



Endoplasmic Reticulum Stress in the Keratinocytes Contributes to Chronic Itch by Activation of Lipocalin 2/MC4R/TRPV1 Pathway

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Abstract Endoplasmic reticulum (ER) stress plays a significant role in chronic pain, but its potential involvement in chronic itch remains largely unexplored and poorly understood. In the current study, we investigated whether ER stress signaling in keratinocytes contributes to the pathogenesis of chronic itch. Our behavioral tests showed that the ER stress inhibitor 4-PBA attenuated itch-related behaviors in both acute and chronic itching mouse models, and reduced compound 48/80 and serotonin-induced activity of dorsal root ganglion (DRG) neurons. qPCR and western blotting revealed that the ER stress-related proteins and Lipocalin-2

(LCN2) were significantly elevated in the affected skin under chronic itch conditions and in cultured keratinocyte HaCaT cells and mice skin keratinocytes. The ELISA test showed that the level of LCN2 increased significantly in plasma but not in DRG tissue, from both acetone-ether-water (AEW) induced dry skin and imiquimod (IMQ) induced psoriasis model mice. Current clamp recording demonstrated that LCN2 induced hyperexcitability in dorsal root ganglia neurons, which could be abolished by HS024, the inhibitor of melanocortin receptor 4 (MC4R). In addition, pharmacological inhibition of transient receptor potential vanilloid 1 (TRPV1) or TRPV1 knockout blocked LCN2-induced hyperexcitability in DRG neurons. In conclusion, this study demonstrated that keratinocyte ER stress is involved in chronic itch genesis by releasing LCN2, which sensitized primary sensory neurons *via* TRPV1. These findings

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suggested that inhibition of ER stress in keratinocytes could be a promising therapeutic strategy for treating chronic itch.

Keywords Itch · Keratinocyte · Endoplasmic reticulum stress · LCN2 · TRPV1

Introduction

Pruritus is an unpleasant sensory experience that predominantly affects both the skin and mucous membranes. Chronic pruritus, characterized by its persistence beyond six weeks, represents a challenging and often debilitating condition with currently limited therapeutic efficacy, and has a serious impact on patients' quality of life [1–3]. As one of the most prevalent clinical manifestations, it frequently coexists with a wide spectrum of dermatological conditions and systemic disorders, posing substantial diagnostic and therapeutic challenges in clinical practice [4, 5]. The etiology of chronic pruritus is complex, involving both intrinsic and extrinsic factors. Intrinsic factors may include chronic infections, impaired blood circulation, hormonal or metabolic changes, and genetic predispositions such as allergies. Extrinsic factors are highly diverse and may encompass food allergens, airborne particles, chemical irritants, and animal dander [6].

Peripheral itch signals are primarily sensed by free nerve fiber endings in the epidermis of the skin. However, other non-neuronal cell types are also involved in the initiation and transmission of itch [7]. Keratinocytes, the main epithelial cells that make up the stratified epidermis of the skin, can be directly activated by endogenous or exogenous pruritogens or neuropeptides, such as histamine, calcitonin gene-related peptide (CGRP), and substance P. Keratinocytes are capable of modulating the activity of primary sensory neurons and immune cells by secreting a variety of inflammatory factors, such as thymic stromal lymphopoietin (TSLP) and interleukin 31 (IL-31) [8, 9]. In addition, physical impairment of the skin barrier facilitates the penetration of exogenous pruritogens into the skin, consequently exacerbating pruritus and inflammatory responses [10].

ER stress occurs when various stress signals, such as oxidative stress or disturbances in calcium homeostasis within the ER, lead to the accumulation of unfolded proteins that exceed the cell's ability to process and manage them [11, 12]. In response, the unfolded protein response (UPR) is activated to restore ER function, initiating a series of mechanisms aimed at re-establishing ER homeostasis, often by reducing overall protein synthesis. The UPR is triggered through the coordinated action of three key ER transmembrane stress sensors: inositol-requiring enzyme 1 α (IRE1 α , also known as ERN1), PKR-like ER kinase (PERK, also known as EIF2AK3), and activating transcription

factor 6 α (ATF6 α) [11, 13, 14]. These sensors activate transcription factors, including ATF6, X-box binding protein 1 (XBP-1), and activating transcription factor 4 (ATF4), which induce a number of genes with corresponding *cis*-elements, including Bip/GRP78, HRD1, PDI, and CHOP/GADD153 [15–17]. Pro-inflammatory stimuli like reactive oxygen species (ROS), TLR ligands, and cytokines can trigger ER stress, and in turn, ER stress, resulting in UPR activation, can further amplify the inflammatory response [18]. Evidences suggest that ER stress plays a significant role in the differentiation and proliferation of keratinocytes, which is associated with various skin conditions, including psoriasis [19, 20]. However, it remains unclear whether ER stress in keratinocytes contributes to the chronic itch.

LCN2, or neutrophil gelatinase-associated lipocalin, is a secreted protein implicated in diverse physiological and pathological processes [21], particularly chronic pain and pruritus [22, 23]. LCN2 exerts its effects through interactions with multiple receptors, including MC4R, a G protein-coupled receptor widely expressed throughout the central and peripheral nervous systems [24]. Our prior research demonstrated that spinal LCN2-MC4R signaling modulates neuronal excitability and contributes to morphine tolerance development [25]. This signaling pathway involves MC4R-mediated activation of sympathetic preganglionic neurons through protein kinase A-dependent potentiation of the TRPV1 channel [26]. Nevertheless, the potential involvement and mechanistic basis of the LCN2-MC4R-TRPV1 axis in itch sensation remain to be elucidated.

In the current study, we investigated the role of the ER stress signaling pathway in keratinocytes using dry skin or psoriatic mouse models. We investigated molecular mechanisms, particularly focusing on keratinocyte-derived LCN2 and the molecular pathways of skin keratinocytes involved in mediating chronic itch. These mechanisms have been systematically explored to identify novel therapeutic targets, thereby advancing clinical research and facilitating the development of innovative treatments for chronic pruritus.

Materials and Methods

Animals

Adult male C57BL/6J mice (6–8 weeks old) were purchased from the Shanghai SLAC Laboratory Animal Company (Shanghai, China). *Trpv1*^{−/−} mice were purchased from Jackson Lab (Bar Harbor, ME, United States). All animal care and experimental procedures were performed according to the guidelines approved by the ethics committee for the use of experimental animals in the Soochow University Animal Committee. Animals were maintained with a 12 h light/

dark cycle, food and water were available *ad libitum*, and the temperature ($22 \pm 2^\circ\text{C}$) and humidity (40%–60%) were maintained. This animal study also followed the ARRIVE guidelines [27].

Reagents

Compound 48/80 (Cat#: C2313), chloroquine (CQ, Cat#: C6628), and Thapsigargin (Cat#: 67526-95-8) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Endothelin 1 (ET-1, Cat#: 1160) and 5-HT (Cat#: 2451-5 mg) were obtained from Tocris (Bristol, UK). If there were no special instructions, the reagents were dissolved in sterile saline. The following primary antibodies were used: mouse anti-tubulin (1:2000; Cat#: Ab102-02; Vazyme), mouse anti-GAPDH (1:2000; Cat#: MAN1002; MesGen), mouse anti-CHOP (1:1000; Cat#: 2895; CST), rabbit anti-GRP78 (1:1000; Cat#: ab32618; Abcam), Mouse anti-GRP78 (1:500; Cat#: F2702; Selleck), LCN2 (1:1000; Cat#: PA5-18382; Sigma), rabbit anti-XBP-1s (1:1000; Cat#: 12782S; Cell Signaling), rabbit anti-ATF4 (1:1000; Cat#: ab184909; Abcam), rabbit anti-ATF6 (1:1000; Cat#: ab37149; Abcam), Keratin-14 (1:100; Cat#: ab7800; Abcam), and secondary antibodies (1:2000, MesGen). The following fluorescent secondary antibodies were used: goat anti-rabbit (1:1000; Cat#: 4413S; CST), goat anti-mouse (1:1000; Cat#: 4408S; CST).

Neck Model of Acute Itch

The skin on the nape of the mice was shaved 2 days before the experiments. Mice were individually habituated to small plastic chambers (10 cm $\times$ 10 cm $\times$ 12.5 cm) for 30 min, which were on the metal mesh shelf once daily for two consecutive days [28]. On the behavioral testing day, based on our pilot study and previous reports, mice have received an intradermal injection of 50 μL of compound 48/80 (25 and 100 μg) [29], chloroquine (50 and 200 μg) [30, 31], endothelin-1 (30 ng) [28], serotonin (30 μg) [32] and thapsigargin (10, 30 and 100 μg) *via* a 26G needle into the nape of the neck under brief anesthesia by isoflurane. The mice were put back in their chambers and then recorded for 30 min (Sony HDR-CX610). A scratch bout was counted when a mouse lifted the hind paw to scratch the shaved region and returned the paw to the surface or mouth. The video was subsequently played back offline, and scratching behavior was quantified in a blinded manner.

Cheek Model of Acute Itch

The cheek of the mouse was shaved, and the animal was habituated to the testing environment 2 days before the experiments. On the day of behavioral testing, mice

or rats were placed in separate small plastic chambers (10 cm $\times$ 10 cm $\times$ 12.5 cm) on an elevated metal mesh floor for at least 30 min to habituate [28]. Under brief anesthesia with isoflurane, thapsigargin (10 μg) was *i.d.* injected into the cheek of mice. Mice were then immediately returned to their chambers for 30 min video recording. The video was subsequently played back offline, and both scratching and wiping behavior were quantified in a blinded manner. Scratching behavior was defined as lifting of the hind paw to scratch the cheek and the return of the paw back to the floor or mouth. Wiping behavior was defined as a lifting of the forelimbs toward the cheek for a time exceeding one or a few seconds before placing the forelimbs downward.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted by using TRIzol Reagent (Invitrogen, Carlsbad, California) according to the protocol supplied by the manufacturer. Reverse transcription of 1 μg total RNA for synthesizing cDNA was performed by using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA). The Real-time polymerase chain reaction was conducted by using SYBR Green PCR Master Mix (Biotium, Hayward, CA, USA) on an Opticon real-time PCR Detection System (Applied Biosystems, Grand Island, NY). The relative fold of change in expression was calculated by using the $2^{-(\Delta\Delta C_t)}$ method after normalization to GAPDH expression. The primers were synthesized by Genewiz and are shown in Supplementary Table S1 in the supplementary materials.

