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Original Article

Rg3-enriched Korean red ginseng alleviates chloroquine-induced itch and dry skin pruritus in an MrgprA3-dependent manner in mice

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ABSTRACT

Background: Previous studies have found that Korean red ginseng extract (KRG) has antipruritic effects, which can be attributed to the presence of Rg3, one of the most potent ginsenosides. Therefore, Rg3-enriched KRG extract (Rg3EKRG) is anticipated to have enhanced antipruritic effects. The present study was conducted to examine the effects of Rg3EKRG in acute chloroquine (CQ)-induced and chronic dry skin pruritus.

Methods: Calcium imaging technique was used in HE293T cells expressing MrgprA3 and TRPA1 ("MrgprA3/TRPA1") and in primary cultures of mouse dorsal root ganglia (DRG) neurons. Mouse scratching behavior tests were performed on dry skin models. To verify the altered expression of itch-related genes, real-time RNA sequencing analysis and PCR were performed on DRG sections obtained from dry skin models.

Results: Rg3EKRG suppressed CQ-induced intracellular calcium changes to a greater degree than KRG. Rg3EKRG dose-dependently inhibited CQ-induced responses in MrgprA3/TRPA1 cells. Rg3EKRG likely targeted MrgprA3 rather than TRPA1 to exert its inhibitory effect. Further, Rg3EKRG strongly inhibited the scratching behavior in mice induced by acute CQ injection. Importantly, DRG neurons obtained from dry skin mice models showed increased mRNA levels of *MrgprA3*, and treatment with Rg3EKRG alleviated chronic dry skin conditions and suppressed spontaneous scratching behaviors.

Conclusion: The results of the present study imply that Rg3EKRG has a stronger antipruritic effect than KRG, inhibiting both acute CQ-induced and chronic dry skin pruritus in an MrgprA3-dependent manner. Therefore, Rg3EKRG is a potential antipruritic agent that can suppress acute and chronic itching at the peripheral sensory neuronal level.

1. Introduction

Itch is a sensation that evokes the desire to scratch the skin. It is thought that pruritogen, an itch-inducing agent, triggers this sensation by stimulating specific receptors and/or ion channels in itch-mediating sensory neurons present underneath the skin. The most well-known pruritogen is histamine, which evokes an itch sensation by stimulating its cognate receptor (H1 receptor) and subsequently activating the TRPV1 ion channel in sensory neurons.[1] However, there is a consensus that not all itching is mediated by histamines, and distinct histamine-independent itch mechanisms were recently discovered. For example, the antimalarial agent chloroquine (CQ) induces intense itching as a side effect that cannot be suppressed by antihistamine agents.[2,3] It was later revealed that MrgprA3, a member of the mouse Mas-related G protein-coupled receptor (MRGPR) family, senses CQ-induced itch in a histamine-independent manner.[4] Moreover, CQ-induced stimulation of MrgprA3 subsequently activates TRPA1, a non-selective cation channel involved in various sensations, including itch.[5] Although other non-histamine pruritogens and receptors/ion channels have been identified, CQ and MrgprA3 are recognized as representative histamine-independent itch mediators.

Dry skin results from skin barrier dysfunction caused by environmental factors, such as exposure to sunlight, temperature, and humidity.[6–8] In general, dry skin conditions induce chronic itch[9], which is common clinical symptom of skin disorders. Dry skin-

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induced itching is considered to be histamine-independent, as antihistamine treatment is not completely effective.[10] A recent study revealed that innervation by MrgprA3$^+$ itch-sensing sensory neurons is increased under dry skin conditions.[11] It has also been observed that TRPA1 is responsible for dry skin-induced itching.[12]

