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Sensitization of TRPV1 receptors by TNF-α orchestrates the development of vincristine-induced pain

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Abstract. Vincristine is one of the most common anticancer drugs clinically employed in the treatment of various malignancies. A major side effect associated with vincristine is the development of neuropathic pain, which is not readily relieved by available analgesics. Although efforts have been made to identify the pathogenesis of vincristine-induced neuropathic pain, the mechanisms underlying its pathogenesis have not been fully elucidated. In the present study, a neuropathic pain model was established in Sprague-Dawley rats by intraperitoneal injection of vincristine sulfate. The results demonstrated that vincristine administration induced the upregulation of transient receptor potential cation channel subfamily V member 1 (TRPV1) protein expression and current density in dorsal root ganglion (DRG) nociceptive neurons. Consistently, inhibition of TRPV1 with capsazepine alleviated vincristine-induced mechanical allodynia and thermal hyperalgesia in rats. Furthermore, vincristine administration induced the upregulation of tumor necrosis factor (TNF)-α production in DRGs, and inhibition of TNF-α synthesis with thalidomide *in vivo* reversed TRPV1 protein expression, as well as pain hypersensitivity induced by vincristine in rats. The present results suggested that TNF-α could sensitize TRPV1 by promoting its expression, thus leading to mechanical allodynia and thermal hyperalgesia in vincristine-treated rats. Taken together, these findings may enhance our understanding of the pathophysiological mechanisms underlying vincristine-induced pain.

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Introduction

Vincristine is one of the most common anticancer drugs clinically used for the management of leukemia, lymphoma and primary brain cancer (1-3). It can induce neurotoxicity that manifests as painful neuropathy, which significantly decreases the quality of life in patients (4-6). Although efforts have been made to identify the pathogenesis of vincristine-induced neuropathic pain, the mechanisms underlying this remain largely unknown.

Peripheral sensory neurons are more susceptible to vincristine toxicity than central nervous system (CNS) neurons owing to the absence of blood-nerve barrier (7,8). Transient receptor potential cation channel subfamily V member 1 (TRPV1) is a ligand-gated non-selective cation channel which is prominently expressed in nociceptive dorsal root ganglia (DRG) neurons (9,10). Accumulating evidence has suggested that TRPV1 receptor expression and function in DRG neurons are greatly sensitized following tissue inflammation or nerve injury (11,12). Blockade or knockdown of TRPV1 attenuates mechanic allodynia or thermal hyperalgesia in several models of pain (13-15). However, whether TRPV1 contributes to the development of vincristine-induced pain hypersensitivity remains unclear.

Previous studies have demonstrated that glia activation and pronociceptive substances, such as proinflammatory interleukins and tumor necrosis factor (TNF-α), in the spinal cord contribute to vincristine-induced neuropathic pain (16,17). However, it remains unknown whether TNF-α is implicated in the sensitization of primary sensory neurons in vincristine-induced neuropathy. In addition, TNF-α is well-known to potentiate TRPV1 activity, leading to mechanical allodynia and thermal hyperalgesia in DRGs (18,19). Therefore, the present study aimed to investigate whether peripheral TNF-α is responsible for the sensitization of TRPV1 following vincristine administration *in vivo*. The results indicated that vincristine administration induced the upregulation of TRPV1 protein expression and current density in DRG nociceptive neurons. TNF-α sensitized TRPV1 by promoting its expression, thus leading to mechanical allodynia and thermal hyperalgesia in vincristine-treated rats. Taken together, these findings suggest that targeting the TNF-α signaling pathway may be an effective approach for treating vincristine-induced pain.

