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ORIGINAL ARTICLE

# Modulation of $Na_v1.8$ by Lysophosphatidic Acid in the Induction of Bone Cancer Pain

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**Abstract** Given that lysophosphatidic acid (LPA) and the tetrodotoxin-resistant sodium channel $Na_v1.8$ are both involved in bone cancer pain, the present study was designed to investigate whether crosstalk between the LPA receptor $LPA_1$ (also known as EDG2) and $Na_v1.8$ in the dorsal root ganglion (DRG) contributes to the induction of bone cancer pain. We showed that the EDG2 antagonist Ki16198 blocked the mechanical allodynia induced by intrathecal LPA in naïve rats and attenuated mechanical allodynia in a rat model of bone cancer. EDG2 and $Na_v1.8$ expression in $L_{4–6}$ DRGs was upregulated following intrathecal or hindpaw injection of LPA. EDG2 and $Na_v1.8$ expression in ipsilateral $L_{4–6}$ DRGs increased with the development of bone cancer. Furthermore, we showed that EDG2 co-localized with $Na_v1.8$ and LPA remarkably enhanced $Na_v1.8$ currents in DRG neurons, and this was blocked by either a protein kinase C (PKC) inhibitor or a PKCε inhibitor. Overall, we demonstrated the modulation of $Na_v1.8$ by LPA in DRG neurons, and that this probably underlies the peripheral mechanism by which bone cancer pain is induced.

**Keywords** Lysophosphatidic acid · Bone cancer pain · Tetrodotoxin-resistant sodium channel $Na_v1.8$ · Dorsal root ganglion

## Introduction

Bone cancer pain frequently manifests as severe spontaneous pain and breakthrough pain, which strongly impacts patients' quality of life and is difficult to control completely [1–3]. Various cancer cells release algogenic substances such as tumor necrosis factor, bradykinin, nerve growth factor (NGF), and formaldehyde that may sensitize primary afferent neurons or destroy peripheral nerve fibers [4–8].

Lysophosphatidic acid (LPA), a potent signaling lipid secreted by activated blood platelets [9, 10], is found at high concentrations in cancer patients' malignant ascites and blood plasma [11–14], promoting the progression of bone metastases [15]. There are five LPA receptor subtypes, $LPA_{1-5}$, all of which are G protein-coupled receptors [15–17]. Dorsal root ganglion (DRG) neurons mainly express the $LPA_1$ receptor (also known as EDG2) [18]. Our previous studies showed that LPA is involved in the initiation of bone cancer pain *via* sensitizing primary afferent C-fibers [19] and potentiates TRPV1 current *via* a PKC-dependent pathway in the DRG neurons of rats with bone cancer [20].

$Na_v1.8$, a slow-inactivating tetrodotoxin-resistant (TTX-R) voltage-gated sodium channel, is mainly localized in nociceptive small and medium-sized DRG neurons and acts as a key component of the upstroke of the action potential in these neurons [21–27], thus influencing their excitability [22]. $Na_v1.8$ knockdown rats show reduced pain behavior in models of neuropathic pain and

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inflammatory pain [26, 28–30]. Compelling studies have shown that this channel is involved in the development of bone cancer pain [31] and that the $Na_v1.8$ currents are regulated by many inflammatory factors such as prostaglandin E2, NGF, and serotonin [24, 32–34]. What is more, LPA increases TTX-R currents [35].

Taken together, it is reasonable to assume that $Na_v1.8$ may contribute to LPA mechanism underlying the induction of bone cancer pain. Therefore, we designed the present study to investigate whether there is crosstalk between $Na_v1.8$ and the LPA receptor EDG2 in the development of bone cancer pain.

## Materials and Methods

### Animals

Female Sprague-Dawley rats (from the Experimental Animal Center, Nanchang University, China) weighing 80–120 g were used in the patch clamp recording experiments and female Sprague-Dawley rats weighing 180–200 g were used in the rest of the experiments. The 180–200 g rats were divided into three groups: LPA + Ki16198 (1 mmol/L, 50 μL LPA and 1 mmol/L, 50 μL Ki16198); LPA + Control (LPA and 1% DMSO in saline), and Control + Control (saline + 1% DMSO). All the rats were housed three per cage and maintained on a 12:12 h light/dark cycle at ~23 °C with free access to water and food. In all experiments, rats were used only once. All animal handling and experimental procedures were reviewed and approved by the Animal Care Committee of Nanchang University and carried out according to the guidelines of the International Association for the Study of Pain. Animal care, use, and treatment were in accordance with the guidelines and regulations. All efforts were made to minimize the number and suffering of the rats.

### Establishment of Bone Cancer Model

The abdominal cavity of 80-g rats was injected with Walker 256 rat mammary gland carcinoma cells (Walker 256 rat mammary gland carcinoma cells used in the previous study were the same line with those used in our previous papers, provided by the Department of Integrative Medicine and Neurobiology, School of Medicine, Fudan University) for cancer cell culture. To induce bone tumors, carcinoma cells ($10^7$) in 4 μL phosphate-buffered saline (PBS) or 4 μL PBS alone (sham) was injected through the knee joint into the left tibial cavity in Chloral Hydrate anesthetized [300 mg/kg, intraperitoneal (i.p.)] animals.

