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Cellular/Molecular

Zinc Inhibits TRPV1 to Alleviate Chemotherapy-Induced Neuropathic Pain

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Zinc is a transition metal that has a long history of use as an anti-inflammatory agent. It also soothes pain sensations in a number of animal models. However, the effects and mechanisms of zinc on chemotherapy-induced peripheral neuropathy remain unknown. Here we show that locally injected zinc markedly reduces neuropathic pain in male and female mice induced by paclitaxel, a chemotherapy drug, in a TRPV1-dependent manner. Extracellularly applied zinc also inhibits the function of TRPV1 expressed in HEK293 cells and mouse DRG neurons, which requires the presence of zinc-permeable TRPA1 to mediate entry of zinc into the cytoplasm. Moreover, TRPA1 is required for zinc-induced inhibition of TRPV1-mediated acute nociception. Unexpectedly, zinc transporters, but not TRPA1, are required for zinc-induced inhibition of TRPV1-dependent chronic neuropathic pain produced by paclitaxel. Together, our study demonstrates a novel mechanism underlying the analgesic effect of zinc on paclitaxel-induced neuropathic pain that relies on the function of TRPV1.

Key words: neuropathic pain; paclitaxel; TRPA1; TRPV1; zinc

Significance Statement

The chemotherapy-induced peripheral neuropathy is a major limiting factor affecting the chemotherapy patients. There is no effective treatment available currently. We demonstrate that zinc prevents paclitaxel-induced mechanical hypersensitivity via inhibiting the TRPV1 channel, which is involved in the sensitization of peripheral nociceptors in chemotherapy. Zinc transporters in DRG neurons are required for the entry of zinc into the intracellular side, where it inhibits TRPV1. Our study provides insight into the mechanism underlying the pain-soothing effect of zinc and suggests that zinc could be developed to therapeutics for the treatment of chemotherapy-induced peripheral neuropathy.

Introduction

Chemotherapy-induced peripheral neuropathy (CIPN), a highly debilitating symptom without effective treatment, affects >50% of patients undergoing treatment with commonly used chemotherapy drugs, including paclitaxel, vincristine, and oxaliplatin (Addington and Freimer, 2016). The painful neuropathy usually leads to dose reduction or discontinuation of the treatment, resulting in poor treatment outcomes. Currently, how chemotherapies with certain drugs cause pain and neuropathy is not well understood, which has severely limited the development of effective pain therapies (Hershman et al., 2014; Miltenburg and Boogerd, 2014). Therefore, it is critical to understand the molecular and cellular mechanisms underlying the pathogenesis of CIPN to develop effective and safe therapeutics for the treatment of CIPN.

Pain results from complex processing of neural signals at different levels. Transient receptor potential (TRP) channels are molecular sensors for noxious mechanical, chemical, and thermal insults. Activation of peripheral nociceptors by TRP channels, especially the capsaicin receptor TRPV1 and wasabi receptor TRPA1, initiates neurogenic inflammation and pain sensation (Caterina et al., 2000; Lee et al., 2005; Bautista et al., 2006; Huang et al., 2008). TRPV1 is expressed in nearly 60% of peptidergic small-diameter primary nociceptors in the dorsal root ganglia (DRG) and trigeminal ganglia, which sense environmental cues

in the skin and many visceral organs (Xu et al., 2013). TRPV1 is a nonselective cation channel that is activated by capsaicin, noxious heat, acid, and many endogenous ligands as well as plant-derived natural compounds. In addition to direct activation, TRPV1 is also sensitized by activation of GPCRs and tyrosine kinase receptors through intracellular signal transduction pathways involving activation of many protein kinases, including protein kinase C (PKC), protein kinase A (PKA), and phosphoinositide 3-kinase (PI3K). Both activation and sensitization of TRPV1 lead to enhanced pain responses. Both genetic ablation and pharmacological inhibition studies have provided convincing evidence that TRPV1 significantly contributes to both chronic inflammatory pain and neuropathic pain resulting from peripheral nerve injury. Moreover, both TRPV1 and TRPA1 have been shown to be involved in the pathogenesis of CIPN (Boyette-Davis et al., 2015).

Zinc is an essential metal that has been extensively used to promote wound healing in humans for almost 3000 years (Lansdown et al., 2007). It acts as an anti-inflammatory agent and soothes pain sensation in a number of model systems (Safieh-Garabedian et al., 1996; Larson and Kitto, 1999; Liu et al., 1999; Nozaki et al., 2011). Zinc is present in the spinal cord and DRG neurons, including small-diameter nociceptive neurons (Velázquez et al., 1999; Koeppen et al., 2013). Indeed, depletion of vesicular zinc in the spinal cord dorsal horn enhances neuropathic pain in mice (Jo et al., 2008). Moreover, zinc-deficient mice have increased prostaglandin E_2 levels and sensitized nociceptive C fibers, suggesting that endogenous zinc might exert a tonic inhibition on nociception (Izumi et al., 1995). Furthermore, centrally applied zinc attenuates neuropathic pain in mouse models of peripheral neuropathy (Liu et al., 1999; Nozaki et al., 2011) and clinical studies (Pastorfide et al., 1989; Zekavat et al., 2015). However, it is not known whether exogenously applied zinc in the periphery can affect CIPN.

In the present study, we show that local application of zinc inhibits mechanical hypersensitivity induced by paclitaxel treatment in mice in a dose-dependent manner. The zinc-induced analgesic effect is severely attenuated in TRPV1-deficient mice. Extracellular zinc application inhibits capsaicin-evoked intracellular calcium ($[Ca^{2+}]_i$) responses and membrane depolarization in a subset of mouse DRG neurons, and locally injected zinc inhibits capsaicin-evoked acute nociceptive behaviors. Moreover, zinc inhibits capsaicin-induced $[Ca^{2+}]_i$ responses and membrane current intracellularly, and the zinc-sensitive TRPA1 is required for the inhibition of TRPV1 by extracellular zinc. Surprisingly, zinc transporters, but not TRPA1, are required for inhibition of paclitaxel-induced chronic neuropathic pain by zinc. Together, our data demonstrate that TRPV1-mediated pain sensations are inhibited by extracellular zinc entering the cells through either TRPA1 channels or zinc transporters under acute and chronic settings.

Materials and Methods

Animals. Male and female C57BL/6J mice (The Jackson Laboratory), congenic TRPV1 knock-out (KO) (The Jackson Laboratory), and congenic TRPA1 KO mice at the age of 7–10 weeks were used in this study. The TRPA1 KO mice on the C57BL/6J background were described previously (Cruz-Orengo et al., 2008). All animal care and experimental procedures were in accordance with the animal care and use protocol approved by the Institutional Animal Care and Use Committee of University of Texas Health Science Center at Houston and the Institutional Animal Care and Use Committee at Washington University School of Medicine in St. Louis. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath et al., 2010). Mice were housed in a temperature

(24°C)- and humidity (40%–50%)-controlled environment on a 12:12 h dark-light cycle with free access to food and water. All experiments were performed blind with respect to genotypes and treatments.

Paclitaxel treatment and von Frey test. Paclitaxel (TSZ Chem) was administered at a dose of 4 mg/kg intraperitoneally on days 0, 2, 4, and 6 as illustrated in Figure 1A according to previous studies (Matsumoto et al., 2006). Mechanical allodynia was measured as the hindpaw withdrawal response to von Frey hair stimulation using the up-and-down method as described in our previous study (Yin et al., 2013). Intraplantar injection of zinc acetate (ZnAc) (Sigma-Aldrich) was performed on day 7 following the von Frey test.

Intrathecal delivery of siRNA. To knockdown the ZIP family of zinc transporters (ZIPs), the siRNAs specific for mZIP3, mZIP6, and mZIP7 and the siRNA Universal Negative Control were purchased from Sigma-Aldrich, and 0.5 nmol of each was prepared in PBS and mixed with polyethylenimine. After 15 min incubation at room temperature, the mixture was injected intrathecally into mice anesthetized with isoflurane. Animals were used 3 d after the final intrathecal injection.