Western Blotting Analysis

Protein samples were collected by using RIPA lysates containing protease inhibitors and phosphatase inhibitors (Beyotime Biotechnology). To measure the protein concentration, the bicinchoninic acid assay (BCA) method with a total protein assay kit (Thermo Fisher) was used. The Biofuraw TM Pre-cast Gel (Tanon) was used to perform a western blot, and the sample of each well was 40 μg , and then electrophoretically transferred onto polyvinylidene difluoride membrane (PVDF) (Millipore), 100 V for 90 min. The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at 37°C and then incubated with primary antibodies overnight at 4°C . The membranes were washed three times with Tris-buffered saline Tween-20 (TBST), and then incubated with horseradish peroxidase-conjugated secondary antibodies (MesGen) for 1 h at 37°C . Quantitative western blot images were recorded with a fully automated chemiluminescence image analysis system (Tanon). Quantitative analysis was performed with ImageJ 2.x. In brief, rectangular regions of interest were drawn around each target and corresponding loading control band. Integrated density values

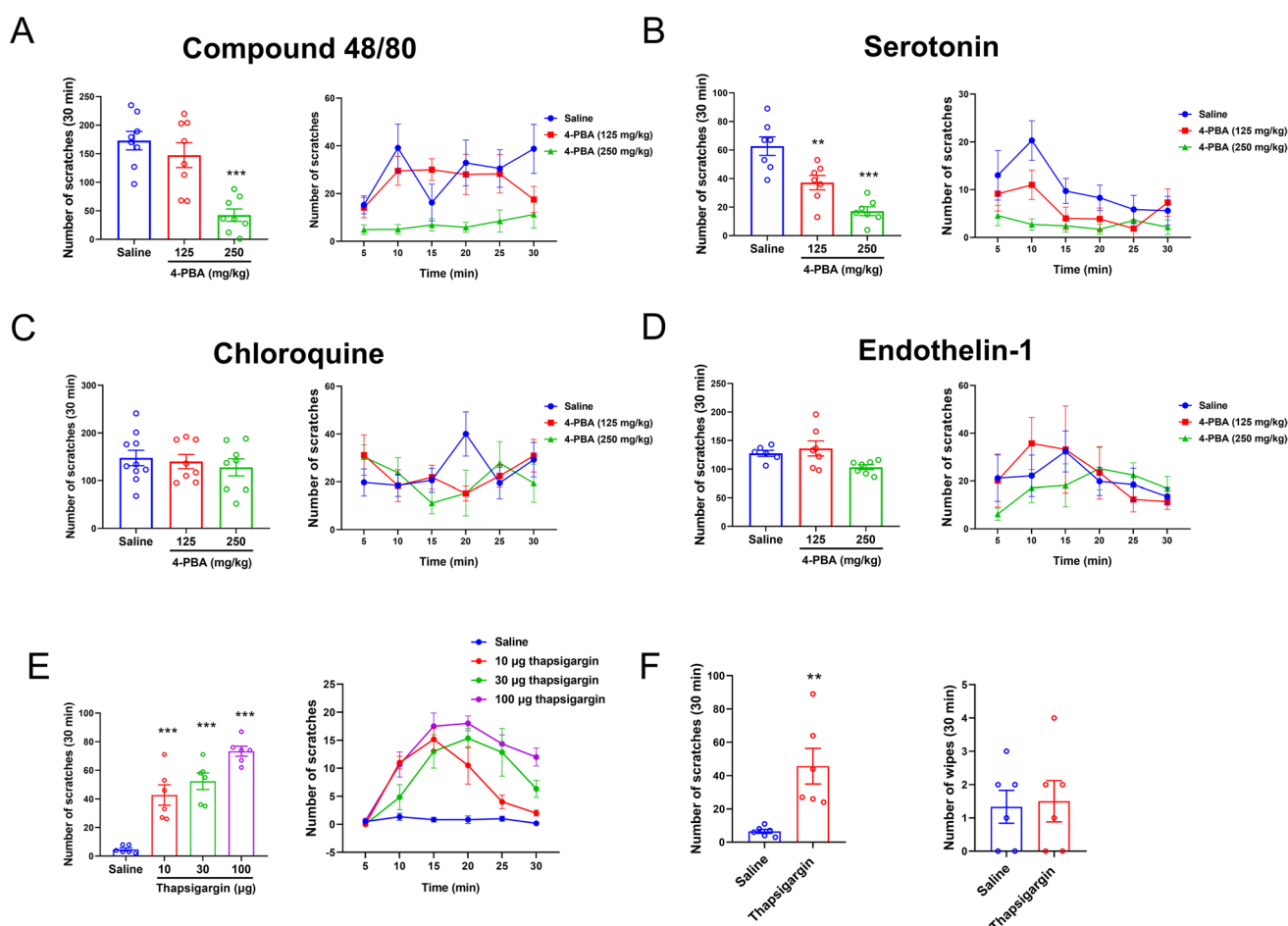


Fig. 1 ER stress is involved in the scratching behaviors in acute itch models. **A–D** The effect of 4-PBA on the total number and time course of scratching behavior events within 30 min induced by i.d. injection of compound 48/80, serotonin, CQ, and ET-1, respectively. ($n = 8–10$, $**P < 0.01$, $***P < 0.001$ vs Saline, one-way ANOVA following Bonferroni's test was used). **E** The total number and time

course of scratching behavior events within 30 min after i.d. injection of thapsigargin in the neck ($n = 6$, $***P < 0.001$ vs Saline, one-way ANOVA following Bonferroni's test or unpaired Student's t test was used). **F** The total number of scratching and wiping behavior events within 30 min after i.d. injection of thapsigargin in the neck ($n = 6$, $**P < 0.01$ vs Saline, unpaired Student's t test).

were measured for each band after local background subtraction. Target protein signals were normalized to their respective loading controls within the same lane. The normalized values for each experimental condition were then expressed relative to the mean of the control group, which was set to 1. Statistical analyses were performed in GraphPad Prism. All data are presented as mean $\pm$ SEM; group comparisons were made using unpaired two-tailed Student's t -tests (for two groups) or one-way ANOVA followed by Dunnett multiple comparison tests (for three groups).

H&E Staining

The skin was perfused with 50 mL of 4% paraformaldehyde in 0.01 mol/L phosphate buffer (pH 7.4), and then the skin was harvested, postfixed in 4% paraformaldehyde for 2–3

days, embedded in paraffin, and cut into 8 μ m sections on a microtome. The sections were stained with hematoxylin and eosin (H&E) using standard procedures. The images were captured by fluorescence microscopy (AXIO SCOPE A1, ZEISS).

For the epidermal thickness measurement, three points were randomly selected along the epidermal-dermal junction in each tissue section, with these points evenly distributed along the epidermal-dermal interface. At each selected measurement point, a straight line was drawn perpendicular to the epidermal-dermal junction, extending vertically from the deepest part of the epidermis to the outermost epidermal layer. The length of each perpendicular line (μ m) was calculated using ImageJ software, representing the epidermal thickness at that measurement point. The average value of three points was recorded as the sample's epidermal thickness.

Immunofluorescence

After mice were perfused with 50 mL of 4% paraformaldehyde in 0.01 mol/L phosphate buffer (pH 7.4), the skin was harvested, postfixed in 4% paraformaldehyde for 6–8 h, transferred to 20% sucrose solution until the tissue reached the bottom, and then transferred to 30% sucrose solution until the tissue reached the bottom. The skin tissues were sectioned at a thickness of 20 μm . The sections were permeabilized in PBST (containing 0.3% Triton X-100) for 10 min and blocked with 5% BSA in PBST for 1 h at room temperature. Then, the sections were incubated with primary anti-GRP78 and anti-LCN2 antibodies overnight at 4°C. The next day, the sections were incubated with primary anti-Keratin-14 antibodies overnight at 4°C. On the third day, the sections were then incubated with anti-rabbit and anti-mouse secondary antibodies for 1 h at room temperature. The images were captured by fluorescence microscopy (AXIO SCOPE A1, ZEISS).

Cell Culture

The immortalized human-derived keratinocyte cell line, HaCaT, was purchased from the China Center for Type Culture Collection (CCTCC). The cells were cultured in Dulbecco modified Eagle's medium (DMEM, Gibco, Grand Island, NY, United States) supplemented with 10% FBS and 1% penicillin/streptomycin (Biosharp, Hefei, China). Cells were cultured at 37°C in a 5% CO₂ atmosphere.

DRG neurons were dissociated from adult C57BL/6J mice and *Trpv1*^{-/-} mice (6–8 weeks-of-age) as previously described [33, 34]. In brief, mice were anesthetized with isoflurane and decapitated. Cervical DRGs were rapidly removed and then digested in DMEM containing collagenase D (0.6 U/mL; Roche, Mannheim, Germany) and dispase II (3.0 U/mL; Roche, Mannheim, Germany) for 40 min at 37°C. The ganglia were then triturated with fire-polished Pasteur pipettes in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA, United States). The dispersed cells were resuspended in F12 (Biological Industries, Beit HaEmek, Israel) medium supplemented with 10% FBS and 1% penicillin/streptomycin (Biosharp, Hefei, China) and plated on coverslips coated with Poly-D-lysine (BBI Lifescience, Shanghai, China). Cell cultures were maintained in regular 95% air and 5% CO₂ at 37°C.

ELISA

LCN2 levels of serum and DRG were measured using a mouse-specific LCN2 enzyme-linked immunosorbent assay (ELISA) kit (Boster Biological Technology, Wuhan, China). For serum preparation, mouse blood samples were allowed to clot at room temperature for 30 min, followed by centrifugation at 3,000×g for 15 min at 4°C to separate serum. The

supernatant was further centrifuged at 12,000×g for 5 min at 4°C to remove cellular debris, and this step was repeated once to ensure serum purity. For DRG tissue processing, freshly isolated DRG tissues were homogenized in ice-cold phosphate-buffered saline (PBS, pH 7.4) using a tissue homogenizer. The homogenates were then centrifuged at 12,000×g for 5 min at 4°C, and the supernatants were collected for LCN2 measurement. LCN2 concentrations were then determined according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader. All samples were analyzed in triplicate, and results were expressed as ng/mL.

