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Activation of acid-sensing ion channel 3 by lysophosphatidylcholine 16:0 mediates psychological stress-induced fibromyalgia-like pain

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ABSTRACT

Objectives Fibromyalgia is commonly considered a stress-related chronic pain disorder, and daily stressors are known triggers. However, the relation between stress and pain development remains poorly defined by clinical approaches. Also, the aetiology remains largely unknown.

Methods We used a newly developed mouse model and lipidomic approaches to probe the causation and explore the biological plausibility for how perceived stress translates into chronic non-inflammatory pain. Clinical and lipidomic investigations of fibromyalgia were conducted for human validation.

Results Using non-painful sound stimuli as psychological stressors, we demonstrated that mice developed long-lasting non-inflammatory hyperalgesia after repeated and intermittent sound stress exposure. Elevated serum malondialdehyde level in stressed mice indicated excessive oxidative stress and lipid oxidative damage. Lipidomics revealed upregulation of lysophosphatidylcholine 16:0 (LPC16:0), a product of lipid oxidisation, in stressed mice. Intramuscular LPC16:0 injection triggered nociceptive responses and a hyperalgesic priming-like effect that caused long-lasting hypersensitivity. Pharmacological or genetic inhibition of acid-sensing ion channel 3 impeded the development of LPC16:0-induced chronic hyperalgesia. Darapladib and antioxidants could effectively alleviate the stress-induced hyperalgesia by inhibiting LPC16:0 synthesis. Clinical investigations showed that excessive oxidative stress and LPC16:0 expression also exist in patients with fibromyalgia. Moreover, LPC16:0 expression was correlated with pain symptoms in patients with high oxidative stress and disease severity.

Conclusions Our study provides experimental evidence for the causal effect of psychological stressors on chronic pain development. The findings identify a possible pathophysiological mechanism of stress-induced chronic non-inflammatory pain at molecular, behavioural and clinical levels that might indicate a new therapeutic approach for fibromyalgia.

Key messages

What is already known about this subject?

- Fibromyalgia is considered as a stress-related chronic pain disorder, and daily stressors are known triggers. However, the causation of stress and disease development remains poorly defined, and the mechanism is largely unknown.

What does this study add?

- This study demonstrates that exposure to repeated and intermittent non-painful sound stress triggered chronic non-inflammatory hyperalgesia in mice, which provides experimental evidence for the causal effect of stress exposure on fibromyalgia-like pain development.
- Excessive oxidative stress inflicted by stress exposure caused lipid oxidisation and subsequent upregulation of LPC16:0, which triggered nociceptive signalling to cause chronic hypersensitivity via activation of acid sensing ion channel 3. Darapladib and antioxidants alleviated the stress-induced chronic hyperalgesia by inhibiting LPC16:0 synthesis.
- Clinical evidence showed that excessive LPC16:0 exists in fibromyalgia cases, and LPC16:0 expression was correlated with pain symptoms in patients with high oxidative stress and disease severity.

How might this impact on clinical practice or future developments?

- The findings identify a possible pathophysiological mechanism of stress-induced chronic non-inflammatory pain, which might indicate a new therapeutic approach for fibromyalgia.

INTRODUCTION

Psychosocial stress is pervasive in modern societies and causatively involved in various illnesses. In addition to psychiatric disorders, stress also results in substantial somatic burden.[1 2] Fibromyalgia (FM) is commonly considered a stress-related disorder characterised by chronic non-inflammatory widespread pain.[1 3] Although the aetiology remains unknown, stress is believed to be an important trigger.[1 3] Numerous lines of clinical evidence suggest that current or preceding stressed status seems to precipitate later development of FM.[3–5] Moreover, high stress levels and significant psychiatric disorders are common in patients with FM.[1 6] Although clinical evidence supports the theoretical link, the cause–effect relation remains poorly defined.[1 3 7] As well, the potential mechanisms underlying these psychophysiological interactions are largely unknown.

In past decades, FM has been increasingly considered a central pain disorder.[3 7] Such attribution not only arises from its distinct psychophysiological trait, but is also compelled by the lack of evidence of peripheral tissue abnormalities.[7 8] Thus far, whether somatosensory inputs are involved in the pain development remains unclear. On the one hand, lack of detectable tissue damage does not support peripheral sensitisation as the cause of hypersensitivity.[8] On the other hand, lack of tissue pathology may not preclude peripheral factors from participating in nociceptive activation.[7 9] Of note, recent clinical evidence revealed C-fibre hyperexcitability in FM, probably contributing to the pathogenesis.[7 10] Nonetheless, such observations still leave the unanswered question of how the aberrant excitability develops in the first place. From the psychophysiological perspective, we also have no clue about whether and how the psychological stressors interact with the somatosensory input.

In translational research, sound stress (SS) is one of the potent psychological stressors used in animal models and has been introduced in pain research for years.[11–13] Currently, SS is commonly used in disease models of inflammation or injury to investigate the enhancing effects of stress on nociceptive responses. Although the established SS model causes stressed states and evidently enhances inflammatory pain behaviours, SS alone does not function to trigger nociceptive activation, and additional pro-inflammatory substances or tissue damage are indispensable for pain induction.[11] Given that FM is characterised by a non-inflammatory condition, the current setting of SS models is not ideal for its translational research. Here, by modifying the original SS protocol, we demonstrate that psychological stressors with repeated and intermittent traits on their own can directly generate long-lasting hyperalgesia in mice. We applied lipidomics and pharmacological approaches to identify the pain mechanism of psychological stress-induced hyperalgesia in stressed animals. Moreover, we conducted clinical and lipidomics investigations of FM to validate mouse discoveries in humans. Understanding the pathophysiology of how these perceived psychological factors engage in the nociceptive process could help in the development of effective therapeutic approaches for FM.

MATERIALS AND METHODS

Animals

Animal care and procedures were approved by the Institutional Animal Care and Use Committee of Academia Sinica (Protocol #16-11-1000). We used adult (8–12-week-old) C57BL/6JNarl mice from the National Laboratory Animal Center (NAR Labs, Taiwan). Female mice were used for most of the studies, and mice of both sexes were used for investigating sexual dimorphism. *Asic3*$^{-/-}$ mice were generated and genotyped as previously described.[14] All efforts were made to minimise the number of animals used and their suffering without compromising the quality of the experiments.

Clinical study

The clinical prospective research was carried out in accordance with the Declaration of Helsinki and was approved by the institutional review board of Kaohsiung Medical University Hospital (KMUHIRB-(I)-20170012). Adult patients with chronic widespread pain in the outpatient department of the hospital were consecutively enrolled over 1 year from 2018 July to 2019 June in the neurological clinic at KMUH. Participants were interviewed and evaluated by an experienced neurologists (CHH), and those fulfilling the 2010 American College of Rheumatology criteria for FM were recruited. Age-matched and sex-matched individuals without chronic pain and mood disorders were also prospectively recruited as healthy controls (HCs). Clinical information was acquired by personal interview and questionnaire, and blood samples were collected for biochemistry and lipidomic analysis. To evaluate the clinical presentation and metabolomic phenotypes without pharmacotherapeutic interference, only patients with newly diagnosed primary FM and without current antidepressant and antiepileptic treatment were recruited for further analysis. All participants were well informed and provided written consent. All patients with a FM diagnosis were followed up for at least 6 months by outpatient services to ensure that no other aetiology was identified.

RESULTS

Repeated and intermittent exposure to stress induces chronic hyperalgesia in mice

In basic research of pain, repeated and intermittent challenge of stimuli is crucial for pain perpetuation.[9 15 16] Therefore, we modified the existing SS setting by increasing stimulus intensity repeatedly and intermittently from once daily to once every 3 hour for six times daily (figure 1A and online supplemental video 1), then evaluated behavioural responses. Strikingly, the modified repeated and intermittent SS (RISS) mouse model induced anxiety-like behaviours and also triggered evident hypersensitivity as compared with the existing model (figure 1B). Follow-up studies after RISS showed significant mechanical and thermal hyperalgesia lasting for more than 4 weeks, and muscle hyperalgesia for 2 weeks (figure 1C). As well, comorbid fatigue-like behaviours were observed after RISS (figure 1D). Daily food consumption was similar between RISS and control mice (online supplemental figure S1A). However, as compared with continuous weight gain in control mice, RISS mice failed to thrive during stress exposure (online supplemental figure S1B). Also, RISS mice developed deficits of nesting behaviours versus controls (online supplemental figure S1C,D). Despite hypersensitivity to mechanical and thermal stimulation, RISS mice showed no hyper-reactivity to auditory stimuli assessed by startle responses, as compared with controls (online supplemental figure S1E). Mice of both sexes developed hyperalgesia after RISS without evident sexual dimorphism (figure 1E,F).

