation (ChIP) assays were carried out using the ChIP Assay Kit (Thermo, United States) (7). L4 and L5 DRG tissues were removed and placed in 1% formaldehyde for 10 minutes to cross-link transcription factors with chromatin. Chromatin extracts containing DNA were fragmented by sonication and digested with micrococcal nuclease. DNA fragments were immunoprecipitated with 6 µg of anti-acetyl-histone H3 antibody or rabbit IgG antibody, and then incubated with preblocked protein G-Sepharose beads overnight at 4°C. Following the complexes were eluted, the precipitated DNA was obtained. Real-time quantitative PCR was performed on the sample as described in the above methods. The following specific PCR primer pairs were designed to amplify the promoter region of Nav1.7 (scn9a): 5'-CTG CGG AAG GAA GAA ATC AG-3' and 5'-TCT CGT GCT TCA AAC TGT GG-3'. Finally, the ratio of ChIP/input in DRG was calculated.

Table 1. *The specific primer sequences.*

GENE	Primer	Sequence
β-actin (rat)	Forward	5′-AGG GAA ATC GTG CGT GAC AT-3′
	Reverse	5′-GAA CCG CTC ATT GCC GAT AG-3′
Nav1.7 (rat)	Forward	5′-CGA TGG GTC ACG ATT TCC TAC-3′
	Reverse	5′-CGT GAA GAA TGA GCC GAA GAT-3′
SIRT1 (rat)	Forward	5′-TTG GCA CCG ATC CTC GAA-3′
	Reverse	5′-ACA GAA ACC CCA GCT CCA-3′

Statistical Analysis

All obtained data were expressed as mean ± SEM, and statistically analyzed using SPSS Version 25.0 (IBM Corporation, Armonk, NY). Data were analyzed by t test (2 independent samples) or one-way analysis of variance followed by Tukey's post hoc test or Dunnett's T3. Data that did not meet normality were analyzed by the permutation test. A *P* value of < 0.05 was considered statistically significant. The sample size was determined by previous publications in painful behavior studies.

Results

Reduction of SIRT1 Expression and Activity in DRG of Oxaliplatin-Induced Neuropathic Pain Rats

It has been reported that the serum SIRT1 level was negatively correlated with pain scores in patients with chronic pain (29). In addition, receiving oral resveratrol (a selective activator of SIRT1) was shown to be a potential therapeutic method in the prevention of oxaliplatin-induced neuropathic pain (11). In this study, to explore whether SIRT1 is involved in the pathogenesis of oxaliplatin-induced neuropathic pain, mechanical paw withdrawal threshold and SIRT1 mRNA and protein expression in the DRG of rats after i.p. injection of oxaliplatin were detected. Consistent with our previous study (30), the results shows that the mechanical withdrawal threshold in oxaliplatin-treated rats was significantly lower than in the vehicle group on days 3, 5, 7, and 10 following i.p. injection (n = 6/group; Fig. 1A), indicating that oxaliplatin-induced mechanical allodynia. In addition, compared with the vehicle-treated rats, oxaliplatin-treated rats showed a statistically significant decrease in SIRT1 mRNA (n = 6/group; Fig. 1B) and protein expression (n = 3/group; Fig. 1C). Meanwhile, SIRT1 activity was also observed to be significantly decreased in the DRG of oxaliplatin-induced neuropathic pain rats (n = 6/group; Fig. 1D). These results suggest that a functional association may exist between SIRT1 and oxaliplatin-induced neuropathic pain. Based on the significant changes of

