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RESEARCH ARTICLE

Lysophosphatidic acid receptor_{1/3} antagonist inhibits the activation of satellite glial cells and reduces acute nociceptive responses

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Abstract

Lysophosphatidic acid (LPA) exerts various biological activities through six characterized G protein-coupled receptors (LPA₁₋₆). While LPA–LPA₁ signaling contributes toward the demyelination and retraction of C-fiber and induces neuropathic pain, the effects of LPA–LPA₁ signaling on acute nociceptive pain is uncertain. This study investigated the role of LPA–LPA₁ signaling in acute nociceptive pain using the formalin test. The pharmacological inhibition of the LPA–LPA₁ axis significantly attenuated formalin-induced nociceptive behavior. The LPA₁ mRNA was expressed in satellite glial cells (SGCs) in dorsal root ganglion (DRG) and was particularly abundant in SGCs surrounding large DRG neurons, which express neurofilament 200. Treatment with LPA_{1/3} receptor (LPA_{1/3}) antagonist inhibited the upregulation of glial markers and inflammatory cytokines in DRG following formalin injection. The LPA_{1/3} antagonist also attenuated phosphorylation of extracellular signal-regulated kinase, especially in SGCs and cyclic AMP response element-binding protein in the dorsal horn following formalin injection. LPA amounts after formalin injection to the footpad were quantified by

Abbreviations: ATX, autotaxin; CREB, cAMP response element-binding protein; DRG, dorsal root ganglion; ERK, extracellular signal-regulated kinase; GFAP, glial fibrillary acidic protein; GS, glutamine synthetase; LPA, lysophosphatidic acid; LPA₁, lysophosphatidic acid receptor 1; LPA₃, lysophosphatidic acid receptor 3; LPC, lysophosphatidyl choline; MPO, myeloperoxidase; MS/MS, tandem mass spectrometry; NF200, neurofilament 200; p-CREB, phosphorylated cAMP response element-binding protein; SGC, satellite glial cell; TRPM8, transient receptor potential melastin 8; TRPV1, transient receptor potential vanilloid 1.

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liquid chromatography/tandem mass spectrometry, and LPA levels were found to be increased in the innervated DRGs. Our results indicate that LPA produced in the innervated DRGs promotes the activation of SGCs through LPA₁, increases the sensitivity of primary neurons, and modulates pain behavior. These results facilitate our understanding of the pathology of acute nociceptive pain and demonstrate the possibility of the LPA₁ on SGCs as a novel target for acute pain control.

KEYWORDS

acute pain, lysophosphatidic acid, neuroglia, nociceptive pain, satellite glial cells

1 | INTRODUCTION

Lysophosphatidic acid (LPA) is a water-soluble bioactive lipid that mediates a wide range of cellular processes, including cell motility and morphological change.^{1–3} LPA is released from abundant diacyl phospholipids upon various extracellular stimuli, undergoes deacylation, and rapidly degrades.³ Therefore, LPA influences the development and progression of the different pathological conditions,⁴ such as cancer,^{5,6} fibrosis,⁷ and inflammation.⁸ Most of the LPA actions are mediated by G protein coupled LPA receptors (LPA₁–LPA₆). LPA₁ receptor (LPA₁) is the most abundant LPA receptor expressed in the nervous system, and numerous reports on neuropathic pain have been published on development and maintenance by LPA₁ signaling. LPA₁ mediates the activation of Rho-Rho-associated protein kinase signaling and demyelination of the dorsal root, causing abnormal hyperalgesia and allodynia.^{9–11} LPA was also shown to directly activate transient receptor potential vanilloid 1 (TRPV1),¹² suggesting that LPA may also contribute to acute pain, but the involvement via the activation of LPA₁ is unknown. Thus, despite the involvement of LPA₁ in the mechanism of neuropathic pain, its role in acute nociceptive pain is still controversial.

Schwann cells and satellite glial cells (SGCs) in dorsal root ganglion (DRG) express LPA₁,^{13,14} and SGCs are known to play an important role in maintaining the homeostasis of DRG neurons.¹⁵ Upon peripheral stimulation, activated neurons release potassium ions, and elevated potassium concentration depolarizes the neighboring neurons to release neurotransmitters, such as ATP and substance P. These neurotransmitters are known to activate SGCs via specific receptors.¹⁵ Activated SGCs reduce the potassium buffering capacity of SGCs, enhancing the formation of gap junction among SGCs,¹⁶ and release proinflammatory cytokines, such as interleukin (IL) –1 β and tumor necrosis factor (TNF) α , further potentiating neuronal excitability. These cross-excitations between neurons and SGCs are essential responses of nociceptive

transmission.^{15,17} However, the influence of LPA₁ on SGCs has never been demonstrated.

The formalin test has long been widely used to assess the analgesic effect as a robust and reliable model of acute nociceptive pain.^{18,19} The subcutaneous injection of formalin into the right hind paw induced a typical biphasic nociceptive response during the 60 min observational period. The 1st phase (0–10 min after formalin injection) of nociceptive response is induced by direct stimulation of nociceptors, and the 2nd phase (11–60 min after formalin injection) is represented by the intensity of peripheral inflammation and central sensitization.^{20,21} Considering that LPA₁ knock-out mice show an abnormal response to acute stress,^{22,23} we examined the effects of the LPA₁/3 receptor (LPA_{1/3}) antagonist instead of LPA₁ knock-out mice.

This study explored the detailed expression site of LPA₁, and investigated the roles of the LPA-LPA₁ axis in formalin-induced acute nociceptive pain. Furthermore, a lipidomic approach was taken to analyze the changes in LPA levels and its precursor, lysophosphatidylcholine (LPC), in the acute phase.

2 | MATERIALS AND METHODS

2.1 | Animals

Male and female C57BL/6JJcl mice purchased from Clea (Tokyo, Japan) were used for all experiments. These mice were housed in the facilities with controlled temperature and humidity, under standard conditions (12 h light–dark cycle; lights on at 6:00 a.m.) with free access to standard rodent chow and water. The ethics committee of the University of Tokyo approved all animal experiments (approval No. P17-025). Male wild-type mice were used to avoid the sex difference, the adaptation of the receptor loss, and abnormal central nervous system development in LPA receptor knock-out mice.²²

2.2 | Drug

LPA_{1/3} antagonist Ki16425 (1001265, Cayman Chemical, MI, USA) was prepared to 60 mg/ml in DMSO and stored at -20°C . As previously reported,²⁴ for intraperitoneal administration (30 mg/kg), 10 μl of Ki16425 in DMSO and 190 μl of sesame oil (196–15385, Wako, Osaka, Japan) per 20 g of mouse body weight were administered by mixing using the vortex right before administration. For local administration (2 mg/kg), 0.67 μl of Ki16425 in DMSO and 4.33 μl of sesame oil per 20 g of mouse body weight were administered.

2.3 | Formalin test

Mice were habituated in an individual observation cage for at least 60 min before the tests. After habituation, 10 μl of 5% formalin (068-03863, Wako, Osaka, Japan) was injected into the dorsal surface of the right hind paw using a 30-gage needle fitted to a 25 μl Hamilton syringe. Each mouse was immediately placed into a transparent observation cage. We counted the seconds of flinching, licking, and biting the right hind paw by eyes and recorded every 5 min for 60 min. The tests and assessments were performed in a blinded manner as described previously.^{19,25}

2.4 | Intrathecal LPA injection

LPA containing oleic acid at the sn-1 position (18:1 LPA) were purchased from Cayman (10010093, Cayman Chemical, MI, USA). 1 nmol of 18:1 LPA was dissolved in 5 μl (200 μM) PBS containing 1% essentially fatty acid free BSA (A6003, Sigma, MI, USA) and 5 μl PBS containing 1% essentially fatty acid free BSA was prepared as vehicle.

For intrathecal injections (i.t.), mice were anesthetized with isoflurane and held by the pelvic girdle. With modifications based on previous papers,^{26,27} a 30-gage needle fitted to a 25 μl Hamilton syringe was inserted into the tissue between the dorsal aspects of lumbar regions 5 and 6. The needle was carefully moved forward to the intervertebral space, and 5 μl of drug solution was injected. After injection, no specific behavior or sign of distress was observed in injected mouse.