Cell culture and transfection. HEK293 cells were obtained from ATCC in 2010 and have been tested to confirm lack of mycoplasma contamination; however, no additional authentication has been performed. Cells were grown as a monolayer using passage numbers <30 and maintained in DMEM (Invitrogen), supplemented with 10% FBS (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37°C with 5% CO_2. The cells were transiently transfected with complementary DNA for mouse TRPV1 (mTRPV1), human TRPA1 (hTRPA1), and hTRPA1-D915A mutant using Lipofectamine 2000 (Invitrogen) with a ratio of 0.3:1. After transfection, cells were maintained in DMEM at 37°C for 24 h before use. The hRPA1-D915A mutant was made using the QuikChange II XL mutagenesis kit (Agilent Technologies), according to the manufacturer's directions and confirmed by DNA sequencing.

Retrograde labeling of paw-innervating DRG neurons. Mice were anesthetized with isoflurane, and 10 μl of 1,1'-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate (FAST DiI) (10 mg/ml in methanol) was injected into the paws of paclitaxel-treated mice. To prevent leakage and labeling of adjacent tissues, the needle was left in place for 30 s after each injection, and any leaked dye was removed with a cotton swab. DRG neurons were isolated 5 d after DiI injection.

Isolation and culture of DRG neurons. Mice were killed by cervical dislocation following CO_2 asphyxia. Spinal columns were removed and placed in ice-cold HBSS; neurons were acutely dissociated and maintained as described previously (Hu et al., 2009). In brief, laminectomies were performed and bilateral DRG or lumbar DRG of DiI-injection side were dissected out. After removal of connective tissues, DRG were transferred to a 1 ml Ca^{2+}/Mg^{2+}-free HBSS containing 2 μl saturated NaHCO3, 0.35 mg L-cysteine, and 20 U papain (Worthington), and incubated at 37°C for 15 min. The suspension of DRG was centrifuged, and the pellet was incubated in 1 ml Ca^{2+}/Mg^{2+}-free HBSS containing 4 mg collagenase Type II and 1.25 mg dispase Type II (Worthington) at 37°C for 15 min. After digestion, neurons were pelleted, resuspended in Neurobasal medium containing 2% B-27 supplement, 1% L-glutamine, 100 units/ml penicillin plus 100 μg/ml streptomycin, and 50 ng/ml nerve growth factor, plated on a 12 mm coverslip coated with poly-L-lysine (10 μg/ml), and cultured under a humidified atmosphere of 5% CO_2/95% air at 37°C for 18–24 h before use.

Patch-clamp recording. Whole-cell patch-clamp recordings were performed using an EPC 10 USB amplifier (HEKA Elektronik) at room temperature (22°C-24°C) on the stage of an inverted phase-contrast microscope equipped with a filter set for green and red fluorescence visualization. Pipettes pulled from borosilicate glass (BF 150-86-10; Sutter Instruments) with a Sutter P-97 pipette puller had resistances of 2–4 MΩ when filled with pipette solution containing the following (in mM): 140 CsCl, 1 EGTA, 1 $MgCl_2$, and 10 HEPES, pH 7.4, and 315 mOsm/L. Cells were continuously perfused with extracellular solution containing the following (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH, and the osmolarity was adjusted to ≈340 mOsm/L with sucrose. The whole-cell membrane currents were recorded using voltage ramp from −100 to 100 mV for 500 ms at holding

potential of 0 mV. For inside-out patch-clamp recordings, both the pipette solution and bath solution contained the following (in mM): 140 CsCl, 1 EGTA, 1 $MgCl_2$, 10 HEPES, pH 7.4. For current-clamp recording, the pipette solution contained the following (in mM): 140 KCl, 1 $MgCl_2$, 1 EGTA, 10 HEPES, and 5 ATP, pH 7.4, and 315 mOsm/L. Data were acquired using PatchMaster software (HEKA Elektronik). Currents were filtered at 2 kHz and digitized at 10 kHz. Data were analyzed and plotted using Clampfit 10 (Molecular Devices). Values are given as mean ± SEM; n indicates the number of measurements.

Calcium imaging. Cultured DRG neurons were loaded with 4 μM Fura-2 AM (Invitrogen) in culture medium at 37°C for 60 min. Cells were then washed three times and incubated in HBSS at room temperature for 30 min before use. Fluorescence at 340 and 380 nm excitation wavelengths was recorded on an inverted Nikon Ti-E microscope equipped with 340 and 380 nm excitation filter wheels using NIS-Elements imaging software (Nikon). Fura-2 ratios (F340/F380) were used to reflect changes in intracellular Ca^{2+} upon stimulation. Values were obtained from 100 to 250 cells in time-lapse images from each coverslip. For FlexStation assays, HEK293 cells transfected with TRPV1 were plated in 96-well plate and maintained for 24–48 h before use. Cells were loaded with Fluo-4 according to the manufacturer's protocol, washed again, and placed on FlexStation 3 (Molecular Devices) to measure fluorescence increase upon addition of agonist in the absence or presence of different zinc compounds as described previously (Luo et al., 2011).

Quantitative RT-PCR. Total RNA was extracted from mouse DRG using the RNeasy kit (QIAGEN) according to the manufacturer's instructions. In brief, the mouse DRG were collected and homogenized. A total of 1 μg RNA was treated with DNase I (Invitrogen), and the cDNA was synthesized *in vitro* using ThermoScript RT-PCR System kit (Invitrogen). The real-time PCR was conducted following the protocol for Fast SYBR Green Master Mix kit (Applied Biosystems) in the StepOnePlus Real-Time PCR System (Applied Biosystems) with primers presented in Table 1. The expression levels of ZIPs were normalized to GAPDH using the $2^{-\Delta CT}$ method.

Nocifensive behavior. Each mouse was placed individually in clear Plexiglas chambers (8 × 8 × 12 cm) and acclimated for at least 1 h to the testing environment before all experiments. To measure pain-related behaviors, the left hind-paws of mice were injected intraplantarly with 10 μl vehicle (0.9% saline + 5% DMSO + 0.5‰ Tween 80; Sigma-Aldrich) with or without chemicals. The protocol for chemicals injection was represented in Figures 1A, E, and 5B. Time spent on nocifensive behavior (flicking and licking injected paw) was recorded for 5 min.

Experimental design and statistical analysis. Both male and female mice were used in this study. All data are presented as mean ± SEM for n independent observations. Statistical analysis was performed using GraphPad Prism 5.0 software. Unpaired Student's t test was used to analyze statistical significance between two groups (see Figs. 1E, 4A–D, 5E,H, and 6C). One-way ANOVA was used where more than two groups were compared (see Figs. 2C,D, 3B,C). Repeated-measures ANOVA was used to compare differences between multiple groups occurring over time (Figs. 1B–D, 5A, and 6B). The *post hoc* Bonferroni test was per-

Table 1. Primer sequences used for the measurement of ZIPs in mouse DRG with real time RT-PCR[a]

Name	Forward (5′ →3′)	Reverse (5′ →3′)	Length (bp)
mZIP1	TGCTTGTGTCCTGGTCTTCTC	ACAGGCTGACTGCCAGAATG	140
mZIP2	AGCCGCTGGCACGTTTTTATA	ACCCAGCAGCCACACAGCTA	104
mZIP3	TGTCAGCTTCTCCTATGGCTTGT	GGATCCCGCCTGCACTAATA	64
mZIP4	TCTGAGAAAGATGGGCCTTGTAG	TGTTTGGACTGTCGGAGATTG	89
mZIP5	TCCAGTGGCCTCAGCACTA	AGCAGCTTCCGAAAGGATAA	107
mZIP6	ACAGGGATATTCATCGGGCATTA	AACCAGAGCGACATACATGAACAAG	90
mZIP7	TGCTGCCTGAGCTATTGAGAGA	AATCAGTACCATCATGGCAACAC	92
mZIP8	AACAATTGCCTGGATGATCAC	CAAAGTACAAGATGCCCCAATC	82
mZIP9	TAAGAGCAGTAAAGAAGCCCTTTCA	ACGGTGGCAACATAAAGAAATC	90
mZIP10	GCCCTTCACCAGAGACCAATAA	CCTCCTGACCTTCACTGACTTCA	65
mZIP11	CTCACCTGGGTGCTACAGAAGAC	CAATGCAGGGTCCAAGTTCA	62
mZIP12	AATGTGCCAGCCTCCAACA	TTACTAGGCCATCTGCAAAATTGTG	100
mZIP13	TGCTTGCCAACACCATAGACAAC	CAGAAGCCCGATCTTTTTGC	83
mZIP14	TCTGCCAGGAGGATGAGAAG	ATAATGGAGAAGCCGGTTAGG	85
mGAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	100