To test the predictive validity of the RISS model, we systemically administered analgesics (pregabalin, morphine and diclofenac) and tested their pharmacotherapeutic effects (figure 1G). Intraperitoneal injection of pregabalin dose-dependently reversed the hyperalgesia, but morphine and diclofenac had no significant analgesic effects.

Levels of plasma corticosterone and epinephrine levels were significantly increased after RISS versus controls (online supplemental figure S2A). Haematological and serum biochemistry studies showed no significant difference between groups (online supplemental figure S2B).

RISS activates primary afferent neurons

To investigate the histopathology of RISS-treated mice, we used H&E staining of muscle tissues. Gastrocnemius muscle sections in RISS mice showed intact myofascial structure and vascular channels without evidence of tissue injury or inflammation (online supplemental figure S2C). Also, serum levels of tumour necrosis factor-alpha showed no significant difference between groups (online supplemental figure S2D). The intramuscular pH values did not differ between RISS mice and controls (online supplemental figure S2E). Given that histopathological studies demonstrated no local tissue damage, we next used

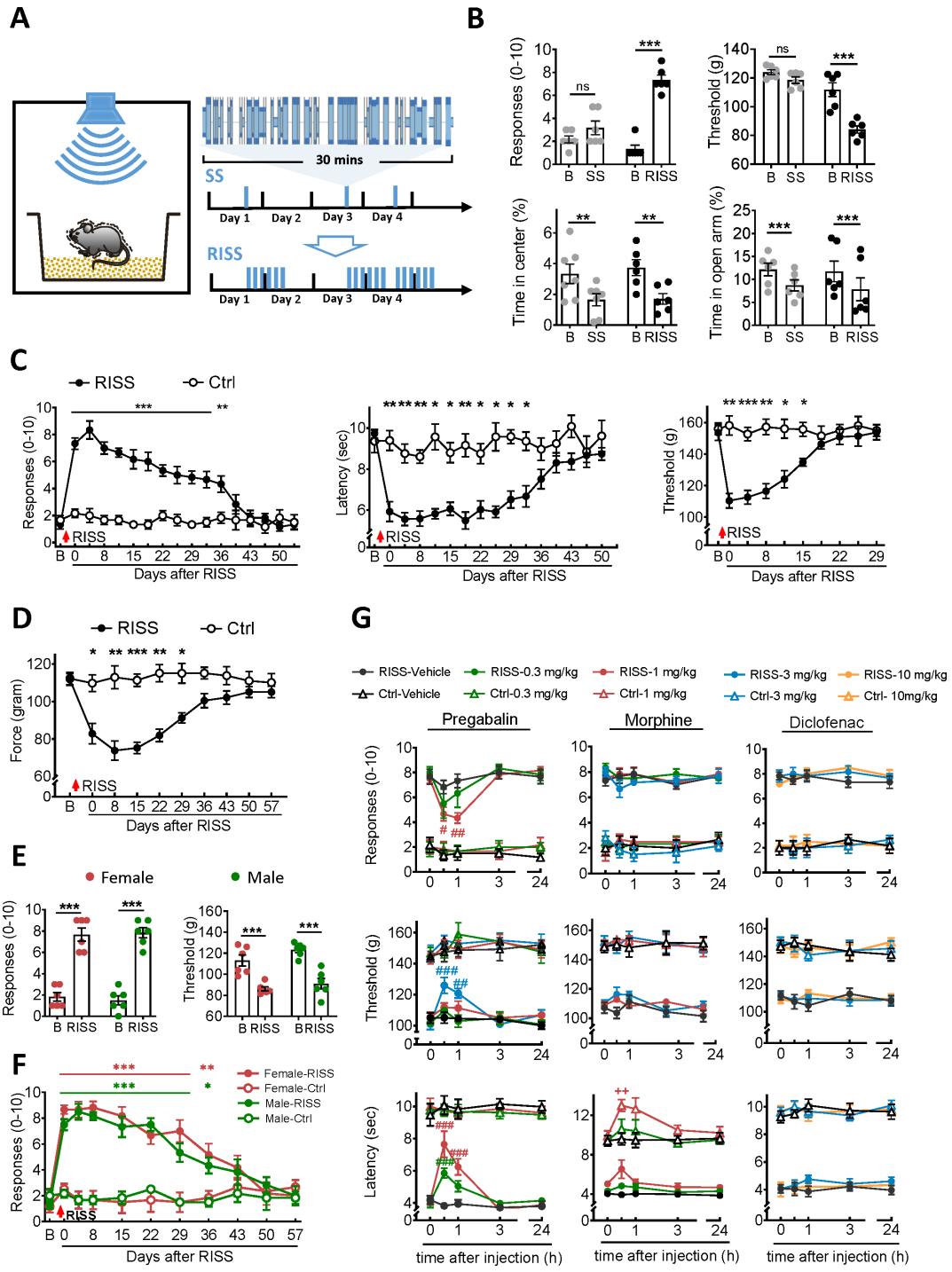

Figure 1 RISS induces fibromyalgia-like pain behaviours in mice. (A) Schematic representation of the RISS protocol: mice were placed in a bedding cage under a speaker emitting continuous pure tone stimuli with randomly varied frequencies (5–19 kHz), duration (5 s or 10 s) and amplitude (0–100 dB) for 30 min as a set. In the original sound stress (SS) protocol, the stimulus set was given once a day on days 1, 3 and 4. The RISS protocol intensified the stimulus intensity by repeating the stimulus set every 3 hour for six times overnight (from 16:00 to 10:00) on the same day. (B) Sensory-related and anxiety-related behaviour assessed in mice at day 0 post SS and RISS exposure. Upper left, mechanical hyperalgesia measured by von Frey test. Upper right, muscle hyperalgesia measured as muscle withdrawal threshold. Lower left and right, anxiety state measured as duration of time spent in the centre area of the open field test and in the open arm of the plus maze test (n=6). (C) Time course of pain behaviours after RISS. Left, mechanical hyperalgesia. Middle, thermal hyperalgesia measured by Hargreaves test. Right, muscle hyperalgesia (n=6). (D) Time course of muscle fatigue-like behaviours after RISS measured by grip force test (n=6). (E) Hyperalgesic behaviour assessed in mice of both sexes at day 0 after RISS. Left, mechanical hyperalgesia measured by von Frey test. Right, muscle hyperalgesia measured as muscle withdrawal threshold (n=6). (F) Time course of pain behaviours assessed by von Frey test in female and male mice after RISS (n=6). (G) Analgesic effect of pregabalin, morphine and diclofenac on RISS-induced pain (n=6). Upper panel, mechanical hyperalgesia. Middle, muscle hyperalgesia. Lower, thermal hyperalgesia. Data are mean±SEM. B, basal status. ns, non-significant. Ctrl, control. *p<0.05, **p<0.01, ***p<0.001 compared with B (B and E) or Ctrl (C, D and F). #p<0.05, ##p<0.01, ###p<0.001 compared with RISS-Vehicle injection groups (E). ++p<0.01 compared with Ctrl-Vehicle injection groups (G) (all two-way ANOVA). For complete statistical analyses, please refer to online supplemental results. RISS, repeated and intermittent sound stress.

immunohistochemical analysis of L4 dorsal root ganglia (DRG) to probe nociceptive processing. Activating transcription factor 3 (ATF-3) was first examined as a surrogate marker of nerve injury, but the immunoreactivity remained negative (online supplemental figure S2F,G).[17] We further examined phosphorylated extracellular signal-regulated kinase (pERK) as a marker of neuronal activation to determine whether the hypersensitivity responses originated from the peripheral nociceptive input (figure 2A).[18] pERK expression was significantly higher in the RISS than control group, with no difference between the SS and control groups (figure 2B). To evaluate the distribution range of the activated signals, we next examined pERK expression in DRG neurons from different spinal segments, including cervical, thoracic and lumbar regions (figure 2C,D). As compared with controls, all sampled RISS DRG neurons showed increased pERK expression, which suggests the widespread distribution of signalling activation after RISS.