2.5 | RNA extraction and real-time PCR

Tissues were isolated from mice and immediately submerged in RNA later (Thermo Fisher Scientific, MA,

USA). Total RNA was extracted by TRIzol (Ambion, MA, USA) from mice's spinal cord, DRG, sciatic nerve, footpad, kidney, and lung. DRGs were isolated carefully using a stereoscopic microscope (M80, Leica Microsystems, Wetzlar, Germany), as previously described.²⁸ DRGs were homogenized with Eppendorf pestles (33522, Kisker Biotech, Steinfurt, Germany),²⁹ and the others with Micro Smash (MS-100, TOMY, Tokyo, Japan). Each cDNA was synthesized by QuantiTect Reverse Transcription (QIAGEN, Venlo, Netherlands) from 0.5 μg total RNA. These products were quantified by PCR using FastStart Essential DNA Green Master (Roche, Basel, Switzerland). Real-time PCR was performed by Light Cycler (Roche Diagnostics, Tokyo, Japan). The cycling conditions for all primers were 10 min at 95°C , 45 cycles of 10 s at 95°C , 10 s at 60°C , and 10 s at 72°C . Rsp18 mRNA was used as an internal standard. The PCR primer for mouse sequences were as follows: LPAR1 (*Lpar1*) upstream region, 5'-CTGCCTCTACTTCCAGCCCTGTAA-3', and downstream, 5'-TGCTCACTGTGTTCCATTCTGTGG-3'; LPAR3 (*Lpar3*) upstream region, 5'-GGCTCCCATGAAGCTAATGAAG-3', and downstream, 5'-TTCATGACGGAGTTGAGCAGTG-3'; ATX (*ENPP2*) upstream region, 5'-TC TAGCATCCCAGAGCACCT-3', and downstream, 5'-CGTTTGAAGGCAGGGTACAT-3'; PA-PLA1 (*Ddhd1*) upstream region, 5'-GACTCCGCATTGGAAGTGA-3', and downstream, 5'-ACGTGACGGCTGACCAATAG-3'; LPP3 (*Plpp3*) upstream region, 5'-CACGGGATTGTACGGGTAT-3', and downstream, 5'-GAGGTCGGACACGAAGAACA-3'; GFAP (*Glial fibrillary acidic protein*) upstream region, 5'-CATCGAGATCGCCACCTACA-3', and downstream, 5'-CAGGCTGGTTTCTCGGATCT-3'; TNF α (*Tnf*) upstream region, 5'-CGTCAGCCGATTTGCTATCT-3', and downstream, 5'-CGGACTCCGCAAAGTCTAAG-3'; IL-1 β (*IL1B*) upstream region, 5'-CCTTCCAGGATGAGGACATGA-3', and downstream, 5'-TGAGTCACAGAGGATGGGCTC-3'; r18s (*Rps18*) upstream region, 5'-TTCTGGCCAACGGTCTAGACAAC-3', and downstream, 5'-CCAGTGGTCTTGGTGTGCTGA-3'.

2.6 | Immunohistochemistry and RNA scope analysis

2.6.1 | Preparation of sections

Formalin fixed paraffin-embedded DRG and spinal cord

Mice were anesthetized by intraperitoneal injection of pentobarbital sodium 0.25 mg/kg and sedated by sevoflurane and fixed by perfusing through the left cardiac ventricle with 0.1 M PBS, followed by 4% paraformaldehyde (PFA) flush.

L4–6 lumbar segments and DRGs were collected cautiously and immersed in 4% PFA containing one tablet of PhosSTOP (04906845001, Roche, Basel, Switzerland) per 10 ml. After 4 h of fixation, they were kept in 70% ethanol until paraffin infiltration. Tissues were embedded in paraffin blocks and sliced to 5 μ m.

Formalin fixed paraffin-embedded section for foot

Feet were cut at the lower fibula and immersed in 4% PFA to fix for 1 week at room temperature firmly. Tissues were decalcified and embedded in paraffin blocks after 0.05% celloidin treatment and sliced to 5 μ m.

Fixed frozen section for DRG

DRGs were collected as previously described and placed in freshly prepared 4% PFA for 28 h at 4°C.²⁵ The tissue was immersed in 10% sucrose in PBS at 4°C for 10 min, followed by the same procedures in 20% sucrose in PBS and 30% sucrose in PBS. The tissue blocks frozen in the OTC compound were cut to a thickness of 8 μ m.

2.7 | Immunohistochemistry for p-cAMP-response element-binding in dorsal horn

The immunohistochemical analysis of p-CREB (cAMP-response element-binding) was performed using spinal cord formalin fixed paraffin-embedded (FFPE) samples. After deparaffinization, epitope retrieval was performed by autoclave in 0.01 M citric buffer (pH 6.0). The endogenous peroxidase activity was quenched by incubating the samples with 3% hydrogen peroxide in PBS for 30 min. The sections were incubated overnight at 4°C with rabbit anti-phospho-CREB (1:400, #9198, Cell signaling, MA, USA) as primary antibody. The antibody was diluted in PBS containing 2% BSA.

Then, sections were incubated with simple stain MAX-POR (SSMAX, Nichirei Bioscience, Tokyo, Japan) for 30 min and diaminobenzidine solution for 1 min. The slides were covered with VectaMount AQ (Vector Laboratories, CA, USA), and images were captured under 100 $\times$ magnification with a BZ-X700 microscope (KEYENCE, Osaka, Japan). Neurons positive for p-CREB in the dorsal horn of the spinal cord gray matter were counted by a blinded investigator using ImageJ software (National Institutes of Health, MD, USA).

2.8 | Fluorescent immunohistochemistry for phosphorylated ERK1/2 (p-ERK) and glutamine synthase in DRG

The double staining of p-ERK and glutamine synthase (GS) immunohistochemical analysis was performed using DRG

FFPE samples. After deparaffinization, antigen retrieval was performed by autoclave at 120°C for 20 min in 0.01 M citric buffer (pH 6.0). Next, general blocking was performed for 60 min using 5% BSA/PBS containing 10% goat serum (S-1000, Vector, CA, USA) in a humidified chamber. Sections were then incubated with purified anti-rabbit phospho-p44/42 MAPK (ERK1/2) antibody (1:250, #4370, Cell signaling technology, MA, USA) and anti-mouse-GS antibody (1:200, GT7711, Invitrogen, CA, USA) in 1% BSA/PBS containing 0.03% triton for 1 h at room temperature.

Sections were washed once with PBST (PBS with 0.05% Tween[®] 20) and twice with PBS. Next, goat anti-rabbit (1:400, 20033-0, CF555, Biotium, CA, USA) and goat anti-mouse (1:400, A11001, 488, Lifetech, MA, USA) antibodies were applied as secondary antibodies for 1.5 h at room temperature with light shading. Finally, sections were washed, mounted onto slides with mounting medium (Vectashield, H-1500, VECTOR), and cover slipped.

2.9 | Combining RNAscope with Fluorescent immunostaining

Fixed frozen slides were post-fixed by immersing in pre-chilled 4% PFA for 15 min and dehydrated using a pre-determined method. The sections were incubated with hydrogen peroxide (H₂O₂) for 10 min at room temperature, and then target retrieval was done with RNA scope[®] Target Retrieval Reagent (322000, ACD) at 98°C for 5 min. The sections were pretreated with Protease III for 30 min at 40°C. RNAscope Probe-Lpar1 (Cat No. 318591) was used to visualize *Lpar1* mRNA according to the manufacturer's protocol.

After the RNA scope procedure, sections were incubated with 10% goat serum (S-1000, Vector, CA, USA) and 5% BSA in PBS for general blocking for 1 h in a humidified chamber. After the tip solution was removed, each primary antibody was applied. Anti-NF200 (1:400, mouse, MAB5266, Merck, NJ, USA), peripherin (1:50, mouse, MAB1527, Sigma, MO, USA), and Glutamine Synthetase (1:200, mouse, MA5-27750, Invitrogen) antibodies were used. Goat anti-rabbit (1:400, A21070, 633, Invitrogen or 1:400, 20033-0, CF555, Biotium), goat anti-mouse (1:400, A10321, 594, Lifetech or 1:400, A11001, 488, Lifetech), goat anti-rat (1:400, A21247, 647, Lifetech or 1:400, A21434, 555, Invitrogen) antibodies were used as secondary antibodies. Sections were rinsed, mounted onto slides with mounting medium (Vectashield, H-1500, VECTOR), and cover slipped.

2.10 | Image analysis

The fluorescent signal was detected by a confocal laser microscope (TCS-SP5; Leica, Wetzlar, Germany). Digital

images were obtained under the same exposure conditions. To detect the spots of mRNA and objectively assess the colocalization with respective immunohistochemistry (IHC), we used the programs Bioapplication Colocalization V4.1 and Spots detector V4 (Thermo fisher science, MA, USA).

2.11 | Paw swelling

The vernier caliper (Dial thickness gage G-1A, Ozaki, Tokyo, Japan) was used to measure the maximum thickness of the paw. The results were expressed as the difference in paw thickness of the injected side and contralateral side.²⁵

2.12 | Myeloperoxidase assay

Mice footpad tissues were homogenized in 500 μ l of 50 mM potassium phosphate buffer (pH 7.4) and then homogenized on ice. Homogenized samples were frozen, thawed, and then centrifuged at 10 000 g for 10 min at 4°C. The samples' total protein concentrations were determined using the BCA Assay (Nacalai Tesque, Kyoto, Japan), and the samples were diluted to 0.4 mg/ml. Twenty μ l (8 μ g protein) of the diluted sample was transferred to a 96-well plate containing 100 μ l of reaction buffer [50 mM potassium phosphate buffer (pH 6.0), 0.157 mg/ml o-dianisidine (Sigma-Aldrich, MO, USA), and 0.0005% hydrogen peroxide]. Absorbance at 450 nm was measured after 25 min of incubation. Serial dilutions of commercially available myeloperoxidase (MPO; 475911, EMD Millipore, MA, USA) were used as the standards.^{25,30}

2.13 | Flow cytometry for footpad

Analysis of immune cell infiltration/recruitment was carried out as previously described.³¹ Briefly, tissue was minced and digested with a mixture of 1 mg/ml collagenase A and 2 mg/ml Dispase II (Roche Applied Sciences) in Hepes-buffered saline solution (HBSS) for 120 min at 37°C. After digestion, cells were triturated by pipette, washed with HBSS and 0.5% BSA, filtered through a 40- μ m mesh, and blocked with rat anti-CD16/CD32 (Fc block, 1:10 hybridoma supernatant) on ice for 5 min. The cells were then incubated with anti-CD11b-PE (1:800), anti-Ly6G-APC, and anti-Ly6C-FITC (1:200 unless noted, BioLegend, CA, USA) and analyzed by a flow cytometer Cytoflex (Beckman Coulter, CA, USA). Flow cytometry data were analyzed by FlowJo software (Becton, Dickinson and Company, NJ, USA).