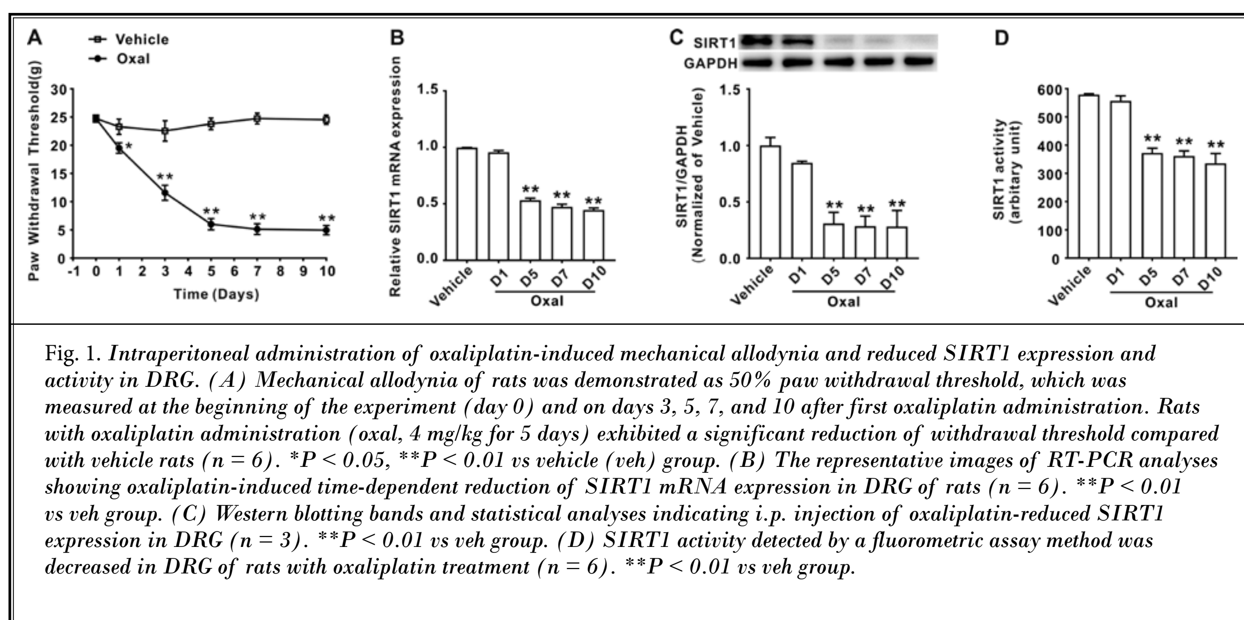

Fig. 1. *Intraperitoneal administration of oxaliplatin-induced mechanical allodynia and reduced SIRT1 expression and activity in DRG. (A) Mechanical allodynia of rats was demonstrated as 50% paw withdrawal threshold, which was measured at the beginning of the experiment (day 0) and on days 3, 5, 7, and 10 after first oxaliplatin administration. Rats with oxaliplatin administration (oxal, 4 mg/kg for 5 days) exhibited a significant reduction of withdrawal threshold compared with vehicle rats (n = 6). *P < 0.05, **P < 0.01 vs vehicle (veh) group. (B) The representative images of RT-PCR analyses showing oxaliplatin-induced time-dependent reduction of SIRT1 mRNA expression in DRG of rats (n = 6). **P < 0.01 vs veh group. (C) Western blotting bands and statistical analyses indicating i.p. injection of oxaliplatin-reduced SIRT1 expression in DRG (n = 3). **P < 0.01 vs veh group. (D) SIRT1 activity detected by a fluorometric assay method was decreased in DRG of rats with oxaliplatin treatment (n = 6). **P < 0.01 vs veh group.*

pain-related behavior and the significant reduction of SIRT1 expression and activity on day 7, we focused all subsequent experiments on day 7.

Reduction of SIRT1 in DRG Mediated Oxaliplatin-Induced Neuropathic Pain

Our previous study (7) has shown that intrathecal injection of resveratrol significantly upregulated SIRT1 expression in the spinal dorsal horn and suppressed bortezomib-induced neuropathic pain. Other study also indicates that receiving daily oral resveratrol attenuated oxaliplatin-induced mechanical and thermal allodynia in rats (11). To further determine the role of SIRT1 in oxaliplatin-induced neuropathic pain, we investigated the effect of resveratrol on the expression and activity of SIRT1 in DRG and the paw withdrawal threshold of oxaliplatin-treated rats. We find that intrathecal administration of resveratrol could indeed increase the expression of SIRT1 mRNA (n = 6/group) and protein (n = 3/group), with increased activity of SIRT1 in DRG of rats (n = 6/group) on day 7 following oxaliplatin treatment (Figs. 2A-2C). In line with the increase of SIRT1 expression and activity, mechanical allodynia was markedly suppressed in oxaliplatin-treated rats with resveratrol injection (n = 6/group; Fig. 2D). These findings suggest that upregulation of SIRT1 by resveratrol ameliorate the mechanical allodynia induced by oxaliplatin.