Calcium Imaging

DRG neurons were used for calcium imaging after an overnight culture. The cells were loaded with 1.5 $\mu\text{g/mL}$ Fluo-2 (1:1000, Thermo Fisher) and 0.01% F-127 (w/v; Invitrogen) for 30 min in a 37°C incubator with 5% CO₂. Images were captured *via* a CCD camera (PCO, Germany) integrated with an inverted microscope (Nikon, Japan). The excitation of Fluo-2 was facilitated by an alternating light source (PTI, USA) emitting wavelength of 340 nm and 380 nm. In chambers equipped with a custom four-channel perfusion valve control system, neurons were infused with calcium imaging buffer (CIB) (130 mmol NaCl, 5.6 mmol/L KCl, 2.6 mmol/L CaCl₂, 1.2 mmol/L MgCl₂, 10 mmol/L HEPES, and 5.6 mmol/L d-glucose at pH 7.4) to baseline, then infused with the test solution, and finally with 56 mmol/L KCl for 1 min and infused with CIB to baseline.

For the quantitative analysis of calcium imaging, the fluorescence ratio ($F = F_{340}/F_{380}$) was calculated after background subtraction to minimize artifacts related to dye loading, cell thickness, and excitation light fluctuations. For data normalization, the magnitude of Ca²⁺ responses was expressed as $\Delta F/F_0$, where F_0 represents the mean baseline ratio calculated during a stable period of 0–30 s prior to agonist application. A positive response to an agonist was defined as a peak increase in F_{340}/F_{380} of at least 20% of the maximal amplitude induced by 56 mmol/L KCl and/or an absolute change in the ratio value of ≥ 0.2 .

Patch Clamping Recording

DRG neurons were recorded after culturing for 16–24 h as described previously [33, 34]. Neurons were visualized under an upright infrared-differential interference contrast microscope (BX51WI, Olympus), and small-diameter DRG neurons (<25 μm) were chosen for whole-cell patch clamp recording at room temperature. Data were acquired using a Multiclamp 700B amplifier (Molecular Devices, USA) driven by a personal computer equipped with pClamp 10 software (Axon Instruments, USA). DRG neurons were recorded with fire-polished,

borosilicate glass patch pipettes (5–8 MΩ), which were pulled from borosilicate glass capillaries (Sutter Instrument, Novato, CA, United States) using a Sutter P-1000 puller (Sutter Instrument, Novato, CA, United States). The bath solution consisted of 140 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L MgCl₂, 2 mmol/L CaCl₂, and 10 mmol/L HEPES, pH 7.3. The pipette solution consisted of 30 mmol/L KCl, 110 mmol/L potassium gluconate, 0.5 mmol/L EGTA, 5 mmol/L HEPES, and 3 mmol/L Mg-ATP, pH 7.3. Action potentials (APs) were evoked by depolarizing current steps with long- or short-time durations (1,000 ms or 2 ms). Long current steps were used to test AP rheobases, and the number of evoked APs was determined. Short current steps were used to trigger single action potentials to calculate the shape properties of APs. All signals obtained were sampled at 20 kHz, low-pass filtered at 5 kHz, and analyzed offline.

Statistical Analysis

Data were analyzed using GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA) and Clampfit 10.3 (Molecular Devices, San Jose, CA, USA). All data were expressed as the mean ± SEM. Statistical analysis was performed by Student's *t*-test, one-way analysis of variance (ANOVA) followed by Dunnett multiple comparison tests, or two-way repeated-measures ANOVA followed by post hoc Bonferroni's multiple comparison tests as appropriately indicated. *P* < 0.05 was considered to be statistically significant (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

Results

ER Stress Inhibitor 4-PBA Alleviated Compound 48/80 and Serotonin-Induced Acute Itch

First, to determine the role of ER stress in acute itch, we investigated the effect of 4-PBA, a pharmacological inhibitor of ER stress, in models of acute itch induced by diverse endogenous pruritogens. Mice were intraperitoneally injected with 4-PBA (125 mg/kg and 250 mg/kg) half an hour before intradermal injection of pruritogens in the nape of the neck. Total scratching bouts induced by compound 48/80 (100 μg) and serotonin (30 μg) were significantly decreased by 4-PBA in a dose-dependent manner (for compound 48/80, $F_{(2, 21)} = 16.83$, *P* < 0.001, Fig. 1A; for serotonin, $F_{(2, 18)} = 20.27$, *P* < 0.001; Fig. 1B). 4-PBA at a dose of 250 mg/kg significantly decreased the number of scratching bouts in all the intervals (Fig. 1A&B). However, 4-PBA showed no significant inhibitory effect on chloroquine and endothelin-1-induced itch ($F_{(2, 23)} = 0.3735$, *P* = 0.6924, Fig. 1C; $F_{(2, 17)} = 3.874$, *P* = 0.0411; Fig. 1D). To further elucidate the role of ER stress in acute itch, we administered thapsigargin, a well-characterized ER stress inducer, through intradermal (i.d.) injection into the nape of the neck to investigate whether cutaneous ER stress activation can directly elicit itch responses. The results showed

that thapsigargin can induce significant scratching behavior in a dose-dependent manner ($F_{(3, 20)} = 33.75$, *P* < 0.001, Fig. 1E). Consistent with above results, intradermal administration of thapsigargin into the cheek of the mice elicited robust scratching responses but not wiping behaviors (*P* < 0.01, Fig. 1F), demonstrating that cutaneous ER stress specifically mediates pruritic signaling rather than nociceptive processing.

4-PBA Alleviated Compound 48/80 and Serotonin-Induced Activity of DRG Neurons

Both compound 48/80 and serotonin can directly act on neurons. To further investigate the effect of ER stress inhibition on compound 48/80 and serotonin-induced neuron activity, we then performed calcium imaging with or without 4-PBA incubation. The results showed that 1 mmol/L 4-PBA significantly reduced the response amplitude of DRG neurons induced by compound 48/80 ($t_4 = 7.839$, *P* = 0.0014, Fig. 2A–D), as well as induced by serotonin ($t_4 = 4.434$, *P* = 0.0114, Fig. 2E–H). These results indicate that ER stress is involved in the activation effect of compound 48/80 and serotonin on DRG neurons.

ER Stress Inhibitor 4-PBA Alleviated Chronic Itch

Next, we measured the role of ER stress in chronic itch by using two chronic itch models. The dry-skin mouse model was successfully established through twice-daily application of AEW treatment over a consecutive seven-day period, and the psoriasis models were established by IMQ cream once per day. 4-PBA was intraperitoneally injected half an hour before every AEW treatment or imiquimod cream application, respectively. 50 mg/kg 4-PBA significantly reduced spontaneous itch in both models (for AEW model, $F_{(12, 124)} = 4.357$, *P* < 0.0001; for psoriasis model, $F_{(4, 50)} = 3.058$, *P* = 0.0249; Fig. 3A, B), whereas the low-dose 4-PBA had no significant effect. Interestingly, histopathological analysis revealed that administration of 4-PBA at both 20 mg/kg and 50 mg/kg doses significantly attenuated the AEW-induced epidermal hyperplasia. ($F_{(2, 20)} = 9.569$; *P* = 0.0012; Fig. 3C, D). Similarly, H&E staining showed 50 mg/kg 4-PBA significantly reduced the skin epidermal thickening in the psoriasis model ($t_{16} = 8.255$; *P* = 0.0144; Fig. 3E, F). These results indicate that ER stress may play a pivotal role in the pathogenesis of itch.

ER Stress was Activated in the Skin Tissue of Chronic Itch Model Mice

To determine whether chronic itch is associated with ER stress activation in cutaneous cells, we first performed qPCR to measure the transcriptional levels of key ER stress