2.14 | Neutrophil depletion and flow cytometry for peripheral blood

Mice received two rounds of intraperitoneal injections of 100 μ g of Ly6G antibody 24 and 48 h before the formalin test. In addition, peripheral blood was collected with heparin and analyzed by flow cytometry to confirm neutrophil depletion. Briefly, cells were stained with anti-Ly6G-FITC (1:200), anti-Gr-1-FITC (1:200), anti-CD45.2-PCP antibody (1:200), and anti-CD11b-APC (1:200) and analyzed on a Cytoflex. In addition, flow cytometry data were analyzed by using FlowJo.

2.15 | Mass spectrometry

2.15.1 | LC-MS/MS analysis in DRGs

Methanol containing internal standards (IS)s (17:0 LPA, 0.10 μ mol/l and 17:0 LPC, 1 μ mol/l) (100 μ l) was added to the mouse tissue (3 DRGs) in a 1.5 ml siliconized tube. After sonication in an ultrasonic bath for 15 min, the samples were centrifuged at 16 400 $\times$ g for 10 min at 4°C. Then, the supernatant was subjected to LC-MS/MS analysis and was also used for phosphorus quantification using the molybdenum blue colorimetric method. LPAs and LPCs in DRG in naïve, 20 min and 2 h after LC-MS/MS measured the formalin injection, modified with the previous method.^{11,32,33}

The LC-MS/MS system consisted of Vanquish HPLC (Thermo Fisher Scientific, San Jose, CA, USA) and a TSQ Altis triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). LC separation was performed using a CAPCELL PAK C18 ACR (1.5 mm i.d. $\times$ 100 mm, 3 μ m particle size; Osaka Soda) with the gradient elution of the mobile phase A: 5 mmol/l-ammonium formate in water and B: 5 mmol/l-ammonium formate in water/acetonitrile, 5/95 (v/v%). The parameters for ionization and multiple reaction monitoring (MRM) analysis of LPAs and LPCs were optimized in this system based on previous reports.^{11,32} The area ratio (peak area of analyte/peak area of IS) was calculated by Xcalibur software (version 4.2.47, Thermo Fisher Scientific). Area ratios of LPA and LPC were corrected using the total phosphorous.

2.15.2 | LC-MS/MS analysis in the footpad

LPAs and LPCs in hind paw tissues at 2 h after the formalin injection were measured by LC-MS/MS, modified with the previous method.^{11,32,33} Methanol containing 0.1% formic acid and internal standards (IS)s (17:0 LPA, 0.10 μ mol/l and 17:0 LPC, 0.49 μ mol/l) (200 μ l) was added

to the mouse tissue (approximately 20 mg) in 2.0 ml tube. After homogenization with beads at 5000 rpm for 20 s (2.8 mm zirconium oxide beads) twice and sonication in an ultrasonic bath for 10 min, the samples were centrifuged at 16 400× g for 20 min at 4°C. Then, the supernatant (150 µl) was transferred to a 1.5-ml sample tube and stored at −80°C until the analysis. The sample (10 µl) was subjected to LC-MS/MS.

The LC-MS/MS system consisted of NANO SPACE SI-II (Osaka Soda, Osaka, Japan) and a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). It was operated in positive ion mode for electrospray ionization (ESI). The condition of LC, ionization parameters, and the MRM for the MS/MS analysis of LPAs and LPCs were performed by the modified method of previous reports.^{11,32} LC separation was performed using a CAPCELL PAK C8 DD (1.5 mm i.d. × 150 mm, 3 µm particle size; Osaka Soda) with the gradient elution of the mobile phase A: 5 mmol/l-ammonium formate in water and B: 5 mmol/l-ammonium formate in water/acetonitrile, 5/95 (v/v%). The area ratio (peak area of analyte/peak area of IS) was calculated by Xcalibur software (version 4.2.47, Thermo Fisher Scientific).

2.16 | Data analysis

Multiple groups were compared using ANOVA with a Bonferroni or a Tukey post hoc test. In addition, two groups were tested with the two-tailed unpaired Student *t*-test.

Data processing and statistical analysis were performed using Prism 8 (GraphPad Software, CA, USA). All data are presented as means ± SEM. Values of *p* < .05 were considered statistically significant.

3 | RESULTS

3.1 | Blockade of LPA signaling attenuated the formalin-induced acute nociceptive behavior

To determine whether the LPA_{1/3} receptor (LPA_{1/3}) is involved in acute nociceptive pain, an LPA_{1/3} antagonist, Ki16425, was pre-administrated by intraperitoneal (i.p.) (Figure 1A,B), planter injection (ipl.) (Figure 1C) before the formalin test. From 15 to 20 min after the formalin injection, a significant difference between the vehicle and i.p. group was observed. The total duration time of nociceptive behavior of the 2nd phase (Figure 1D–F) was significantly attenuated by either i.p. in both sexes or ipl. treatments in male with the LPA_{1/3} antagonist compared

with their respective vehicle group. The total duration time of nociceptive responses of 1st phase was comparable between the vehicle group and the LPA_{1/3} antagonist group. In addition, compared to males and females, females showed significantly increased total duration time of nociceptive responses of 2nd phase in the vehicle group.

Thermal responses (Hot plate and Hargreaves test) and mechanical responses (Von Frey test) were examined in a non-inflammatory state to confirm the effect of the compound on the arousal level. Pretreatment with the LPA_{1/3} antagonist did not affect these behavior tests (data not shown). Furthermore, there were no sedative effects, allodynia, or thermal hypersensitivity by compounds' administration themselves compared with vehicle administration. In addition, there was also no significant difference in the behavioral test before and after administration of compounds and vehicle. These results indicate that LPA_{1/3} antagonist is involved in acute nociceptive responses after formalin administration at the inflammatory states.

3.2 | *Lpar1* mRNA was detected in SGCs, especially in SGCs surrounding the myelinated neuron in DRG

The LPA_{1/3} antagonist we used in this study, Ki16425, does not penetrate the blood-brain barrier and blood-spinal cord barrier.³⁴ Thus, the central nervous system is not the direct target of this drug. Considering that the neuron-rich areas of primary afferents in DRGs are composed of highly windowed capillaries,³⁵ we hypothesized that the analgesic effect of this drug acts in highly vascularized DRGs and/or in post-inflammatory peripheral tissue with abundant blood flow. Quantitative comparison of the *Lpar* mRNA expression in various organs showed significantly higher *Lpar1* and *Lpar3* expression in DRG than in lung, kidney, foot, and spinal cord (Figure 2A,B). Therefore, we confirmed the localization of *Lpar1* in DRG by the RNA scope technology and immunostaining with cell-specific markers. We used glutamine synthase (GS) as a marker of SGCs (Figure 2E), NF200 as a marker of myelinated neuron (Figure 2F), and peripherin as a marker of unmyelinated neuron (Figure 2G). *Lpar1* mRNA was detected abundantly in DRG and most of the *Lpar1* mRNA was co-stained with GS (Figure 2E). Quantification using the programs, Bio application Colocalization V4.1 and Spots detector V4 (Thermo fisher science, MA, USA), showed that 88.3% of the *Lpar1* mRNA signals were co-localized with GS positive signal (Figure 2C). It is known that plural SGCs envelop a single neuron to form a functional unit around a given neuron.¹⁵ Because SGCs affect the neurons in their unit, we studied the profiles of neurons surrounded