Korean ginseng, the root of *Panax ginseng* Meyer, has been used for more than a thousand years in East Asian countries as a traditional herbal remedy to recover strength or improve health conditions.[13] Korean red ginseng (KRG) is a steamed and dried Korean ginseng that contains various ginsenosides, which are its main active ingredients. Certain ginsenosides such as Rg2, Rg3, Rg5, and Rh1 are considered specific for KRG because their concentrations are particularly increased during the KRG steaming process.[14,15] Among these KRG-specific ginsenosides, the ginsenoside Rg3 has various beneficial effects, including anti-cancer[16], anti-depressive[17,18], anti-oxidant[19], and anti-inflammatory effects.[20]

Importantly, our team has previously shown that KRG and its active ingredient Rg3 also have anti-pruritic effects.[21] It was shown in detail that Rg3 has strong inhibitory effects on CQ-induced, MrgprA3-dependent TRPA1 activation and suppresses CQ-induced scratching behavior in mice. However, it is still unclear which molecule, MrgprA3 or TRPA1, is the target for the inhibitory effects of KRG and Rg3. Furthermore, whether KRG and Rg3 may be beneficial in treating chronic dry skin-induced pruritis, unlike acute CQ-induced itch conditions, needs to be determined.

Rg3-enriched KRG extract (Rg3EKRG) has been gaining increasing interest because of its enhanced biological activities. Therefore, the present study aimed to determine: A) Whether Rg3EKRG is better than KRG in terms of inhibiting CQ-induced pruritus; B) The target molecule of Rg3EKRG in CQ-induced responses; C) Whether Rg3EKRG can suppress pruritus resulting from chronic conditions such as dry skin. Further, the present study elucidated the putative antipruritic mechanism and effects of Rg3EKRG in both CQ-induced acute itching conditions and dry skin-induced chronic pruritus.

2. Methods

2.1. Reagents and materials

KRG extract and Rg3EKRG were provided by the Korea Ginseng Corporation (Seoul, Korea). To obtain KRG, the roots of 6-year-old red ginseng were harvested in Korea, and fresh ginseng was steamed at 90–100°C for 3 h, followed by drying at 50–80°C. KRG extract was then prepared by subjecting the solution of red ginseng extract to 85–90°C for 8 h in hot circulating water thrice. Further, the KRG extract obtained in the previous step was used to prepare Rg3EKRG, which was extracted in seven 12-hour cycles of hot circulating water (87°C) and two 3-hour cycles of hot circulating ethanol (65°C). The extract was concentrated at 50–60°C and spray dried. The ginsenoside contents in KRG and Rg3EKRG were analyzed using high-performance liquid chromatography (Korea Ginseng Corporation).

CQ and allyl isothiocyanate (AITC) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2. Cell culture and transfection of cDNA

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin (Hyclone, Thermoscientific, South Logan, UT) at 37°C in a humidified atmosphere with 5% CO_2 condition. Cells were co-transfected with mouse *MrgprA3* (NM_153067) and human *TRPA1* (NM_007332) cDNAs using the FuGENE® HD Transfection Reagent (Promega, Wisconsin, USA) following the manufacturer's protocol. Further experiments were performed 24 h after transfection.

2.3. Primary culture of dorsal root ganglia (DRG)

Mouse DRGs were cultured as described previously.[22] Briefly, DRGs were dissected and collected from adult mice and cultured in neurobasal medium (Gibco, Life technologies, Greenland, NY) containing 10% FBS, 50–100 ng/mL nerve growth factor (Invitrogen, Gaithersburg, MD, USA), and 100 U/mL penicillin-streptomycin solution (Hyclone, Thermoscientific, South Logan, UT). Dissected DRGs were incubated with 1 mg/mL collagenase (Worthington Biochemical, Lakewood, NJ) for 30 min at 37°C, followed by an additional incubation with 2.5 mg/mL trypsin (Gibco, Life Technologies, Greenland, NY) for 30 min at 37°C. Dissociated cells were plated in poly L-lysine-coated 8-well chambers (Lab-Tek^{TM}, Thermo Scientific) and grown for 3 or more days at 37°C in 95% air/5% CO_2.