### von-Frey Test for Mechanical Allodynia

Rats were first placed individually into a Plexiglas chamber for 30 min acclimation as described previously [36]. As in our previous studies [19, 20, 37], the hindpaw withdrawal threshold (PWT) was determined by a calibrated series of von Frey hairs (1, 2, 4, 6, 8, 10, 15, and 26 g; Stoelting, Wood Dale, IL), applied in ascending order for 3 s to the center of the plantar surface of the left hindpaw. A positive response was considered only when the hindpaw was completely lifted off the platform. Each force was repeated 5 times at 10-s intervals. The lowest force to induce at least 3 responses out of 5 tests was defined as the PWT.

### Western Blotting

The $L_{4–6}$ DRGs from sham, cancer, and drug-treated rats were rapidly collected after the animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and homogenized in lysis buffer (12.5 μL/mg tissue) containing protease inhibitor (Roche, Mannheim, Germany) and phenylmethylsulphonyl fluoride (Sigma, St. Louis, MO). The protein concentrations were assessed with BCA assays (Pierce Biotechnology Inc., Rockford, IL). A protein sample (20 μg) was loaded onto each lane, separated using 8% SDS-PAGE, and then transferred to polyvinylidene fluoride membranes. After blocking in 5% nonfat dry milk for 2 h at room temperature (RT), the membranes were incubated overnight at 4°C with rabbit anti-EDG2 primary antibody (1:400, Novus Biologicals Inc., Littleton, CO), rabbit anti-$Na_v1.8$ primary antibody (1:2000, Alomone Labs Ltd, Jerusalem, Israel), or mouse anti-tubulin primary antibody (1:5000, Sigma) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at RT. Finally, signals were detected with enhanced chemiluminescence (Pierce Biotechnology Inc., Rockford, IL) and visualized with the ChemiDoc XRs system (Bio-Rad Laboratories Inc., Richmond, CA). The tubulin level was used as loading control, and EDG2 or $Na_v1.8$ expression was normalized against the tubulin level. EDG2/tubulin or $Na_v1.8$/tubulin in the DRGs from the treatment group was normalized against those in the control group. All Western blot analysis was repeated at least 3 times.

### Immunohistochemistry

After an overdose of urethane (2 g/kg, i.p.), animals were perfused intracardially with normal saline followed by 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4, 4 °C). The DRGs of the $L_{4–6}$ segments were removed, post-fixed in the same fixative (4 h, 4 °C), and then immersed in a 10%–

30% gradient of sucrose in PBS for cryoprotection (24–48 h, 4 °C). DRG sections at 7 μm (to detect $Na_v1.8$ and EDG2 co-localization) were cut on a cryostat (Leica 1900, Leica, Wetzlar, Hesse, Germany) and processed for immunofluorescence. After blocking with 10% donkey serum in 0.01 mol/L PBS (pH 7.4) with 0.3% Triton X-100 for 1 h at RT, two adjacent sections were each incubated overnight at 4 °C with rabbit anti-$Na_v1.8$ (1:2000, Alomone) and rabbit anti-EDG2 (1:50, Novus Biologicals) primary antibodies in PBS with 1% normal donkey serum and 0.3% Triton X-100. Following three 15-min rinses in 0.01 mol/L PBS, the sections were incubated with Alex Fluor 546- and Alex Fluor 488-conjugated secondary antibodies for 2 h at 4 °C, respectively, and then washed in PBS. After coverslipping with 50% glycerin in 0.01 mol/L PBS, the sections were observed under a confocal laser scanning microscope (FV1000, Olympus, Tokyo, Japan). Images were captured with FV10-ASW software. Omission of primary antibody served as a negative control.

### Preparation of DRG Neurons

$L_{4–6}$ DRG neurons were acutely dissociated from 80–100 g rats as described previously [38–40]. Anesthetized with ether, the rats were rapidly decapitated. The DRGs were removed and incubated in Dulbecco's modified Eagle's medium (Gibco Life Technologies, Grand Island, NY) saturated with a $CO_2/O_2$ mixture, containing 2.67 mg/mL collagenase (type IA, Sigma, St. Louis, MO) and 1 mg/mL trypsin (type I, Sigma), for 35 min at 37 °C. After enzyme treatment, the DRGs were washed with standard external solution (in mmol/L, 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4) and gently triturated with a fine fired-polished Pasteur pipette to dissociate single cells. Neurons were plated onto glass coverslips in culture dishes, and then incubated in standard external solution for recording at RT. All experiments were carried out within 2–8 h after plating and each coverslip was used only once.

### Patch-Clamp Recordings

Whole-cell voltage-clamp recordings were made at RT (23 ± 1°C) using an Axonpatch 200B amplifier (Molecular Devices, LLC Sunnyvale, CA). Only small (< 25 μm) DRG neurons with resting membrane potentials more negative than −50 mV were selected for study. Microelectrodes (N51A borosilicate glass, Sutter Instruments Co., Novato, CA) were pulled on a P97 puller (Sutter Instruments). Microelectrodes with resistances of 2–6 MΩ were selected and filled with (in mmol/L): 140 CsF, 1 $MgCl_2$, 2.5 $Na_2ATP$, 1 EGTA, and 10 HEPES, adjusted to pH 7.2 with CsOH. The data were sampled at 10 kHz and low-pass filtered at 2 kHz. The external solution contained (in mmol/L): 32 NaCl, 1 $MgCl_2$, 20 TEA-Cl, 105 choline-Cl, 1 $CaCl_2$, 0.1 $CdCl_2$, 10 HEPES, 0.0005 TTX, and 10 glucose; pH was adjusted to 7.4 with NaOH. $Na_v1.8$ currents were evoked by 50-ms depolarizing pulses in DRG neurons held at −60 mV. The peak $Na_v1.8$ currents were determined by a voltage-clamp protocol of depolarizing steps from −55 mV to +40 mV (50 ms, at 5-mV increments).