Materials and methods

Animals. Experiments were performed on 195 adult male Sprague-Dawley rats aged 2 months (weight, 220±20 g) from Beijing Weitong Lihua Experimental Animal Co., Ltd. (Beijing, China). All rats were housed in separated cages with free access to food and water under a 12-h light/dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). The room temperature was kept at 24±1˚C, with a relative humidity of 50±5%. All animal experimental procedures were conducted in accordance with the guidelines of the International Association for the Study of Pain (20) and were approved by the Animal Experimentation Ethics Committee (AEEC) of Henan University (Kaifeng, China). Vincristine was used to induce neuropathy as previously described (20). Briefly, 75 μg/kg of vincristine sulfate (Hospira, Lake Forest, IL, USA) was administered once daily intraperitoneally (i.p.) for 5 consecutive days, paused for 2 days, then subsequently administered for another 5 consecutive days. For the control group, the same volume of normal saline was administered by the same schedule.

Behavioral analysis. The 50% paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) were performed as described in our previous studies (11,21). All behavioral experiments were performed blinded to treatment group. For mechanical sensitivity, all rats were placed in transparent boxes on an elevated metal mesh floor and allowed a 20 min period for habituation prior to examination. A series of von Frey hairs (0.41-15.1 g; Stoelting Co., Wood Dale, IL, USA) were applied perpendicular to the plantar surface for 3-5 sec of each hind paw. The 50% PWT was determined using the Dixon's up-down method (22). For testing thermal nociception, all rats were allowed to acclimate for a minimum of 30 min in a transparent, square, bottomless acrylic box (22x12x22 cm). Then, PWLs were measured with radiant light focused on the left plantar surface of the hind paw. A maximal cutoff time of 30 sec was applied to avoid unnecessary tissue damage. The measurements of PWL were repeated three times with 3-5 min intervals between consecutive tests.

The first behavioral experiment was performed to examine whether vincristine induced nociceptive behaviors in normal rats. Vincristine (75 μg/kg, i.p. injection) was administered once a day for 5 days, paused for 2 days, then resumed for an additional 5 days. Pain behaviors were evaluated prior to drug injection and on days 1, 3, 7, 10, 14, and 21 post-vincristine administration.

To examine whether inhibition of TRPV1 with capsazepine (CPZ) attenuated vincristine-induced nociceptive behaviors in normal rats, effects of CPZ (30 mg/kg; i.p. injection) were measured at 2, 6, 12, 24 and 48 h post-administration.

To explore whether inhibition of TNF-α synthesis alleviated vincristine-induced nociceptive behaviors, the TNF-α synthesis inhibitor, thalidomide (50 mg/kg), was injected i.p. 2 h prior to vincristine administration once a day for 5 days, paused for 2 days, then resumed for an additional 5 days, and pain hypersensitivity was examined on day 14.

Western blot analysis. Under deep anesthesia with isoflurane (2-3% in 1 l/min oxygen), the rats L4-L5 DRGs were removed and stored at -80˚C until use. Samples were homogenized in ice-chilled lysis buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail. Then, the homogenates were centrifuged at 10,000 x g for 10 min at 4˚C. Following centrifugation, the supernatant was collected and the concentration of total protein was determined with a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal amount of protein samples were denatured and then analyzed by western blotting.

Preparation of membrane fractions was performed in DRG neurons as previously described (11,23). In brief, L4 and L5 DRGs were harvested and homogenized in ice-cold lysis buffer containing 10 mM Tris (pH 8.1), 0.3 M sucrose, 2 mM EDTA, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail. Homogenates were placed on ice for 1 h before centrifugation at 1,000 x g for 7 min at 4˚C to remove nuclei and intact cells. The pellet was rehomogenized and spun again under the same conditions. The supernatants from the two low-speed spins were combined and centrifuged at 120,000 x g for 1 h at 4˚C. The pellet, containing the total membrane fraction, was suspended in 0.2 M KCl/10 mM HEPES (pH 7.4). To solubilize the membrane fraction, an equal volume of 5% Triton X-100/10 mM HEPES (pH 7.4) was added to the samples and the suspension was kept on ice for 1 h. The unsolubilized material was pelleted by centrifugation at 10,000 x g for 10 min at 4˚C, and the supernatant was analyzed by western blotting.