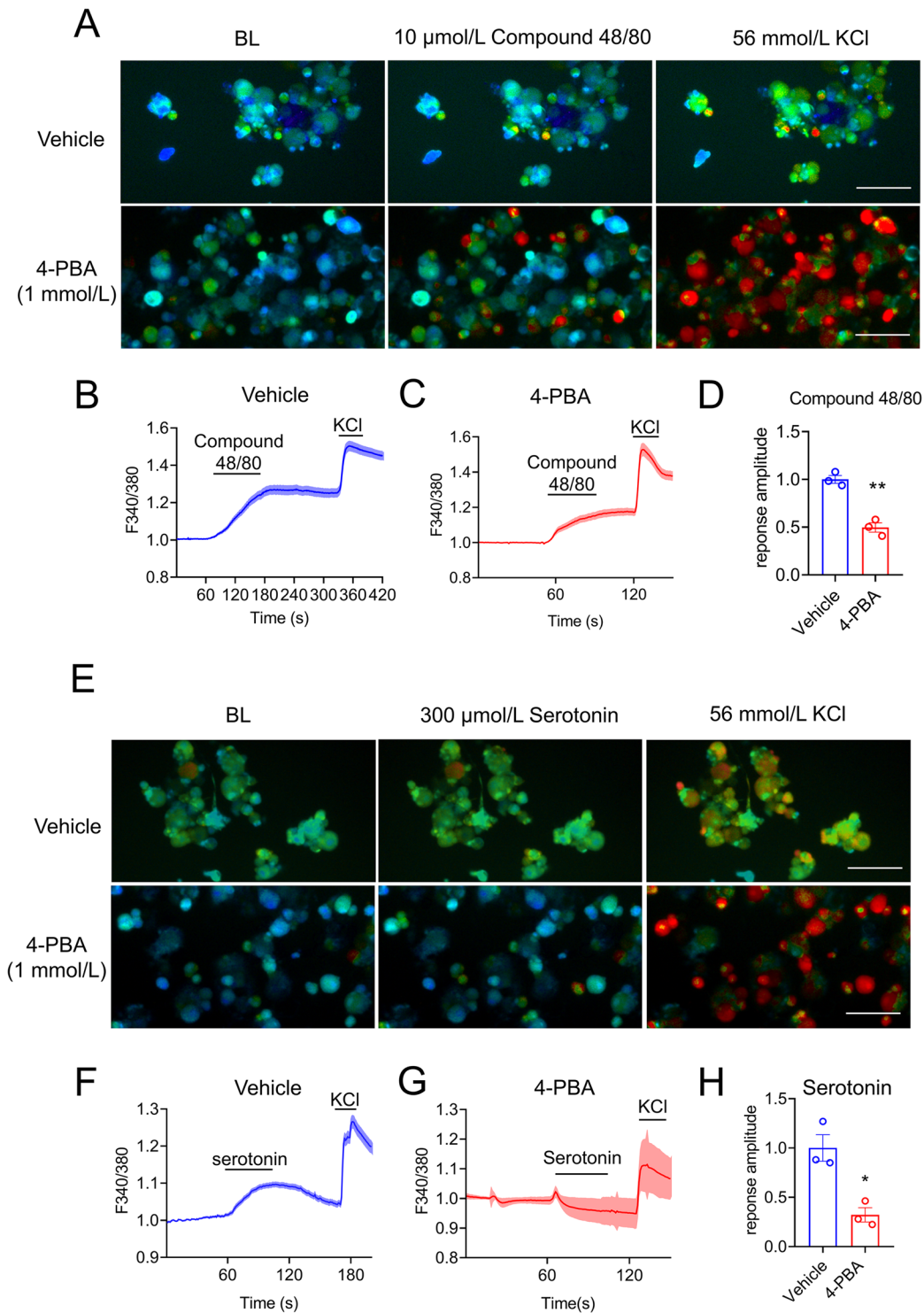


Fig. 2 ER stress is involved in compound 48/80 and serotonin-induced neuron activity. **A** Representative images of compound 48/80 induced activation of DRG neurons without or with 4-PBA incubation. **B**, **C** Median Ca^{2+} transients of DRG neurons induced by compound 48/80 without or with 4-PBA incubation, respectively. ($n = 85$, median $\pm$ 95% CI). **D** The effect of 4-PBA incubation on response amplitude of DRG neurons induced by compound 48/80 (** $P < 0.01$ vs Vehicle, $n = 3$,

unpaired Student's t test). **E** Representative images of serotonin-induced activation of DRG neurons without or with 4-PBA incubation. **F**, **G** Median Ca^{2+} transients of DRG neurons induced by serotonin without or with 4-PBA incubation, respectively. ($n = 85$, median $\pm$ 95% CI). **H** The effect of 4-PBA incubation on response amplitude of DRG neurons induced by serotonin (* $P < 0.05$ vs Vehicle, $n = 3$, unpaired Student's t test). Scale bar: 25 μ m.

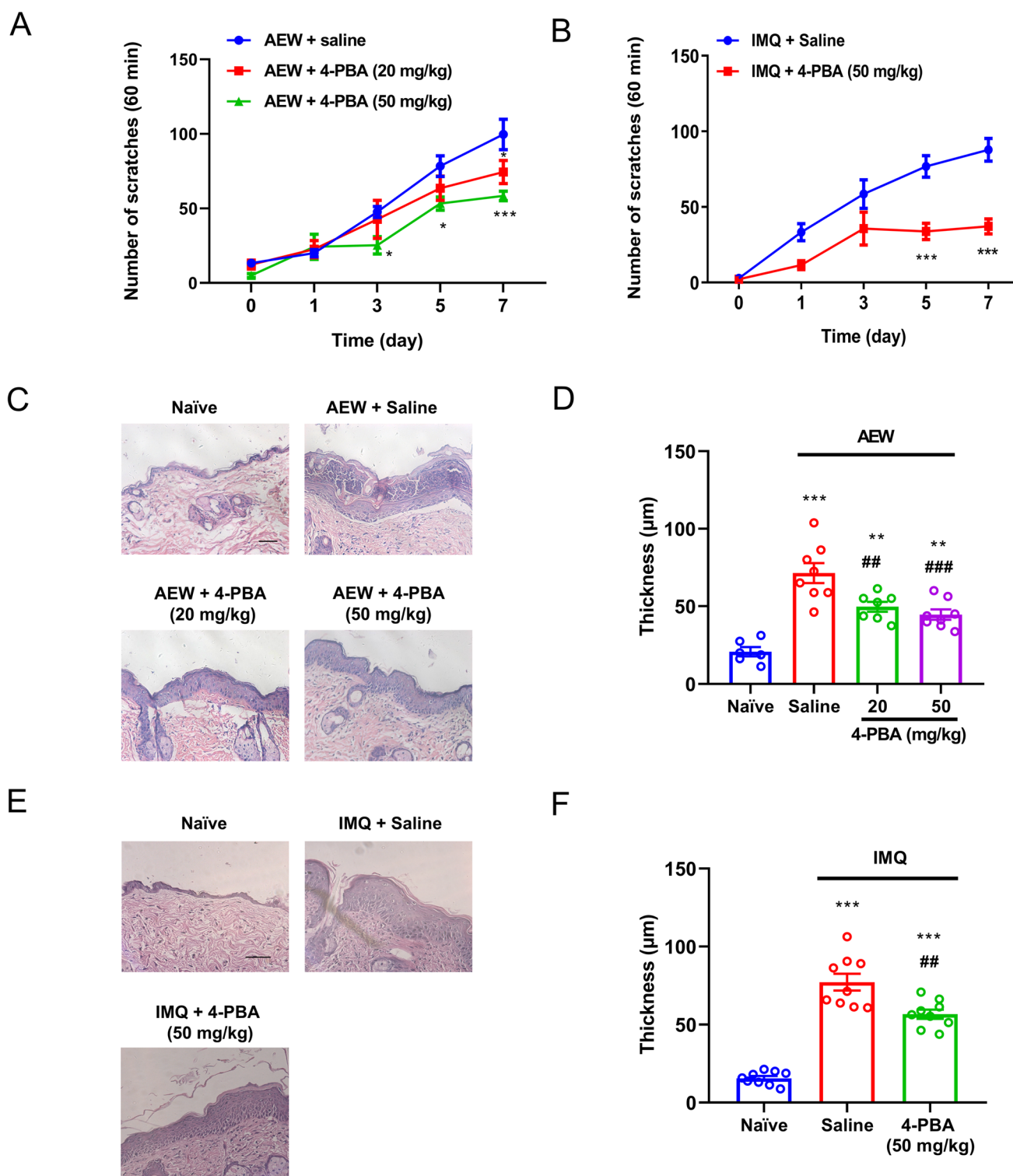


Fig. 3 ER stress is involved in the scratching behaviors in chronic itch models. **A** The effect of 4-PBA on the scratching behaviors in the AEW-induced dry skin model ($n = 7-8$, * $P < 0.05$, *** $P < 0.001$ vs Saline, two-way repeated measures ANOVA with Bonferroni test was used). **B** The effect of 4-PBA on the scratching behaviors in the imiquimod-induced psoriasis model. ($n = 6$, *** $P < 0.001$ vs Saline, two-way repeated measures ANOVA with Bonferroni test was used).

C, D The effect of 4-PBA on the thickness of the skin in the dry skin model ($n = 6-8$, ** $P < 0.01$, *** $P < 0.001$ vs Naïve, ## $P < 0.01$, ### $P < 0.001$ vs Saline, one-way ANOVA following Bonferroni's test). **E, F** The effect of 4-PBA on the thickness of the skin in the psoriasis model ($n = 9$, *** $P < 0.001$ vs Naïve, ## $P < 0.01$ vs Saline, one-way ANOVA following Bonferroni's test). Scale bar: 100 μm.

markers in the lesional cervical skin tissues obtained from the AEW-induced dry skin model mice and psoriasis model mice. Compared to control group (abbreviated as “ctrl”) mice, the ER stress-related genes *Grp78*, *Xbp1s*, *Atf6*, *Atf4* and *Chop* were increased significantly in AEW treated mice (for *Grp78*, $t_6 = 2.974$, $P = 0.0248$; for *Xbp1s*, $t_6 = 3.454$, $P = 0.0136$; for *Atf6*, $t_6 = 2.871$, $P = 0.0284$; for *Atf4*, $t_6 = 3.484$, $P = 0.0131$; for *Chop*, $t_6 = 4.479$, $P = 0.0042$; Fig. 4A) and IMQ treated mice (for *Grp78*, $t_6 = 9.313$, $P < 0.001$; for *Xbp1s*, $t_6 = 3.654$, $P = 0.0107$; for *Atf6*, $t_6 = 4.885$, $P = 0.0028$; for *Atf4*, $t_6 = 7.285$, $P < 0.001$; for *Chop*, $t_6 = 6.231$, $P < 0.001$; Fig. 4B). We also performed western blot to detect whether there were changes in above ER stress-related proteins. The results showed that the levels of GRP78, XBP1s, and ATF4 in both AEW treated mice (for GRP78, $t_6 = 5.785$, $P < 0.001$; for XBP1s, $t_6 = 5.159$, $P < 0.001$; for ATF4, $t_6 = 7.305$, $P < 0.001$; Supplementary Fig. S1A, B) and IMQ treated mice (for GRP78, $t_6 = 6.232$, $P < 0.001$; for XBP1s, $t_6 = 3.919$, $P = 0.0044$; for ATF4, $t_6 = 2.466$, $P = 0.0389$; Supplementary Fig. S1C, D) compared to those in the control group.