To further map the signalling distribution in sensory neuron subtypes, we co-stained various neuronal markers with pERK (figure 2E,F,G). Of the pERK-positive DRG neurons, 42.6%, 30.6% and 31.5% colocalised with calcitonin gene-related protein (CGRP), substance P (SP) (both markers of peptidergic nociceptive neurons) and isolectin B4 (IB4; a marker for non-peptidergic neurons), respectively. Conversely, 51.4%, 32.1% and 26.2% of CGRP-positive, SP-positive and IB4-positive neurons, respectively, immunostained for pERK. In comparison, few pERK-positive neurons (1.37%) co-stained with neurofilament 200 (N52; a marker for myelinated neurons), and only a small amount of N52-postive neurons (2.0%) colocalised with pERK. The unique distribution patterns of pERK suggested that the nociceptive activation by RISS mainly involved small unmyelinated C-fibres but not large myelinated A-fibre neurons. To test whether RISS would induce neuron activation in the spinal cord that may contribute to central sensitisation, immunoreactivities of pERK (online supplemental figure S3A,B) and c-fos (online supplemental figure S3C,D) in that spinal cord of RISS mice were examined, and both showed significantly increased expression as compared with controls.

RISS incurs endogenous oxidative stress and results in lipid oxidisation

Both human and animal studies indicated that exposure to psychological distress is closely related to increasing oxidative stress.[1 19–21] Patients with FM are under higher oxidative status than HCs, as indicated by significantly higher plasma lipid peroxidation assessed by malondialdehyde (MDA) level.[22] To assess the RISS effect on oxidative status, we used the same method to assay lipid oxidisation in RISS mice (figure 3A). After RISS, MDA level was significantly higher in stressed than control mice, which suggests that RISS results in oxidative stress and reactive oxygen species (ROS)-dependent lipid damage.

To investigate the potential source of the oxidative stress, we used fluorescent indicators (CM-H_2DCFDA) to detect the presence of ROS in peripheral tissue. Muscular tissues from RISS mice showed comparable levels of fluorescence intensity as their controls, which suggests no ROS accumulation in muscle tissues (figure 3B,C). Leucocytes function to generate ROS for phagocytic purposes, so we next used ROS indicators to examine ROS levels in blood leucocytes by flow cytometry (figure 3D). Immediately after RISS, ROS levels increased significantly in all leucocyte populations, and soon decreased to baseline in 24 hours. In addition to excessive oxidative stress within leucocytes, RISS also increased the serum level of hydrogen peroxide (H_2O_2) (figure 3E).

Lipidomics implicates upregulation of LPC16:0 after RISS

Excessive oxidative stress causes direct damage to lipids.[23] Clinical metabolomics identified upregulated products of lipid oxidisation in FM patients, and suggested that these oxidised lipids might participate in algogenesis.[24 25] However, the roles of these dysregulated lipids in pathogenesis remain inconclusive. To explore the mechanism of RISS pain, we used untargeted mass spectrometry-based metabolomic study with serum of RISS mice.[26] We first hypothesised that certain dysregulated metabolites exist during the hyperalgesia period and result in long-term nociceptive activation. To identify potential algesic substances, we used time-series lipidomic analysis at different times in accordance with the temporal course of hyperalgesic behavioural changes: acute (P4), subacute (P14), chronic (P28) and recovery (P56) stages. Principle component analysis showed no outlier or intrinsic distribution, so all samples were recruited for analysis (online supplemental results and figure S4A). Changes in peak intensity of all variables between the RISS and basal groups were compared by fold-change analysis, and lipids with significantly increasing levels at each time were screened out (online supplemental table S1).

The identified lipids were mainly LPCs, phosphatidylcholines (PCs), sphingomyelin (SM) and ceramides (Cer). Their changing trends of intensity over time were further related to the course of behavioural changes. Although specific lipids showed differential intensity at each stage, none of the dysregulated metabolites retained the intensity throughout the hyperalgesic period from P4 to P28.

Given no metabolite with enduring intensity, we next hypothesised that dysregulated metabolites for algogenesis are elicited transiently during RISS and then decompose over time. Accordingly, the targeted metabolites should be at highest levels at the acute stage (P4) and thus more detectable than afterward. To identify the potential targets, we used orthogonal partial least squares discriminant analysis (OPLS-DA) with the basal and P4 groups to classify the metabolic phenotypes, and an S-plot was created to determine the discriminative lipids for P4 (figure 3F,G). Among the 14 metabolites selected by OPLS-DA, six showed a significant increase in peak intensity based on fold-change analyses (online supplemental table S1). In addition to considering their discriminative abilities, we considered the variation patterns of lipids from P4 to P56; the lipids with variation trends of timely emergence after RISS and gradual attenuation over time were preferentially recruited for advanced investigation. In summary, we identified lipids with the following conditions as targeted metabolites: (1) high discriminative values for P4 by S-plot, (2) significant incremental changes by fold-change analysis and (3) accordant variation in trends of intensity with the hyperalgesic behavioural changes.

Among the detected lipids, LPC16:0, LPC18:2 and PC36:3 were eligible candidates, with upregulation by 1.37-fold, 1.35-fold and 1.19-fold of the basal status, respectively (online supplementary figure S4B). From the temporal frame of hyperalgesic behaviours, dysregulated metabolites for nociceptive activation should be detectable as early as at P0. Therefore, we next pushed forward the lipidomic profiling to the hyper-acute phase (P0) to assess the timely performance of the candidates (online supplemental results and figure S4C). Lipids with significant upregulation at P0 were screened out (online supplemental table S2) and referred to the candidates from P4. Among the