[a]mZIP, Mouse zinc transporter of ZIP family; mGAPDH, mouse glyceraldehyde 3-phosphate dehydrogenase.
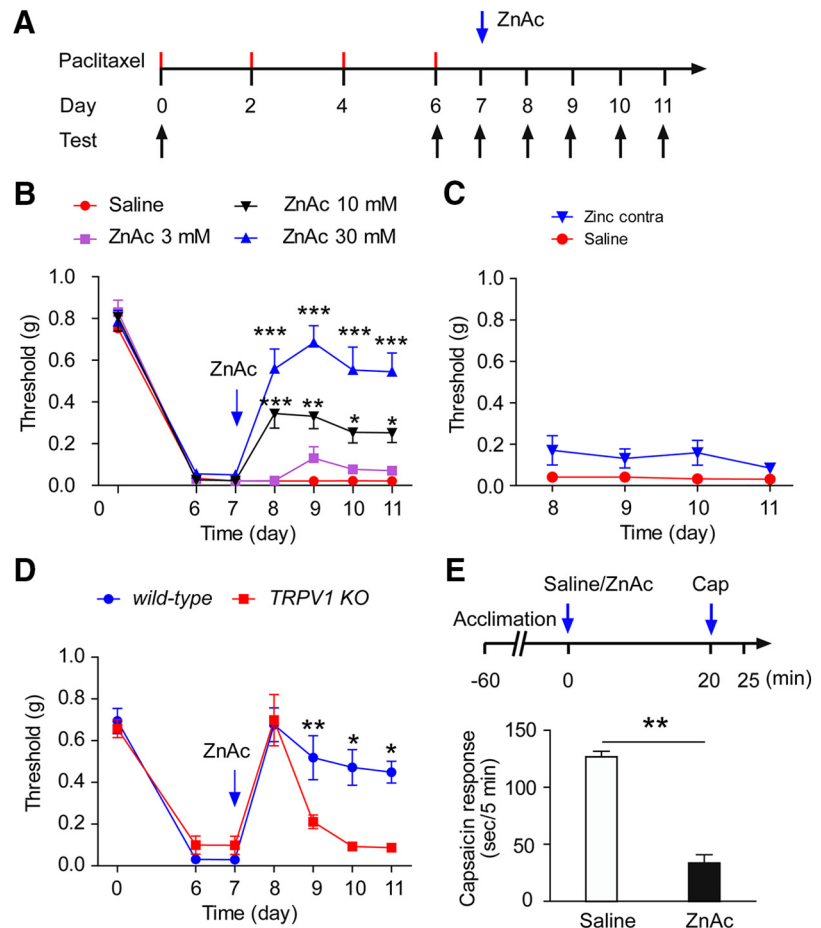

Figure 1. Zinc inhibits paclitaxel-induced mechanical hypersensitivity in a TRPV1-dependent manner. *A*, Schematic protocol of induction of mechanical hypersensitivity by paclitaxel and administration of zinc. Paclitaxel (4 mg/kg) was administered through intraperitoneal injections at days 0, 2, 4, and 6. ZnAc (3, 10, or 30 mM, 10 μl) was injected into the plantar of paclitaxel-treated mice at day 7. Black arrows indicate the dates when von Frey tests were performed. *B*, Dose–dependent inhibition of paclitaxel-induced mechanical hypersensitivity by ZnAc. *$p < 0.05$ versus saline group (repeated-measures ANOVA). **$p < 0.01$ versus saline group (repeated-measures ANOVA). ***$p < 0.001$ versus saline group (repeated-measures ANOVA). $n = 5$ mice in each group. *C*, Time courses show the hypersensitivity induced by paclitaxel in the contralateral paws of ZnAc- and saline-injected mice. $n = 5$ mice in each group. *D*, Time courses represent the effect of ZnAc on paclitaxel-induced mechanical hypersensitivity in both wild-type ($n = 7$ mice) and TRPV1 KO ($n = 5$ mice) mice. *$p < 0.05$ versus wild-type group (repeated-measures ANOVA). **$p < 0.01$ versus wild-type group (repeated-measures ANOVA). *E*, Top, Schematic protocol of ZnAc inhibition of capsaicin-elicited acute nocifensive responses. Bottom, Bar chart represents the inhibition of ZnAc on capsaicin (0.5 μg, 10 μl)-induced acute nociception. **$p < 0.01$ versus saline group (Student's t test). $n = 7$ mice for saline; and $n = 6$ mice for ZnAc.
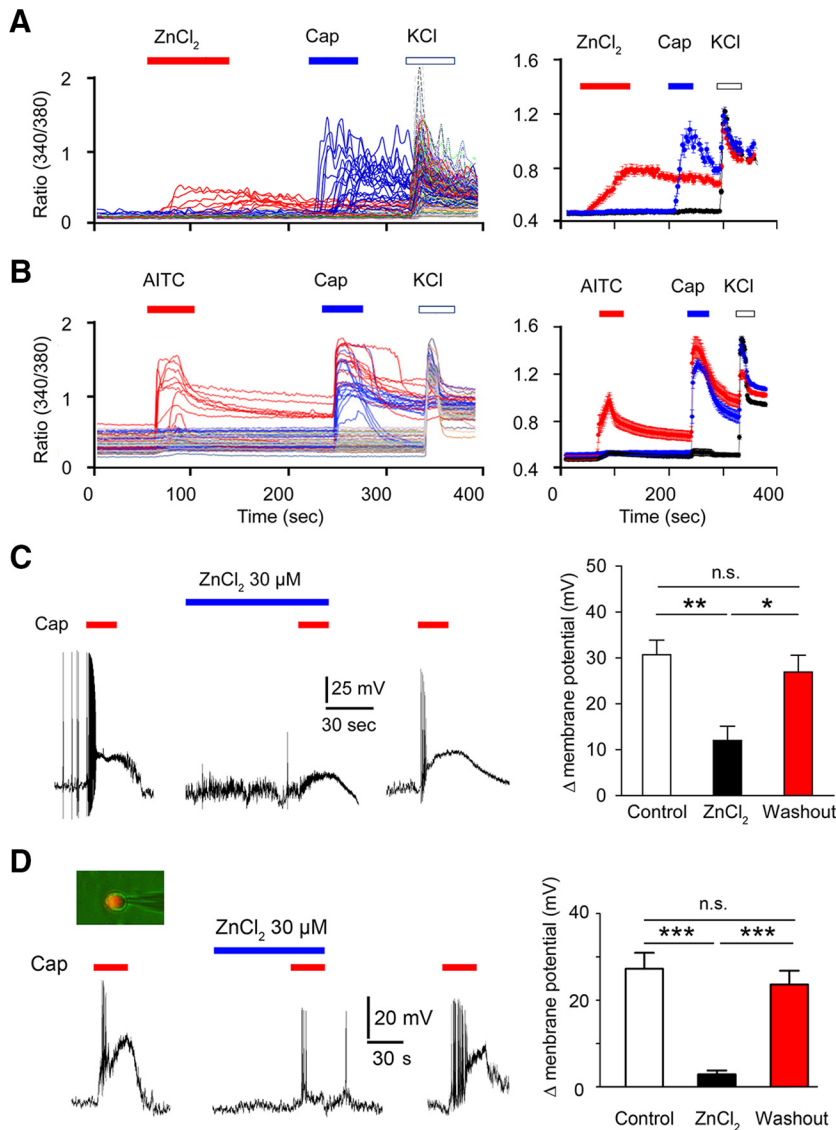
Figure 2. Zinc inhibits capsaicin-induced $[Ca^{2+}]_i$ response in a subset of DRG neurons. ***A***, Ratiometric Ca^{2+} imaging of cultured wild-type mouse DRG neurons. Each trace corresponds to fluorescence in a single neuron. Neurons were exposed to 30 μM $ZnCl_2$, 1 μM capsaicin, and 100 mM KCl for the indicated times (colored bars). Red represents zinc-responsive neurons, which showed no response to subsequent capsaicin application. Blue represents zinc-insensitive neurons, which responded to subsequent capsaicin application. The remaining traces were recorded from the zinc- and capsaicin-insensitive cells. Right, Averaged responses of the three subsets of DRG neurons. ***B***, Ca^{2+} imaging of wild-type DRG neurons, exposed to 100 μM AITC, 1 μM capsaicin, and 100 mM KCl for the indicated times. Red represents AITC-responsive cells, which responded to subsequent capsaicin application. Blue represents AITC-insensitive neurons, which also responded to subsequent capsaicin application. The remaining traces indicate the AITC- and capsaicin-insensitive neurons. Right, Averaged responses of the three subsets of DRG neurons. ***C***, Representative traces and summarized data showing capsaicin-induced membrane depolarization and action potential firing before (left), during (middle), and after (right) the application of zinc in an untreated wild-type mouse DRG neuron. Horizontal bars represent the indicated time course of chemical applications. n.s., Not significant versus control group. *$p < 0.05$ versus washout group (ANOVA). **$p < 0.01$ versus control group (ANOVA). $n = 7$ cells in each group. ***D***, Representative traces and summarized data showing the effect of $ZnCl_2$ on capsaicin-induced membrane depolarization and action potential firing in a DiI-labeled DRG neuron from a paclitaxel-treated mouse. Inset, Representative picture of the DiI-labeled DRG neuron in which the voltage traces in ***D*** were recorded. n.s., Not significant versus control group. ***$p < 0.001$ versus control and washout group (ANOVA). $n = 6$ cells in each group.