For western blot analysis, Equal amounts of protein samples (40 μg) were separated through SDS-PAGE using 10% running gels and then transferred to polyvinylidene fluoride membranes. After blocking with 5% nonfat milk in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) for 60 min at room temperature, the membrane was probed with the following primary antibodies overnight at 4˚C: anti-TRPV1 (1:200; cat. no. sc-28759; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-β-actin (1:1,000; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.), anti-TNF-α (1:500; cat. no. sc-52746; Santa Cruz Biotechnology, Inc.), and anti-transferrin receptor (TfR; 1:1,000; cat. no. 13-6800; Thermo Fisher Scientific, Inc.). The blots were washed in TBST and then incubated in horseradish peroxidase-conjugated goat anti-rabbit (1:1,000, sc-2054; Santa Cruz Biotechnology, Inc.)/mouse IgG secondary antibody (1:1,000, sc-2973; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence detection kit (Pierce; Thermo Fisher Scientific, Inc.) followed by autoradiography using Hyperfilm MP (GE Healthcare Life Sciences, Little Chalfont, UK). The average density of each band was quantified using Quality One system (version 4.6.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized with that of β-actin or TfR bands.

Acute dissociation of DRG neurons. Neurons were isolated from ipsilateral L4 and L5 DRG of adult rats using previously described methods (11). Briefly, DRGs were removed from each rat and minced in cold, oxygenated Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.). The DRGs were digested with collagenase type IA (3 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 45 min, followed by 0.25% trypsin (Sigma-Aldrich; Merck

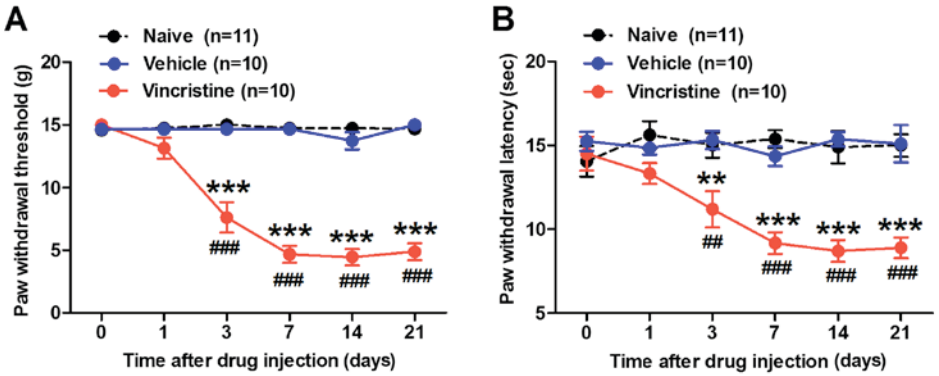

Figure 1. Changes in (A) 50% paw withdrawal threshold to von Frey filaments and (B) paw withdrawal latency to radiant heat in rats following administration of vincristine. **P<0.01 and ***P<0.001 compared with naive group; ##P<0.01 and ###P<0.001 compared with vehicle-treated group.

KGaA) for another 12 min at 37˚C. After terminating the enzymatic treatment by adding DMEM/10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), ganglia were dissociated by trituration with fire-polished glass pipettes. Isolated cells were plated on poly-D-lysine-coated glass coverslips placed in 24-well sterile tissue culture plates, and kept in a 5% CO_2 incubator at 37˚C for 2 h prior to patch clamp recording.

Whole-cell patch recording of TRPV1 current in dissociated DRG neurons. Whole-cell patch recording was performed as previously described (11). Two h after plating, the dissociated DRG neurons were perfused with extracellular solution containing 128 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 5 mM $MgCl_2$, 5.55 mM glucose, and 20 mM HEPES, adjusted to pH 7.4 with NaOH. Patch pipettes had a resistance of 5-8 MΩ when filled with internal solution containing 100 mM KCl, 10 mM EGTA, 40 mM HEPES, 5 mM $MgCl_2$, and 1 mM Na_2ATP, adjusted to pH 7.3 with KOH. Under voltage-clamp recording, neurons were clamped at -70 mV, and series resistance was compensated to >75%. The membrane capacitance was read from the amplifier by patch master software (version 2x69; HEKA Elektronik Dr Schulze GmbH, Lambrecht/Pfalz, Germany) to determine the size of neurons and to calculate the current density. The agonist-evoked TRPV1 currents were measured by adding capsaicin (0.5 μM for 3 sec). Origin software 8.5 (OriginLab Corporation, Northampton, MA, USA) was used for data analysis.