Moreover, we used a small interfering RNA SIRT1 siRNA to knockdown the expression of SIRT1 in the DRG of naive rats and then observed the paw withdrawal threshold. Results showed that SIRT1 siRNA treatment reduced the expressions of SIRT1 mRNA (n = 6/group; Fig. 3A) and protein levels in naive rats (n = 3/group; Fig. 3B), which confirmed an effective knockdown of SIRT1 expression. Contrasted from the role of SIRT1 activator resveratrol, we found that reduction of DRG SIRT1 by SIRT1 siRNA induced mechanical allodynia in naive rats (n = 6/group; Fig. 3C). Altogether, these results indicate that the reduction of SIRT1 in the DRG of rats induces pain-related behavior.

SIRT1 Mediated Oxaliplatin-Induced Neuropathic Pain by Enhancing DRG Neuron Hyperexcitability via Epigenetic Upregulation of Nav1.7 Expression

It is known that decreased SIRT1-mediated central sensitization contributes to diabetic neuropathic pain via regulating synaptic plasticity of spinal dorsal horn neurons (12). To further determine the role of SIRT1 in peripheral sensitization, we investigated the neuronal excitability of DRG during oxaliplatin-induced neuropathic pain. The results showed that the action potential firing frequency of DRG neurons was increased in oxaliplatin-induced neuropathic pain rats on day 7 following oxaliplatin treatment and activation of SIRT1 by resveratrol reversed the increase (n = 8 neurons from 3 rats per group; Fig. 4A). Studies (31-33) have shown that Nav1.7 abundantly expressed in DRG neurons, regulates neuronal excitability and the peripheral sensitization