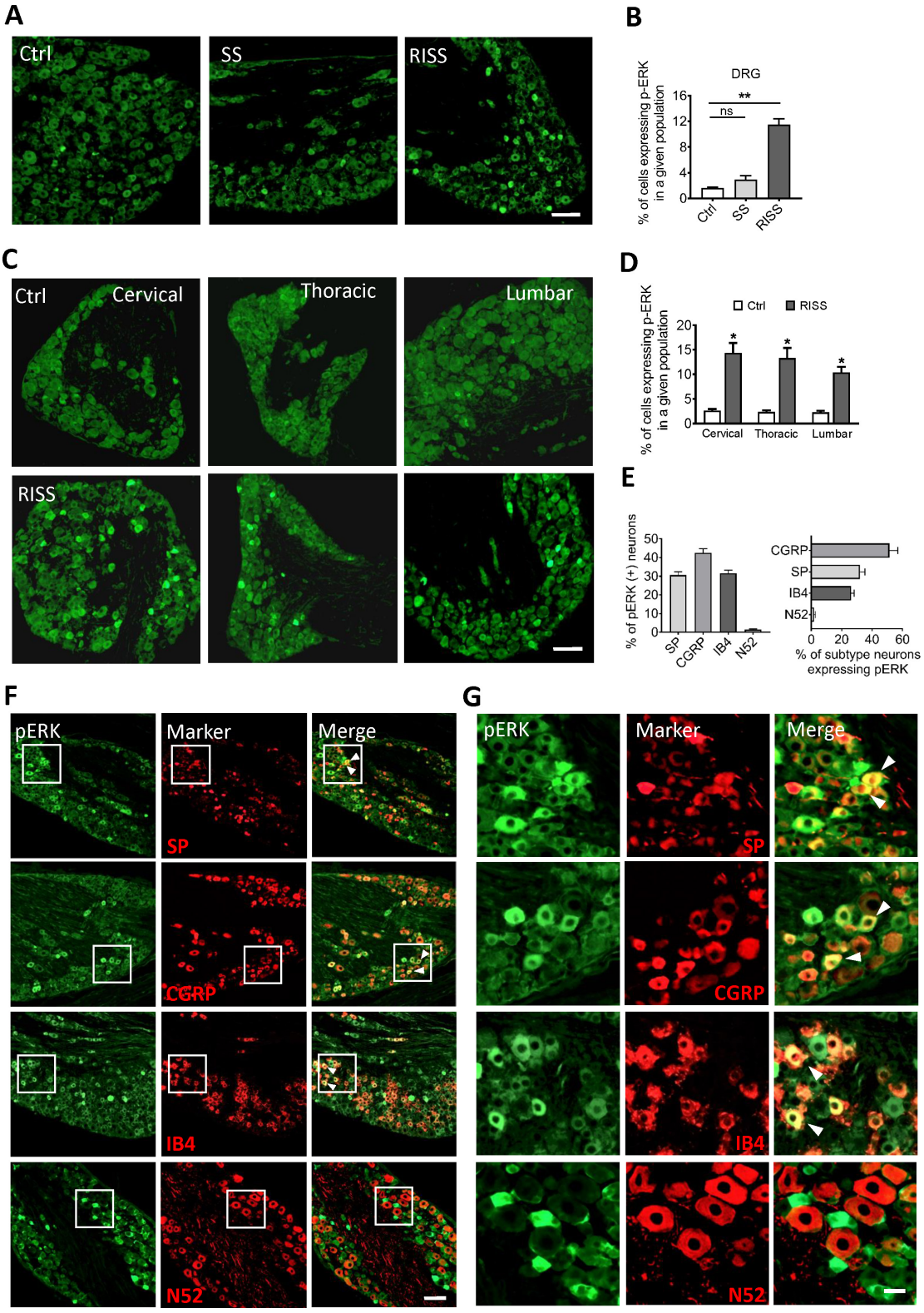

Figure 2 Upregulated expression of phosphorylated extracellular signal-regulated kinase (pERK) after RISS in lumbar dorsal root ganglia (DRG) of mice at post-RISS day 0. (A) Representative imaging of pERK expression of L4 DRG neurons in control, SS and RISS mice. Arrowheads indicate examples of positive pERK expression. (B) Quantitative analysis of expression of pERK in L4 DRG neurons (n=3) (Kruskal-Wallis test). (C) Representative imaging of pERK expression of cervical (C8), thoracic (T12) and lumbar (L4) DRG neurons in control and RISS mice. Scale bar, 100 µm. (D) Quantification of pERK expression in control and RISS mice (n=3 mice) (Mann-Whitney test). (E) Quantitative analysis of pERK colocalisation with sensory neurons markers. Left, percentage of pERK-positive DRG neurons immunostained for substance P (SP), calcitonin gene-related peptide (CGRP), isolectin B4 (IB4) and neurofilament 200 (N52). Right, percentage of CGRP-positive, SP-positive, IB4-positive and N52-positive neurons expressing pERK (n=3). (F) Representative double immunofluorescence staining showed colocalisation of pERK with SP, CGRP, IB4 and N52 in L4 DRG of RISS mice. Arrowheads indicate examples of colocalised neuronal profiles. Scale bar, 100 µm. (G) Magnified images of (F). Scale bar, 25 µm. Ctrl, control. Data are mean±SEM (n=3 mice). *p<0.05, **p<0.01 compared with controls. SS, sound stress; RISS, repeated and intermittent sound stress.

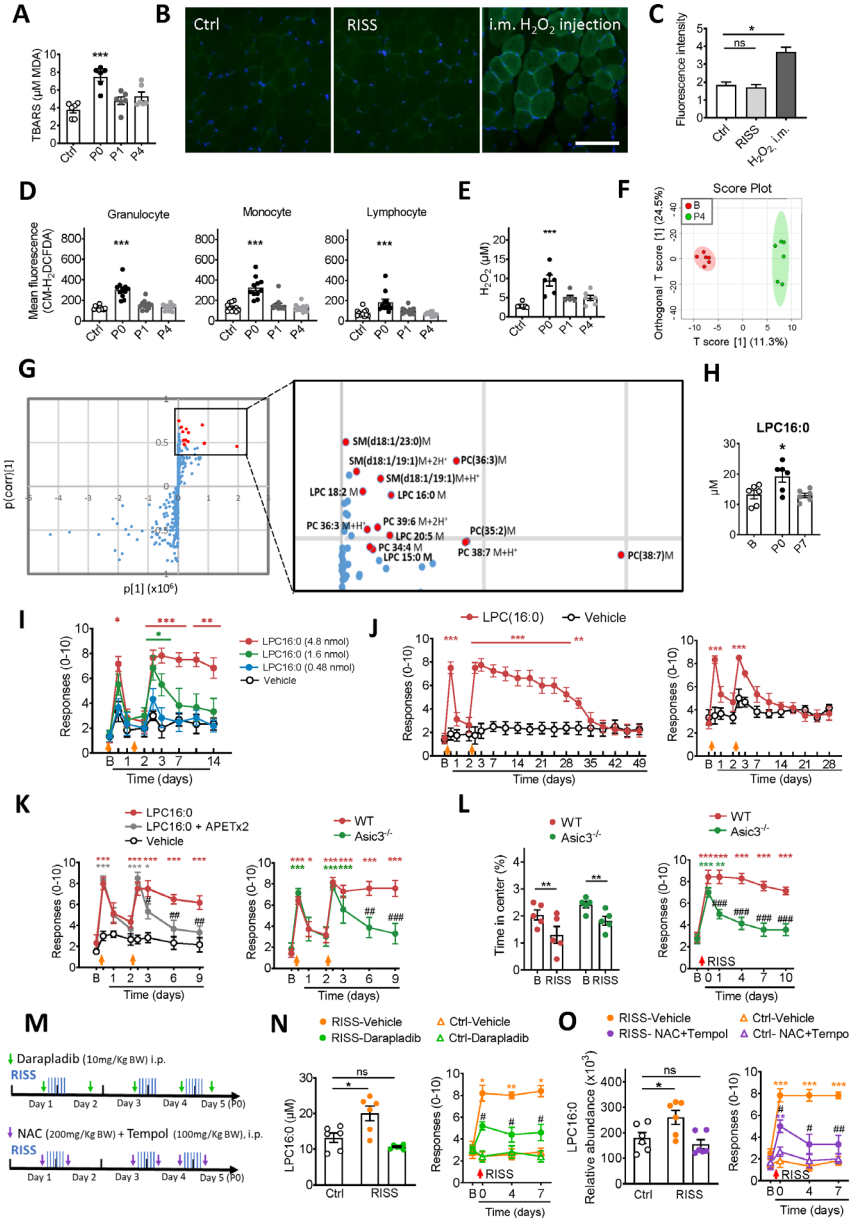

Figure 3 LPC16:0 resulting from lipid oxidisation inflicts RISS-induced chronic hyperalgesia via activating ASIC3. (A) Serum malondialdehyde (MDA) level assayed by TBARS method in RISS mice compared with controls (n=6). (B) Representative imaging of ROS expression in gastrocnemius cross-sections at P0 in control and RISS groups. Tissue ROS levels were measured by CM-H_2DCFDA staining. Scale bar, 80 µm. (C) Quantification of ROS expression in gastrocnemius muscles of mice. Positive controls were with intramuscular H_2O_2 injection (n=3). (D) RISS effects on blood leucocyte oxidative status (n=11). Intracellular ROS levels were measured with CM-H_2DCFDA staining using flow cytometry. (E) RISS effects on serum H_2O_2 level (n=6). (F) Score plot of OPLS-DA distinguishing basal and RISS (P4) groups based on serum lipidomic profiling (R^2Y=0.71, Q^2=0.166) (n=6). (G) S-plot of OPLS-DA and the differentiating metabolites identified for P4 (marked with red colour). The fold change analysis and variation trends of the selected lipids were presented in online supplemental figure S4B. (H) Quantitative analysis of LPC16:0 by QqQ MS (n=6). (I) Dose-dependent effects of repeated LPC16:0 injection on mechanical hypersensitivity as assessed by von Frey test in WT mice. Mice received two intramuscular injections (1 day apart; orange arrows) of neutral saline containing LPC16:0 (4.8 nmol, 1.6 nmol and 0.48 nmol) or vehicle (n=6). (J) Effect of repeated intramuscular (left; n=8) and intraplantar (right; n=6) LPC16:0 injection (4.8 nmole; orange arrows) on mechanical hyperalgesia as assessed by von Frey test. (K) Effects of pharmacological (left; n=6) and genetic (right; n=7) inhibition of ASIC3 on the chronification of mechanical hyperalgesia induced by repeated LPC16:0 injection (orange arrows). APETx2, ASIC3 inhibitor. (L) Effect of RISS on anxiety-like (open field test; n=5) and pain behaviours (von Frey test; n=7) in WT and *Asic3*$^{-/-}$ mice at P0. (M) Schematic presentation of drug administration. (N) Left, effect of darapladib on serum LPC16:0 level in RISS mice measured by QqQ MS at P0 (n=6). Right, effect of darapladib on the RISS-induced hyperalgesia as assessed by von Frey test (n=5). (O) Left, effect of NAC and Tempol on serum LPC16:0 level in RISS mice as assessed by relative quantitative analysis with LC-MS at P0 (n=6). Right, effect of NAC and Tempol on the RISS-induced chronic hyperalgesia (n=6). B, basal status. Ctrl, control; i.p., intraperitoneal injection; LPC, lysophosphatidylcholine; NAC, N-acetylcysteine. Data are mean±SEM. *p<0.05, **p<0.01, ***p<0.001 compared with Ctrl or B. #p<0.05. ##p<0.01, ###p<0.001 compared with LPC injection (K left), WT mice (K right and L) or RISS-Vehicle injection (N, O) (Kruskal-Wallis test or ANOVA). For complete statistical analyses, please refer to online supplemental results. ASIC3, acid sensing ion channel 3; OPLS-DA, orthogonal partial least squares discriminant analysis; PCs, phosphatidylcholines; QqQ MS, triple quadrupole mass spectrometry; RISS, repeated and intermittent sound stress; ROS, reactive oxygen species; SM, sphingomyelin; TBARS, thiobarbituric acid reactive substances.