### Reagents

All reagents for patch-clamp recording and intrathecal (i.t.) and subcutaneous injections were from Sigma, except that the EDG2 inhibitor Ki16198 was from Selleck (Selleck Chemicals, Houston, TX) and the PKCε inhibitor $\varepsilon V_{1–2}$ was from Biomol (Plymouth Meeting, PA). All reagents were dissolved in saline (at least 1000-fold the working concentration) as stock solutions stored at −20 °C and the working concentrations were prepared on the day of the experiment. The reagent concentrations used were based on previous studies. LPA was continuously applied near the neurons for 1 min using an ALA-VM8 perfusion system (ALA Scientific Instruments, Westbury, NY). Ki16198 or the PKC inhibitor bisindolylmaleimide (BIM) was added to the chamber 30 min before and during the perfusion of LPA at a concentration based on a previous study [41]. $\varepsilon V_{1–2}$ was delivered intracellularly *via* the recording electrode.

### Data Analysis

Student's *t*-test was used to analyze all of the data. The criterion of significance was set at $P < 0.05$ and all data are presented as mean ± SEM.

## Results

### LPA-Induced Pain Behavior and Upregulation of EDG2 and $Na_v1.8$ in DRGs of Normal Rats

Two hours after i.t. administration, mechanical allodynia was tested using von Frey filaments. The results showed that PWTs in the Control + Control group did not differ before and after injection. In the LPA + Control group, compared with baseline, the PWT was significantly decreased 2 h after injection. Further, the LPA + Control group showed a lower PWT than the LPA + Ki16198 group (Fig. 1A).

Meanwhile, EDG2 and $Na_v1.8$ expression in $L_{4–6}$ DRGs was examined 2 h after i.t. administration. Compared with the Control + Control group, EDG2 expression was upregulated in the LPA + Control group, and this was blocked by the EDG2 antagonist Ki16198 (Fig. 1B, C).