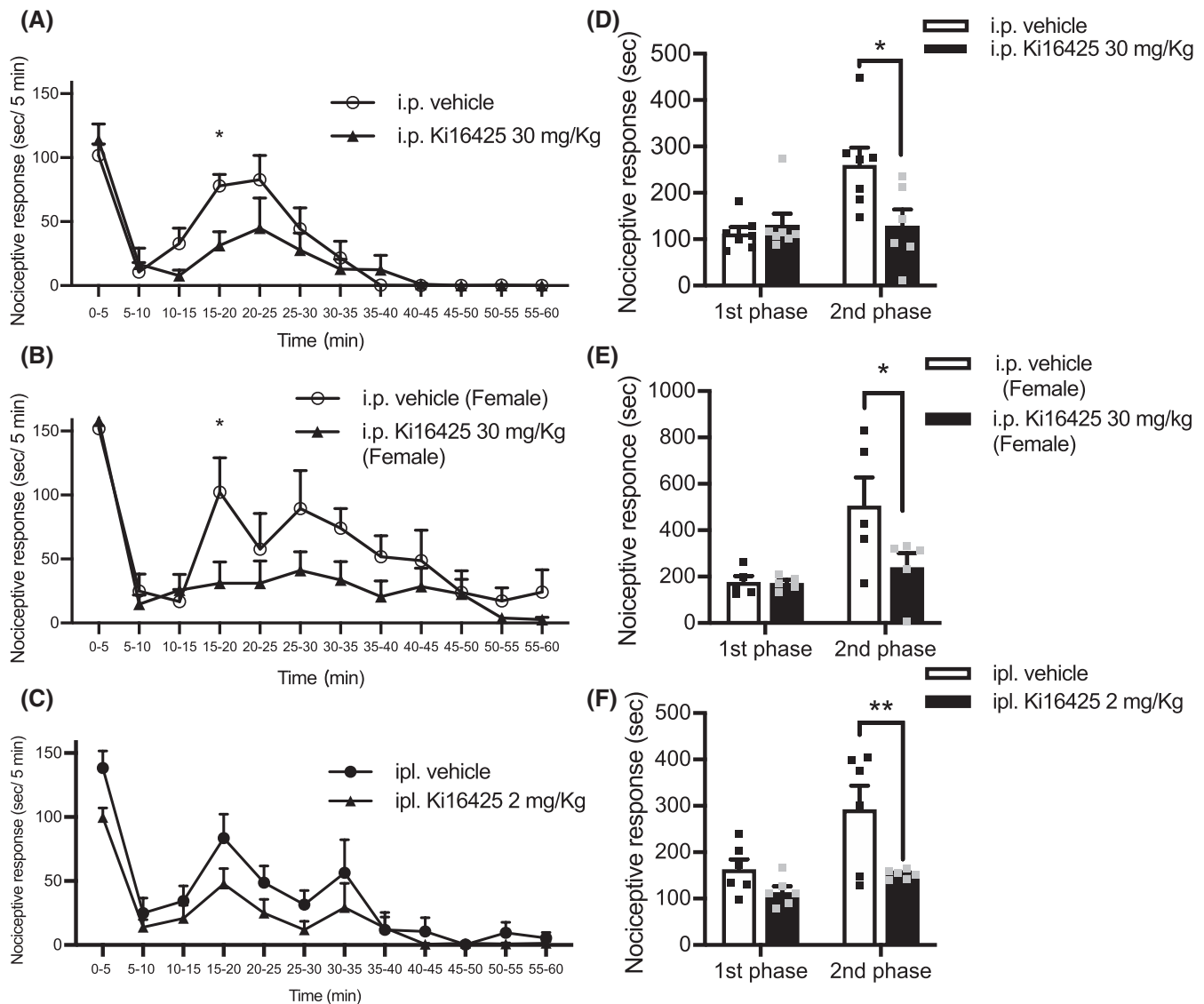


FIGURE 1 Systemic and local LPA_{1/3} antagonist (Ki16425) administration attenuated formalin-induced nociceptive responses in the second phase. (A–C) Time course of nociceptive response time in every 5 min after formalin injection. Mice were pretreated with LPA_{1/3} antagonist Ki16425 in systemic (A: in male, B: in female), and local injection (C). (* $p < .05$, vs. vehicle mice, two-way ANOVA with Bonferroni). (D–F) Total duration time of nociceptive responses during the 1st (0–10 min) and the 2nd phases (11–60 min) (* $p < .05$, ** $p < .01$ vs. vehicle, two-way ANOVA with Bonferroni). [LPA_{1/3} antagonist Ki16425 systemic administration in male, $n = 7$ (D) and in female, $n = 5$ (E), $n = 7$, local administration in male (F), $n = 6$]

by SGCs that express *Lpar1* mRNA. The fluorescent signal intensities of *Lpar1* expression per unit (15 pixels around each neuron) area was significantly higher in the surround of myelinated neuron (NF200 positive) than that in the surround of unmyelinated neuron (peripherin positive) (Figure 2D). These results indicate that *Lpar1* mRNA was present in SGCs, especially in SGCs surrounding myelinated neurons.

Next, the expression of autotaxin (ATX) (Figure 2H) and *papla1α* (Figure 2I), two LPA-producing enzymes, and *lipid phosphate phosphatase 3* (*LPP3*) (Figure 2J), an LPA-degrading enzyme, were examined before and after formalin injection. There was no change in the expression

of *papla1α* and *LPP3* mRNA, but a significant increase of the ATX mRNA expression (Figure 2H) was observed after formalin administration.

3.3 | LPA_{1/3} antagonist decreased the phosphorylated ERK1/2 (p-ERK) in SGCs and phosphorylated CREB in dorsal horn

We examined the effect of the LPA_{1/3} antagonist on the cellular activation of DRGs following formalin injection. Co-immunostaining analyses with anti-p-ERK

antibody and anti-GS antibody were performed on DRG of three groups, naïve (Figure 3A), vehicle-pretreated (Figure 3B), and LPA_{1/3} antagonist-pretreated group (Figure 3C) following formalin injection. P-ERK signals were detected in both neurons and SGCs. Formalin injection significantly increased p-ERK and GS co-stained area (Figure 3D, the vehicle group), which was significantly decreased in the LPA_{1/3} antagonist-pretreated group. However, the number of p-ERK-positive neurons was not affected by treatment with LPA_{1/3} antagonist (Figure 3E). These results suggest that the signaling of LPA-LPA_{1/3} is involved in the cell activation shown by ERK phosphorylation in SGCs, rather than neurons in DRGs.

Next, CREB signaling in the dorsal horn (DH) was assessed by immunohistochemical staining with an antibody to phosphorylated CREB (p-CREB) at Ser133. It is reported that CREB is phosphorylated rapidly and bilaterally and is involved in the priming of hyperalgesia.³⁶ Five minutes after formalin injection, the L4–5 spinal cord was harvested and stained with an anti-p-CREB antibody (Figure 3F). The percentage of p-CREB-positive areas in the lamina 1–5 layers of DH were compared among the naïve group, vehicle-pretreated group, and LPA_{1/3} antagonist-pretreated group. P-CREB positive area increased following formalin injection in all groups, and the LPA_{1/3} antagonist significantly attenuated the percentage of the positive area compared with the vehicle-pretreated group (Figure 3G).

These results indicated that the LPA-LPA_{1/3} signaling is involved in acute pain processing through the activation of SGCs, not only through the DRG neuron, and has the effect of sensitizing the CREB phosphorylation in DH.

3.4 | Treatment with LPA_{1/3} antagonist reduced proinflammatory cytokines expression in the DRG

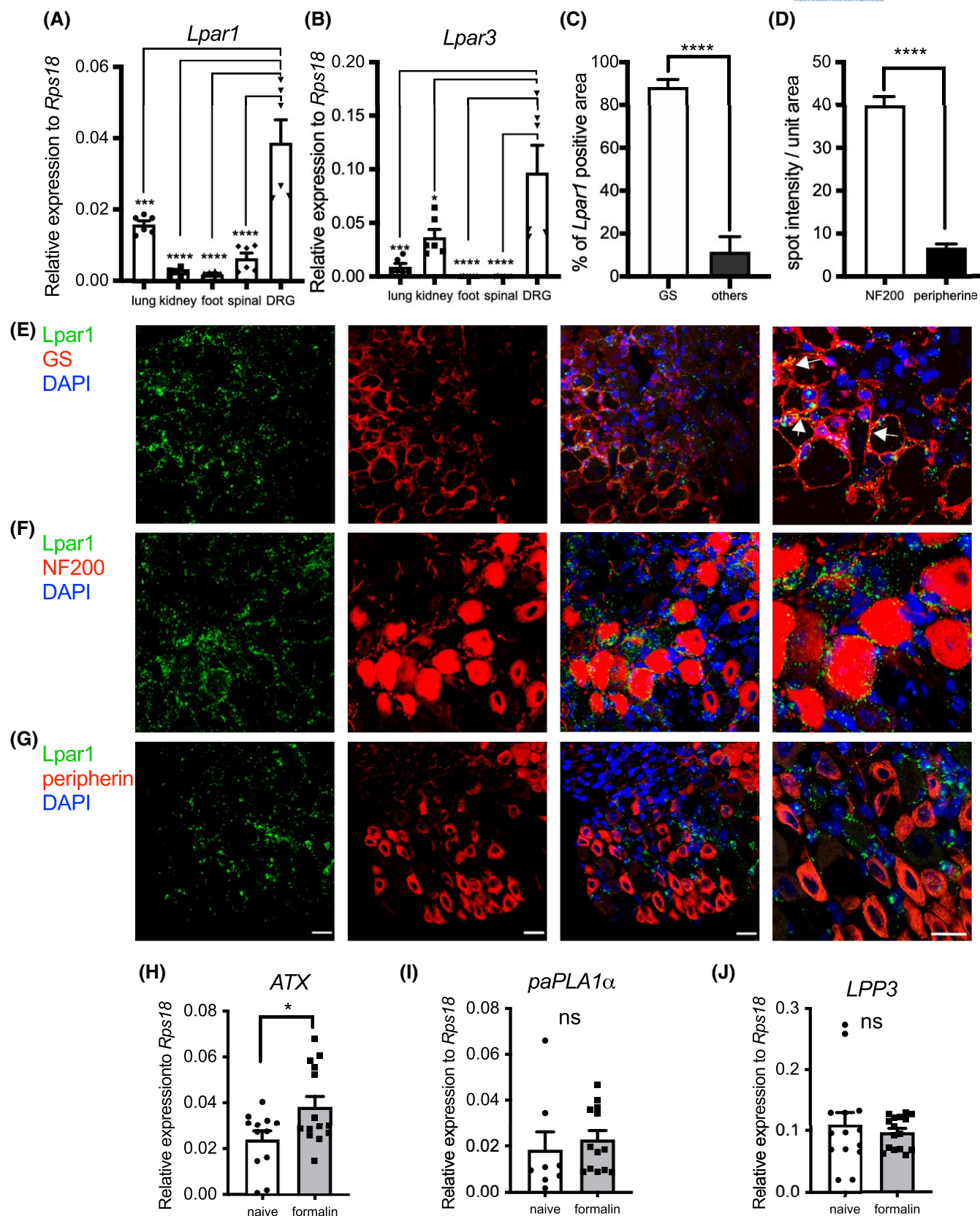
Activated SGCs exhibit the upregulation of GFAP and enhance neuronal excitability by releasing