three candidates, only LPC 16:0 showed incremental change (1.47-fold; p=0.002) and thus was recruited for further quantitative analysis. Because all the upregulated metabolites at P0 are potential pain initiators, the metabolites with a significant increase in level with available calibration standards, including LPC18:0 and SM(d18:1/18:0), were also recruited for quantitative profiling. Quantitative analysis revealed a significant upregulation of LPC16:0 (1.45-fold; p=0.019), but not LPC18:0 (1.07-fold; p=0.877) or SM(d18:1/18:0) (0.75-fold; p=0.543) (figure 3H, and online supplemental figure S4D).

Repeated LPC16:0 injection induces chronic hyperalgesia by activating acid sensing ion channel 3 (ASIC3)

LPCs participate in cell signalling and function as precursory nociceptive substances.[27 28] To determine whether LPC16:0 contributes to chronic pain development, we injected LPC16:0 into the left hindlimb of WT mice via the intramuscular or intraplantar route on the analogy of repeated acidic saline injection model by Sluka *et al*, then evaluated mechanical hypersensitivity.[11] Intramuscular injection of LPC16:0 dose-dependently evoked hyperalgesic responses (figure 3I). Single intramuscular LPC injection induced transient bilateral hyperalgesia, and repeated intramuscular injection 2 days apart induced long-lasting hyperalgesic changes (figure 3J). By comparison, vehicle-treated mice showed no mechanical hypersensitivity. Immunostaining analysis showed increased pERK expression after LPC injection as compared with vehicle treatment (online supplemental figure S5A,B). Of note, intramuscular administration of LPC16:0 not only evoked nociceptive activation but also triggered a hyperalgesic priming-like effect as observed in repeated intramuscular acid injection pain models[16]; although both routes of LPC16:0 injection evoked acute hypersensitivity, only repeated intramuscular injection led to long-lasting hyperalgesic changes (figure 3J). Unlike LPC16:0 injection, with LPC18:0 or SM(d18:1/18:0) injection, the priming-like effects and long-lasting hyperalgesia were not observed (online supplemental figure S5C).

ERK is known as an important contributor to central sensitisation.[29] To determine whether ERK activity participates in the hyperalgesic priming-like effects in RISS mice, we used intrathecal administration of U0126, a mitogen-activated ERK inhibitor, or its inactive analogue U0124 30 min before the first LPC16:0 injection. U0126 prevented the LPC16:0-induced acute hyperalgesia, and also impeded the development of chronic hyperalgesia induced by the second LPC16:0 injection (online supplemental figure S5D). Moreover, we tested the analgesic effects of pregabalin, morphine and diclofenac to evaluate the face validity of the repeated LPC16:0 injection model. The injected mice responded to pregabalin treatment but not morphine or diclofenac, which suggested that the LPC16:0 injection model shared similar pharmacotherapeutic features with the RISS model (online supplemental figure S5E).

In the neurobiological research of chronic widespread pain, ASIC3 has long been considered an important research target.[7] In addition to protons, other endogenous substances such as arachidonic acid and lactate have been reported as modulators of ASIC3. Of note, a recent study showed that certain LPCs, such as LPC16:0 and LPC18:1,[28] are able to directly activate ASIC3 channels in the absence of extracellular pH variations. To determine the contribution of ASIC3 to the RISS-induced chronic hyperalgesia, APETx2 (a selective ASIC3 antagonist) was co-injected with LPC16:0 intramuscularly, then behavioural responses were evaluated (figure 3K). As compared with vehicle treatment, transient hyperalgesia was observed with LPC or LPC-APETx2 co-injection. However, chronic hypersensitivity was significantly reduced in APETx2-treated mice. Also, we gave repeated intramuscular injection of LPC16:0 in WT and *Asic3*$^{-/-}$ mice and evaluated mechanical hyperalgesia. Similarly, both WT and *Asic3*$^{-/-}$ mice showed transient hypersensitivity after a single LPC16:0 injection, but the chronic hyperalgesic changes in WT animals were significantly reduced in *Asic3*$^{-/-}$ mice after repeated LPC injections (figure 3K). In addition, LPC16:0 injection-induced pERK expression was reduced after pharmacological and genetic inhibition of ASIC3 (online supplemental figure S5F-I). We further exposed WT and *Asic3*$^{-/-}$ mice to RISS, then evaluated behavioural responses (figure 3L). Both groups showed anxiety-like behaviours and transient hypersensitivity after RISS, but the chronic hyperalgesic changes in WT mice were significantly attenuated in *Asic3*$^{-/-}$ mice. Likewise, the ratio of pERK-positive DRG neurons induced by RISS was significantly decreased in *Asic3*$^{-/-}$ mice (online supplemental figure S5J,K).

In addition to catalytic synthesis by phospholipase A_2 during inflammatory reaction, LPCs can be produced as direct consequences of lipid oxidisation under non-inflammatory conditions, via hydrolysis of oxidised PCs (ox-PCs) by platelet-activating factor acetylhydrolase (PAF-AH) or spontaneous deacylation of ox-PCs by ROS attack.[30 31] To investigate whether PAF-AH participates in the LPC generation of the RISS model, we gave darapladib (a selective PAF-AH inhibitor) once daily (10 mg/kg; i.p.) throughout the RISS procedure (figure 3M), then evaluated LPC16:0 levels and pain behaviours. As compared with vehicle, darapladib significantly reduced LPC16:0 production after RISS and prevented the development of chronic hypersensitivity (figure 3N).

ROS also elicited LPC generation by spontaneous deacylation of ox-PCs under physiological conditions (37°C and pH 7.4).[30] In this non-catalytic pathway, using ROS scavengers to reduce the production of ox-PCs may be feasible for blocking LPC generation. To investigate the potential analgesic effect of ROS scavengers, we systemically administered antioxidants (N-acetylcysteine, 200 mg/kg; Tempol, 100 mg/kg; i.p.) throughout the RISS procedure (figure 3M). Antioxidants significantly reduced the oxidative stress of the serum (evaluated by MDA and H_2O_2 levels) and leucocytes in RISS mice (online supplemental figure S5L,M). Antioxidants also effectively reduced serum LPC16:0 levels and rescued the development of chronic hyperalgesia (figure 3O). In vehicle-treated RISS mice, neither the LPC16:0 amount nor hypersensitivity development was affected.