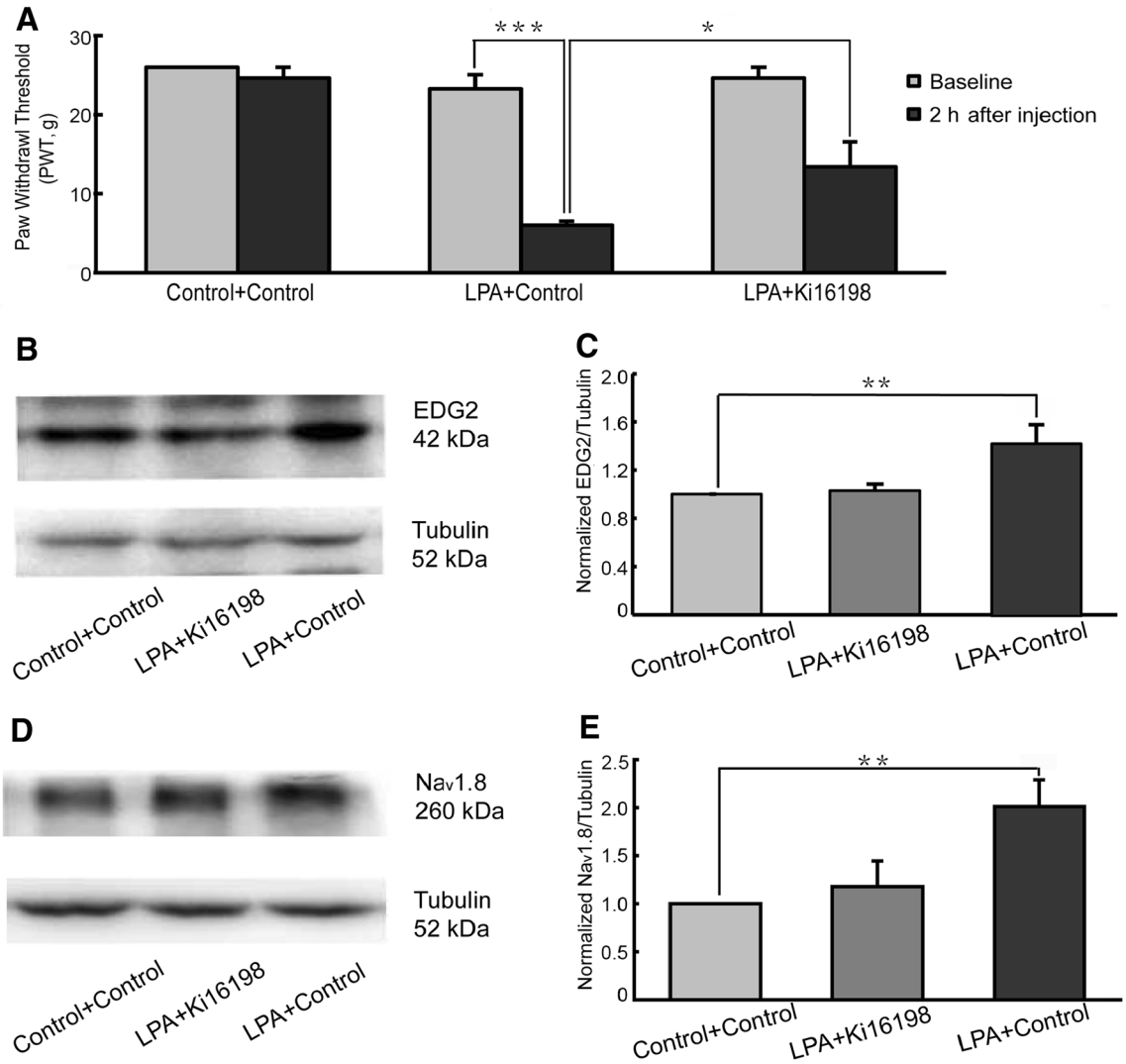


**Fig. 1** Pain behavior and EDG2 and $Na_v1.8$ expression after intrathecal injection of LPA. **A** LPA decreased PWTs ($n$ = 8), and this was attenuated by the EDG2 antagonist Ki16198. **B, C** LPA up-regulated EDG2 expression in $L_{4–6}$ DRGs ($n$ = 6). **D, E** $Na_v1.8$ expression on $L_{4–6}$ DRGs increased after LPA injection ($n$ = 6). *$P$ <0.05, **$P$ <0.01, ***$P$ <0.001.

The level of EDG2 was similar in the LPA + Ki16198 and Control + Control groups. Similarly, $Na_v1.8$ expression was significantly higher in the LPA + Control group than in the Control + Control group. Also, the upregulation of $Na_v1.8$ expression was blocked by Ki16198; the LPA + Ki16198 group had an $Na_v1.8$ level similar to that in the Control + Control group (Fig. 1D, E).

To investigate its direct action on peripheral afferent fibers, LPA was subcutaneously injected into the hindpaw and 2 h later, EDG2 and $Na_v1.8$ expression was assessed in the ipsilateral $L_{4–6}$ DRGs. EDG2 expression was significantly higher in the LPA + Control group than in the Control + Control group, and this was blocked by Ki16198. There was no statistical difference in the EDG2 level between the Control + Control group and the LPA + Ki16198 group (Supplemental Fig. 1A, B). Similar results were obtained for $Na_v1.8$ expression. $Na_v1.8$ expression was higher in the LPA + Control group than in the Control + Control group and its expression in the Control + Control group was similar to that in the LPA + Ki16198 group, indicating that LPA-induced upregulation of $Na_v1.8$ expression was blocked by Ki16198 (Supplemental Fig. 1C, D).

## Bone Cancer-Induced Upregulation of EDG2 and $Na_v1.8$ in DRGs

The levels of EDG2 and $Na_v1.8$ expression were examined in the ipsilateral DRGs at the $L_{4–6}$ spinal segments on post-tumor days (PTDs) 3, 7, and 14. Western-blotting results showed that expression of EDG2 was higher in rats with bone cancer than in sham rats on PTDs 7 and 14, but not on PTD 3 (Fig. 2A, B). Similarly, $Na_v1.8$ was significantly

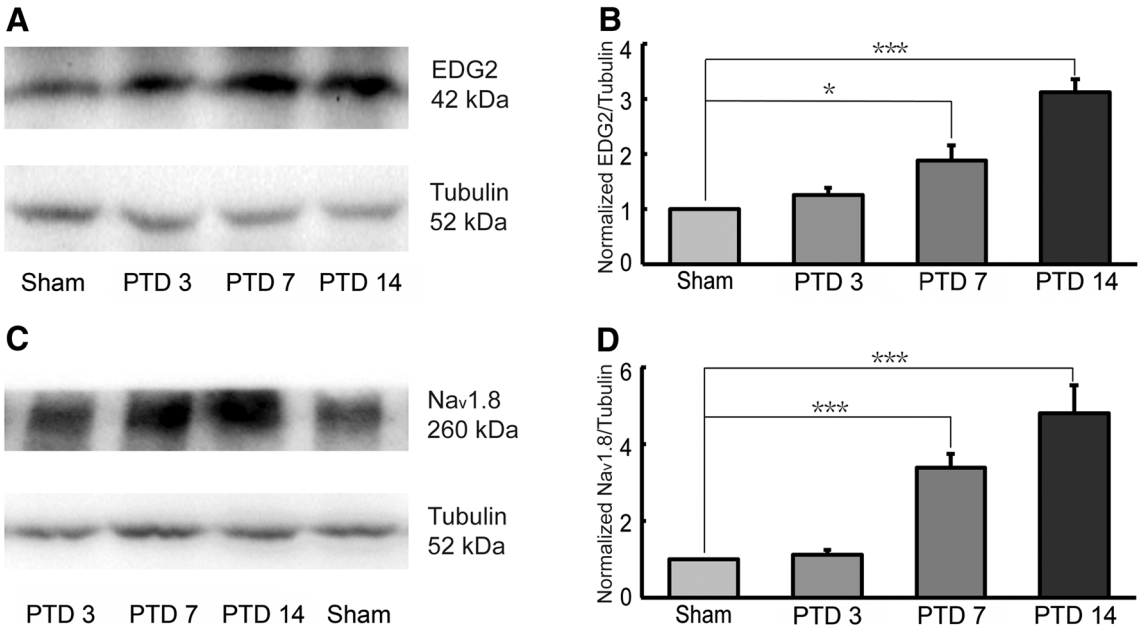


**Fig. 2** Time-courses of EDG2 and $Na_v1.8$ expression on ipsilateral $L_{4–6}$ DRGs after cancer cell inoculation. EDG2 (**A**, **B**, $n = 6$) and $Na_v1.8$ (**C**, **D**, $n = 6$) expression were both increased in PTD 7 and PTD 14 rats. $^*P < 0.05$, $^{***}P < 0.001$.

upregulated on PTDs 7 and 14, but not on PTD 3, compared with sham rats (Fig. 2C, D).