formed following ANOVA analysis. A p value of <0.05 was considered significantly different.

Results

Zinc inhibits paclitaxel-induced mechanical hypersensitivity in a TRPV1-dependent manner

Chemotherapy-induced neuropathic pain is a major dose-limiting adverse effect in cancer chemotherapy, and lacks effective treat-

ments. Because zinc is used to soothe pain and has shown promising effects in attenuating neuropathic pain caused by peripheral nerve injury (Liu et al., 1999; Nozaki et al., 2011), we explored the possibility that zinc might attenuate paclitaxel-induced mechanical hypersensitivity by applying ZnAc to the paw of paclitaxel-treated mice through intraplantar injections. After four paclitaxel injections (Fig. 1A), mice developed mechanical allodynia, which was markedly attenuated by administration of ZnAc in a dose-dependent manner ($F_{(3,96)} = 54.24$, $p < 0.0001$; Fig. 1B). Moreover, the effect of ZnAc lasted for >4 d after a single injection (Fig. 1B). On the other hand, ZnAc did not significantly inhibit paclitaxel-induced hypersensitivity of contralateral paws ($F_{(1,24)} = 3.42$, $p = 0.101$; Fig. 1C), suggesting that ZnAc mainly exerts the inhibitory effect locally. Previous studies have demonstrated that enhanced expression and function of TRPV1 are tightly correlated with paclitaxel-induced mechanical hypersensitivity, suggesting that targeting TRPV1 for inhibition could be a novel approach for alleviating chemotherapy-induced neuropathic pain (Anand et al., 2010; Chen et al., 2011; Hara et al., 2013; Li et al., 2015; Sisignano et al., 2016). We thus sought to determine whether inhibition of paclitaxel-induced mechanical hypersensitivity by zinc is related to the inhibition of TRPV1 function. The data showed that the zinc-induced sustained inhibitory effect was markedly attenuated in TRPV1 KO mice compared with wild-type mice ($F_{(1,60)} = 9.56$, $p = 0.011$; Fig. 1D). It should be noted that TRPV1 deficiency did not abolish the analgesic effect of zinc within the first 24 h after zinc administration, which suggests that TRPV1-independent pathways are involved in the early phase of zinc inhibition of paclitaxel-induced neuropathic pain. These results suggest that TRPV1 is critically involved in zinc-induced analgesic effect in paclitaxel-induced neuropathic pain. Furthermore, when we applied ZnAc 20 min before intraplantar injections of capsaicin (Fig. 1E), the capsaicin-induced acute nocifensive behaviors were also substantially reduced ($t_{(14)} = 5.43$, $p = 0.002$; Fig. 1E). Together, these results suggest that zinc inhibits both acute and chronic pain sensations mediated by TRPV1.

Extracellular zinc inhibits capsaicin response in DRG neurons

To elucidate the cellular basis of the inhibitory effect of zinc on TRPV1, we compared capsaicin-evoked $[Ca^{2+}]_i$ response in cultured mouse DRG neurons with and without pretreatment of $ZnCl_2$ (Sigma-Aldrich). Upon administration of extracellular
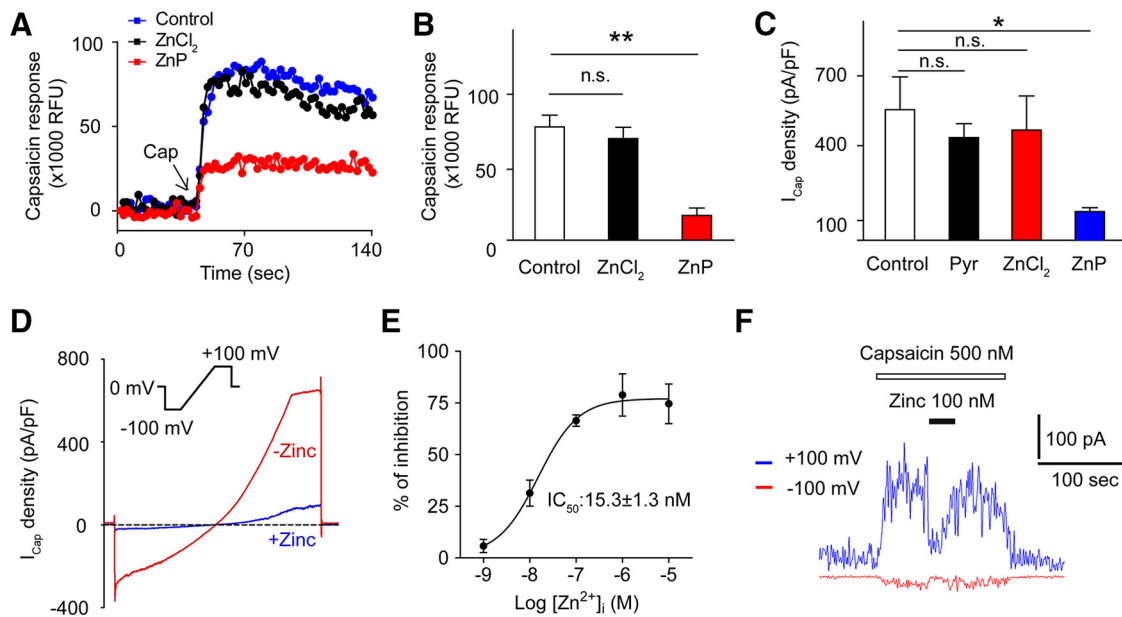
Figure 3. Intracellular, but not extracellular, zinc inhibits capsaicin-induced responses. **A**, Representative traces showing $[Ca^{2+}]_i$ responses induced by capsaicin in the presence of buffer (blue circle), $ZnCl_2$ (black circle), and ZnP (red circle) assayed by Flexstation. **B**, Bar graph represents the summarized data in **A**. **$p < 0.01$ versus control group. n.s., Not significant versus control group (ANOVA). $n = 6$ for each condition. **C**, Bar graph represents capsaicin-activated whole-cell current densities at the holding potential of +100 mV in TRPV1-expressing HEK293 cells in the presence of extracellular Pyr (30 μM), $ZnCl_2$ (30 μM), and ZnP (30 μM). *$p < 0.05$ versus control group. n.s., Not significant versus control group (ANOVA). $n = 5$ cells in each group. **D**, Representative current-voltage curves of capsaicin-activated membrane currents in response to voltage ramps from −100 mV to +100 mV (insert) in the absence (red) or presence (blue) of 100 nM zinc in the recording pipette. **E**, Concentration-response curve for the inhibitory effect of intracellular zinc was fitted with the logistic equation: $Y = Y_{min} + (Y_{max} - Y_{min})/(1 + 10^{\wedge}[(\log EC_{50} - X) \times \text{Hill slope}])$, where Y is the response at a given concentration, Y_{max} and Y_{min} are the maximum and minimum response, X is the logarithmic value of the concentration, and Hill slope is the slope factor of the curve. EC_{50} is the concentration that gives a response halfway between Y_{max} and Y_{min}. **F**, Representative traces show the capsaicin (500 nM)-activated macroscopic membrane currents from an inside-out membrane patch at holding potentials of −100 mV and +100 mV; 100 nM zinc nearly abolished the capsaicin-activated current when applied to the inside of the membrane. Horizontal bars represent the time course of chemical applications.