proinflammatory cytokines.³⁷ Therefore, to determine the involvement of LPA_{1/3} to the activation of SGCs following formalin injection, the transcriptional levels of GFAP, TNF α , and interleukin (IL), -1β were compared between the vehicle-pretreated group and LPA_{1/3} antagonist-pretreated group. As expected, there were significant increases in *GFAP* (Figure 4A), *TNF α* (Figure 4B), and *IL-1 β* (Figure 4C) mRNA expression following formalin injection, and the increments were significantly suppressed in LPA_{1/3} antagonist-pretreated group compared with the vehicle-pretreated group. Next, to confirm the effect of LPA on the activation of the SGCs, we created a model with increased LPA levels in DRG by injecting 18:1 LPA into the intrathecal space of mice. The DRGs were harvested two hours after intrathecal injection, and transcription levels of *GFAP* (Figure 4D), *TNF α* (Figure 4E), and *IL-1 β* (Figure 4F) were compared to the vehicle-injected group. Intrathecal injection of 18:1 LPA increased the mRNA expression of *GFAP*, *TNF α* , and *IL-1 β* in DRGs, with significant increases in *GFAP* and *TNF α* compared to the vehicle-injected group.

3.5 | LPA_{1/3} antagonist attenuated infiltration of neutrophils into the footpad caused by formalin injection

To examine the effect of the LPA_{1/3} antagonist on peripheral inflammation, we measured the thickness of the most swollen part of the mouse footpad following formalin injection. The paw swelling was significantly attenuated in the LPA_{1/3} antagonist group compared with the vehicle group (Figure 5A). The MPO (an enzyme expressed by neutrophils) assays and counts of infiltrated neutrophils were performed in the peripheral tissue to investigate the involvement of neutrophils. Formalin injection increased the MPO activity of both groups, and LPA_{1/3} antagonist significantly suppressed the MPO activity (Figure 5B). Neutrophils (CD11b⁺Ly6G⁺ cells) infiltration increased both in the vehicle and LPA_{1/3} antagonist groups at 2 h after formalin injection and was

FIGURE 2 LPA₁ receptor mRNA was abundantly localized in satellite glial cells (SGCs) especially surrounding the myelinated neuron in DRG. The mRNA expression levels of *Lpar1* (A) and *Lpar3* (B) by organ. ($n = 5-7$, * $p < .05$, ** $p < .01$, **** $p < .0001$ vs. expression in DRG, one-way ANOVA with Bonferroni). (C) 88.3% $\pm$ 5% signals of *Lpar1* mRNA were co-stained with GS (SGCs marker). Graph showed the percentage of co-stained with GS and others. ($n = 5$, **** $p < .0001$ unpaired t). (D) The signal intensity in 15 pixels around NF200-positive and peripherin positive cells was divided by the ring area using the program of Bioapplication®. The graph shows signal intensity per unit, with a more intense signal for *Lpar1* around the NF200 (myelinated neural marker). ($n = 157-236$, **** $p < .0001$, unpaired t). (E) A representative co-staining with IHC with RNAscope of *Lpar1* (Green), DAPI (Blue), and GS (Red). White arrows indicate co-staining for GS and *Lpar1* signals. A representative co-staining of *Lpar1* (Green), DAPI (Blue), and (F) NF200 (Red) or (G) peripherin (Red). Scale bar = 25 μ m. (H) The mRNA expression levels of *ATX* (H), *paPLA1 α* (I), *LPP3* (J) in the L5 DRG after injecting formalin to the footpad. ($n = 9$, * $p < .05$, vs. naïve DRG, unpaired t)



significantly inhibited in the LPA_{1/3} antagonist group either with i.p. or i.p.l. administration (Figure 5C). The RNA scope technique showed the expression of *Lpar1*

in eccrine glands, vascular endothelial cells, fibroblasts, and keratinocytes near the basal layer in the foot (Supporting Information Figure S1).