2.4. Calcium imaging

Intracellular calcium levels were determined using a calcium imaging technique as previously described.[23] Briefly, the culture medium was replaced with a normal bath solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, and 5.5 mM HEPES [pH 7.4]) containing Fluo-3 AM (2 μM, Invitrogen) and 0.1% Pluronic F-127 (Invitrogen). After incubation for 40 min, the bath solution containing Fluo-3 AM was replaced with a normal bath solution, and 1 mM CQ was applied to the cells to induce calcium influx. The cells were pre-incubated with KRG and Rg3EKRG 5 min prior to CQ application. Fluorescence intensities were measured at 488 nm with an interval of 1.5 s using an inverted microscope (ECLIPSE Ti-U; Nikon, Tokyo, Japan). Intracellular calcium changes were expressed as the F/F_0 ratio, where F_0 is the fluorescence intensity at 0 s. Image analysis was performed using ImageJ (NIH) with custom scripts for automatic cell counting, fluorescence intensity calculations, and ratiometric image production.

2.5. In vivo *scratching behavior test*

All protocols for animal experiments were approved by the Institutional Animal Care and Use Committee of Gachon University (GIACUC-R2020002) and were performed in accordance with the guidelines for the Care and Use of Laboratory Animals issued by Gachon University. Ten-week-old male ICR mice were purchased from Orient (Gyeonggi-do, Korea), following which an additional 1 week was required for acclimation. To induce scratching, 200 μg of CQ dissolved in saline was injected subcutaneously into the nape of the mice. Rg3EKRG (25 mg/kg or 50 mg/kg) was orally administered to mice 1 h prior to CQ administration. After CQ injection, mouse behaviors were video-recorded for 1 h using a camcorder (HDR-CX560, Sony, Japan). The recorded videos were played later, and the bouts of scratching were counted by an experienced observer. Mice that used their hind limbs to scratch near the injected area were considered to be exhibiting bouts of scratching.[24]

2.6. Acetone/ethanol/water animal mouse model

After shaving the back of the nape, mice were randomly assigned to the acetone/ethanol/water (AEW) group (n = 6) or the control group (n = 6). The nape of the mice from the AEW group was treated with acetone and diethyl ether (1:1) for 15 s, followed by water treatment for 60 s. The treatment was repeated twice a day (at 10 AM and 5 PM) for five consecutive days, as previously described.[8] The mice in the control group were treated with water instead of acetone/diethyl ether.

2.7. RNA sequencing (RNA-seq) data analysis

For control and test RNAs, library construction was performed using the QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Inc., Austria) according to the manufacturer's instructions. Briefly, 500 ng of total RNA was prepared, an oligo-dT primer containing an Illumina-compatible sequence at its 5′ end was hybridized to the RNA, and reverse transcription was performed. After degradation of the RNA template, the synthesis of second strand was initiated by random primers containing an Illumina-compatible linker sequence at its 5′ end. The double-stranded library was purified using magnetic beads to remove all the reaction components. The library was amplified to add complete adapter sequences required for cluster generation. High-throughput sequencing was performed as single-end 75 sequencing using a NextSeq 500 (Illumina, Inc., USA). QuantSeq 3 mRNA-Seq reads were aligned using Bowtie2.[25] Differentially expressed genes were determined based on counts from unique and multiple alignments using coverage in Bedtools.[26] Read count data were processed based on the quantile normalization method using EdgeR within R of the Bioconductor software.

2.8. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). For calcium imaging data, total cell numbers from at least three separate experiments were acquired and designated as "*n*." Student's t-test was used for comparison between two groups. For comparison of more than three groups, one-way analysis of variance with the Holm-Sidak multiple comparison *post-hoc* test was used. Fisher's exact test was used to compare the responsiveness of DRG neurons. Statistical analysis was performed using Prism 9 (GraphPad).