$ZnCl_2$, three populations of neurons responded differentially to subsequent applications of capsaicin: the first group of neurons had no or negligible $[Ca^{2+}]_i$ response to both $ZnCl_2$ and capsaicin but had a large response to KCl (Fig. 2A, black traces); the second group of neurons had no or negligible response to $ZnCl_2$ but retained a large capsaicin response (Fig. 2A, blue traces); and the third group of neurons had a large $[Ca^{2+}]_i$ response to $ZnCl_2$ but a negligible response to capsaicin (Fig. 2A, red traces). These results indicate that neurons exhibiting large responses to $ZnCl_2$ have severely attenuated capsaicin responses. Based on previous studies showing that TRPA1 coexpresses with TRPV1 (Story et al., 2003; Bautista et al., 2005) and TRPA1 mediates $ZnCl_2$-elicited $[Ca^{2+}]_i$ response in DRG neurons (Andersson et al., 2009; Hu et al., 2009), we speculated that activation of TRPA1 by $ZnCl_2$ inhibits TRPV1 function in the same DRG neurons. Unexpectedly, neurons that responded to preapplied allyl isothiocyanate (AITC) (Fig. 2B, red traces) still retained a large capsaicin-evoked $[Ca^{2+}]_i$ response, supporting reports that TRPA1 is expressed by a subset of TRPV1-expressing nociceptors (Story et al., 2003; Jordt et al., 2004), but activation of TRPA1 in general does not inhibit TRPV1 function. Therefore, zinc-induced inhibition of TRPV1 in DRG neurons is a unique property of zinc, separable from consequences resulting from activation of TRPA1 by other activators, such as AITC. We further examined the effect of zinc on capsaicin-induced excitation of DRG neurons using current-clamp recording. Consistent with Ca^{2+} imaging and behavioral results, capsaicin evoked a large membrane potential depolarization and robust action potential firing, which was significantly inhibited by pretreatment with extracellular $ZnCl_2$ ($F_{(2,18)} =$

8.81, $p = 0.002$; Fig. 2C). To determine whether zinc could inhibit TRPV1-mediated excitation of DRG neurons specifically innervating paws of paclitaxel-treated mice, we injected retrograde tracing dye DiI into the paws of paclitaxel-treated mice and examined the effect of $ZnCl_2$ on capsaicin-induced excitation of the DiI-labeled DRG neurons. Indeed, $ZnCl_2$ also severely reduced capsaicin-induced membrane depolarization of the labeled DRG neurons from mice treated with paclitaxel ($F_{(2,15)} = 22.20$, $p < 0.0001$; Fig. 2D), suggesting that zinc likely suppresses TRPV1 function in DRG neurons innervating both skin and visceral organs (Vysotskaya et al., 2014). Moreover, $ZnCl_2$ can inhibit TRPV1 function under normal conditions and in CIPN.

Zinc acts intracellularly to inhibit TRPV1
To further investigate the mechanism underlying zinc inhibition of TRPV1, we tested the effect of $ZnCl_2$ on TRPV1 heterologously expressed in HEK293 cells. Unexpectedly, we did not detect an inhibitory effect of $ZnCl_2$ on capsaicin-induced $[Ca^{2+}]_i$ response and membrane current when $ZnCl_2$ was applied extracellularly, suggesting that extracellular zinc does not inhibit TRPV1 function and the lack of inhibition of extracellular $ZnCl_2$ might be due to the lack of entry route for zinc into the HEK293 cells. To test this hypothesis, we used zinc pyrithione (ZnP), a zinc ionophore that allows zinc entry into cells (Andersson et al., 2009). Indeed, although pyrithione alone had no effect on the capsaicin-induced $[Ca^{2+}]_i$ response, ZnP exhibited a strong inhibition of capsaicin response when applied to the extracellular site, suggesting that zinc indeed acts intracellularly to inhibit TRPV1 function ($F_{(2,15)} = 19.73$, $p < 0.0001$; Fig. 3A,B), which is consistent with a previous report that zinc inhibited acid-evoked TRPV1 current intracellu-
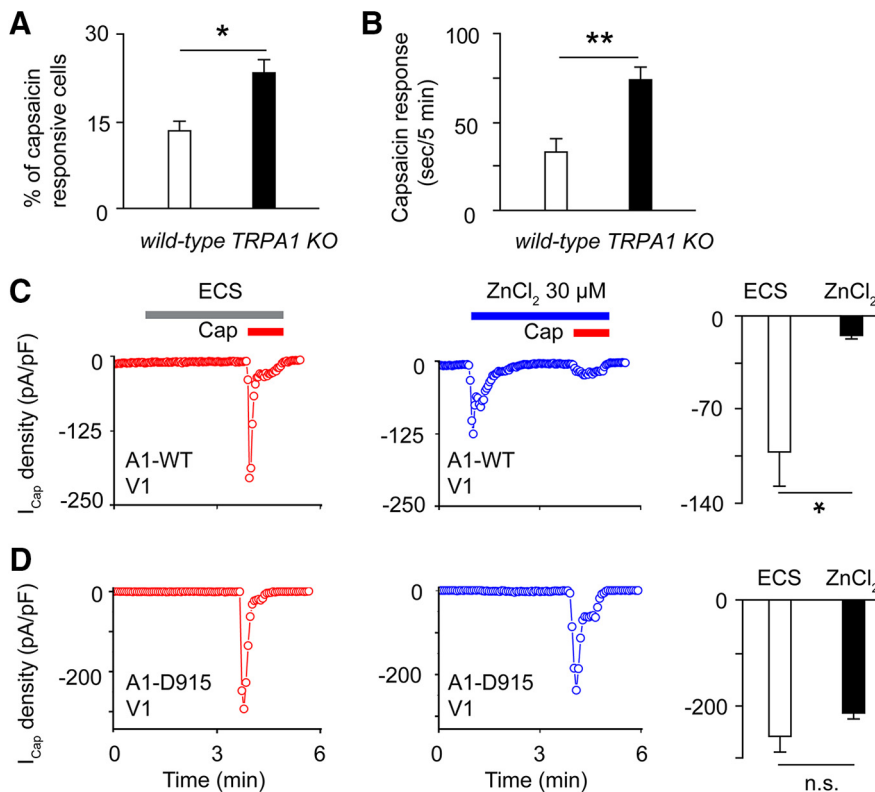
Figure 4. TRPA1 is required for the inhibitory effect of zinc on TRPV1-mediated acute responses. **A**, Bar graph represents the percentages of wild-type and TRPA1 KO DRG neurons responding to capsaicin in the presence of extracellular zinc (30 μM). *$p <$ 0.05 versus wild-type group (Student's t test). $n = 5$ coverslips in each group. **B**, Bar graph represents the effects of pretreatment with zinc (30 mM, 10 μl) on capsaicin (0.5 μg, 10 μl)-induced nociceptive behaviors in wild-type and TRPA1 KO mice. **$p <$ 0.01 versus wild-type group (Student's t test). $n = 7$ mice for the wild-type group and $n = 6$ mice for the TRPA1 KO group. **C**, Capsaicin (1 μM)-activated inward currents in the presence of normal extracellular buffer (ECS, left) or ECS plus zinc (30 μM, middle) in HEK293 cells coexpressing TRPV1 and wild-type TRPA1. Right, Summarized data. *$p <$ 0.05 versus ECS group (Student's t test). $n = 6$ cells in each group. **D**, Capsaicin (1 μM)-activated inward currents in the presence of ECS (left) or ECS plus zinc (30 μM, middle) in HEK293 cells coexpressing TRPV1 and the TRPA1 zinc permeation mutant (D915). The summarized data are shown on the right. n.s., Not significant versus ECS group (Student's t test). $n = 6$ cells in each group.