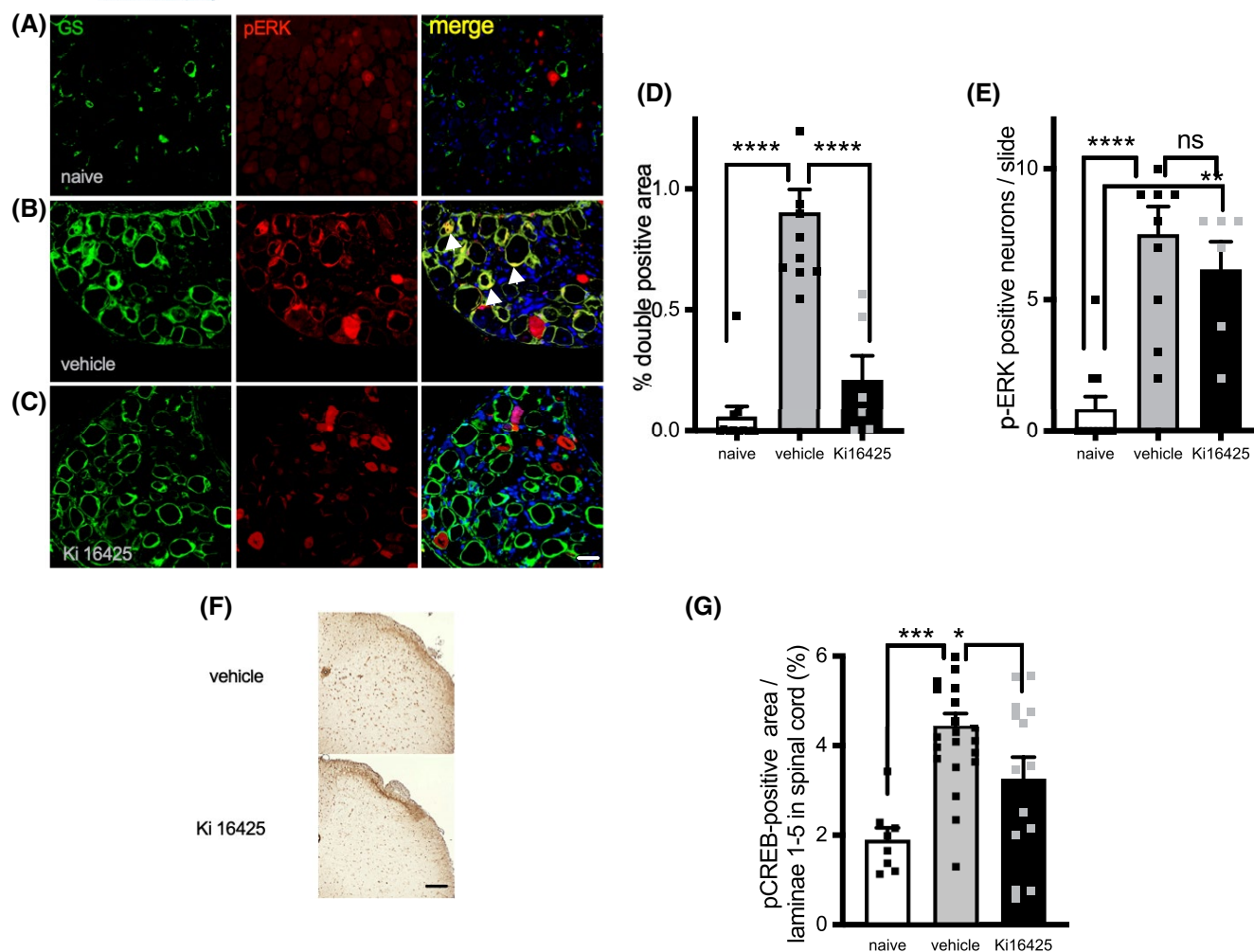


FIGURE 3 LPA_{1/3} antagonist inhibited the phosphorylation of ERKs in SGC but not of primary neurons in DRG and suppressed CREB phosphorylation of secondary neurons in DH. Representative IHC in mouse DRG of GS (green), phosphorylated ERKs (red), and merge (yellow). (A) shows naïve DRG, (B) shows 2 h after formalin injection pretreatment with vehicle or (C) LPA_{1/3} antagonist. White arrows indicate co-staining for GS and p-ERK. Scale bar = 25 μ m. (D) The quantification of the % of GS and p-ERK co-staining area in A–C. (E) The number of p-ERK positive neuron from A to C. (D, E) ($n = 7–11$) Data were analyzed by one-way ANOVA with Turkey, ** $p < .01$, **** $p < .0001$. (F) The representative images of IHC of p-CREB in L4–6 DH of mice 5 min after formalin injection into the footpad. The upper one is DH with the vehicle, and the lower one is with LPA_{1/3} antagonist. Scale bar = 100 μ m. (G). The quantification of (F), the % of p-CREB-positive neurons in the subregion of laminae 1–5 in DH (L4–L6) $n = 5$ and 2–4 slides for a mouse (Data were analyzed by one-way ANOVA with Turkey's multiple-comparison, * $p < .05$, *** $p < .001$)

The experiments were conducted to clarify whether the attenuated neutrophil infiltration by the LPA_{1/3} antagonist correlates with the attenuation of nociceptive responses. Neutrophil was successfully depleted in all animals intraperitoneal injected with the Ly6G antibody (200 μ g/mouse) (Figure 5D). However, the LPA_{1/3} antagonist administration similarly attenuated the 2nd phase of formalin-induced nociceptive response in neutrophil depletion and control groups (Figure 5E). Moreover, the differences in the nociceptive responses between the neutrophil depletion and control groups were not significant. These results indicate that the analgesic effect of the LPA_{1/3} antagonist does not depend on the number of neutrophils infiltrated into the formalin-injected footpad.

3.6 | Formalin injection to the footpad increased the LPA concentration in the innervated DRG

Our results have indicated that LPA_{1/3} receptor signaling in DRG modulates the transmission of formalin-induced acute nociceptive responses. Next, we measured LPA levels using LC-MS/MS to know whether formalin injection to the footpad increased LPA within the innervated DRG. Bilateral DRGs of L3–5 were collected from mice before, 20 min or 2 h after formalin injection in the right hind paw. The amounts of LPA (Figure 6A, Supporting Information Figure S2A) and its precursor LPC (Figure 6B, Supporting Information Figure S2B) were measured by LC-MS/MS.

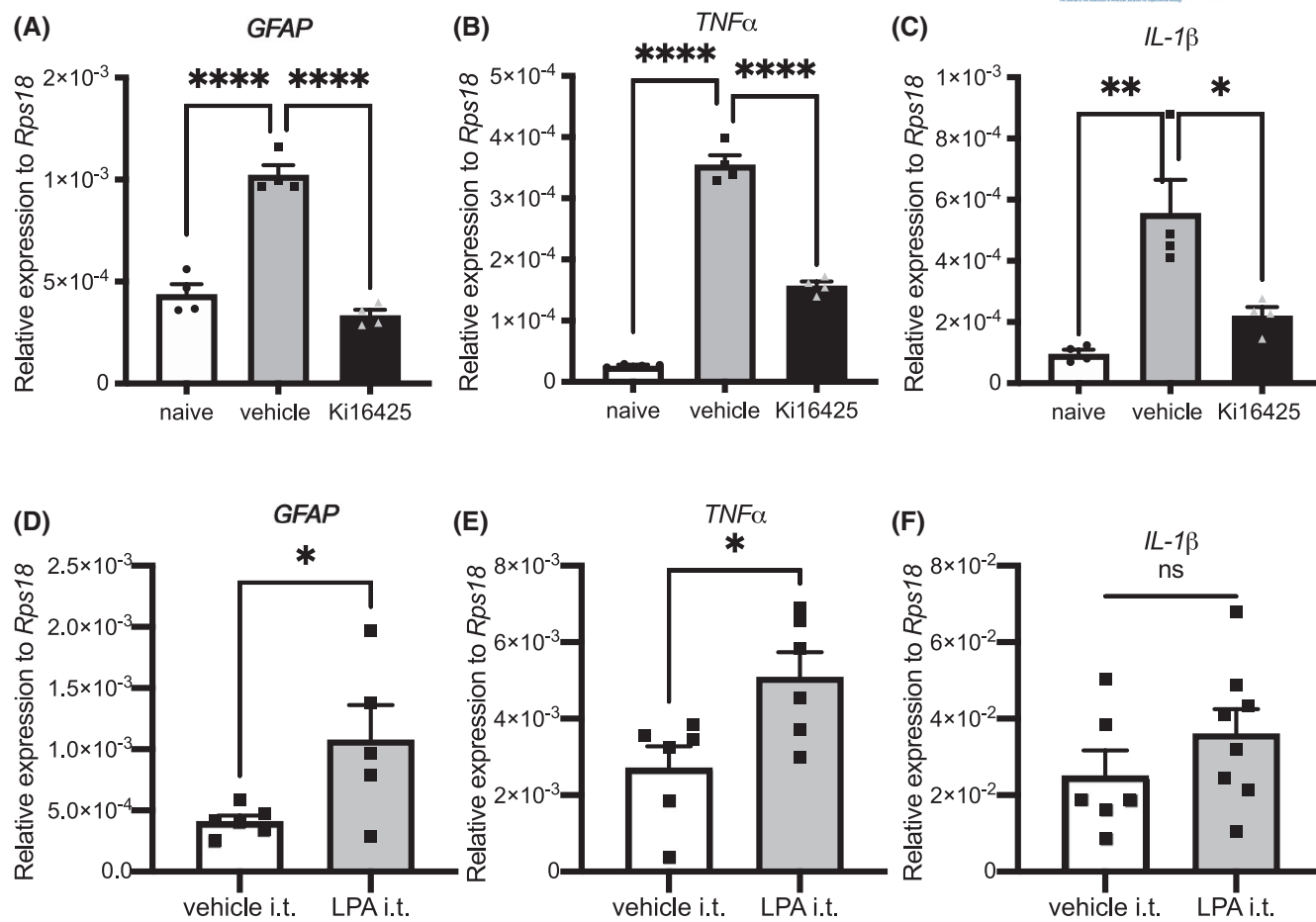


FIGURE 4 LPA_{1/3} antagonists inhibited upregulation of inflammatory cytokine and glial marker in DRGs after formalin injection, and intrathecal injection of 18:1 LPA increased these markers. The *GFAP*(A), *TNFα*(B), *IL-1β*(C) mRNA expression levels from DRG of naïve, 2 h after formalin injection to the right hind paw with vehicle or LPA_{1/3} antagonist. ($n = 4-9$, * $p < .05$, ** $p < .01$, **** $p < .0001$ vehicle vs LPA_{1/3} antagonist, one-way ANOVA with Bonferroni). The *GFAP*(D), *TNFα*(E), *IL-1β*(F) mRNA expression levels from DRG of 2 h after intrathecal injection with vehicle or 18:1 LPA ($n = 6-8$, * $p < .05$, vehicle i.t. vs. LPA i.t., unpaired t -test)

The levels of 16:0 (contra and ipsi ** $p < .01$), 18:0 (contra and ipsi * $p < .05$), and 18:1 (contra ** $p < .01$, ipsi * $p < .05$) and total LPA (Figure 6A, contra and ipsi $^{++++}p < .0001$) were increased 2 h after formalin injection bilaterally and approximately doubled compared to naïve mice. The levels of unsaturated LPA, such as 16:1, 18:2, and 20:4 LPA in bilateral DRGs of 2 h after formalin injection, were slightly higher than naïve mice.

In addition, the levels of 16:0 LPC (See graph, Supporting Information Figure S2B, contra * $p < .05$) and total LPC (Figure 6B, contra $^{++++}p < .0001$, ipsi $^{+++}p < .001$) in DRGs of 2 h after formalin injection were higher than the levels of the naïve side. On the other hand, LPA levels in bilateral DRGs of 20 min after formalin injection did not change significantly.

The amounts of LPA and its precursor LPC in the footpad samples were also quantified using LC/MS/MS. Although neither molecular species of LPA increased significantly (data not shown), the levels of 16:0, 18:2, and 22:6 LPC were significantly elevated in the tissue sample

of the ipsilateral side compared with that of the naïve and contralateral side (Supporting Information Figure S2C) after formalin injection.

4 | DISCUSSION

This study demonstrated that inhibition of LPA_{1/3} signaling significantly suppressed formalin-induced acute nociceptive responses by inhibiting the activation of SGCs. In addition, we revealed that formalin injection in the footpad increases LPA levels in its innervated DRGs for the acute period. LPA produced in DRGs could promote the activation of SGCs through LPA₁, increase the sensitivity of primary neurons, and modulate pain behavior.