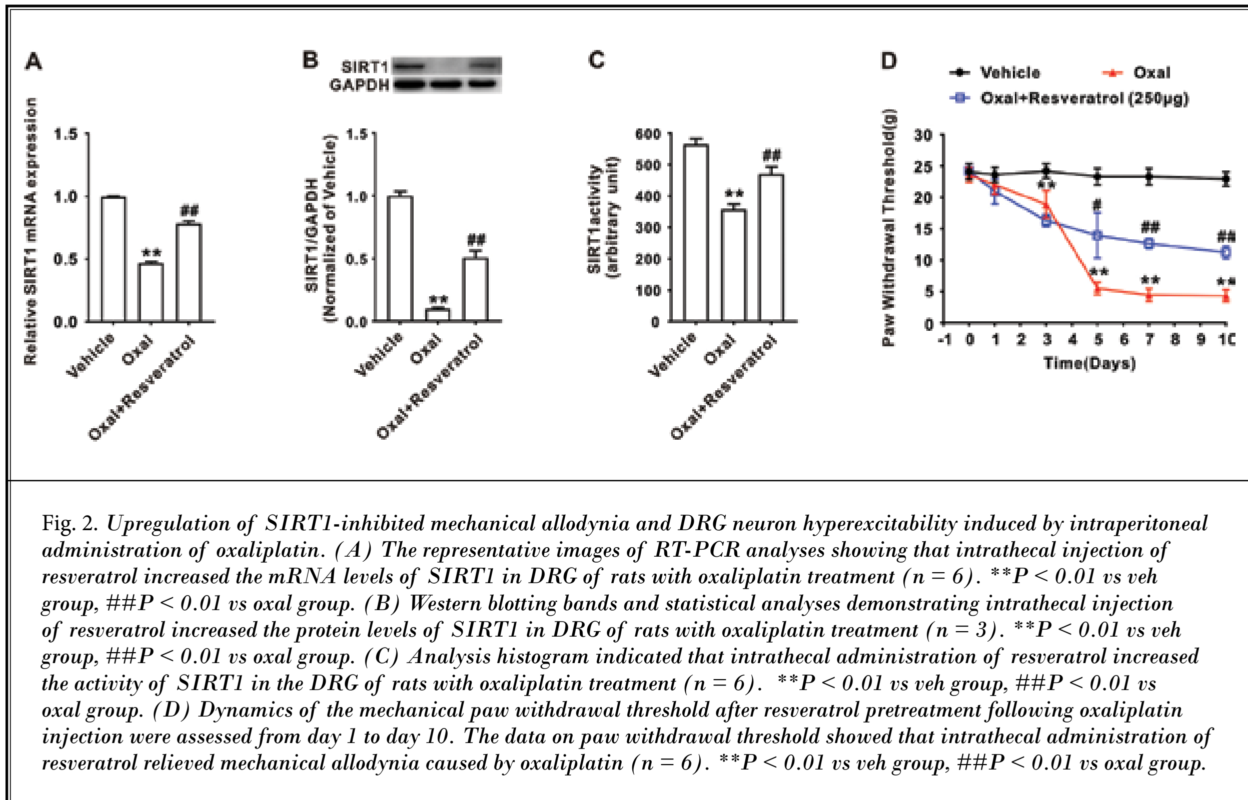

Fig. 2. *Upregulation of SIRT1-inhibited mechanical allodynia and DRG neuron hyperexcitability induced by intraperitoneal administration of oxaliplatin. (A) The representative images of RT-PCR analyses showing that intrathecal injection of resveratrol increased the mRNA levels of SIRT1 in DRG of rats with oxaliplatin treatment (n = 6). **P < 0.01 vs veh group, ##P < 0.01 vs oxal group. (B) Western blotting bands and statistical analyses demonstrating intrathecal injection of resveratrol increased the protein levels of SIRT1 in DRG of rats with oxaliplatin treatment (n = 3). **P < 0.01 vs veh group, ##P < 0.01 vs oxal group. (C) Analysis histogram indicated that intrathecal administration of resveratrol increased the activity of SIRT1 in the DRG of rats with oxaliplatin treatment (n = 6). **P < 0.01 vs veh group, ##P < 0.01 vs oxal group. (D) Dynamics of the mechanical paw withdrawal threshold after resveratrol pretreatment following oxaliplatin injection were assessed from day 1 to day 10. The data on paw withdrawal threshold showed that intrathecal administration of resveratrol relieved mechanical allodynia caused by oxaliplatin (n = 6). **P < 0.01 vs veh group, ##P < 0.01 vs oxal group.*