The EDG2 antagonist Ki16198 (1 mmol/L, 50 μL) or 50 μL of 1% DMSO (control) was injected i.t. the day before cancer cell implantation, as well as on PTDs 2, 4, 7, 10, 13, and 16. The sham rats received saline injections. On PTD 16, $Na_v1.8$ and EDG2 expression were assessed in the ipsilateral $L_{4–6}$ DRGs and pain behavior was assessed by ipsilateral PWTs with von Frey filaments. Compared with the sham group, EDG2 expression was strongly up-regulated in cancer rats that received control injections. In the Ki16198 group (Cancer + Ki16198), the upregulation of EDG2 was blocked (Fig. 3A, C). Similarly, $Na_v1.8$ expression was increased in the Cancer + Control group, while it did not change significantly in the Cancer + Ki16198 group, compared to the sham group (Fig. 3B, D). Meanwhile, PWTs in the Cancer + Control and Sham groups were 2.35 ± 0.33 g and 22.33 ± 2.31 g, respectively (Fig. 3E), indicating a reduction of PWTs by bone cancer. However, this reduction was reversed by Ki16198 injection in the Cancer + Ki16198 group. These results suggested that Ki16198 significantly attenuated the upregulation of EDG2 and $Na_v1.8$ expression and mechanical allodynia in rats with bone cancer.

### Co-localization of $Na_v1.8$ with EDG2 and Potentiation of $Na_v1.8$ Currents by LPA in DRG Neurons

To explore the roles of EDG2 and $Na_v1.8$ in the development of bone cancer pain, their co-localization and interaction were investigated. According to our previous study [27], immunofluorescence staining of two adjacent sections (7 μm) from an $L_4$ DRG with $Na_v1.8$ antibody and EDG2 antibody reveals their co-localization in the same neurons. With this method, we found that $Na_v1.8$ and EDG2 were widely co-localized in DRG neurons (Fig. 4).

Given their co-localization, we explored whether LPA modulates the $Na_v1.8$ channel. Whole-cell patch-clamp recordings were performed on isolated small-diameter (< 25 μm) DRG neurons in which the membrane potential was held at −60 mV to inhibit $Na_v1.9$ currents and leave $Na_v1.8$ intact [27]. A voltage-clamp protocol (depolarizing steps from −55 mV to +40 mV, 50 ms, 5 mV increments) was used to generate $Na_v1.8$ currents (Fig. 5A). According to the current–voltage curve (Fig. 5B), the peak amplitude of $Na_v1.8$ currents was elicited at −15 mV in most recordings. Three minutes after DRG neurons were perfused with 10 μmol/L LPA for 1 min, the $Na_v1.8$ currents were potentiated by 51 ± 0.14% in the neurons recorded (Fig. 5C, D).

As a G-protein coupled receptor, EDG2 interacts with the $G_i$, $G_q$, and $G_{12}$ families, activating their downstream mitogen-activated protein kinase (MAPK), PKC, and Rho-Rho kinase pathways, while inhibiting the protein kinase A (PKA) pathway [15]. Previous studies have suggested that the PKC-dependent pathways, especially the PKCε pathway, is involved not only in $Na_v1.8$ channel modulation [34, 40, 42, 43], but also in the potentiation of the TRPV1 channel by LPA [20].