To further verify that ER stress was involved in chronic itch, we tested the effect of 4-PBA on AEW-treated dry-skin model mice and IMQ-treated psoriasis model mice. Compared with the control group, the mRNA expression levels of *Grp78*, *Xbp1s*, *Atf6*, *Atf4*, *Chop* in the neck skin were significantly decreased by 50 mg/kg 4-PBA in dry-skin model mice (for *Grp78*, $t_6 = 6.233$, $P = 0.0008$; for *Xbp1s*, $t_6 = 6.752$, $P = 0.0005$; for *Atf6*, $t_6 = 4.531$, $P = 0.0040$; for *Atf4*, $t_6 = 6.038$, $P = 0.0009$; for *Chop*, $t_6 = 11.12$, $P < 0.0001$; Fig. 4C) and psoriasis model mice (for *Grp78*, $t_6 = 4.862$, $P = 0.0083$; for *Xbp1s*, $t_6 = 4.740$, $P = 0.0090$; for *Atf6*, $t_6 = 4.885$, $P = 0.0131$; for *Atf4*, $t_6 = 4.328$, $P = 0.0124$; for *Chop*, $t_6 = 18.22$, $P < 0.001$; Fig. 4D). These results indicate that ER stress is activated in the lesioned skin of dry skin and psoriasis model mice.

To further confirm that ER stress was activated in keratinocytes, we next performed immunofluorescence staining with skin samples from both dry skin and psoriasis mouse models. Compared to Naïve group, it's obvious that the expression of GRP78 increased significantly in the two itch models ($F_{(2,15)} = 143.4$, for AEW, $P < 0.001$; for IMQ, $P < 0.001$), and GRP78 expression was partially co-localized with keratin-14 (K14), a keratinocyte marker, in the epidermal layer (Fig. 4 E, F).

To assess whether pruritogens directly trigger ER stress in keratinocytes, we examined the effects of imiquimod on ER stress markers in the human keratinocyte cell line HaCaT *in vitro*. After 24-hour treatment of imiquimod, the mRNA expression levels of *Grp78*, *Xbp1s*, *Atf4*, *Chop*, *Atf6*, significantly increased (for *Grp78*, $t_6 = 14.38$, $P = 0.0001$; for *Xbp1s*, $t_6 = 14.36$, $P = 0.0001$; for *Atf4*, $t_6 = 10.66$, $P = 0.0004$; for *Chop*, $t_6 = 15.06$, $P = 0.0001$; for *Atf6*, $t_6 =$

20.01, $P < 0.0001$; Fig. 4G). Similarly, western blot verified that the level of phosphorylated IRE1 α (p-IRE1 α), GRP78 and XBP1s were significantly increased (for p-IRE1 α , $t_6 = 2.943$, $P = 0.0147$; for GRP78, $t_6 = 2.559$, $P = 0.0307$; for XBP1s, $t_6 = 2.501$, $P = 0.0314$; Supplementary Fig. S2A, B). Then we tested the effect of 4-PBA to verify the imiquimod-induced ER stress in keratinocytes. 4-PBA (3 μ mol/L) significantly reversed the imiquimod-induced increase of *GRP78*, *ATF4*, *XBP1s*, *CHOP* in keratinocytes (for GRP78, $F_{(3,8)} = 10.21$, $P = 0.0041$; for ATF4, $F_{(3,8)} = 6.778$, $P = 0.0138$; for XBP1s, $F_{(3,8)} = 12.95$, $P = 0.0019$; for CHOP, $F_{(3,8)} = 14.79$, $P = 0.0013$; Fig. 4H). Western blot also showed that 4-PBA significantly reduced imiquimod-induced upregulation of GRP78, ATF4, and CHOP in keratinocytes (for GRP78, $F_{(2,15)} = 20.97$, $P < 0.001$; for ATF4, $F_{(2,10)} = 10.63$, $P = 0.0033$; for CHOP, $F_{(2,6)} = 14.14$, $P = 0.0054$; Supplementary Fig. S2C, D). These results suggest that ER stress-associated pathways in keratinocytes are activated by imiquimod directly, rather than as a secondary response after activation of the immune system.

ER Stress-Induced Keratinocyte LCN2 Secretion is Involved in Itch

Recent studies have identified specific ER stress signaling pathways as the key driver of LCN2 expression in several tumor cell types. To investigate the cellular origin of LCN2 in mouse skin, we next performed immunofluorescence staining to determine its specific localization and assess its potential derivation from keratinocytes. The results showed that LCN2 expression was predominantly co-localized with K14 in the epidermal layer. Consistently, the intensity of LCN2 immunofluorescence was increased in dry skin and psoriasis models compared to naïve mice ($F_{(2,15)} = 40.81$, for AEW, $P < 0.001$; for IMQ, $P < 0.001$; Fig. 5A, B). Subsequently, to investigate the potential involvement of LCN2 in chronic pruritic pathologies, we quantitatively assessed LCN2 transcriptional levels in lesional skin from both dry skin and psoriatic mouse models, particularly focusing on its keratinocyte-specific expression patterns during disease progression. The results showed that LCN2 mRNA in the skin of both models were significantly upregulated compared with the naïve mice (for AEW, $F_{(3,12)} = 146.9$, $P < 0.001$; for IMQ, $F_{(2,9)} = 27.19$, $P < 0.001$; Fig. 5C, D), which can be reversed by 50 mg/kg 4-PBA treatment (for AEW, $F_{(3,12)} = 146.9$, $P < 0.001$; for IMQ, $F_{(2,9)} = 27.19$, $P = 0.0088$).

Meanwhile, after being treated with imiquimod for 24 h, the mRNA of LCN2 was significantly upregulated in the HaCaT cell line ($F_{(2,6)} = 52.98$, $P < 0.001$; Fig. 5E). The protein level of LCN2 was also increased by imiquimod treatment ($F_{(2,12)} = 13.48$, $P < 0.001$; Fig. 5F, G). Consistent

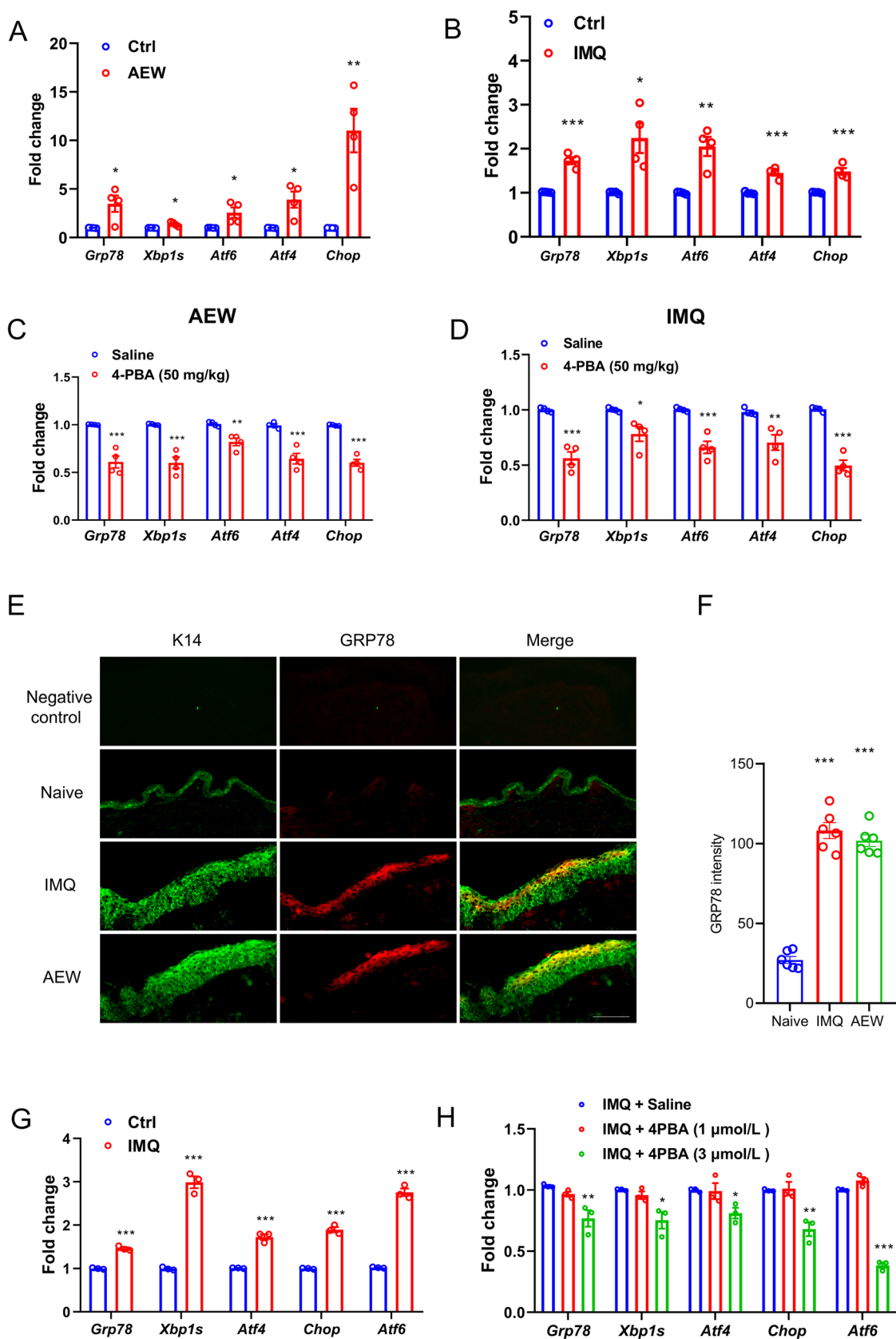


Fig. 4 ER stress-related signaling pathway is activated in the skin of chronic itch models, and imiquimod-treated HaCaT cells *in vitro*. **A** qPCR of ER stress-related genes *grp78*, *xbp1s*, *atf6*, and *atf4* in the affected skin treated with AEW. ($n = 4$, $*P < 0.05$, $**P < 0.01$, multiple t tests.) **B** qPCR of ER stress-related genes *grp78*, *xbp1s*, *atf6*, and *atf4* in the affected skin treated with imiquimod ($n = 4$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, multiple t tests). **C** The effect of 4-PBA on the expression of ER stress-related genes *grp78*, *xbp1s*, *atf6*, and *atf4* in the affected skin treated with AEW ($n = 4$, $**P < 0.01$, $***P < 0.001$, multiple t tests). **D** The effect of 4-PBA on the expression of ER stress-related genes *grp78*, *xbp1s*, *atf6*, and *atf4* in the affected skin treated with imiquimod ($n = 4$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, multiple t tests). **E, F** Immunofluorescence of keratin-14 and GRP78 in the skin of AEW or imiquimod mice models, respectively ($n = 6$, $***P < 0.001$, vs Naïve, one-way ANOVA following Bonferroni's test). Scale bar: 100 μm . **G** qPCR of ER stress-related genes *grp78*, *xbp1s*, *atf4*, *chop*, and *atf6* in HaCaT cells with or without the treatment of imiquimod ($n = 3$, $***P < 0.001$ vs Ctrl, multiple t tests). **H** The effect of 4-PBA on the expression of ER stress-related genes was tested by qPCR ($n = 3$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, one-way ANOVA following Bonferroni's test). Ctrl, control group.

with the results *in vivo*, the upregulation of LCN2 mRNA and protein caused by imiquimod was both significantly reduced by 4-PBA (Fig. 5E–G) ($F_{(2,6)} = 52.98$, $P = 0.0087$; $F_{(2,12)} = 13.48$, $P = 0.0072$). These results suggest that LCN2 production is closely associated with ER stress activation in keratinocytes.