Statistical analysis. Statistical analyses were performed with GraphPad Prism 5 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). All data were presented as mean ± standard error of the mean. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test or two-way ANOVA followed by Bonferroni's post hoc test were used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Vincristine-evoked mechanical allodynia and thermal hyperalgesia. To confirm the establishment of the vincristine-induced pain model, the 50% PWT and PWL to the radiant heat stimulation were measured prior to vincristine administration and on days 1, 3, 7, 10, 14, and 21 post-administration. Consistent with previous reports (17,20), the results demonstrated that rats in the vincristine-treated group exhibited significant mechanical allodynia and thermal hyperalgesia (Fig. 1). For example, the 50% PWT to von Frey filaments in the left hind paw in the vincristine-treated rats decreased from 15.00 g to 4.66±0.59 g on day 7 and 4.8±0.67 g on day 21 post-vincristine administration (P<0.001; Fig. 1A). The PWL in the vincristine-treated rats decreased from 14.50±1.02 sec to 9.17±0.64 sec on day 7 and 8.88±0.63 sec on day 21 post-administration (Fig. 1B). By contrast, the 50% PWT and PWL of the left hind paw in the vehicle-injected or naive rats exhibited no significant differences during the time course of the experiments (Fig. 1A and B, respectively).

Functional upregulation of TRPV1 in DRGs in vincristine-treated rats. Functional upregulation of TRPV1 has been implicated in different models of pathological pain (11,15,18). To evaluate whether TRPV1 may contribute to vincristine-induced pain, the total protein expression levels of TRPV1 in DRGs collected from vehicle or vincristine-treated rats were examined. Using western blot analysis, the results demonstrated that the expression levels of TRPV1 total protein in L4 and L5 DRGs increased significantly from day 7 to day 14 in vincristine-treated rats compared with vehicle-treated rats (P<0.05; n=7; Fig. 2A). Considering that membrane insertion of TRPV1 receptors in DRG neurons is important for the development of pain hypersensitivity, expression of membrane-bound TRPV1 receptors in DRGs was examined following vincristine administration. Using western blot analysis of membrane-specific fractions, the results demonstrated that the membrane expression of TRPV1 in L4 and L5 DRGs increased significantly from day 7 to day 14 in vincristine-treated rats compared with vehicle-treated rats (P<0.01; Fig. 2B). To determine the functional alteration of TRPV1 receptors in nociceptive DRG neurons in vincristine-treated rats, the TRPV1 receptor currents in DRG neurons were then measured with whole-cell patch clamp recording. As expected, TRPV1 current density was also significantly increased in DRG neurons from vincristine-treated rats compared with vehicle-treated rats (64.64±8.81 in vincristine-treated rats vs. 34.36±4.29 in vehicle-treated rats; P<0.01; Fig. 2C and D).

CPZ alleviates mechanical pain hypersensitivity in vincristine-treated rats. To examine whether the functional