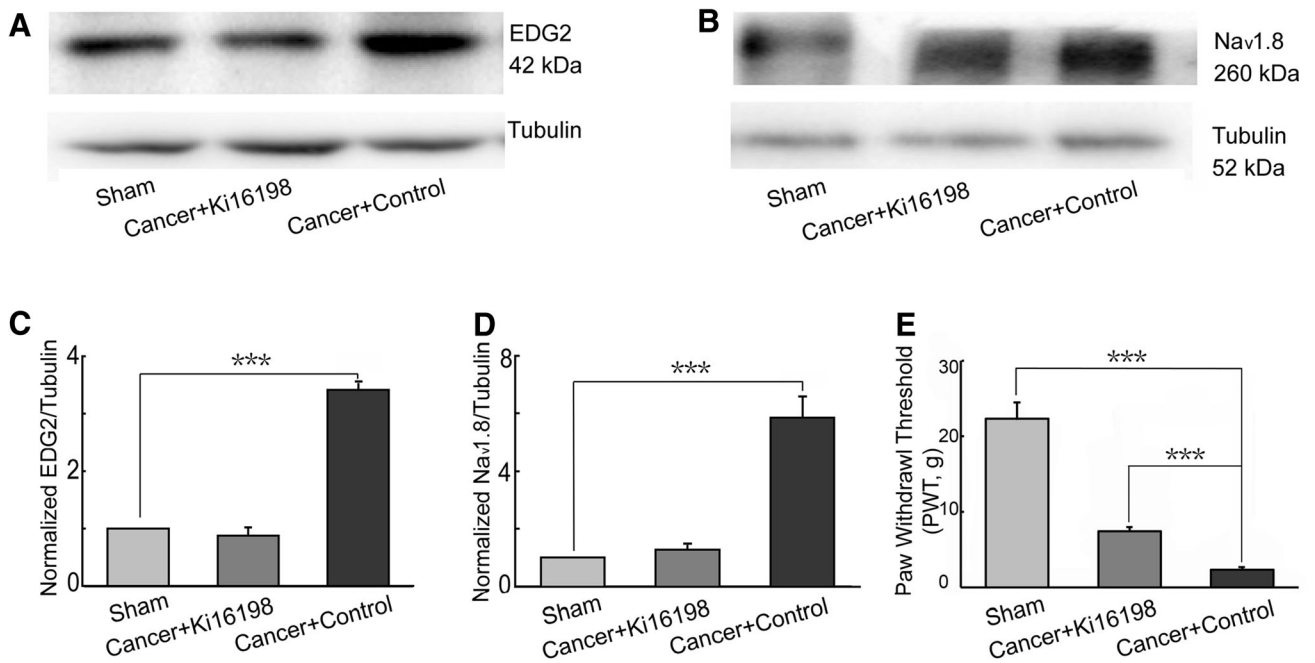


**Fig. 3** Effects of Ki16198 on EDG2 and $Na_v1.8$ upregulation and pain behavior of rats with bone cancer. **A, C** Ki16198 blocked the upregulation of EDG2 expression on ipsilateral $L_{4–6}$ DRGs of rats with cancer ($n$ = 6). **B, D** Increased $Na_v1.8$ expression on ipsilateral $L_{4–6}$ DRGs of rats with cancer was also blocked by Ki16198 ($n$ = 6). **E** Ki16198 attenuated mechanical allodynia in rats with cancer ($n$ = 6). ***$P$ <0.001.

In the present study, the PKC inhibitor BIM and the PKCε inhibitor $\varepsilon V_{1–2}$ were used to explore the downstream molecules that contribute to potentiation of the $Na_v1.8$ current by LPA. Among the 7 neurons tested, BIM (1 μmol/L, 30 min) incubation blocked the LPA-induced potentiation of $Na_v1.8$ currents (Fig. 6A, B,). When $\varepsilon V_{1–2}$ (200 μmol/L) was delivered *via* the recording electrode 5 min before recording, LPA failed to increase the $Na_v1.8$ currents (Fig. 6C, D) in all 8 of the neurons recorded.

## Discussion

LPA is secreted by activated platelets, as well as tumors and their surrounding tissues [15, 44, 45], mediating a wide range of effects such as the proliferation, migration, and survival of cancer cells [12, 46, 47]. Our previous study demonstrated that LPA is involved in the induction of bone cancer pain by interacting with TRPV1, an important pain-related factor widely expressed in small DRG neurons [20].

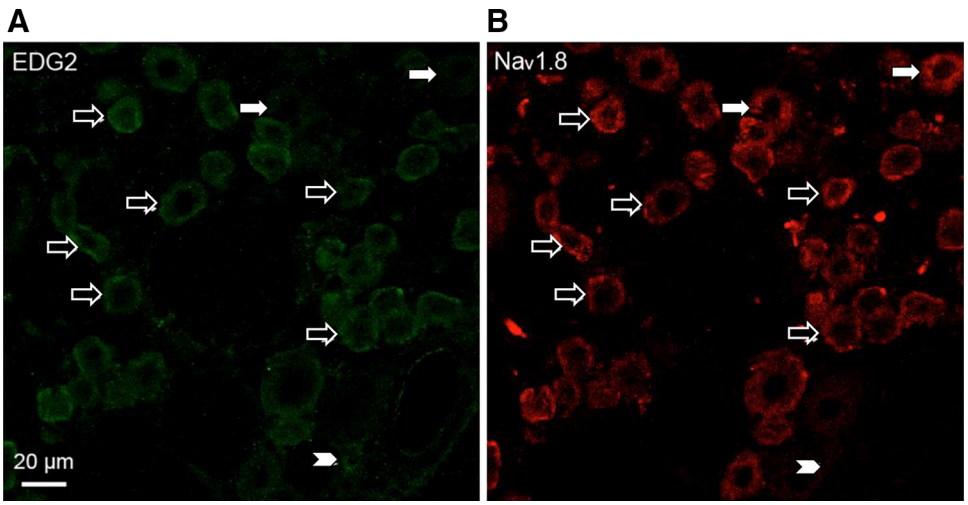


**Fig. 4** Co-localization of $Na_v1.8$ and EDG2 in DRG neurons. **A, B** Double immunofluorescence staining showing $Na_v1.8$ and EDG2 co-localized in DRG neurons (open arrows). Neurons expressing EDG2 but not $Na_v1.8$ and $Na_v1.8$-positive but EDG2-negative neurons are indicated by arrowheads and filled arrows, respectively.