Our result showed that LPA₁ was expressed in SGCs, especially in SGCs surrounding myelinated/NF200-positive neurons, and that pharmacological inhibition of LPA_{1/3} suppressed the activation of SGCs and expression of proinflammatory cytokines in DRG. SGCs

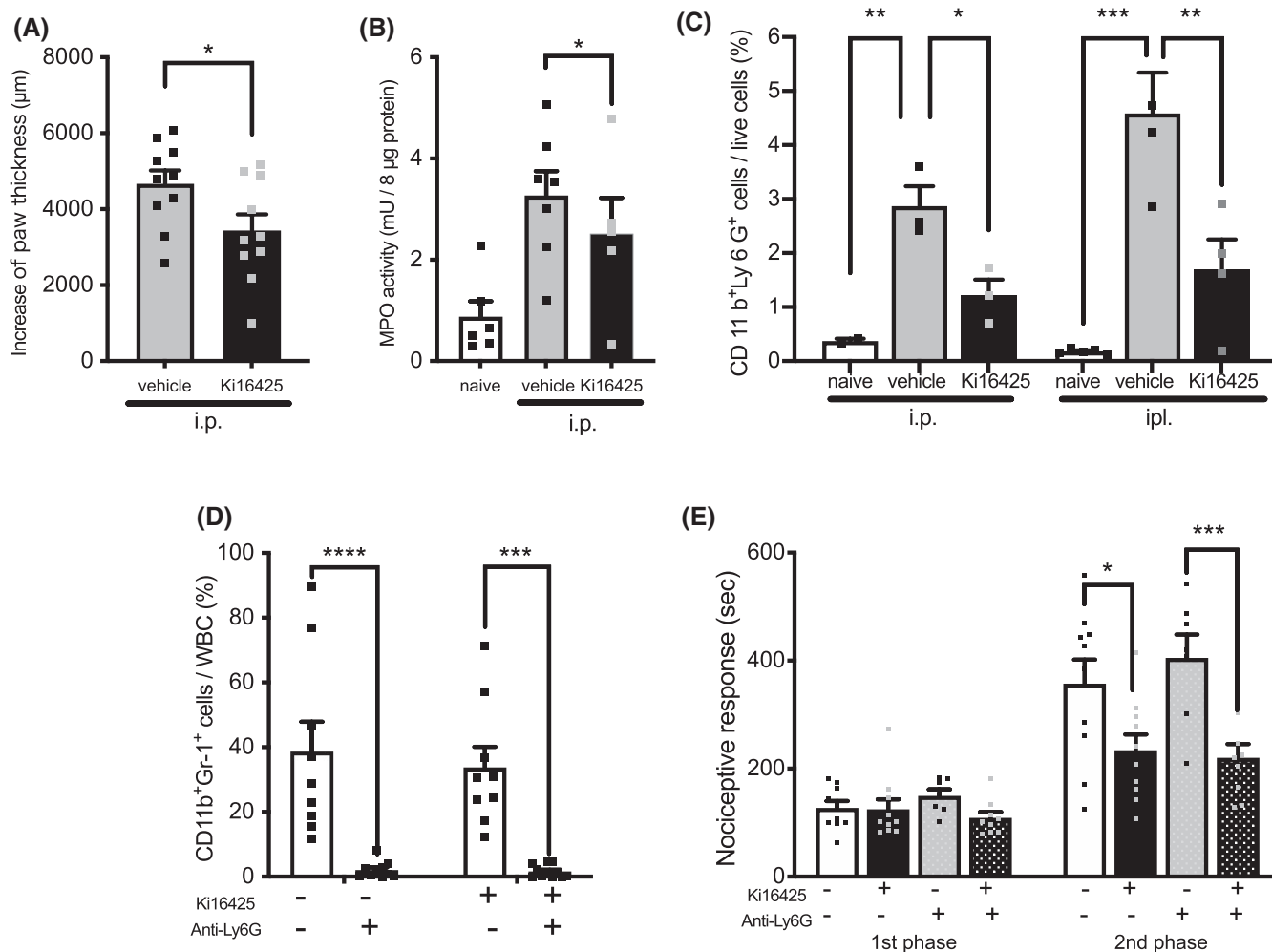


FIGURE 5 LPA_{1/3} antagonists attenuated neutrophils infiltration in the paw after formalin injection. (A) The thickness of the most swollen part of the mouse footpad 2 h after formalin injection ($n = 10$, * $p < .05$, unpaired t -test). (B) Myeloperoxidase (MPO) activity in the right hind paw 1 h after formalin injection ($n = 5-7$, * $p < .05$ vehicle vs. LPA_{1/3} antagonist, ANOVA with Bonferroni's multiple comparisons test). (C) The percentage of CD11b⁺Ly6G⁺ cells/live cells in the right hind paw tissue 2 h after formalin injection was analyzed by flow cytometry ($n = 3$, ** $p < .01$ vehicle vs. LPA_{1/3} antagonist administration, one-way ANOVA with Bonferroni's multiple comparisons test). (D) The percentage of CD11b⁺Gr1⁺ cells/live cells in peripheral was analyzed by flow cytometry, control vs neutrophil depletion ($n = 18-21$, *** $p = .001$, **** $p < .0001$, t -test). (E) Total duration time of nociceptive behaviors during the 1st and the 2nd phases, from control and LPA_{1/3} agonist with and without neutrophil depletion ($n = 8-10$, * $p = .0176$, *** $p = .0005$ two-way ANOVA with Bonferroni's)

are the glial cells in the DRG that wraps the neuronal cell body as envelopes. DRG neurons and SGCs form a distinct morphological unit representing the basis for their intense bidirectional communication via the gap junction.³⁷ Recently, activated SGCs have been shown to contribute to the pathogenesis of most pain in animal studies. Regarding chronic inflammatory pain, for example, expression of GFAP increased in SGCs surrounding injured neurons in the complete Freund's adjuvant (CFA)-induced monoarthritis model³⁸ and blocking of gap junction of SGCs ameliorated CFA-induced inflammatory pain.¹⁵ Furthermore, acute nociception induced by intraplantar injection of capsaicin was inhibited by the P2X7 receptor (expressed exclusively by SGCs) antagonist and a gap junction

blocker.¹⁶ These studies support that SGCs activation is a common feature in pain models and an effective target for various analgesic drugs. LPA_{1/3} antagonists also suppressed the expression of *TNFα* and *IL-1β* mRNA in DRGs. Previous studies revealed the release of these proinflammatory cytokines from DRG after injury on the innervation site.^{39,40} SGCs-derived these proinflammatory cytokines may act on neurons, which increase neuronal excitability.¹⁵ Our results suggest that LPA-LPA₁ signaling is involved in cytokine production, most likely from SGCs, and affects neuronal excitability.

It was reported that 18:1 LPA as well as ATP and capsaicin, induced calcium (Ca^{2+}) mobilization, which depends on phospholipase C in cultured sensory neurons