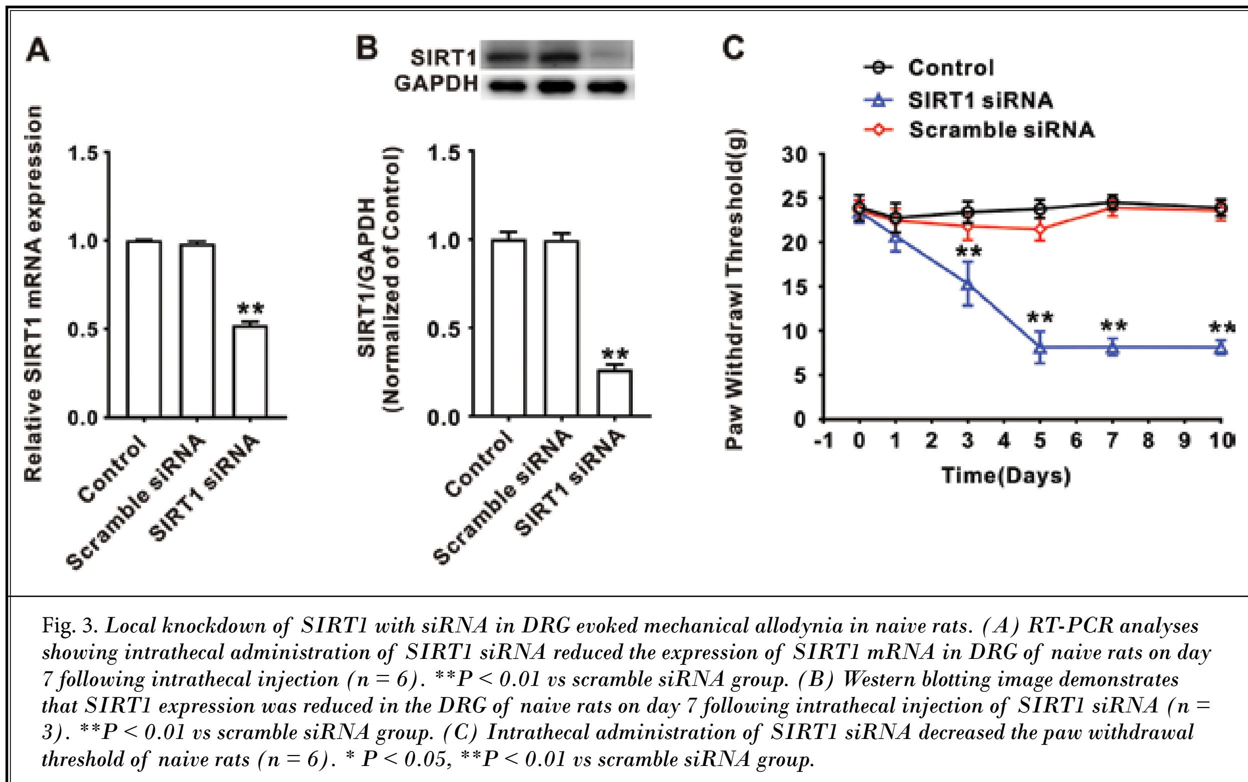

Fig. 3. *Local knockdown of SIRT1 with siRNA in DRG evoked mechanical allodynia in naive rats. (A) RT-PCR analyses showing intrathecal administration of SIRT1 siRNA reduced the expression of SIRT1 mRNA in DRG of naive rats on day 7 following intrathecal injection (n = 6). **P < 0.01 vs scramble siRNA group. (B) Western blotting image demonstrates that SIRT1 expression was reduced in the DRG of naive rats on day 7 following intrathecal injection of SIRT1 siRNA (n = 3). **P < 0.01 vs scramble siRNA group. (C) Intrathecal administration of SIRT1 siRNA decreased the paw withdrawal threshold of naive rats (n = 6). * P < 0.05, **P < 0.01 vs scramble siRNA group.*