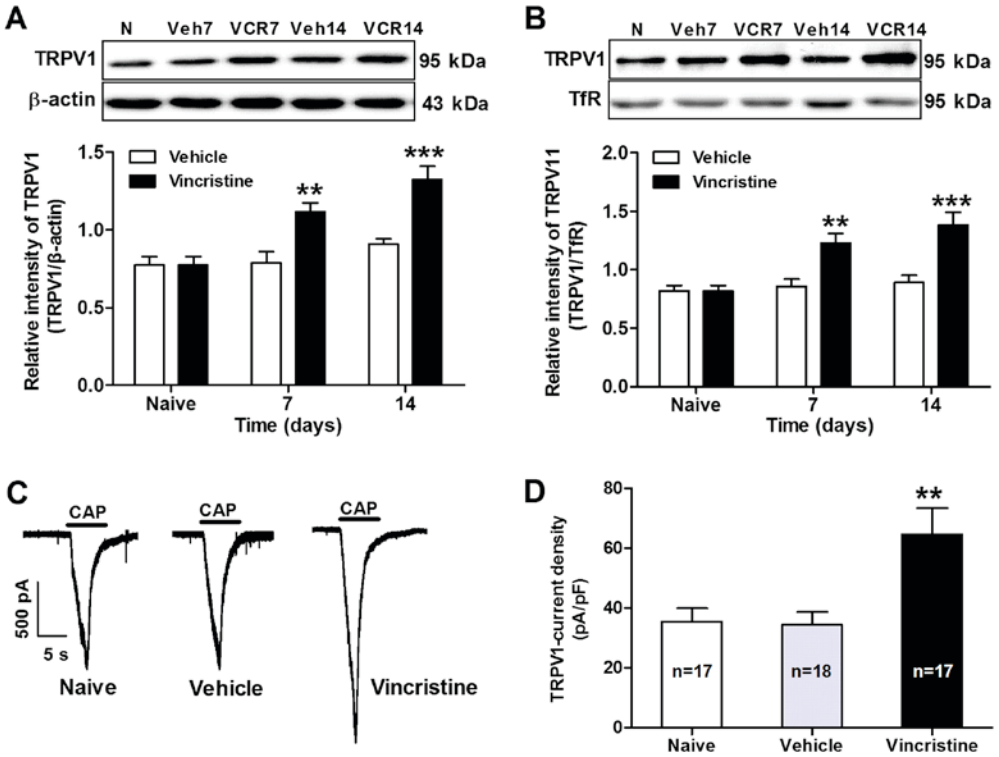

Figure 2. Functional upregulation of TRPV1 in vincristine-treated rat DRGs. (A) Representative blot images and quantification from western blot analysis of TRPV1 total protein expression levels in ipsilateral L4 and L5 DRGs (n=7/group). (B) Representative blot images and quantification from western blot analysis of TRPV1 membrane-bound protein expression levels in ipsilateral L4 and L5 DRGs (n=7/group). (C) Representative TRPV1 currents evoked by application of 0.5 μM CAP for 3 sec in naive, vehicle-treated and vincristine-treated rat DRG neurons. (D) Quantification of the current density (pA/pF) of agonist-evoked TRPV1 currents. CAP-evoked TRPV1 currents were significantly enhanced in vincristine-treated rat DRG neurons. **P<0.01 and ***P<0.001 compared with vehicle-treated group. TRPV1, transient receptor potential cation channel subfamily V member 1; DRG, dorsal root ganglions; CAP, capsaicin; Veh, vehicle; VCR, vincristine; TfR, transferrin receptor.

potentiation of TRPV1 may have induced pain hypersensitivity in vincristine-treated rats, the present study examined whether CPZ, a TRPV1 receptor antagonist, could attenuate vincristine-induced pain in rats. CPZ or vehicle (DMSO) was intraperitoneally delivered to vincristine-treated rats on day 14, and both the PWT and PWL were measured at 2, 6, 12, 24 and 48 h post-CPZ or vehicle administration. As illustrated in Fig. 3, administration of CPZ could prominently reverse the vincristine-induced decrease in both PWT (Fig. 3A) and PWL (Fig. 3B). These data provided direct evidence that TRPV1 receptors contribute to vincristine-induced pain hypersensitivity in rats.