larly in rat vagal bronchopulmonary sensory neurons (Vysotskaya et al., 2014). This conclusion is also supported by the findings that capsaicin-activated whole-cell currents were severely reduced by application of extracellular ZnP but not pyrithione or ZnCl$_2$ ($F_{(3,16)} = 6.05$, $p = 0.006$; Fig. 3C). To further test this hypothesis, we applied ZnCl$_2$ (with a final free Zn^{2+} concentration of 100 nM calculated using Maxchelator program) directly to the cytoplasmic side of TRPV1-expressing HEK293 cells by recording pipette dialysis. Consistent with the ZnP result, intracellular ZnCl$_2$ reduced capsaicin-activated whole-cell currents at both positive and negative membrane potentials in a concentration-dependent manner (Fig. 3D,E). The concentration-response curve of Zn^{2+} inhibition reveals an IC$_{50}$ value of ~15 nM (Fig. 3E). In line with these findings, when applied directly to the cytoplasmic side of the membrane, zinc inhibited capsaicin-induced macroscopic current in inside-out patches isolated from the TRPV1-expressing HEK293 cells (Fig. 3F).

TPRA1 mediates the inhibitory effect of extracellular zinc on capsaicin-induced nociception

The finding that zinc acts intracellularly to inhibit TRPV1 raises the question about the route of zinc entry into DRG neurons. Our previous studies have identified TRPA1 as a major route for zinc entry into primary nociceptors because genetic ablation of TRPA1

function abolished the large zinc influx in DRG neurons (Hu et al., 2009). In addition, TRPA1 is selectively expressed by ~50% of TRPV1-positive primary sensory nociceptors (Story et al., 2003; Jordt et al., 2004; Bautista et al., 2005). We thus asked whether TRPA1 is required for inhibition of TRPV1 in DRG neurons by extracellular zinc. To test this hypothesis, we first examined the proportion of DRG neurons responding to capsaicin in both wild-type and TRPA1 KO mice in the presence of extracellular ZnCl$_2$. As shown in Figure 4A, deficiency of TRPA1 function significantly increased the proportion of capsaicin-responsive DRG neurons in the presence of extracellular ZnCl$_2$ ($t_{(8)} = 3.52$, $p = 0.023$), suggesting that TRPA1 is involved in the inhibition of capsaicin response by extracellular ZnCl$_2$. Consistent with this finding, zinc also exhibited significantly less inhibitory effect on capsaicin-evoked nocifensive response in the TRPA1 KO mice compared with the wild-type mice ($t_{(11)} = 4.003$, $p = 0.002$; Fig. 4B).

To further elucidate the role of TRPA1 in extracellular zinc-induced inhibition of TRPV1, we examined ZnCl$_2$-induced inhibition of capsaicin response in HEK293 cells coexpressing TRPV1 and TRPA1. In marked contrast to the finding that capsaicin response in HEK293 cells expressing TRPV1 alone was not inhibited by extracellular ZnCl$_2$ (Fig. 3C), capsaicin-induced current was substantially inhibited by extracellular ZnCl$_2$ in HEK293 cells cotransfected with TRPV1 and TRPA1

constructs ($t_{(10)} = 3.11$, $p = 0.023$; Fig. 4C), suggesting that TRPA1 can indeed serve as an important zinc carrier mediating zinc influx for inhibition of TRPV1 function in the same cell. We further investigated whether zinc permeation mutation of TRPA1 affects the inhibitory effect of zinc on capsaicin response by coexpressing TRPV1 and mutant TRPA1-D915A, which is functional but lacks zinc permeability (Hu et al., 2009). As expected, capsaicin-induced current was not significantly inhibited by extracellular zinc in HEK293 cells expressing TRPV1 and mutant TRPA1-D915A ($t_{(10)} = 1.366$, $p = 0.199$; Fig. 4D). Combined, these results demonstrate that TRPA1 serves as a cellular zinc entry route for the zinc inhibition of TRPV1function.

Inhibition of paclitaxel-induced chronic neuropathic pain by zinc requires zinc transporters but not TRPA1