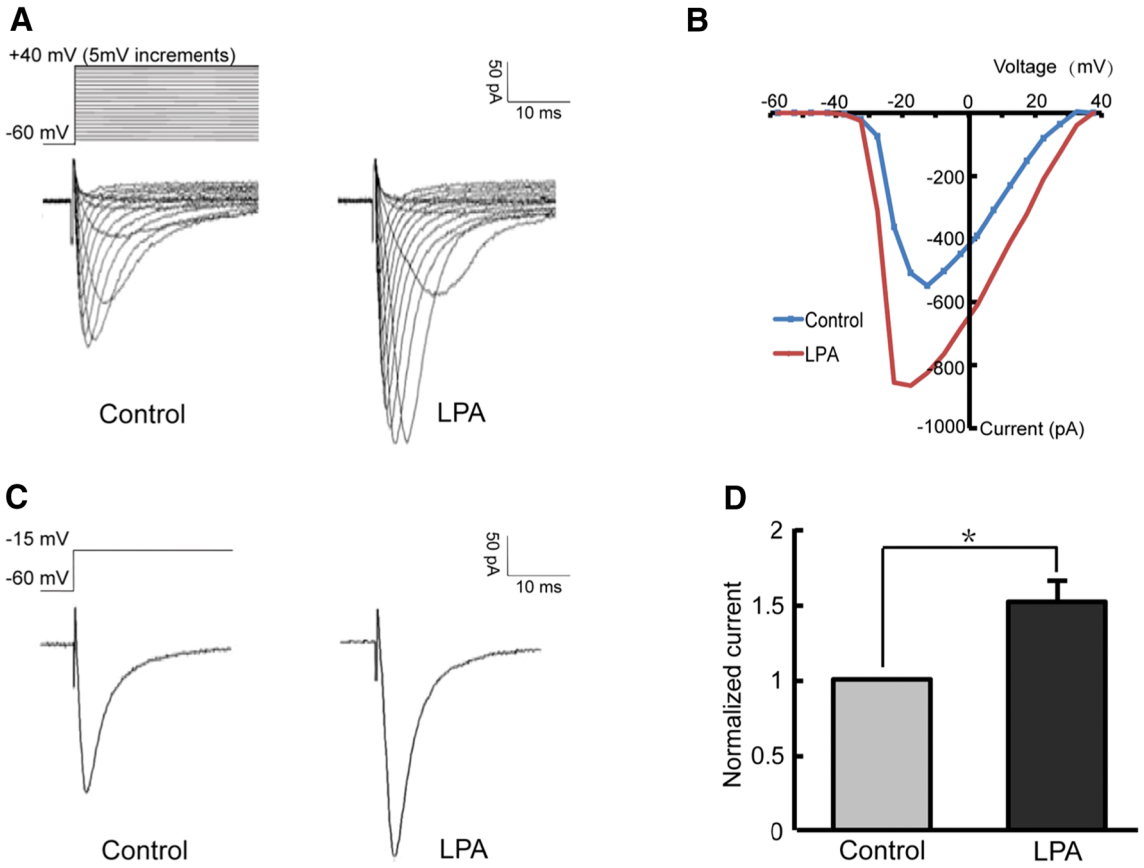


**Fig. 5** Potentiation of $Na_v1.8$ currents by LPA. **A** Representative $Na_v1.8$ currents recorded before (left) and after (right) LPA perfusion. **B** *I-V* curves of $Na_v1.8$ currents. **C, D** Peak amplitude of $Na_v1.8$ currents increased after LPA perfusion ($n = 11$). *$P < 0.05$.

$Na_v1.8$, another key pain signaling molecule in primary afferent neurons, is a TTX-R sodium channel primarily localized in nociceptors [21, 23]. In the present study, we showed that LPA facilitates the $Na_v1.8$ channel in DRG neurons, providing a new peripheral LPA mechanism underlying the induction of bone cancer pain.

The present results showed that i.t. injection of LPA induced upregulation of both the $LPA_1$ receptor EDG2 and $Na_v1.8$ expression in the $L_{4–6}$ DRGs of normal rats, and this was completely blocked by the EDG2 antagonist Ki16198. Functionally, i.t. injection of LPA decreased the PWT, and this was partially, but not completely, blocked by Ki16198, suggesting that LPA potentiates the excitability of the DRG neurons that innervate the hind-paw. This may be related to the upregulation of $Na_v1.8$ in $L_{4–6}$ DRGs, while other channels such as TRPV1 may also be involved in this effect. As EDG2 is also expressed in the spinal cord [15, 18], LPA injected i.t. may act directly on DRG neurons and/or *via* a spinal mechanism to up-regulate both EDG2 and $Na_v1.8$ expression in $L_{4–6}$ DRGs and induce allodynia.

It has been documented that bone innervation in the hind-limbs is predominantly from thinly-myelinated A-δ and unmyelinated C-fibers, originating from medium and small DRG nociceptor neurons, respectively [48]. Algogenic substances released by carcinoma cells and tumor stroma have been suggested to sensitize or directly activate peripheral nociceptive sensory neurons [49]. Our previous study revealed that LPA sensitizes sural C-fibers that innervate the hindpaw [19]. In the present work, we found that after intraplantar injection of LPA, the EDG2 and $Na_v1.8$ levels were greatly increased, and this was blocked by Ki16198. These results suggested that LPA may amplify the C fiber-mediated nociceptive information, with the excitability of small-sized DRG neurons be enhanced and the EDG2 and $Na_v1.8$ levels increased, thus sensitizing nociceptive DRG neurons and contributing to pain.

In addition to up-regulating the $Na_v1.8$ level in DRGs, LPA also potentiated the $Na_v1.8$ currents in isolated DRG neurons. This result for the first time identified the co-localization of EDG2 and $Na_v1.8$ in DRG neurons, providing a basis for their interaction. In patch-clamp recordings, LPA greatly increased the $Na_v1.8$ currents in small DRG neurons, reflecting that more $Na_v1.8$ channels were opened after LPA perfusion, resulting in potentiation of the excitability of DRG neurons.