Sensitization of TRPV1 by TNF-α orchestrates the development of vincristine-induced pain. Previous studies have demonstrated that TNF-α is a key mediator in inflammatory and neuropathic hyperalgesia (24,25). An increase of TNF-α protein levels in the spinal cord has been observed following administration of vincristine in rats, and inhibition of TNF-α synthesis attenuated neuropathic pain caused by vincristine (16,17). To explore whether TNF-α has a role in promoting functional upregulation of TRPV1 in DRG neurons in vincristine-treated rats, the present study first examined the expression levels of TNF-α in the DRGs in vincristine-treated rats using western blot analysis. As presented in Fig. 4A and B, expression of TNF-α in L4 and L5 DRGs increased markedly from day 7 to day 14 in vincristine-treated rats compared with vehicle-treated rats. Next, the TNF-α synthesis inhibitor thalidomide (50 mg/kg) was administered to the rats 2 h prior to vincristine administration and TRPV1 expression was examined on day 14. Using western blot analysis, the results demonstrated that the total (Fig. 4C) and membrane-bound (Fig. 4D) TRPV1 expression levels in L4 and L5 DRGs decreased significantly on day 14 post-thalidomide administration. In addition, the pain behaviors were evaluated in the thalidomide-treated rats. The results demonstrated that thalidomide administration significantly inhibited the vincristine-induced decreases in PWT (11.07±1.51 g in thalidomide+vincristine-treated rats vs. 4.87±0.65 g in vincristine alone-treated rats; P<0.001; Fig. 4E) and in PWL (11.98±0.99 sec in thalidomide+vincristine-treated rats vs. 8.32±0.39 sec in vincristine alone-treated rats; P<0.01; Fig. 4F) in vincristine-treated rats on day 14. These results indicated that TNF-α has a positive role in promoting functional upregulation of TRPV1 in DRG neurons in vincristine-induced neuropathic rats.

Discussion

Pain is an early and especially disabling symptom of vincristine-induced peripheral neurotoxicity, which reduces the quality of daily life in cancer patients (26,27). DRGs are hypothesized to be one of the preferential sites in which chemotherapy neurotoxicity occurs because of the absence of blood-nerve barrier (7,8). As is well-known, one of the pathologic mechanisms of pain is associated with sensitization of primary sensory neurons. Cumulative evidence indicates that TRPV1 expression and

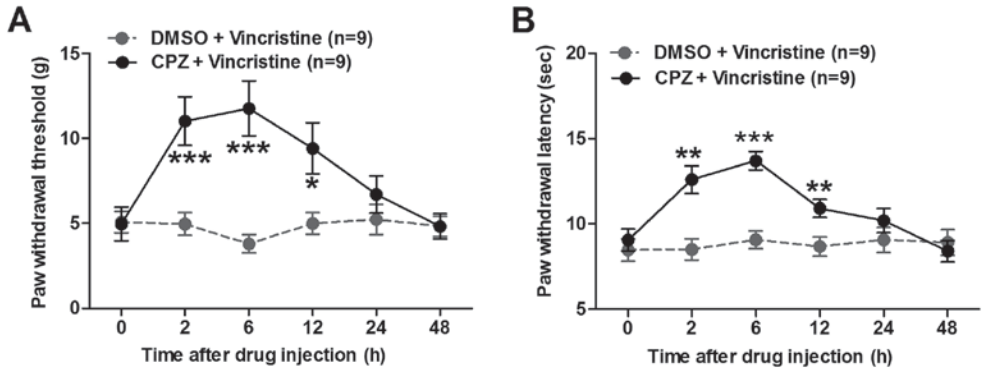

Figure 3. Effects of CPZ administration, a TRPV1 antagonist, on mechanical allodynia and thermal hyperalgesia in vincristine-induced neuropathic rats. CPZ administration dramatically alleviated the vincristine-induced mechanical allodynia and thermal hyperalgesia as measured by (A) paw withdrawal threshold and (B) paw withdrawal latency, respectively, in vincristine-treated rats. *P<0.05, **P<0.01 and ***P<0.001 compared with vehicle-treated group. CPZ, capsazepine; TRPV1, transient receptor potential cation channel subfamily V member 1; DMSO, dimethyl sulfoxide.