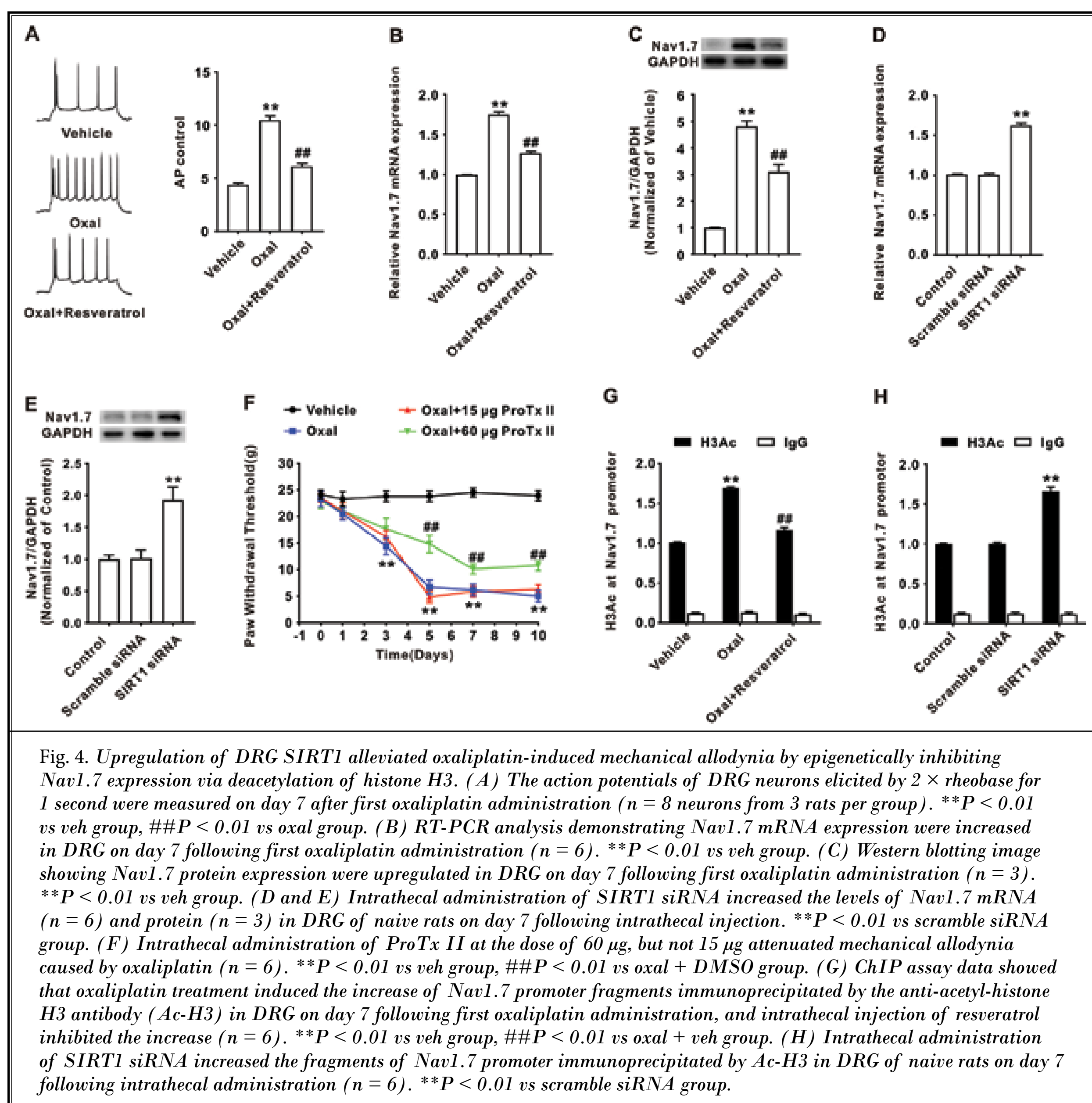

Fig. 4. *Upregulation of DRG SIRT1 alleviated oxaliplatin-induced mechanical allodynia by epigenetically inhibiting Nav1.7 expression via deacetylation of histone H3. (A) The action potentials of DRG neurons elicited by 2 × rheobase for 1 second were measured on day 7 after first oxaliplatin administration (n = 8 neurons from 3 rats per group). **P < 0.01 vs veh group, ##P < 0.01 vs oxal group. (B) RT-PCR analysis demonstrating Nav1.7 mRNA expression were increased in DRG on day 7 following first oxaliplatin administration (n = 6). **P < 0.01 vs veh group. (C) Western blotting image showing Nav1.7 protein expression were upregulated in DRG on day 7 following first oxaliplatin administration (n = 3). **P < 0.01 vs veh group. (D and E) Intrathecal administration of SIRT1 siRNA increased the levels of Nav1.7 mRNA (n = 6) and protein (n = 3) in DRG of naive rats on day 7 following intrathecal injection. **P < 0.01 vs scramble siRNA group. (F) Intrathecal administration of ProTx II at the dose of 60 μg, but not 15 μg attenuated mechanical allodynia caused by oxaliplatin (n = 6). **P < 0.01 vs veh group, ##P < 0.01 vs oxal + DMSO group. (G) ChIP assay data showed that oxaliplatin treatment induced the increase of Nav1.7 promoter fragments immunoprecipitated by the anti-acetyl-histone H3 antibody (Ac-H3) in DRG on day 7 following first oxaliplatin administration, and intrathecal injection of resveratrol inhibited the increase (n = 6). **P < 0.01 vs veh group, ##P < 0.01 vs oxal + veh group. (H) Intrathecal administration of SIRT1 siRNA increased the fragments of Nav1.7 promoter immunoprecipitated by Ac-H3 in DRG of naive rats on day 7 following intrathecal administration (n = 6). **P < 0.01 vs scramble siRNA group.*

of pain. Next, we detected the expression of Nav1.7 to explore the relationships between Nav1.7 and SIRT1 in neuropathic pain induced by oxaliplatin. These results indicated that the level of Nav1.7 mRNA (n = 6/group) and protein (n = 3/group) were significantly elevated on day 7 in the DRG of oxaliplatin-induced neuropathic pain rats and the increase of Nav1.7 was suppressed by resveratrol (Figs. 4B and 4C). Moreover, the levels of Nav1.7 mRNA (n = 6/group) and protein (n = 3/group) were elevated in the DRG of naive rats with intrathecal injection of SIRT1 siRNA (Figs. 4D and 4E). Besides, intrathecal administration of a selective Nav1.7 channel blocker, ProTx II, attenuated oxaliplatin-induced mechanical allodynia (n = 6/group; Fig. 4F). These results suggest that SIRT1 reduction via upregulation of Nav1.7 may enhance the neuronal excitability of DRG and subsequently contributes to oxaliplatin-induced neuropathic pain.