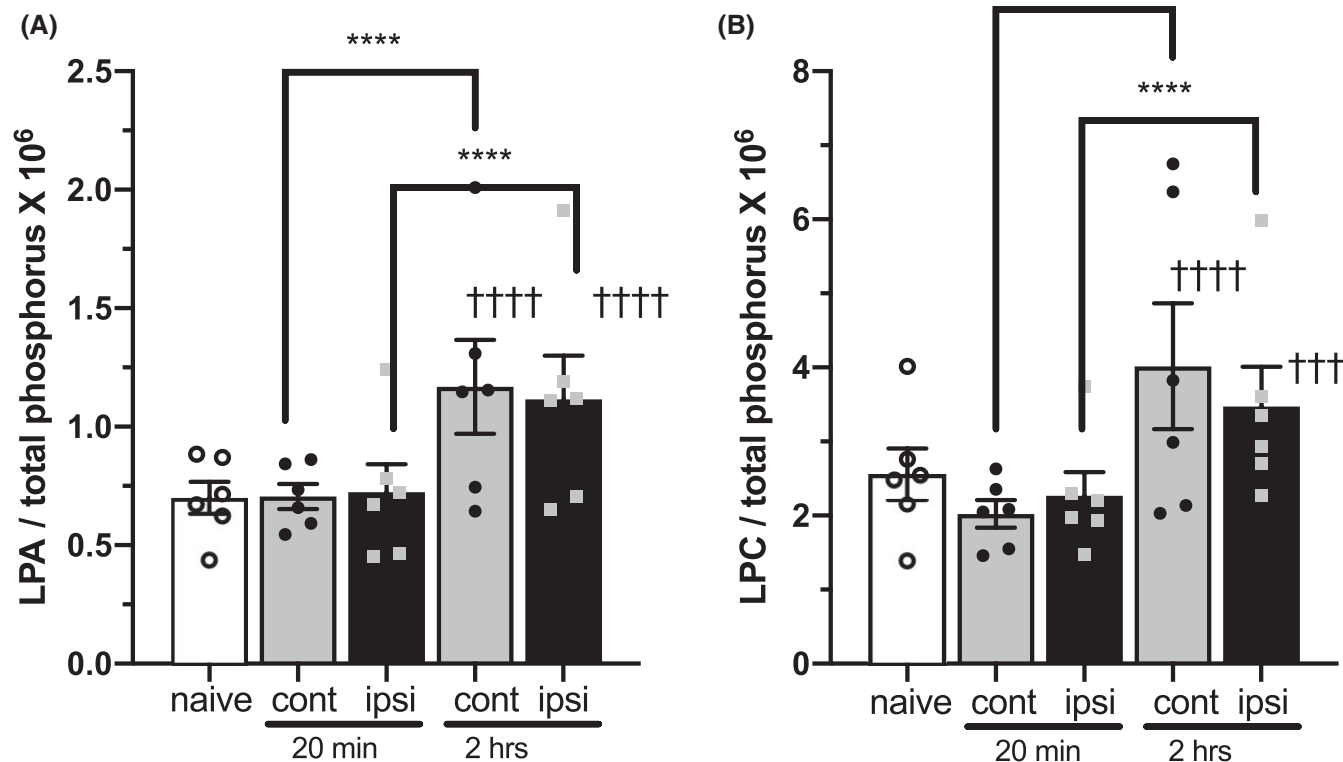


FIGURE 6 Formalin injection to the footpad increased the LPA concentration in the innervated DRG. Using LC-MS/MS, total LPA (A) and total LPC (B) levels were measured from bilateral L3–5 DRGs of naïve, 20 min, and 2 h after formalin injection to the right hind paw. Total LPA (A) and total LPC (B) were adjusted using the total phosphorous, respectively. ($n = 6$, †††† $p < .0001$ vs naïve, *** $p < .001$, **** $p < .0001$, vs. corresponding DRG with different time, two-way ANOVA with Bonferroni)

and SGCs.¹³ Activation of ERK1/2 and the Ca^{2+} mobilization is known to promote the transcriptional activity of CREB and involved in a variety of cellular processes such as neuron-glia communication.^{36,41} We detected a marked increase of LPA in the innervated DRG to the injured paw and increased ERK phosphorylation. Then, the subsequent phosphorylation of CREB in the DH of the spinal cord could lead to central sensitization. Note that the phosphorylation of ERK in SGCs but not in neurons was suppressed by $\text{LPA}_{1/3}$ antagonist treatment. It is conceivable that the activation of SGCs surrounding A-fiber neurons is crucial to initiate the subsequent bidirectional communication between SGC-SGC and SGC-A-fiber neurons communicating fast nociceptive responses. Further studies are required to elucidate the process of amplification of the afferent signal via LPA_1 -mediated SGCs activation. LPA was reported as a direct ligand of TRPV1 and induced Ca^{2+} mobilization in DRG neurons.¹² Conversely, it was reported that LPA elevated Ca^{2+} concentration in the DRG even in TRPV1 deficient mice, and LPA activates a large fraction of SGCs rather than neurons.¹³ Our results also confirmed that $\text{LPA-LPA}_{1/3}$ signaling is involved in the cellular responses in SGCs, but not in DRG neurons alone. $\text{LPA-LPA}_{1/3}$ signaling contributes to acute pain via

activation of SGCs and might be a potent target for the pain.

Pharmacological inhibition of $\text{LPA}_{1/3}$ suppressed the neutrophil infiltration at the peripheral site. Upon exposure to LPA, endothelial cells increase vascular permeability and release CXCL1, both of which contribute to the recruitment of neutrophils.^{42,43} Our results showing that *Lpar1* mRNA was expressed in endothelial cells, and LPA receptor antagonists inhibited the infiltration of Ly6G-positive cells support the previous reports.^{44,45} We speculated that the neutrophil infiltration driven by $\text{LPA-LPA}_{1/3}$ signaling facilitates the peripheral sensitization and nociceptive responses. However, neutrophil depletion did not affect the nociceptive behavior in the presence and absence of $\text{LPA}_{1/3}$ antagonists. It was also reported that nociceptive response was not abrogated simply by suppressing neutrophil infiltration.⁴⁶ Thus, although $\text{LPA}_{1/3}$ signaling triggered neutrophil infiltration, the effect of neutrophils on nociceptive responses was not potent, at least in our experiments, and we propose that $\text{LPA}_{1/3}$ signaling in SGCs is mainly involved in acute nociceptive pain independently of neutrophils. Our results suggest that LPA-LPA_1 signaling in SGCs is essential to enhance nociceptive response.

For the first time, we have revealed that formalin injection in the footpad increases LPA levels in its innervated DRGs of mice for the acute period. Two enzymes, ATX and PA-PLA1 α , which synthesize LPA extracellularly, were expressed in DRG. LPC, a substrate of ATX, also increased by formalin injection in DRG, and might contribute the increased LPA production by ATX to stimulate LPA_{1/3}. However, it should be noted that the major LPA species that increased in DRG was LPA 18:0, which is a poor agonist for each LPA receptor^{47,48} and cannot be substantially produced by ATX.⁴⁹ As most of the LPAs detected in tissues in our study are known to locate intracellularly as an intermediate for synthesizing other glycerolipids. Thus, increased LPA by formalin injection may be used for the generation of glycerolipid in injured DRG. Therefore, further study is required for the significance of the LPA production in DRG.

Interestingly, there was an increase in LPA in contralateral DRGs to a similar extent to the ipsilateral DRG. Many reports show that unilateral stimulus evokes contralateral responses of DRG even though exact mechanisms are still unclear.^{50–53} After unilateral neuropathy, cytokines such as TNF α and IL-10 have been altered in the DRG of the contralateral side and ipsilateral side,⁵² and SGCs in DRG are shown to be involved in the bilateral TNF α increase.⁵³ During inflammatory pain, pain-related glycosphingolipid biosynthesis was increased in DRGs bilaterally.⁵¹

Rimola et al. reported that peripheral LPC injection contributed to the activation of TRPV1 and TRPM8 in sensory neurons and was involved in the mechanism of oxaliplatin-induced peripheral pain.⁵⁴ LPC increases with damage and inflammation and is involved in the increase of LPA, as a substrate for conversion to LPA by the lysophospholipase D activity of ATX. LPA also increases with damage and inflammation in parallel. As well as increase of LPA levels, formalin injection in the footpad increases LPC levels in its innervated DRGs during the acute period (Figure 6, Supporting Information Figure S2). To date, at least six GPCRs have been identified for LPA, and each receptor signaling mechanism in neuronal and non-neuronal cells are being elucidated. On the other hand, LPC-specific receptors have not been identified. LPA converted from LPC following tissue injury functions as a unique receptor-mediated signaling molecule and could activate a LPAR, LPA1 of SGC, play a crucial role of signaling of acute pain modulation as shown in this study.

Ki16425 selectively blocks both LPA₁ ($K_i = 0.34 \mu\text{M}$) and LPA₃ ($K_i = 0.93 \mu\text{M}$),⁵⁵ and in this study, we focused on LPA₁ in relation to acute nociceptive pain, which has been featured in SGCs. It has been reported that not only LPA₁ but also LPA₃ is expressed in DRG of mice,¹³ and studies focusing on LPA₃ are required in the future. There are reports showing that pain inhibition in paclitaxel-induced

neuropathic pain models was observed in LPA3KO as well as LPA1KO.^{56,57} Single-cell RNA-sequence and subsequent cluster analyses showed that LPA₁ was assigned by cluster analysis as an eigengene of the glia gene module, while LPA₃ was neuronal gene module but not with glia of DRGs.^{58,59} In our study, LPA_{1/3} antagonist suppressed phosphorylation of ERK in SGC which express LPA₁ but not in neuron which might express LPA₃ (Figure 3). These results indicated that the inhibitory effect of LPA_{1/3} antagonist was observed on LPA₁ rather than LPA₃. We propose that LPA₁-mediated activation of SGC was mainly involved in the induction of pain in this model.

In this study, male and female mice were used to observe the acute phase of the pain response and confirmed the pain inhibition in both sexes. However, all other experiments were conducted on males only. Additional detailed studies should be conducted to determine the core mechanisms following SGCs and neuronal activation, focusing on gender-distinct experiments.

In summary, we demonstrated that the LPA_{1/3} antagonist attenuated the spontaneous nociceptive response, activation of SGCs in the DRG, and peripheral inflammation during formalin-induced tissue injury pain. In addition, increase of LPA levels was detected in the innervated DRGs at acute nociceptive phase. The acute nociceptive pain response is an essential response for the body's defense, and LPA-LPA₁ axis in the SGCs was found to be involved in this initial biological response. The persistence of these responses lead to hyperalgesia and unbearable pathological pain condition, and appropriate initial pain management is essential. LPA-LPA₁ axis could be a novel therapeutic target for the acute nociceptive pain next to NSAIDs and acetaminophen.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Yoko Hoshino, Toshiaki Okuno, Takehiko Yokomizo, and Nobuko Ito designed and performed experiments, analyzed data, and wrote the manuscript. Daisuke Saigusa and Kuniyuki Kano performed the lipid analysis. Shota Yamamoto and Hideo Shindou performed experiments of intrathecal injection. Kanji Uchida, Junken Aoki, and Takehiko Yokomizo designed the study; and all authors assisted in editing the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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