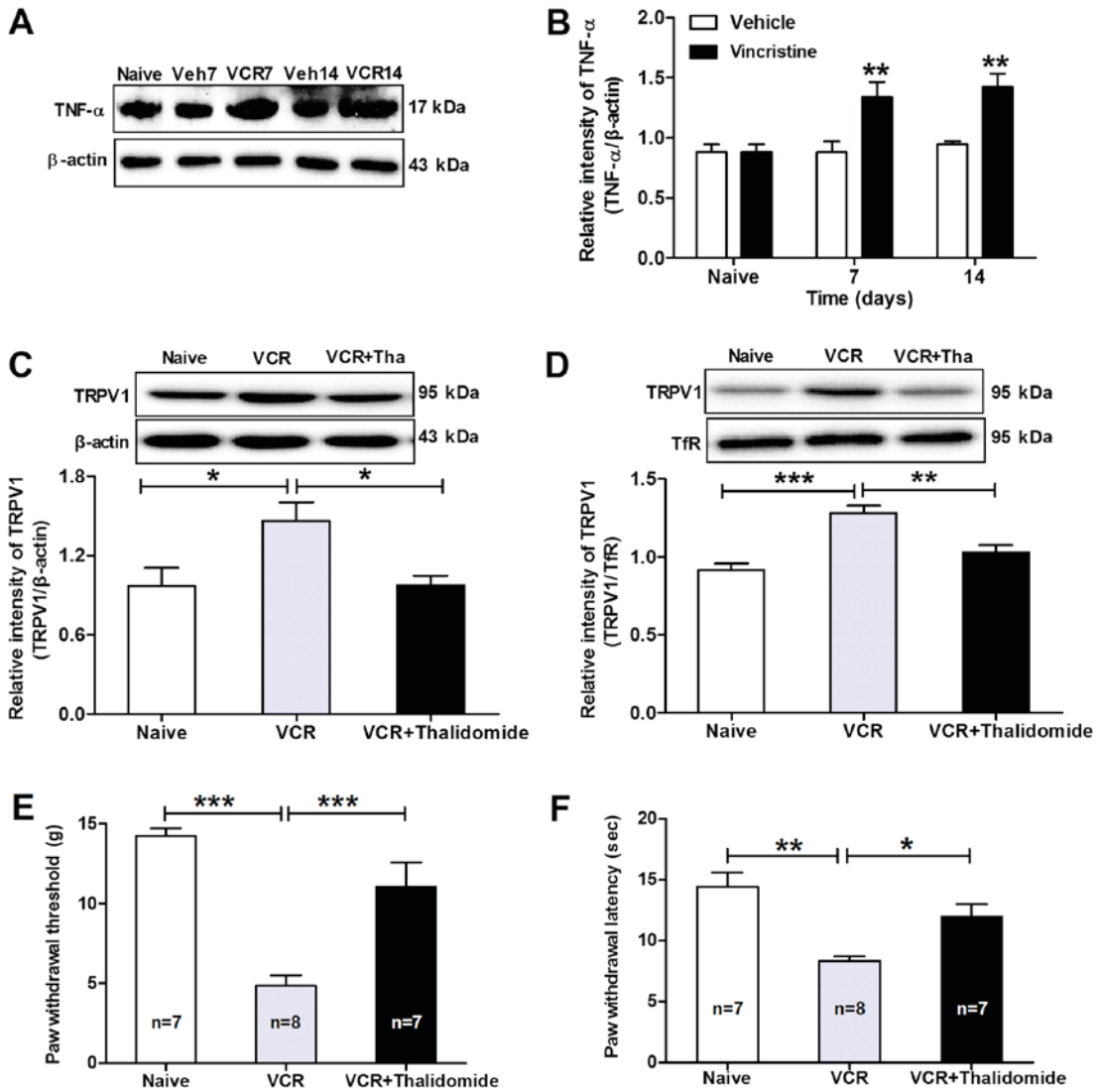

Figure 4. TNF-α promotes functional upregulation of TRPV1 in DRG neurons in vincristine-treated rats. (A) Representative images and (B) quantification from western blot analysis of TNF-α protein expression levels in the DRGs of vincristine-treated rats. TNF-α protein expression levels increased significantly from day 7 to day 14 in vincristine-treated rats compared with vehicle-treated rats. **P<0.01 compared with vehicle-treated group (n=5/group). (C) Western blot analysis of TRPV1 total and (D) membrane-bound protein expression levels in ipsilateral L4 and L5 DRGs following treatment with thalidomide in vincristine-treated rats. β-actin and TfR were used as internal controls for total and membrane-bound protein, respectively. *P<0.05, **P<0.01 and ***P<0.001, with comparisons indicated by brackets (n=5/group). (E) Effects of pre-treatment with thalidomide on the mechanical allodynia and (F) thermal hyperalgesia of vincristine-treated rats. Note that thalidomide pre-treatment significantly prevented the vincristine-induced decreases in paw withdrawal threshold and paw withdrawal latency in vincristine rats. *P<0.05, **P<0.01 and ***P<0.001, with comparisons indicated by brackets. TNF, tumor necrosis factor; TRPV1, transient receptor potential cation channel subfamily V member 1; DRG, dorsal root ganglions; TfR, transferrin receptor; Veh, vehicle; VCR, vincristine.

function in DRGs is greatly sensitized following tissue inflammation or nerve injury, thus leading to peripheral sensitization and pain hypersensitivity (12,14). Therefore, the present study examined the changes in TRPV1 expression and function in DRG sensory neurons. As expected, the results revealed that the expression as well as the function of TRPV1 receptors was significantly increased in DRGs in vincristine-treated rats. Furthermore, administration of the TRPV1 antagonist CPZ significantly attenuated vincristine-induced mechanic allodynia and thermal hyperalgesia. Collectively, these results indicated that TRPV1 contributes to vincristine-induced pain sensation. To the best of our knowledge, this is the first report providing a direct link between functional upregulation of TRPV1 and vincristine-induced pain behaviors.