To further figure out whether keratinocyte-derived LCN2 traveled to the DRG *via* blood, we next performed an ELISA test with plasma and DRG tissue from both AEW and IMQ model mice. The results showed that the level of LCN2 increased significantly in plasma ($t_{10} = 2.522$, $P = 0.0303$; $t_{10} = 2.365$, $P = 0.0396$; Fig. 5 H, I) but not DRG from the two itch model mice (Fig. 5 J, K). So, the results above indicate that LCN2 is increased in keratinocytes of both itch models, and keratinocytes secreting LCN2 may target DRG neurons through serum transportation.

LCN2 Sensitized DRG Neurons *via* MC4R

Emerging evidence revealed that LCN2 plays a significant role in modulating sensory processing, particularly in pain perception, pruritus signaling, and neuronal excitability regulation within the nervous system [35]. We next employed a calcium image to test the effect of LCN2 on primary sensory neurons. Perfusion of recombinant LCN2 at the concentration of 1 $\mu\text{mol/L}$ triggered calcium signaling in approximately two-thirds of DRG neurons of wild-type mice (Fig. 6A–C). Melanocortin-4 receptor, a G protein-coupled receptor widely expressed in the nervous system, could bind to LCN2 and enhance neuronal excitability. We tested the effect of LCN2 in the presence of HS024, the inhibitor of MC4R, to determine whether the effect of LCN2 is dependent on MC4R. As shown in the results, the effects of LCN2

were completely abolished when the neurons were perfused with HS024 (Fig. 6A–C).

Subsequently, we employed patch-clamp recording techniques to assess the excitability of small-diameter DRG neurons (diameter $< 25 \mu\text{m}$), comparing conditions with and without pre-incubation of recombinant LCN2 *in vitro*. Our results showed that LCN2 significantly increased the APs of DRG neurons evoked by depolarizing current steps, which were abolished in the presence of HS024 (Fig. 6D–F). Neurons exhibited hyperexcitability after LCN2 incubation compared with the control group (abbreviated as “ctrl”), characterized by significantly lower rheobase, and HS024 also restored the rheobase to the normal state (Fig. 6G). There were no significant change on the membrane resistance (RM), resting membrane potential (RMP), and amplitude of APs (AMP) after LCN2 incubation (Fig. 6H–J). Meanwhile, we observed that the amplitude of afterhyperpolarization (AHP) was enlarged after LCN2 incubation, and HS024 abolished this effect (Fig. 6K). The other properties of the action potentials were not significantly different between the control group and the LCN2-treated group (Fig. 6L–N). These findings collectively demonstrate that MC4R serves as a critical mediator of LCN2-induced modulation of DRG neuronal excitability.

Since distinct itch conditions may engage different subsets of DRG neurons, we then conducted calcium imaging to determine whether these itch types share overlapping neuronal populations. The results showed that LCN2 triggered calcium signaling in approximately 66.7% (18 out of 27 cells across three independent experiments) of DRG neurons that respond to serotonin, and in approximately 25.3% (21 out of 83 cells) of neurons that respond to compound 48/80, but compound 48/80 and serotonin almost activate the same subsets of DRG neurons (Supplementary Fig. S3).

TRPV1 is Essential in Mediating LCN2-Induced Hyperexcitability in DRG Neurons

Given that TRPV1 and transient receptor potential ankyrin 1 (TRPA1) are highly expressed in small-diameter DRG neurons and play crucial roles in itch signal transduction [36, 37], we next investigated whether TRPA1 and TRPV1 channels are involved in LCN2-induced neuronal hyperexcitability. Capsaicin, a highly selective agonist of TRPV1, triggered calcium signaling in approximately 95% of the neurons that respond to LCN2 (83 out of 88 cells across three independent experiments), while allyl isothiocyanate (AITC), the activator of TRPA1, only triggered calcium signaling in approximately 50% of the neurons that respond to LCN2 (Fig. 7A–E). Meanwhile, capsazepine, the inhibitor of TRPV1, completely blocked the calcium signaling induced by LCN2 (Fig. 7F, G).

Consistently, LCN2 did not evoke calcium signaling in the DRG neurons from TRPV1 knockout mice (Fig. 7H, I). Notably, blocking TRPA1 with HC030031 or knocking

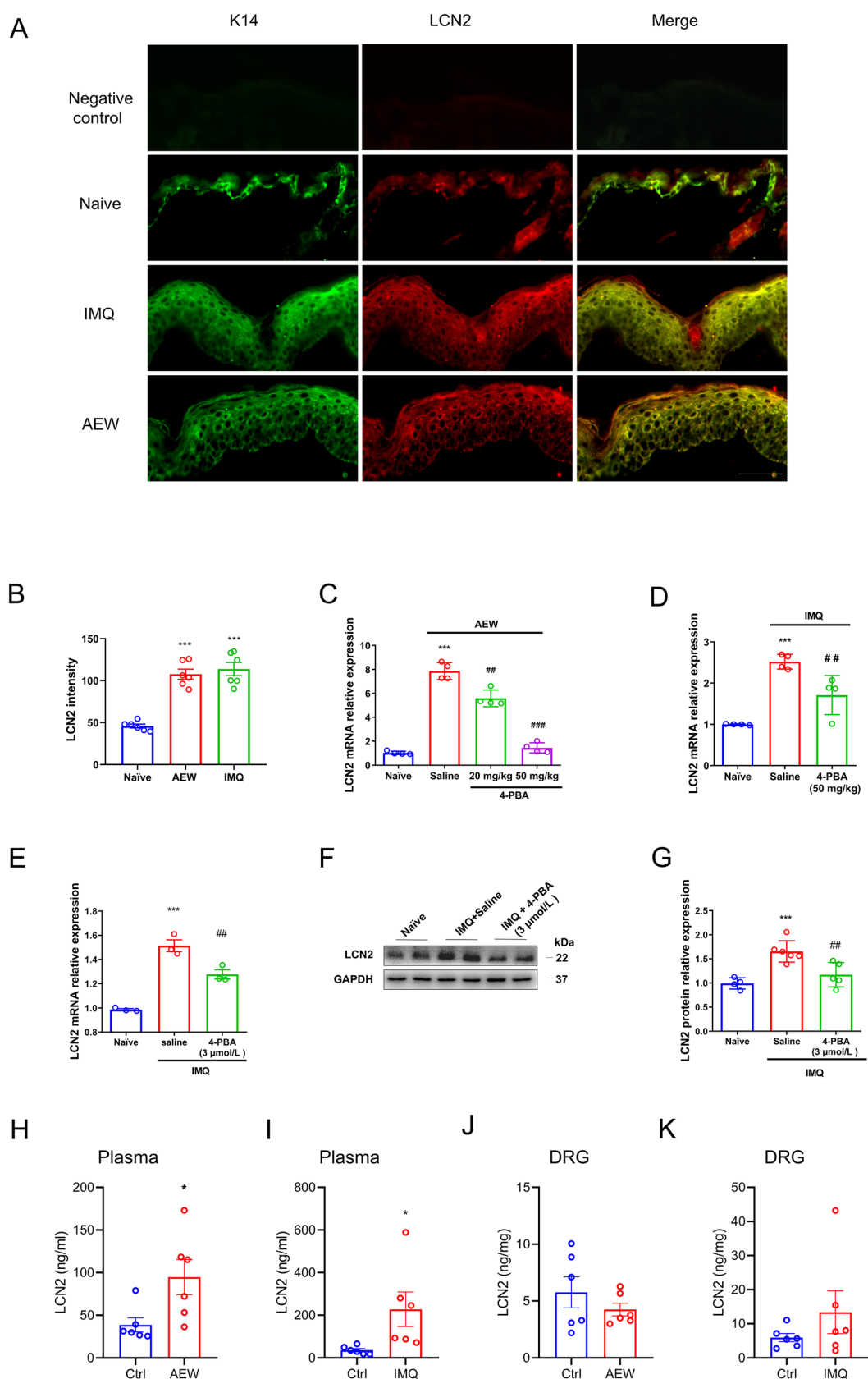


Fig. 5 The expression of LCN2 in the affected skin in chronic itch models. **A, B** Immunofluorescence of LCN2 in the skin of AEW and imiquimod mice models, respectively ($n = 5-6$, $*P < 0.05$, $***P < 0.001$, one-way ANOVA following Bonferroni's test). Scale bar: 100 μm . **C, D** qPCR of LCN2 in the skin of AEW and imiquimod-treated mice with or without 4-PBA intervention, respectively ($n = 4$, $***P < 0.001$ vs sham, $##P < 0.01$, $###P < 0.001$ vs saline, one-way ANOVA following Bonferroni's test). **E-G** qPCR and Western blot of LCN2 in the HaCaT cells treated with imiquimod with or without 4-PBA intervention, respectively ($n = 3-6$, $***P < 0.001$ vs CTRL, $##P < 0.01$ vs saline, one-way ANOVA following Bonferroni's test). **H, I** ELISA test on the serum level of LCN2 in AEW and IMQ model mice, respectively ($n = 5-6$, $*P < 0.05$ vs Ctrl, unpaired Student's t test). **J, K** ELISA test on the level of LCN2 in DRG tissue from AEW and IMQ model mice, respectively ($n = 6$, unpaired Student's t test). Ctrl, control group.

out TRPA1 did not exhibit similar effects (Supplementary Fig. S4). Next, we used patch clamp to confirm the role of TRPV1 by testing the effect of LCN2 on the small DRG neurons from TRPV1 knockout mice (Fig. 7J). LCN2 had no effect on the AP firing and other electrophysiological properties in the DRG neurons of TRPV1 knockout mice (Fig. 7K-S). These findings indicate that TRPV1 plays a crucial role in mediating LCN2-induced DRG neuronal activity.