Increased LPC16:0 expression in FM patients correlated with pain symptoms

Our mouse model revealed how LPC16:0 triggered long-lasting bilateral hyperalgesia after stress exposure. Patients with FM manifest chronic widespread pain with daily stressors as known triggers, and may also have excessive expression of these nociceptive oxidised lipids. To test this prediction, we examined 31 newly diagnosed FM patients and 30 age-matched and sex-matched HCs (online supplemental figure S6 and table S3). Patients with FM perceived higher psychological stress as assessed by the Perceived Stress Scale and were under higher oxidative stress as assessed by MDA level than controls (figure 4A). We used a lipidomic analysis to compare the lipidomic expression between groups. Changes in peak intensity for all variables between patients and controls were compared by fold-change analysis, and lipids with significantly increased levels in the disease group were screened out (figure 4B and

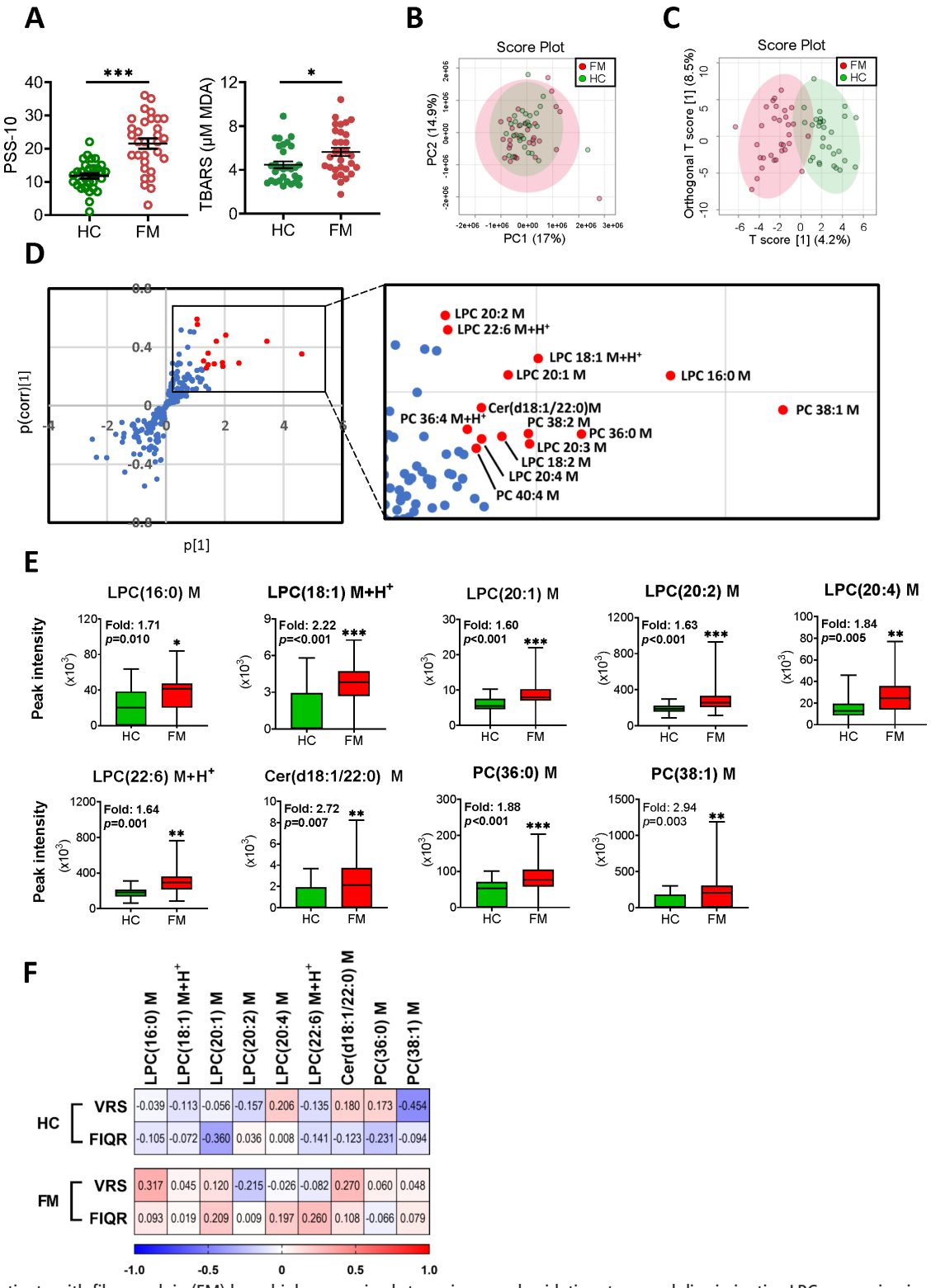

Figure 4 Patients with fibromyalgia (FM) have higher perceived stress, increased oxidative stress and discriminative LPC expression in plasma. (A) Left, psychological stress levels in FM patients (n=31) and healthy controls (HCs) (n=30) as assessed by the Perceived Stress Scale-10 (PSS-10) (unpaired t test). Right, plasma malondialdehyde (MDA) level assayed by the thiobarbituric acid reactive substances (TBARS) method in FM patients and HCs (unpaired t test). (B) Score plot of principal component analysis based on lipidomic analysis of plasma from FM patients and HCs. (C) Score plot of orthogonal partial least squares discriminant analysis (OPLS-DA) distinguishing FM and HC groups based on plasma lipidomic profiling (R^2Y=0.593, Q^2=0.149). (D) S-plot of OPLS-DA and the differentiating metabolites identified for FM (marked with red colour). (E) Box-and-whisker plots of discriminative lipids for FM and their fold change in peak intensity (Mann-Whitney test). Whiskers represent minimum and maximum values. (F) Correlation matrix between lipid expression of peak intensity and FM symptom measures (VRS and FIQR Scores) in the HC and FM groups (Spearman's rank correlation test). Spearman correlation coefficients are labelled in each cell. FIQR, the Revised Fibromyalgia Impact Questionnaire; VRS, Verbal Rating Scale of pain. Data are mean±SEM. *p<0.05, **p<0.01, ***p<0.001 compared with HC. For complete statistical analyses, please refer to online supplemental results. cer, ceramides; LPC, lysophosphatidylcholine; PCs, phosphatidylcholines.

online supplemental table S4). The identified lipids were mainly various LPCs as well as PCs and Cer.

OPLS-DA was next used to identify the most discriminative metabolites for disease (figure 4C,D). Among the 14 identified targets, nine showed a significant increase in peak intensity based on fold-change analyses (figure 4E). We evaluated whether the metabolite phenotypes were associated with clinical manifestations and found pain scores from Verbal Rating Scale (VRS) and disease severity assessed by the Revised Fibromyalgia Impact Questionnaire (FIQR) correlated with the peak intensity expression of the identified lipids (figure 4F). We found no distinct positive correlation between lipid expression and symptoms in HCs, but modest correlation between LPC16:0 and pain intensity in patients with FM.

To investigate whether a metabolomic phenotypic difference exists within patients with different disease severity, we used K-means cluster analysis to further divide the cohort into two subgroups based on disease severity assessed by the FIQR (figure 5A). The resulting FM subgroups did not differ in sex or age (online supplemental table S5). Overall FIQR Scores were significantly higher in cluster 2 than cluster 1 (70.65±11.46 vs 41.49±8.90), as were scores on all the FIQR domains (daily functions, overall impact and symptom intensity). Moreover, cluster 2 manifested significantly higher pain intensity and wider pain distribution than cluster 1 (figure 5B). Hence, cluster 2 was classified as the group with severe symptoms (FM-S), and cluster 1 with mild symptoms (FM-M). Of note, the FM-S group had significantly higher oxidative stress levels than HCs, with no significant difference observed between the FM-M and HC groups (figure 5C). We next compared lipidomic expression of the identified lipids between subgroups (figure 5D). In accordance with the biological gradation of disease severity, the HC, FM-M and FM-S groups showed an increasing trend of peak intensity expression of all identified metabolites except PC36:0. To evaluate whether the metabolite expression was associated with symptom severity and to compare the presentation of groups, we assessed the correlations between symptom measures (VRS and FIQR Scores) and expression of peak intensity (figure 5E and online supplemental figure S7A,B). In the FM-M group, LPC20:4 expression was positively correlated with FIQR Score, with no correlation of other metabolites with clinical parameters. By comparison, the expression of several metabolites in the FM-S group showed fair correlation with clinical symptoms. FM-S patients with higher expression of LPC16:0, LPC18:1, LPC22:6 and Cer(d18:1/22:0) showed higher disease severity as indicated by FIQR Score. Also, those with higher expression of LPC16:0 and Cer(d18:1/22:0) had higher VRS Score. Moreover, the expression of LPC16:0 in FM-S patients was significantly correlated with VRS Scores, which suggests that LPC16:0 might participate in the pain phenotype modulation (figure 5E).

DISCUSSION

Our animal model provides direct experimental evidence that psychological stressors can have a causative role in generating chronic hypersensitivity. Our study also identified a possible pathophysiology of the stress-induced pain with evidence spanning molecular, pharmacological, behavioural and clinical levels in mouse stress models and clinical cases. Peripheral nociceptive activation triggered by an oxidised lipid, LPC16:0, after stress exposure generates the stress-induced chronic non-inflammatory pain (figure 6). With clinical lipidomic approaches, we validated that FM patients exhibited excess expression of LPC16:0, which was correlated with pain symptoms in those with increased oxidative stress and high disease severity. Taken together, excessive expression of LPCs can be detected by clinical lipidomics and is correlated with pain severity in FM patients with increased oxidative stress and symptom severity, as predicted by our mouse model. In this sense, the products of lipid oxidisation, especially LPC16:0, might be a pathological signature and a therapeutic target for FM.