A growing number of studies have demonstrated that TNF-α serves a vital role in the development of pathological pain (16,28,29). For example, TNF-α is overexpressed in the spinal cord or DRGs when chronic pain happens, while administration of TNF-α elicits ectopic discharges in DRG neurons and pain hypersensitivity in normal animals (16,30-32). In addition, treatment with a TNF-α antibody or TNF receptor antagonist ameliorates pain hypersensitivity in several models of pathological pain (16,30-32). In agreement with these reports, the present study also demonstrated that TNF-α was upregulated in DRGs in vincristine-treated rats. In addition, the present results demonstrated that thalidomide, an inhibitor of TNF-α synthesis, attenuated vincristine-induced TRPV1 total and membrane protein expression. Obviously, TNF-α not only promotes TRPV1 expression at a translational level, but may also induce the directed TRPV1 trafficking to the surface membrane in DRGs in vincristine-treated rats. Consistent with the present results, emerging evidence has suggested that TNF-α overexpression in sensory neurons results in increased cyclin-dependent kinase 5 activity which phosphorylates TRPV1 at Thr^{406} and promotes its surface localization (19,33). In addition to regulating TRPV1 membrane translocation, TNF-α has been reported to increase TRPV1 total protein levels via a translational regulation. For example, long-term incubation of DRG neurons with TNF-α significantly increases the proportion of TRPV1-immunoreactive neurons through the activation of extracellular signal-regulated kinase signaling pathway (34). Hence, it can be concluded that TNF-α sensitizes TRPV1 by increasing TRPV1 expression and by mobilizing new channels to the neuronal surface in vincristine-induced pain hypersensitivity. Taken together, the present results suggested that TNF-α could sensitize TRPV1 by promoting its expression, thus leading to mechanical allodynia and thermal hyperalgesia in vincristine-treated rats. These findings may enhance the current understanding of the pathophysiological mechanisms underlying vincristine-induced pain.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

YW, WL and JH conducted the electrophysiological studies and participated in the design of the study. CF, HH and JH participated in the behavioral test and performed the statistical analysis. JW, XL and SW performed the western blotting, and part of the behavioral test. SQX participated in the design of the study. DF conceived of the study, participated in its design and coordination, and drafted the article. TL performed the statistical analysis and participated in the preparation of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experimental procedures were carried out in accordance with the guidelines of the International Association for the Study of Pain and were approved by the Animal Experimentation Ethics Committee of Henan University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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