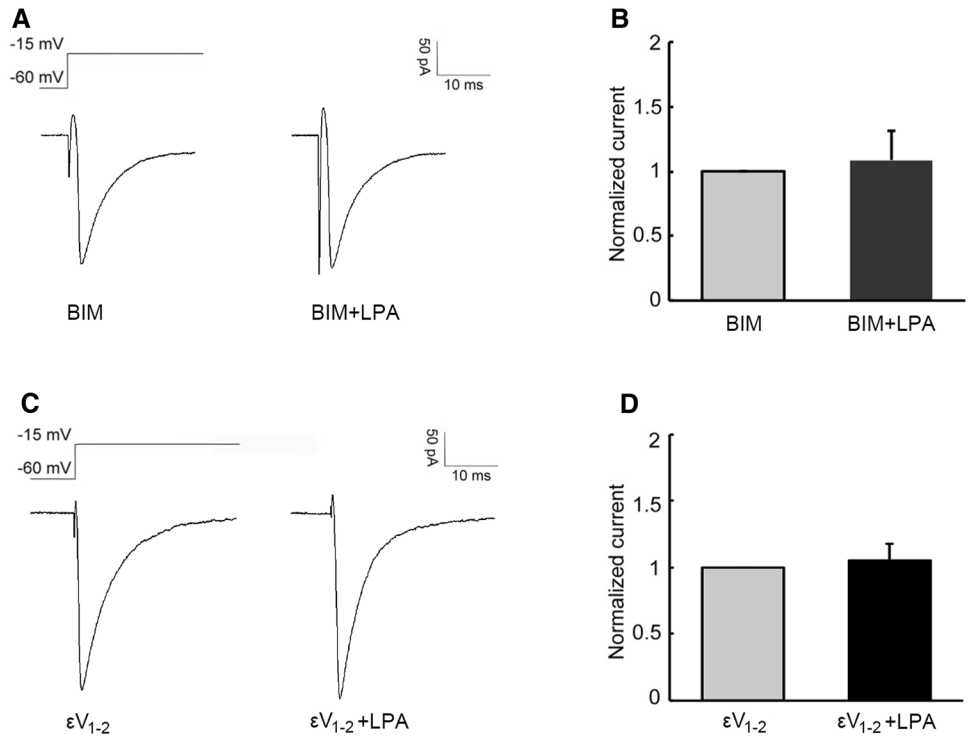


**Fig. 6** PKC, especially PKCε, is involved in the LPA-induced potentiation of $Na_v1.8$ currents. **A, B** After BIM incubation, LPA failed to increase the amplitude of $Na_v1.8$ currents ($P > 0.05$). **C, D** With $\varepsilon V_{1-2}$ delivery, the amplitude of $Na_v1.8$ currents remained unchanged after LPA perfusion ($P > 0.05$).

In rats with bone cancer, the up-regulated EDG2 and $Na_v1.8$ expression had a similar time-course in ipsilateral DRGs. EDG2 and $Na_v1.8$ expression was assessed at three time points, 3, 7, and 14 days after cancer cell inoculation, and they were significantly increased at 7 and 14 days. In particular, i.t. injection of Ki16198 blocked the upregulation of EDG2 and $Na_v1.8$ expression in rats with bone cancer, further demonstrating that the up-regulation of EDG2 and $Na_v1.8$ was associated with LPA. It has been reported that $Na_v1.8$ influences the excitability of small and medium nociceptive DRG neurons [22], and an increase in $Na_v1.8$ expression contributes to mechanical allodynia [26, 28, 30, 31, 50]. It is plausible that as cancer develops, LPA released by carcinoma cells and the tumor stroma increases the excitability of peripheral C-fibers, as well as the EDG2 and $Na_v1.8$ expression, resulting in the enhancement of $Na_v1.8$ currents, further increasing the excitability of DRG neurons and thus causing mechanical allodynia. Correspondingly, our results showed that cancer-induced bone pain was attenuated by i.t. injection of Ki16198, indicating that blocking the $LPA_1$ receptor EDG2 partially prevents the sensitization of DRG neurons by LPA. However, as the cancer-induced bone pain was not totally blocked by i.t. Ki16198, other targets such as TRPV1 may also be involved in the effect of LPA. Taken together, it is conceivable that excessive LPA in rats with cancer activates unmyelinated peripheral sensory nerve fibers and sensitizes DRG neurons *via* increasing the activity and expression level of $Na_v1.8$ channels.

The $LPA_1$ receptor EDG2 is a G-protein coupled receptor [15], which is able to interact with three G protein families, $G_i$, $G_q$, and $G_{12}$, generating their downstream propagation through the MAPK, PKC, and Rho-Rho kinase pathways, while inhibiting the PKA pathway [15], further triggering a wide range of signaling molecules underlying modulation of the excitability of DRG neurons. Compelling evidence has shown that the $Na_v1.8$ channel is modulated by a PKC-dependent pathway [34, 42, 43], and PKC activation enhances TTX-R currents in DRG neurons[33]. Our previous results demonstrated that the PKC signal pathway is involved in the interaction between LPA and TRPV1 in the induction of bone cancer pain [20]. Also, neurokinin-1, a G-protein coupled receptor, potentiates $Na_v1.8$ currents *via* the PKCε pathway [39, 40]. Therefore, it is reasonable to assume that the PKC signal pathway participates in the LPA-induced sensitization of the $Na_v1.8$ channel and up-regulation of $Na_v1.8$ expression in rats with bone cancer. Our supplemental experiments showed that both the PKC inhibitor BIM and the PKCε inhibitor $\varepsilon V_{1-2}$ blocked the potentiation of $Na_v1.8$ currents by LPA. In addition, given that cancer pain is a complicated symptom with inflammatory and neuropathic components [48] and

LPA is important for the initiation of neuropathic pain *via* the Rho-Rho kinase pathway [18, 51], it is probable that LPA is involved in bone cancer pain by modulating several effectors through different intracellular signal pathways.

Taken together, LPA is involved in bone cancer pain *via* facilitating the TTX-R sodium channel $Na_v1.8$ in nociceptive primary sensory neurons, acting directly on DRG neurons and/or *via* a spinal mechanism, which probably constitutes a peripheral mechanism by which bone cancer pain develops.

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