Our findings suggest that LPC16:0 mediates RISS-induced chronic hyperalgesia by activating ASIC3. Under excessive oxidative states, LPCs are aberrantly generated from ox-PCs by PAF-AH catalyzation or spontaneous deacylation.[30 31] The exclusive production of LPC16:0 after lipid oxidisation probably results from the substrate specificity of PAF-AH for palmitic acid (C16:0)-containing ox-PCs.[32] The abundance of palmitic acids in the fatty acid composition of plasma phospholipids may also contribute to this result.[33] Underpinning the role of LPC16:0 in the pathogenesis of the stress-related pain disorders is the concordant findings from our clinical investigations and the past clinical lipidomic reports.[24 25] The clinical lipidomic evidence strengthens the face validity of our model, and also helps to support the bio-significance of LPC16:0 in the clinical conditions.

LPCs are known to trigger various signalling pathways. In addition to ASIC3, LPCs also activates G-protein-coupled receptors.[34] Besides, lysophosphatidic acids (LPAs), the direct downstream metabolites of LPCs, are known potent nociceptive substances capable of activating various LPA receptors as well as TRPV1 (transient receptor potential cation channel subfamily V member 1).[27 35] The acute hypersensitivity observed in *Asic3*$^{-/-}$ mice suggested that the nociceptive responses induced by LPC16:0 are not exclusive to ASIC3, but likely involve other ASIC3-independent mechanisms.

We demonstrated that darapladib, a PAF-AH inhibitor, can effectively attenuate the RISS-induced chronic hyperalgesia. Darapladib was originally designed for antiatherogenic purpose by blocking LPC synthesis.[31] Since PAF-AH mediates both PAF degradation during inflammation and LPC synthesis during lipid oxidisation, darapladib may have dual but opposite roles in the nociceptive process depending on the situation.[31] Darapladib was less likely considered for analgesic purposes, given that PAF is a potent inflammatory mediator, and thus inhibiting its hydrolysis could aggravate nociceptive responses.[36] However, under oxidative but non-inflammatory conditions, such as RISS, our study indicated that darapladib has a predominantly antinociceptive role by preventing LPC generation.

One major unanswered question in this study is that the upregulation of LPC16:0 and the correlations between LPC expression and symptom severity were observed only in part of the FM patients (FM-S). In those without oxidative stress who manifested minor symptoms (FM-M), the LPC upregulation and clinical relevance were indistinct. Such a clinical divergence may result from patient heterogeneity, because FM is commonly considered a group of diseases rather than a single disease. Other mechanisms may also mediate the stress-related chronic non-inflammatory pain, and the behavioural and metabolomic scenario of RISS mice may only represent part of the disease phenotypes. Another possibility is that the timing of metabolic sampling causes this clinical divergence. In the RISS model, the expression of excessive oxidative stress and LPC16:0 upregulation was observed only in the first few days after stress exposure, but the pain behaviours were observed for much longer (5 weeks). Given that the LPC16:0-induced priming effects and pain sensitisation are completed in the early stage of pain processing, the

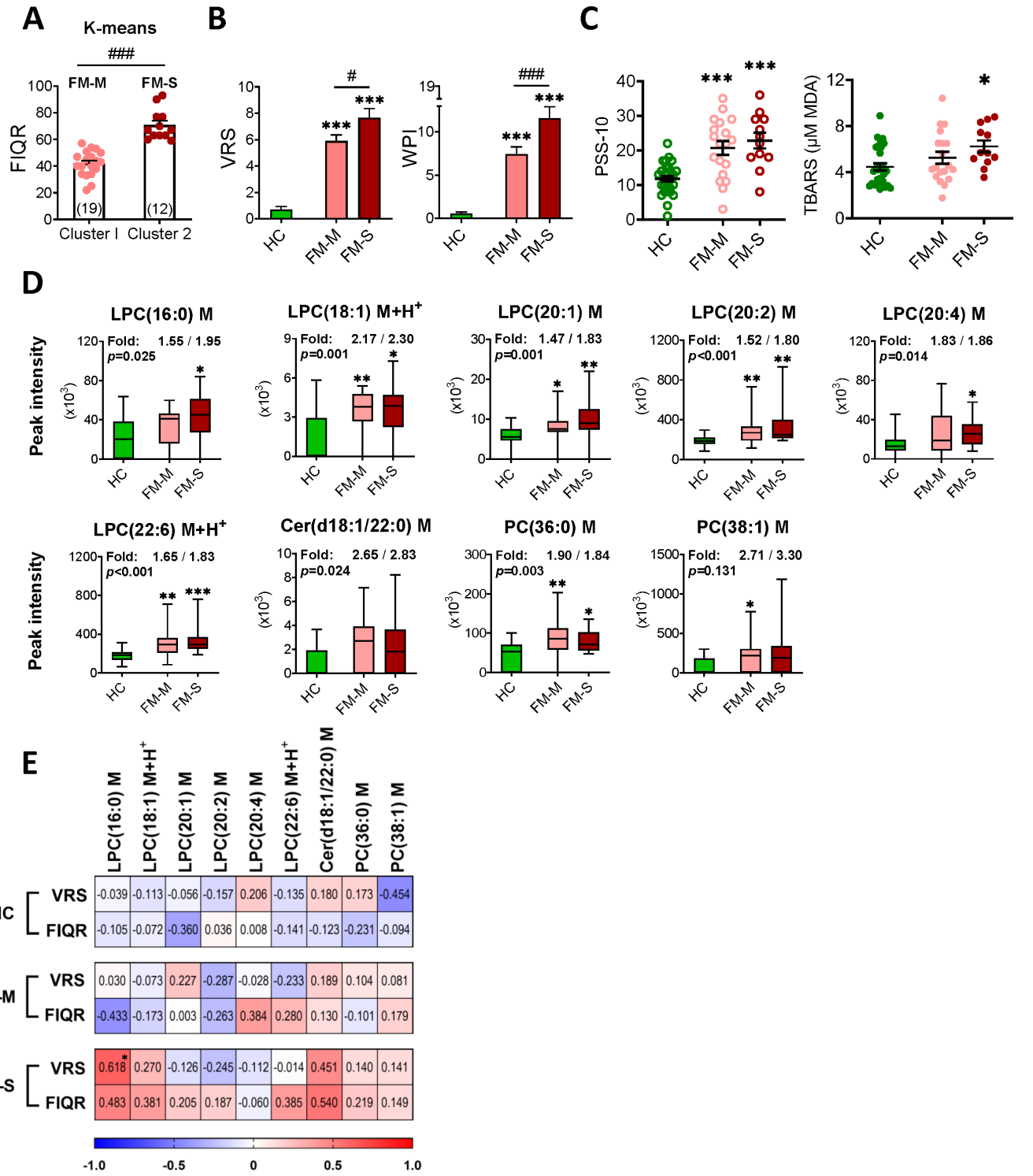

Figure 5 Expression of LPC16:0 and other lipids are correlated with FM symptoms in patients with increased oxidative stress and high disease severity. (A) Subgrouping FM patients based on disease severity by cluster analysis. Patients were classified into two subgroups (cluster 1 and 2) based on FIQR Score with K-means cluster analysis. FIQR Scores were higher in cluster 2 (n=12) than cluster 1 (n=19). Patients of cluster 2 were thus designated as the group with severe symptoms (FM-S), and those of cluster 1 with mild symptoms (FM-M) (unpaired t test). (B) Left, pain intensity assessed by Verbal Rating Scale (VRS; 0–10) among the HC (n=30), FM-M (n=19) and FM-S (n=12) groups. Right, extent of pain diffuseness assessed by Widespread Pain Index (WPI; 0–19) (one-way ANOVA). (C) Left, psychological stress levels among HC, FM-M and FM-S groups as assessed by the Perceived Stress Scale-10 (PSS-10). Right, plasma malondialdehyde (MDA) level assayed by the thiobarbituric acid reactive substances (TBARS) method (one-way ANOVA). (D) Box-and-whisker plots of peak intensity and fold change of lipid expression in the FM-M and FM-S subgroups versus HCs (Kruskal-Wallis test). (E) Correlation matrix between lipid expression of peak intensity and clinical symptom measures (VRS and FIQR Scores) (Spearman's rank correlation test). Spearman correlation coefficients are labelled in each cell. Statistical details and scatter plots were presented in online supplemental figureS7A,B. Data are mean±SEM *p<0.05, **p<0.01, ***p<0.001 compared with HC (A–D) or with significant relevance (E). #p<0.05, ###p<0.001 comparing FM-S with FM-M. For complete statistical analyses, please refer to online supplemental results. cer, ceramides; FIQR, the Revised Fibromyalgia Impact Questionnaire; FM, fibromyalgia; HC, healthy control; LPC, lysophosphatidylcholine; pc, phosphatidylcholine.