Although the expression of Nav1.7 has been known to be regulated by the histone acetylation during

inflammatory hyperalgesia (34), whether the decrease of SIRT1 mediated Nav1.7 upregulation by regulating histone H3 acetylation levels at the Nav1.7 promoter regions remains unclear. To explore this question, we observed H3 acetylation levels at the Nav1.7 promoter regions by ChIP assays. The results showed that Nav1.7 promoter fragments immunoprecipitated by the anti-acetyl-histone H3 antibody were increased in the DRG of oxaliplatin-induced neuropathic pain rats and activation of SIRT1 by resveratrol reversed the increase (n = 6/group; Fig. 4G). However, SIRT1 siRNA treatment enhanced the amount of anti-acetyl-histone H3 antibody-precipitated Nav1.7 promoter fragments in the DRG of naive rats (n = 6/group; Fig. 4H). Collectively, these data suggest that upregulation of Nav1.7 expression in DRG results from reduction of SIRT1-mediated histone H3 hyperacetylation at Nav1.7 promoter regions, thereby contributing to oxaliplatin-induced neuropathic pain.

Discussion

The underlying mechanisms for the development and maintenance of oxaliplatin-induced neuropathic pain remain poorly understood. Previous studies (7,21,23) from ours and others have shown that SIRT1-mediated epigenetic upregulation of pain-related gene expression attenuated bortezomib or diabetes-induced neuropathic pain via increasing the acetylation of histone. Moreover, an oral SIRT1-activating molecule, resveratrol, has been reported to prevent oxaliplatin-induced neuropathic pain (11). These studies (7,11,21,23) demonstrate that SIRT1-mediated epigenetic regulation may play a momentous role in the development of oxaliplatin-induced neuropathic pain. Here, we found that oxaliplatin administration reduced the expression and activity of SIRT1 in DRG, which enhanced the excitability of DRG neurons and promoted the genesis of neuropathic pain by upregulating the level of histone H3 protein acetylation at Nav1.7 promoter regions, thereby promoting Nav1.7 transcription and expression. Moreover, activation of SIRT1 repressed the hyperexcitability of DRG neurons, the upregulation of Nav1.7 via inhibition of histone H3 protein hyperacetylation at the Nav1.7 promoter regions, and correspondingly attenuated oxaliplatin-induced mechanical allodynia. Local knockdown of SIRT1 induced Nav1.7 upregulation in DRG via enhancing histone H3 protein acetylation at the Nav1.7 promoter regions and evoked mechanical allodynia in naive rats. Altogether, our findings provide a potential epigenetic mechanism by which decreased SIRT1 modulates Nav1.7 expression in the DRG, contributing to oxaliplatin-induced neuropathic pain. While the possibility existed that the analgesia induced by intrathecal dosing scheme in the present study might be partially supplemented with its potential effect on the spinal dorsal horn, our results at least showed that reduction of DRG SIRT1-mediated Nav1.7 upregulation was critically involved in oxaliplatin-induced neuropathic pain.

The present study showed that reduction of SIRT1 activity and expression in DRG played a critical role in oxaliplatin-induced neuropathic pain, and restoring activity of SIRT1 in the DRG with resveratrol relieved oxaliplatin-induced mechanical allodynia. These results are consistent with our previous findings that SIRT1 expression was downregulated in the spinal cord of bortezomib-induced neuropathic pain rats, and recovery of SIRT1 activity by resveratrol alleviated the neuropathic pain (7). It is well known that SIRT1 is responsible for deacetylating acetylated histone (35) and mediating ClC-3 membrane trafficking and Cl - current (29), and SIRT1 activator significantly decreases acetyl-histone H3 (36) and pain-related ion channels expression, such as acid-sensing ion channels 3 (14). So, it is possible that ion channel upregulation induced by decreased SIRT1 significantly contribute to neuropathic pain induced by oxaliplatin. However, our present study only focuses on the expression and activity of SIRT1 in DRG during the development and maintenance of oxaliplatin-induced neuropathic pain in rats. Therefore, further studies are needed to determine the mechanism by which SIRT1, expressed in the spinal or supraspinal areas, contributes to the pathophysiology of oxaliplatin-induced neuropathic pain.