Next, we tested whether TRPA1 is also required for zinc inhibition of paclitaxel-induced chronic neuropathic pain. To our surprise, the inhibitory effect of zinc on mechanical hypersensitivity induced by paclitaxel was not significantly different between TRPA1 KO mice and wild-type mice ($F_{(1,60)} = 0.01$, $p = 0.971$; Fig. 5A), suggesting that TRPA1 is not a primary carrier of zinc entry into primary nociceptors to inhibit TRPV1-dependent chronic neuropathic pain induced by paclitaxel. TRPA1 is a highly desensitizing channel, especially in the presence of physiological concentrations of extracellular Ca^{2+} (1–3 mM) (Akopian et al., 2007). The
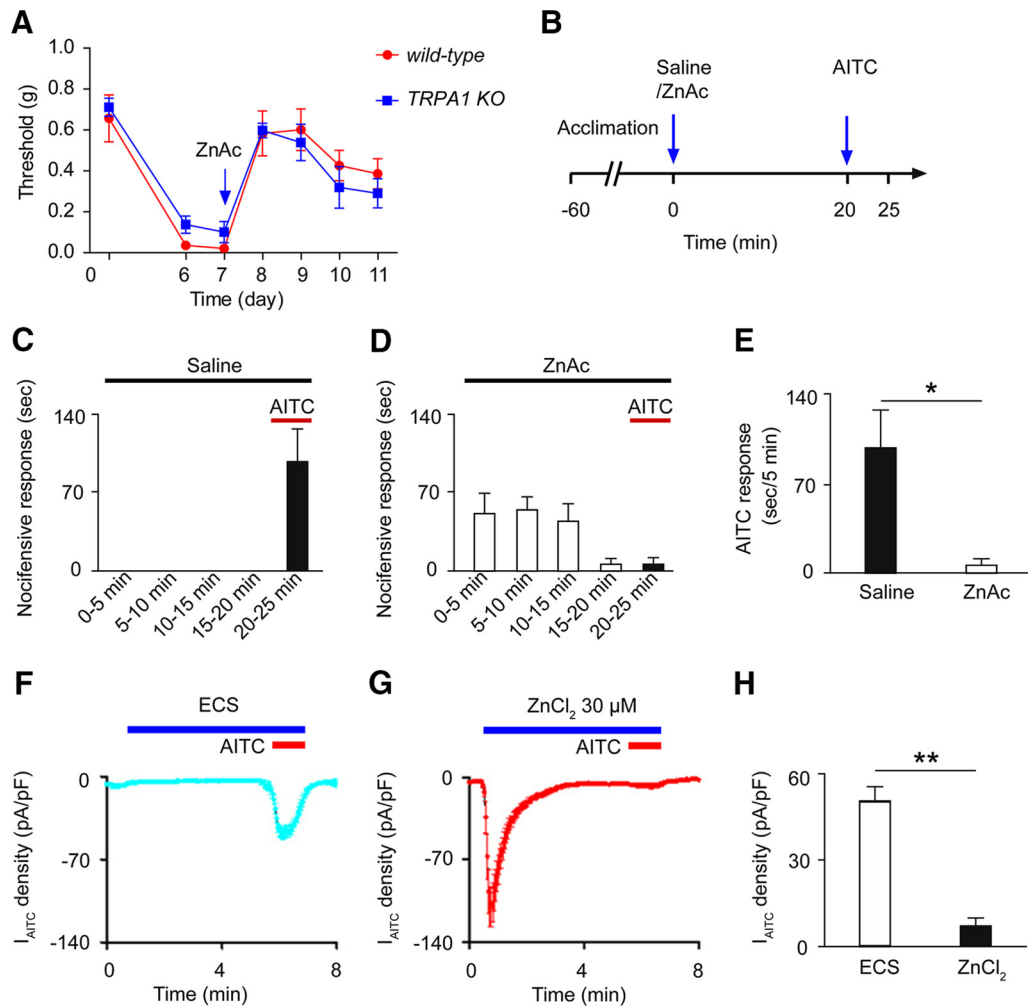
Figure 5. TRPA1 is not involved in zinc inhibition of paclitaxel-induced mechanical hypersensitivity, and zinc strongly desensitizes TRPA1-mediated current and acute nocifensive responses. **A**, Intraplantar injection of ZnAc (30 mM, 10 μl) produced a similar inhibition on paclitaxel-induced mechanical hypersensitivity in both wild-type and TRPA1 KO mice. $n = 6$ mice in each group. **B**, Schematic protocol of zinc-induced inhibition of AITC (75 mM, 10 μl)-elicited acute nocifensive responses. Animals were acclimated for 1 h before ZnAc (30 mM) or saline administration into the paws. Twenty minutes later, AITC (75 mM, 10 μl) was injected into the same paws. The nocifensive responses were recorded for 5 min after AITC administration. **C**, Bar graph demonstrates that AITC elicited a robust nocifensive response in mice pretreated with saline. **D**, Intraplantar injections of ZnAc (30 mM, 10 μl) produced a nocifensive response that gradually desensitized within 20 min after administration. Subsequent applications of AITC (75 mM, 10 μl) did not evoke measurable nocifensive responses. **E**, Bar chart represents AITC (75 mM, 10 μl)-elicited nocifensive responses in mice 20 min after pretreatment with either 10 μl saline or ZnAc (30 mM). *$p < 0.05$ versus saline group (Student's t test). $n = 5$ mice in each group. **F–H**, Extracellular application of zinc (30 μM) (**G**), but not ECS (**F**), nearly abolished membrane current activated by subsequent application of AITC (100 μM) in TRPA1-expressing HEK293 cells. **H**, Summarized data from **F** and **G**. **$p < 0.01$ versus ECS group (Student's t test). $n = 6$ for ECS and $n = 5$ for ZnCl₂.

fact that TRPA1 is involved in the zinc inhibition of capsaicin-induced acute nociception, but not paclitaxel-induced TRPV1-dependent chronic neuropathic pain, raises the possibility that TRPA1 might desensitize over time following initial activation in the presence of sustained high levels of extracellular zinc, making it unavailable for zinc influx. To test this hypothesis, we examined the effect of zinc on AITC-induced nocifensive behaviors. Consistent with our previous study (Hu et al., 2009), intraplantar injections of ZnAc elicited robust nocifensive behaviors lasting for ∼15 min. However, subsequent application of AITC failed to evoke obvious nocifensive behaviors, which is in marked contrast to the robust nociceptive responses induced by AITC in the absence of zinc ($t_{(8)} = 3.186$, $p = 0.013$; Fig. 5B–E). Similarly, AITC-induced membrane current in TRPA1-expresing HEK293 cells was also greatly attenuated by the preapplied zinc, which initially activated a large membrane current ($t_{(9)} = 3.716$, $p = 0.005$; Fig. 5F–H). These results support that zinc-elicited TRPA1 desensitization might account for the ineffectiveness of TRPA1 in mediating zinc inhi-

bition of TRPV1 in the chronic neuropathic pain induced by paclitaxel.

In addition to TRPA1, ZIPs also serve as important entry pathways for extracellular zinc in mammalian cells (Cousins et al., 2006; Kambe et al., 2015). We thus determined the expression of ZIPs in mouse DRG using quantitative RT-PCR. Among 14 known ZIPs, ZIP3, ZIP6, and ZIP7 were the most abundant isoforms in mouse DRG (Fig. 6A). We therefore knocked down the expression of these three ZIPs through intrathecal administration of siRNAs and then examined the inhibitory effect of zinc on paclitaxel-induced mechanical hypersensitivity. Strikingly, zinc-induced inhibitory effect on paclitaxel-induced mechanical hypersensitivity was significantly reduced in mice treated with siRNAs against ZIP3, ZIP6, and ZIP7 compared with mice treated with nontargeting control siRNA only ($F_{(1,48)} = 45.20$, $p < 0.0001$; Fig. 6B), suggesting that these three ZIPs are significantly involved in zinc inhibition of paclitaxel-induced chronic neuropathic pain. Consistent with behavioral studies, the inhibitory effect of zinc on
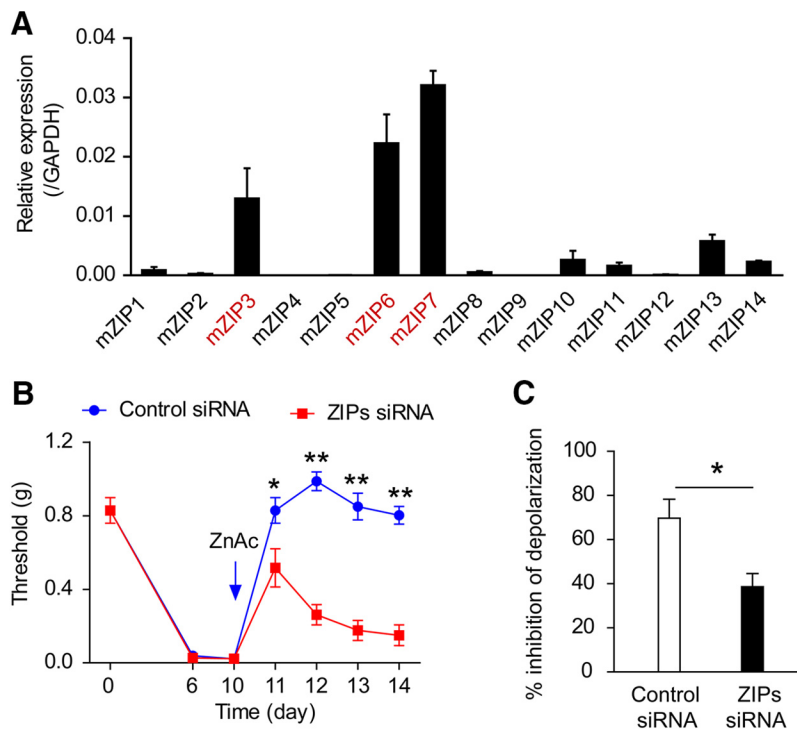
Figure 6. Inhibition of paclitaxel-induced mechanical hypersensitivity by zinc requires ZIPs. **A**, Relative expression of 14 ZIP zinc transporters in mouse DRG. The most abundantly expressed three ZIPs are highlighted in red. **B**, Intrathecal application of siRNAs against ZIP3, ZIP6, and ZIP7, but not control siRNA, severely reduced the inhibitory effect of ZnAc on paclitaxel-induced mechanical hypersensitivity. *$p < 0.05$ versus control siRNA group (repeated-measures ANOVA). **$p < 0.01$ versus control siRNA group (repeated-measures ANOVA). $n = 5$ mice for each group. **C**, Bar chart demonstrates that inhibition of capsaicin-evoked membrane depolarization by zinc in DRG neurons was significantly attenuated by intrathecally applied siRNAs against ZIP3, ZIP6, and ZIP7 compared with that in DRG neurons treated with control siRNA. *$p < 0.05$ versus control siRNA group (Student's t test). $n = 7$ cells for each group.

capsaicin-induced membrane depolarization was also significantly attenuated in DRG neurons isolated from mice treated with siRNAs against selected three ZIPs compared with those from mice treated with nontargeting control siRNA ($t_{(12)} = 3.066$, $p = 0.012$; Fig. 6C). These data strongly support that ZIP3, ZIP6, and ZIP7, but not TRPA1, are required for zinc-induced inhibition on chronic neuropathic pain produced by paclitaxel treatment.