Discussion

The epidermis, primarily composed of proliferating and differentiated keratinocytes, serves as the outermost layer of the skin that directly interfaces with the external environment. Chronic pruritus is closely associated with various dermatological disorders, involving complex interactions among keratinocytes, sensory neurons, and immune system networks that collectively contribute to its pathogenesis [38]. Accumulating evidence has demonstrated the dramatic changes in proliferation and aberrant differentiation of keratinocytes, which may initiate skin inflammation to trigger the onset of inflammatory response in chronic pruritus [39–41]. In the current study, we uncovered the activation of ER stress in keratinocytes in initiating itch sensation by secreting LCN2 and sensitizing primary sensory nerves. We demonstrated that the ER stress inhibitor 4-PBA effectively alleviates itch behaviors and the activity of DRG neurons. The ER stress-associated proteins, including GRP78, XBP1s, and ATF4, were increased in the skin of chronic itch conditions, which demonstrated the presence of ER stress at lesional sites in both psoriatic and dry skin mouse models. Meanwhile, IMQ directly activated ER stress in the cultured keratinocyte HaCaT cells. We further demonstrated that LCN2 expression was significantly upregulated in parallel with ER stress activation, which subsequently sensitized DRG neurons *via* TRPV1. These findings indicate that pharmacological inhibition of ER stress may represent

a novel therapeutic approach for the management of chronic pruritus.

In the current study, we employed four different pruritogens (compound 48/80, serotonin, chloroquine, and ET-1) to induce acute itch in mice. Although these substances induce similar itch phenotypes, our study revealed that the ER stress inhibitor 4-PBA attenuated itch-related behaviors elicited by compound 48/80 and serotonin, but not those induced by chloroquine or ET-1, suggesting that ER stress mediates specific pruriceptive pathways rather than universally contributing to all types of itch. Compound 48/80 is considered a well-known secretagogue that causes mast cell degranulation [42]. Unlike chloroquine or ET-1, which may activate specific neurons directly, compound 48/80 triggers the release of multiple inflammatory mediators, including histamine, leading to subsequent epidermal hyperplasia characterized by increased epidermal thickness and altered keratinocyte proliferation and differentiation [43]. These processes may substantially increase the protein processing burden on the ER stress, given its essential role in protein synthesis, folding, and quality control, consequently leading to the activation of UPR signaling pathways associated with ER stress [19].

Serotonin is a key neurotransmitter that plays a crucial role in regulating mood, appetite, sleep, cognition, and various physiological processes [44, 45]. It has been reported to lead to cell proliferation in fibroblasts, β -cells, adult rat hepatocytes, and osteoblasts *via* 5-HT receptors [46, 47]. Peripheral serotonin is also a key component of the "inflammatory soup" and acts as a potent mediator of itch and pain [48–51]. Dysregulated 5-HT signaling in human skin has been implicated in pruritus associated with atopic dermatitis, psoriasis, allergy, uremia, and cholestasis [52]. While extensive research underscores the role of 5-HT in both acute and chronic itch, the specific 5-HT receptor subtypes may be responsible for transmitting serotonergic pruritic signals. Multiple 5-HT receptor subtypes have been identified in skin keratinocytes, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, 5-HT₃, and 5-HT₇ [53], suggesting a complex role for serotonin in modulating epidermal function. Recent studies have demonstrated that both 5-HT_{2B} and 5-HT₃ receptors are functionally expressed in keratinocytes, which contribute to the modulation of keratinocyte behaviors, encompassing proliferation, migration, and cell death pathways [54, 55]. Meanwhile, serotonin could induce a stress-related reaction in keratinocytes *via* these receptors. This may explain why ER stress plays a critical role in serotonin-induced acute itch. Ultimately, the selective involvement of ER stress in specific types of pruritus provides new insights for developing targeted therapeutic strategies based on the distinct mechanisms of different pruritogens. While our *in vivo* data suggest that ER stress contributes to itch induced by compound 48/80 and serotonin, based on the inhibitory effect of 4-PBA on behaviors and neuronal activation, whether

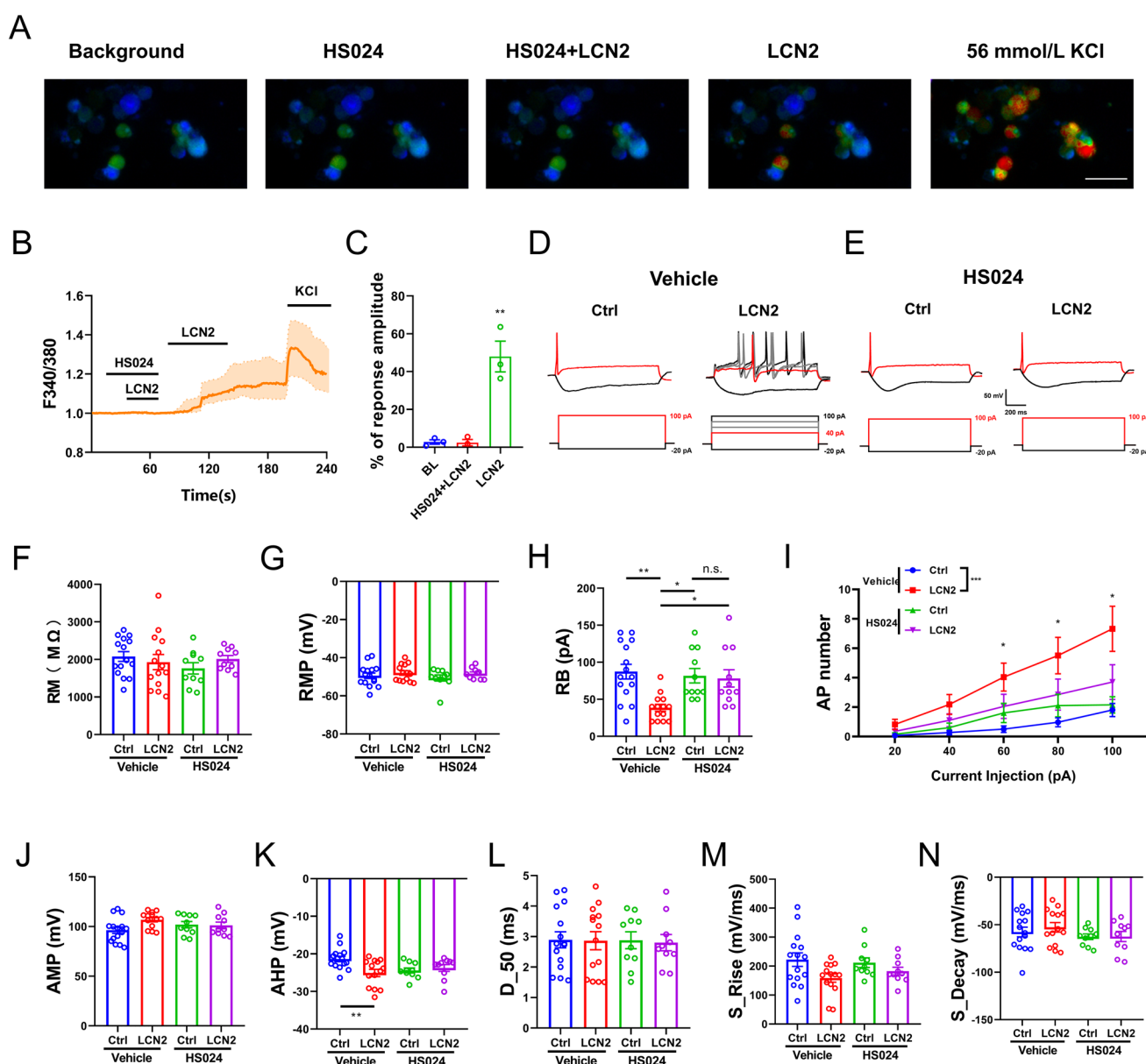


Fig. 6 Keratinocyte-derived LCN2 contributes to the excitability of DRG neurons. **A** Representative images of DRG neurons responding to 1 μ mol/L recombinant LCN2 with or without perfusion of HS024. **B** Median Ca^{2+} transients of DRG neurons pre- and post- LCN2 with or without HS024 perfusion in one independent experiment ($n = 33$, median $\pm$ 95%CI). **C** Averaged peak $\Delta F/F$ of calcium activity of DRG neurons responses to LCN2 with or without perfusion of HS024 in three independent experiments (** $P < 0.01$ vs Baseline, one-way ANOVA following Bonferroni's test). **D**, **E** Representative action

potential (AP) traces evoked by current injection with or without LCN2 treatment in the absence or presence of HS024. **F–N** The effect of LCN2 on the membrane resistance (RM), resting membrane potential (RMP), rheobase of AP firing (RB), AP number, amplitude of APs (AMP), afterhyperpolarization (AHP), half width of AP (D_{50}), the maximum rise and decay slope (S_{rise} and S_{decay} , $n = 10$ – 15 , * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA or two-way repeated measures ANOVA following Bonferroni's test). Scale bar: 25 μ m. Ctrl, control group.

these pruritogens directly trigger ER stress in keratinocytes remains unclear. This is a limitation of the current study and needs further investigation.