In this study, we also found that the oxaliplatin treatment markedly enhanced the action potential firing frequency of neurons in DRG and Nav1.7 expression in DRG neurons, and the blockage of the Nav1.7 expression by ProTx II attenuated oxaliplatin-induced mechanical allodynia. These results are consistent with other studies' findings (15,37) that Nav1.7 expression is considerably increased in the DRG of nerve injury or complete Freund's adjuvant-induced chronic pain models and blockage of the Nav1.7 channel relieved the chronic pain. In addition, it is supported by recent evidence that Nav1.7 initiated the upstroke of action potentials in DRG neurons (38) and Nav1.7-mediated neuronal excitability contributes to inflammatory pain and neuropathic pain (20,32,33). Based on the contribution of Nav1.7 to action potential generation (39,40), it may be possible that the increased Nav1.7 in

DRG induced by oxaliplatin contributes to the pathogenesis of neuropathic pain via enhancing action potential generation of DRG neurons. Notably, in this study, we also found that the decrease of SIRT1 in DRG was involved in upregulating Nav1.7 expression. It was consistent with the previous evidence that the activity and expression of SIRT1 were reduced in the DRG of CCI rodent models, and recovery of activity by resveratrol attenuated the neuropathic pain via suppressing Nav1.7 expression (15).

Furthermore, in our study, SIRT1 reduction was shown to mediate histone H3 hyperacetylation at the Nav1.7 promoter region in DRG after the oxaliplatin injection. It was supported by the findings that SIRT1 regulated the histone acetylation level in the promoter region of target genes, such as SIRT1, bound to histone H3K9 at the p53 promoter region and decreased H3K9 acetylation (41). A previous study (34) also showed that histone H3 hyperacetylation at the promoter region of Nav1.7 gene was involved in regulating Nav1.7 expression. In addition, the present study indicated that upregulation of SIRT1 expression in DRG inhibited histone H3 hyperacetylation at the promoter region of the Nav1.7 gene and Nav1.7 upregulation induced by oxaliplatin, and downregulation of SIRT1 expression in DRG increased the acetylation of histone H3 in the Nav1.7 promoter region and the expression of Nav1.7 in DRG of naive rats. These findings suggested that SIRT1 reduction upregulated Nav1.7 expression in the DRG via enhancing histone H3 acetylation in the Nav1.7 promoter region following oxaliplatin treatment. It was consistent with the previous report that SIRT1 reduction increased histone H3 acetylation in Grm1/5 promoter region, consequently upregulated the expression of mGluR1/5 in the spinal cord of diabetic rats, thereby contributing to neuropathic pain (21).

Limitations

However, there were several limitations in the present study. On one hand, DRG overexpression of SIRT1 should be performed to reversely confirm the conclusion that SIRT1 conducts analgesia effect through epigenetically regulating Nav1.7. On the other hand, we only detected the expression of Nav1.7 and histone H3 acetylation at the promoter region of the Nav1.7 gene after the resveratrol treatment. Whether histone H3 hyperacetylation could result in further changes in Nav1.7 upregulation, depolarization, inward currents, and excitability of DRG neurons would be explored in the future. Meanwhile the present study hasn't come to the relationship between oral resveratrol and intrathecal injection of resveratrol in attenuating oxaliplatin-induced neuropathic pain, which will be investigated in the following studies. In addition, further studies in terms of the analgesia, safety, and tolerance of resveratrol on a large amount of preclinical tests and clinical trials are highly demanded.

Conclusions

These findings suggest that reduction of SIRT1-mediated epigenetic upregulation of Nav1.7 in the DRG contributes to oxaliplatin-induced neuropathic pain in rats. The intrathecal drug delivery treatment of activating SIRT1 might be a novel therapeutic option for oxaliplatin-induced neuropathic pain.

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