Discussion

CIPN is a common consequence of chemotherapy that is neither well understood nor effectively treated. It affects >50% of cancer patients treated with commonly used classes of chemotherapy drugs, and has a significant negative impact on treatment outcome and patient's quality of life. Therefore, effective and safe treatment strategies are urgently needed to reduce the often persistent and debilitating effects of painful CIPN. In this study, we have provided evidence that locally injected zinc is effective in reducing mechanical hypersensitivity induced by paclitaxel treatment in a TRPV1-dependent manner, having little effect on the contralateral paws. We further show that extracellular zinc is a potent inhibitor of TRPV1 expressed in DRG neurons both under normal conditions and in CIPN and suppresses capsaicin-elicited acute nocifensive responses. Zinc-induced inhibition of TRPV1 function requires the presence of TRPA1 that facilitates zinc influx, and genetic ablation of TRPA1 function severely reduces the inhibitory effect of zinc on capsaicin responses both *in vitro* and *in vivo*. Unexpectedly, we show that inhibition of paclitaxel-induced neuropathic pain by zinc is not mediated by TRPA1, which might result from the desensitization

property of the channel. Instead, zinc transporters, especially ZIP3, ZIP6, and ZIP7, are critically involved in the zinc inhibition of mechanical hypersensitivity induced by paclitaxel treatment. Our results suggest that zinc suppresses both acute nociception and chemotherapy-induced chronic neuropathic pain through inhibiting TRPV1, although distinct zinc entry routes are required under acute and chronic settings.

Zinc has been widely used as an anti-inflammatory and antiarthritic agent (Cousins and Swerdel, 1985; Whitehouse et al., 1990). Several lines of evidence suggest that exogenously applied zinc has an antihyperalgesic effect in a number of inflammatory and neuropathic pain model systems (Safieh-Garabedian et al., 1996; Larson and Kitto, 1999; Liu et al., 1999; Nozaki et al., 2011). Although the mechanisms underlying zinc modulation of pain responses are complex, zinc has been shown to regulate functions of many pain-related ion channels, including acid-sensing ion channels (Jiang et al., 2011), TRPV1 (Vysotskaya et al., 2014), and NMDA receptors (Nozaki et al., 2011; Peralta and Huidobro-Toro, 2016). We and others have previously shown that zinc activates and permeates the pain-initiating TRPA1 channels and elicits a nociceptive response (Andersson et al., 2009; Hu et al., 2009; Vysotskaya et al., 2014). Nevertheless, in the present study, we showed that zinc inhibits TRPV1 and suppresses capsaicin-induced excitation of DRG neurons and nociceptive responses, which requires the expression of TRPA1. Several lines of evidence have demonstrated mutual modulation between TRPV1 and TRPA1, which are coexpressed in primary nociceptors (Staruschenko et al., 2010; Spahn et al., 2014; Weng et al., 2015). However, TRPA1 agonist AITC did not inhibit capsaicin responses, suggesting that the inhibitory effect of zinc on TRPV1 is an intrinsic property of zinc, separable from other TRPA1 activators. This is further confirmed by the finding that zinc permeation mutation of TRPA1 abolished the inhibitory effect of zinc on TRPV1. These data indicate that TRPA1 serves as the entry route for extracellular zinc to gain access to the intracellular side of the cell and suppress TRPV1. Furthermore, zinc application markedly desensitizes TRPA1-mediated membrane currents in heterologous cells and AITC-elicited nocifensive responses *in vivo*. These findings might provide distinct mechanisms underlying the antihyperalgesic effect of zinc through inhibition of both TRPA1 and TRPV1.

Although the role of TRPV1 in the pathogenesis of chemotherapy-induced chronic neuropathic pain is still largely unknown, accumulating evidence suggests that both expression and function of TRPV1 are increased upon treatment with commonly used chemotherapy drugs. For instance, TRPV1 expression in both skin and DRG is increased in rats treated with paclitaxel (Hara et al., 2013). Consistent with enhanced expression, treatment with oxaliplatin increases intracellular cAMP and sensitizes responses elicited by capsaicin and icilin in DRG neurons (Anand et al., 2010). Importantly, many exogenous and endogenous TRPV1

inhibitors efficiently reduce chemotherapy-induced neuropathic pain through either direct inhibition of TRPV1 or indirect inhibition of TRPV1 sensitization by signal transduction metabolites (Sałat et al., 2014; Gao et al., 2016; Sisignano et al., 2016). Interestingly, although TRPV1 antagonists have been shown to reduce neuropathic pain (Chen et al., 2011; Li et al., 2015), we found that the mechanical hypersensitivity in wild-type mice was not significantly different from the TRPV1 KO mice when both were treated with paclitaxel, suggesting that there might be a genetic redundancy for paclitaxel-induced pain response in mice.

On the other hand, exogenously applied zinc markedly reduced mechanical hypersensitivity produced by paclitaxel treatment in a TRPV1-dependent manner. Unexpectedly, although the inhibitory effect of zinc on capsaicin-induced response was completely abolished by the absence of TRPA1 or in the presence of the zinc permeation mutation of TRPA1 in heterologous cells, zinc still exhibited inhibitory effect on paclitaxel-induced neuropathic pain in TRPA1 KO mice, indicating that TRPA1 function is not essential or unavailable for the zinc entry under chronic settings. These data are consistent with the findings that zinc desensitizes TRPA1 function both *in vitro* and *in vivo*, and suggest that alternative zinc entry routes may also exist in DRG neurons *in vivo*, such as a large number of zinc transporters, which could mediate the influx of zinc into nociceptors (Cousins et al., 2006). Indeed, we detected abundant expression of ZIP3, ZIP6, and ZIP7 in DRG, and administration of siRNAs against these three ZIPs markedly reduced the inhibitory effect of zinc on paclitaxel-induced mechanical hypersensitivity. Our results suggest that ZIPs are critically involved in zinc entry into nociceptors, which leads to inhibition of TRPV1-dependent neuropathic pain produced by paclitaxel treatment.

Although TRPV1 is also reported to express centrally in the thalamus, locus ceruleus, periaqueductal gray, cortex, and spinal cord (Steenland et al., 2006; Kim et al., 2012), recent studies using TRPV1 reporter mice have shown that TRPV1 is primarily expressed by primary nociceptors with minimal expression in the CNS that is restricted to the area around the caudal hypothalamus (Cavanaugh et al., 2011a, b). Our study demonstrates that zinc inhibits TRPV1-dependent acute and chronic pain responses in primary nociceptors, revealing a novel peripheral mechanism for zinc-mediated analgesic effect. Locally injected zinc could produce a sustained inhibition of TRPV1 and reduce chemotherapy-induced neuropathic pain for >4 d after one single injection. Interestingly, CIPN generally develops with a symmetric, distal, and length-dependent "glove and stocking" distribution affecting predominantly the feet and hands at the extremities. Thus, administration of zinc to localized areas associated with pain might be an effective and safe approach to treat pain without causing systemic side effects, considering that zinc plays a critical role in regulating neurotransmissions in the CNS (Jo et al., 2000; Smart et al., 2004; Wall, 2005; Nakashima and Dyck, 2009; Marger et al., 2014).

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