We also introduced two chronic itch models, the dry skin model induced by AEW and the psoriasis model induced by IMQ, respectively. Our data showed that the ER stress inhibitor 4-PBA significantly alleviated itch-related behaviors

induced by AEW and IMQ, which suggested that the impact of ER stress in keratinocytes occurs in chronic itch. Chronic itch is usually accompanied by skin tissue pathology. ER stress in keratinocytes may emerge during lesion development and exacerbate pathological progression by interacting with the immune system [38]. Keratinocytes can sense exogenous or endogenous pruritogens through various receptors

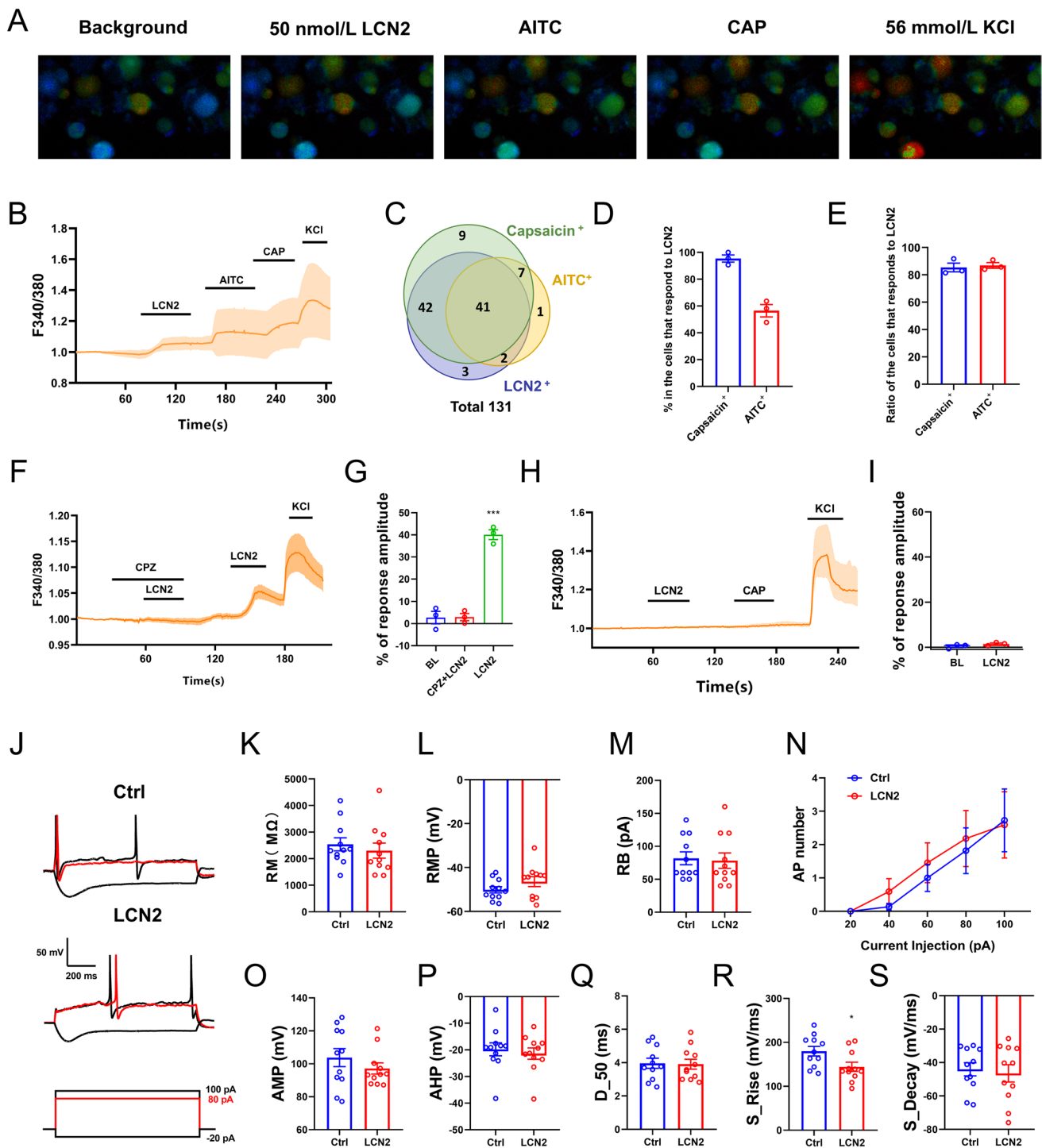


Fig. 7. TRPV1 is involved in LCN2-induced hyperexcitability in DRG neurons. **A** Representative images of DRG neurons responding to recombinant LCN2, capsaicin, and AITC. **B** Median trace of Ca^{2+} transients of DRG neurons pre- and post- LCN2, capsaicin, and AITC pre-fusion ($n = 28$, median $\pm$ 95% CI). **C** Cell distribution and the overlapping activated by LCN2, capsaicin, and AITC. **D** The proportion of LCN2-activated cells among the neurons that respond to capsaicin or AITC. **E** The proportion of capsaicin or AITC-activated cells among the neurons that respond to LCN2. **F, G** The effect of LCN2 on the Ca^{2+} transients when TRPV1 was blocked by capsazepine ($***P < 0.01$ vs Baseline, one-way ANOVA following Bonferroni's test).

H, I The effect of LCN2 on the Ca^{2+} transients in the DRG neurons from TRPV1 knockout mice ($P > 0.05$, one-way ANOVA following Bonferroni's test). **J** Representative traces of APs evoked by current injection with or without LCN2 treatment in the small diameter DRG neurons from TRPV1 knocked out mice. **K–S** The effect of LCN2 on the membrane resistance (RM), resting membrane potential (RMP), rheobase of AP firing (RB), AP number, amplitude of APs (AMP), afterhyperpolarization (AHP), half-width of AP (D_{50}), the maximum rise and decay slope (S_{rise} and S_{decay}). $n = 11$, $P > 0.05$, one-way ANOVA or two-way repeated measures ANOVA following Bonferroni's test). Scale bar: 25 μm . Ctrl, control group.

or ion channels [56]. Our previous study showed that toll-like receptors TLR2 and TLR7 expressed on keratinocytes are activated under either dry skin or psoriasis-associated chronic itch conditions, and when HaCaT cells were treated with the TLR7 agonist IMQ, they exhibited a significant increase in production and secretion of various cytokines and chemokines [57]. Toll-like receptors are sensors of pathogens, which detect microbes and activate transcriptional programs that orchestrate adaptive responses to specific insults, and play an important role in the initiation of immune responses [58, 59]. Evidence has shown that TLR2 and TLR4 signaling pathways can activate key ER stress sensors, including IRE1 α and PERK kinases, along with their downstream effectors XBP1 and ATF6, which are essential to the production of proinflammatory cytokines in macrophages [60]. The current study showed that IMQ directly induced ER stress in cultured HaCaT cells. This process may also be mediated by toll-like receptors. Therefore, it is reasonable that chloroquine might induce itch through an ER stress-independent pathway because chloroquine is considered an inhibitor of toll-like receptors. Notably, nociceptors in the DRG express several types of TLRs, including TLR2, TLR3, TLR4, TLR7, and TLR8 [61]. IMQ induced itch by activating TLR7 in the nociceptor nerve endings directly [28]. This is consistent with our observation in the current study that 4-PBA partially suppressed IMQ-induced itch.

There is a divergence in research regarding the presence of ER stress in psoriatic skin. A study demonstrated that GRP78 was poorly expressed in proliferating keratinocytes in human psoriasis vulgaris tissues [62]. However, recent ultrastructural analysis using transmission electron microscopy (TEM) has revealed significant ER stress morphological alterations, accompanied by upregulated expression of ER stress markers (GRP78, CHOP, and XBP1) in lesional skin of psoriasis vulgaris [20]. In the current study, we specifically observed that the ER stress-associated signaling pathways were activated in the skin of psoriasis mice, despite the possible differences in the pathogenesis between animal models and the disease itself. For the dry skin model, the cutaneous barrier was disrupted by AEW treatment, and pruritogenic mediators, including various cytokines and chemical factors, are released from the affected cutaneous sites. However, the precise mechanisms underlying ER stress-mediated keratinocyte activation in dry skin-associated pruritus remain to be fully elucidated and warrant further investigation.

The most important finding in the current study is that the expression and secretion of LCN2 in keratinocytes were upregulated under dry skin and psoriasis conditions. Studies have shown that elevated LCN2 in the paraventricular thalamic nucleus contributes to depressive behaviors [63], and increased hepatic LCN2 promotes anxiety-like behavior in mice [64]. Here, we showed that ER stress in the skin induces LCN2 release, leading to hyperexcitability of

primary sensory neurons, which aligns with the reports that LCN2 derived from spinal cord astrocytes and microglia is also involved in the central sensitization process of chronic itch and neuropathic pain [65, 66]. A recent study demonstrated that ATF4 and CHOP bind to the LCN2 promoter and are able to induce LCN2 expression, which supports the role of ER stress in LCN2 upregulation [67]. These findings highlighted keratinocyte-derived LCN2 as a mediator in skin inflammation and sensory neuron sensitization.

In the current study, our data showed that the downstream mechanism of LCN2 involves the MC4R and TRPV1 pathway. MC4R inhibition and TRPV1 knockout significantly alleviate LCN2-induced hyperexcitability in DRG neurons, suggesting that TRPV1 is regulated by LCN2 signaling. Our previous work demonstrated that LCN2 enhances neuronal activity in the spinal cord and DRG through MC4R signaling [25], consistent with recent findings that MC4R activation upregulates TRPV1, leading to increased sympathetic neuron activity and blood pressure [26]. TRPV1 is a well-established mediator of chronic itch, responding to pruritogens such as histamine (*via* H1R and H4R) [68, 69], adenosine triphosphate (ATP), lipoxygenase products, and prostaglandins [70, 71]. These results collectively indicate that the LCN2-MC4R-TRPV1 axis plays a pivotal role in amplifying sensory neuron excitability, providing a mechanistic link between keratinocyte-derived LCN2 and chronic itch or pain in skin disorders.

In conclusion, we investigated the role of ER stress in keratinocytes in chronic itch. Our findings revealed that ER stress in keratinocytes may contribute to chronic itch by secreting LCN2 and sensitizing neurons through the activation of TRPV1. This study highlights that targeting ER stress in keratinocytes could offer a promising therapeutic strategy for the treatment of chronic itch.

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Conflict of interest The authors declare that there are no conflicts of interest.

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