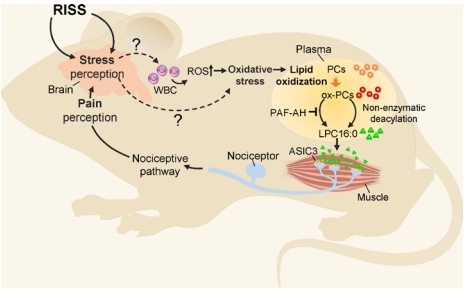

Figure 6 Schematic presentation of the mechanisms of repeated and intermittent sound stress (RISS)-induced chronic hyperalgesia. RISS inflicts excessive oxidative stress and lipid oxidisation, thus resulting in ensuing production of lysophosphatidylcholine (LPC) 16:0 via platelet-activating factor acetylhydrolase (PAF-AH) catalyzation or spontaneous deacylation of ox-PCs. LPC16:0 accordingly evokes nociceptive responses and causes chronic hyperalgesia by activating the acid-sensing ion channel 3 (ASIC3) in muscle tissue. By reducing LPC generation, PAF-AH inhibitor may thus be beneficial in the stress-related pain disorders, such as fibromyalgia. Ox-PCs, oxidised phosphatidylcholines; PCs, phosphatidylcholines; ROS, reactive oxygen species. WBC, white blood cell.

presence of LPC16:0 stimulus is not necessary for maintaining the pain manifestation. Take RISS mice at P14 for example: the pain behaviours remained robust after stress, whereas the incremental changes of MDA and LPC16:0 levels were long gone. In this context, the increasing expression of MDA and LPC16:0 and the correlation between LPC expression and pain phenotypes may not be necessarily detectable in FM patients who currently sustain pain symptoms. Such a prediction can be also applied to the inconsistent results of previous FM studies investigating MDA levels.[22 37] In this sense, MDA levels may help better identify a subset of FM patients who are amenable for PAF-AH inhibitor therapy to prevent LPC synthesis.

In animal research, the chronic hyperalgesia induced by RISS exposure or repeated LPC injection were significantly abolished in *Asic3*$^{-/-}$ mice, which suggests the critical role of ASIC3-containing channels in the chronic pain development. In clinical situations, whether ASIC3 activation participates in the FM development remains undetermined. Clinical research did not suggest a genetic difference in ASIC3 expression between cases and controls.[38] However, the pain phenotype modulation may depend mainly on the metabolomic expression of LPCs rather than ASIC3 expression. ASIC3 has been linked to various pain disorders besides FM in other experimental models, which suggests that its activation may be not unique to the presentation of FM. Although ASIC3 played a key role in the development of stress-induced FM-like pain in our study, its actual pathophysiological association with FM requires future investigation.

Chronic widespread pain is maintained in part by central sensitisation, and FM is commonly considered a central pain disorder.[7] Of note, peripheral inputs may also contribute to central sensitisation development.[39] Our study indicates that the peripheral nociceptive activation triggered by LPC16:0 plays an important role in RISS-induced chronic hypersensitivity, which denotes that the development of stress-induced chronic hyperalgesia cogently involves peripheral inputs. Apart from the peripheral factors, central sensitisation following peripheral nociceptive activation also participated in the chronification of RISS pain, as suggested by the increased immunoreactivity of pERK and c-fos in the spinal cord. Additionally, although the pain generation solely depends on the peripheral mechanism in the LPC injection model, the contributing components of peripheral and central factors in the RISS model could be another situation. Unlike the exclusive contribution of peripheral factors in the LPC16:0-injection model, stress-related psychological factors may also affect central neural modulation and pain processing during the development of RISS pain. The central mechanism of RISS pain should be further investigated, including the involved nociceptive pathways and neuropsychiatric interaction.

We found that repeated intramuscular but not intraplantar LPC injection caused chronic hyperalgesia, so the priming-like effect mainly relies on the muscle afferents. This phenomenon may be ascribed to the higher ASIC3 expression in muscle than skin nociceptors.[40] We also found that RISS dominantly activated pERK in small diameter DRG neurons (figure 2), which suggests that the small fibre neurons mediate the RISS pain rather than those with large myelinating nerves. This finding agrees with the clinical findings of C-fibre dysfunction identified in FM.[10] This particular phenomenon may also explain the diffuse character rather than distinct spatial localisation in FM pain.[7] Furthermore, small fibre neuropathy has been reported in some FM cases.[7] In our model, the negative immunoreactivity of ATF-3 in the DRG neurons did not suggest nerve injury in RISS mice. However, the possibility of neuropathy in the fibre endings cannot be ruled out without comprehensive histopathological inspection. Future studies focusing on the histopathology of nerve fibres and microneurography may be required.

In this study, we chose the injected dosage of LPC16:0 (4.8 nmol; 480 µM) according to a previous report to test its biological effect,[28] whereas the measured levels of LPC16:0 in RISS mice at P0 were relatively lower (~20 µM; figure 3H). Considering the small injection volume (10 µL), drug diffusion in the interstitial space of the hindlimb (~200 µL) can cause considerable dilution.[41 42] Also, transcapillary movement and removal by blood circulation can also affect the drug concentrations after injection. Besides, the peak concentrations of LPC16:0 during the RISS challenges should be higher than what we can measure at P0. In consideration of these factors, the injected dose of LPCs may be reasonably relevant to the circulating concentration as measured in stressed mice.

In the RISS study, we observed upregulation of numerous lipids other than LPC16:0 at P0. Given the limited availability of standard compounds, a comprehensive quantitative analysis or a behavioural test to validate their biological effects in vivo cannot be conducted for all the identified lipids. Therefore, the pathogenic metabolite may not be limited to LPC16:0 but also possibly includes other undetermined lipids. Furthermore, other types of metabolites generated with ROS other than lipids may also induce allodynia. Likewise, in clinical lipidomic profiling, the potential pathogenic substances may not be limited to LPC16:0 but could include other lipids, given that various other lipids also showed significant incremental changes and fair correlation with clinical presentations, such as LPC18:1, LPC 22:6 and cer(d18:1/22:0). Although correlations with statistical significance were observed only between LPC16:0 and VRS Score, the small sample size of FM subgroups may limit the significance level of these tests despite their fair correlation coefficients. Also, the use of metabolite peak intensity rather than the quantitative data for analysis may weaken the interpretation of clinical relevance. Future clinical research with large sample size and quantitative validation is warranted to evaluate the biological significance of these potential targets identified in the study.

Other limitations exist in this study. Although we observed biochemistry and lipidomic findings in both RISS mice and patients in this study, most of the mechanisms were investigated

only in mice. To validate the pain mechanism in humans, further longitudinal observational research is needed to investigate the temporality and interaction among perceived stress, LPC levels and pain presentation in individuals. Also, a clinical interventional study with pharmacotherapeutic trials of PAF-AH inhibitor will help to validate the mechanism identified in our animal model. Additionally, the development of FM depends not just on environmental factors but also on the genetic vulnerability of the individual.[1 3] Therefore, the stress–LPC–diathesis interaction should be further investigated. In addition, although darapladib or ROS scavengers competently reduced serum LPC16:0 levels and rescued chronic hyperalgesia, we cannot definitely rule out whether these drugs affect other targets and involve different antinociceptive pathways. Furthermore, the source of oxidative stress under RISS may be not confined to the overabundance of ROS in immune cells. So far, the underlying mechanisms that connect the perceived stress to cellular oxidative effects remain less understood, and several putative pathways proposed to explain the phenomenon, include mitochondrial dysfunction.[19 20 22] Despite these limitations, our study demonstrates the biological plausibility for how the psychological stressors translate into the somatic pain process, thereby indicating a new therapeutic approach for FM.

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