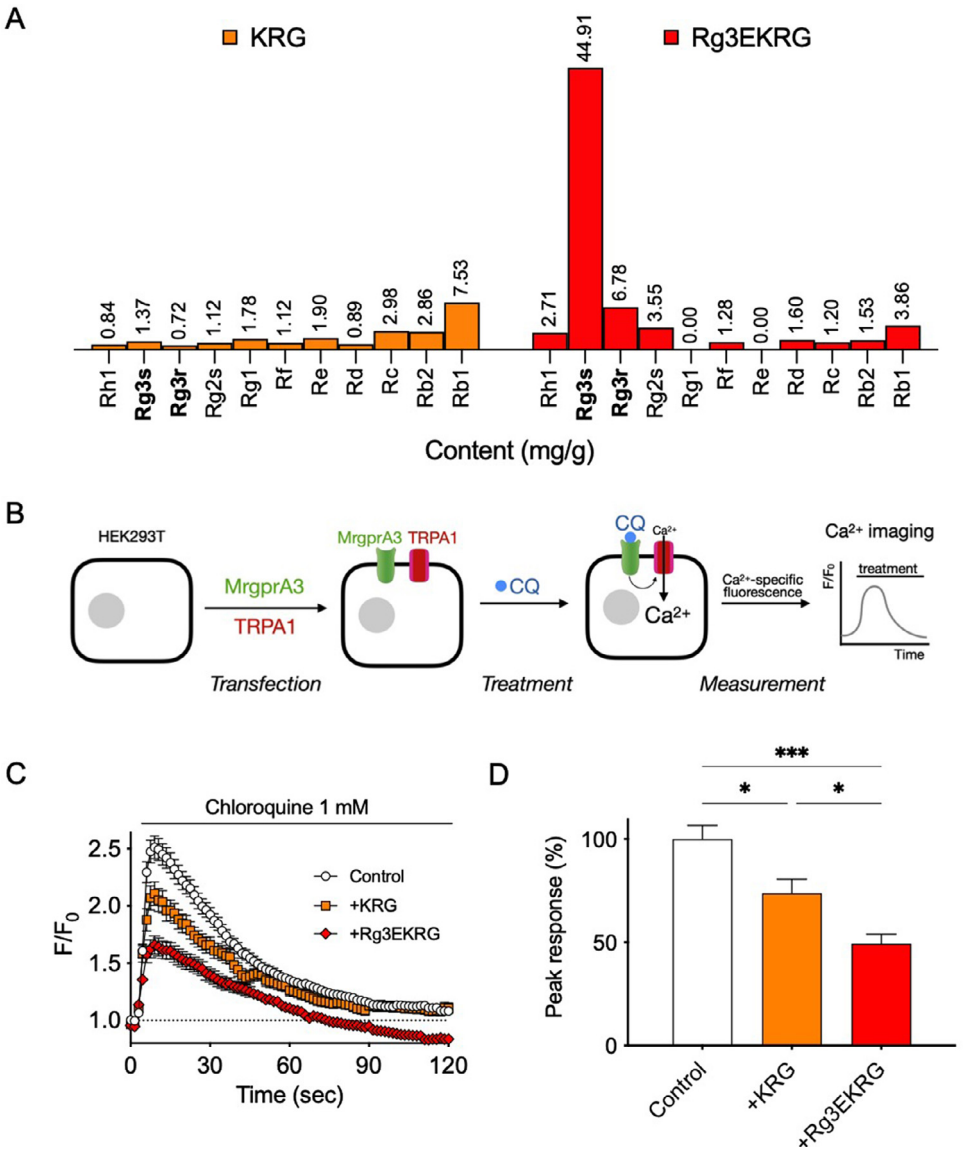

Fig. 1. Rg3EKRG has stronger inhibitory effect on CQ-induced responses than KRG in cells expressing MrgprA3 and TRPA1. (A) Contents of representative ginsenosides in KRG (orange) and Rg3EKRG (red). Notice the increased contents of Rg3s and Rg3r in Rg3EKRG compared to KRG. (B) Schematic illustration of the calcium imaging experiment. The cDNAs of MrgprA3 and TRPA1 were transiently expressed in HEK293T cells ("MrgprA3/TRPA1"). (C) Pretreatment with 100 μg/mL Rg3EKRG (red, "+Rg3EKRG") inhibited 1 mM CQ-induced intracellular calcium increase to a greater degree than 100 μg/mL KRG (orange, "+KRG"). (D) Comparison of the CQ-induced F/F_0 values between control (white, n = 553), pretreated with 100 μg/mL KRG (orange, n = 230), and pretreated with 100 μg/mL Rg3EKRG (red, n = 304) groups. * $p < 0.05$, *** $p < 0.001$

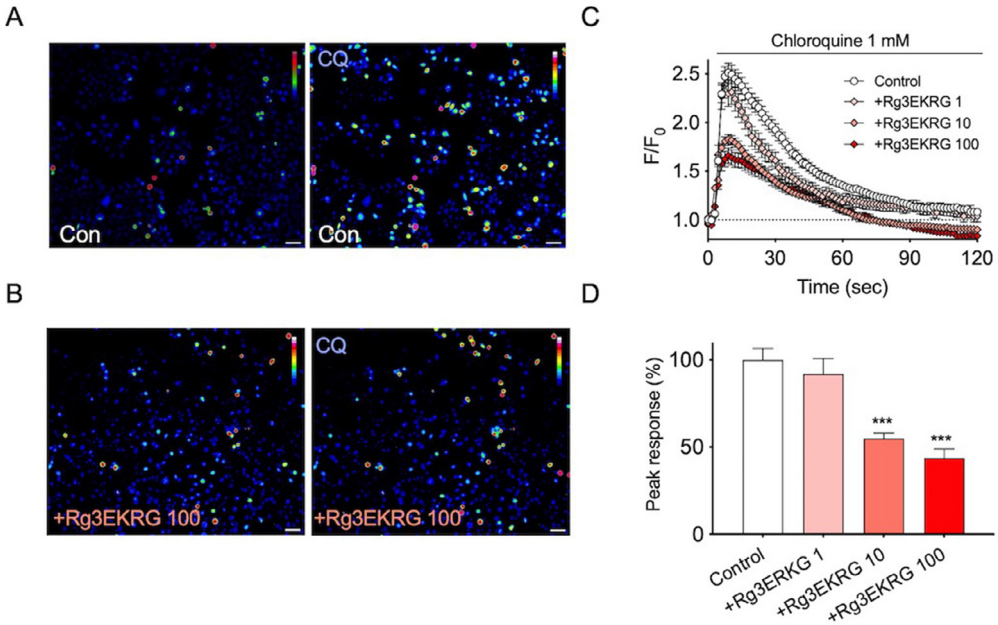

Fig. 2. Rg3EKRG can dose-dependently inhibit CQ-induced intracellular calcium increase in cells expressing MrgprA3 and TRPA1. (A) Representative ratiometric pseudocolor images before (left) and after (right) 1 mM CQ application on MrgprA3/TRPA1-expressing cells. (B) Representative ratiometric pseudocolor images before (left) and after (right) 1 mM CQ application on MrgprA3/TRPA1-expressing cells pretreated with 100 μg/mL Rg3EKRG. Notice that the CQ-induced responses are weaker than those of the control cells. The scale bar indicates 50 μm. (C) Time course graphs of CQ-induced responses in MrgprA3/TRPA1-expressing cells. As the concentration of Rg3EKRG increases, suppression of CQ-induced responses is evident. (D) Comparison of CQ-induced peak F/F_0 values between control (n = 553), 1 μg/mL Rg3EKRG (n = 212), 10 μg/mL Rg3EKRG (n = 511), and 100 μg/mL Rg3EKRG (n = 304) groups. *** $p < 0.001$

3. Results

3.1. Rg3EKRG has stronger inhibitory effect on CQ-induced responses than KRG

First, the ginsenoside contents of KRG and Rg3EKRG were determined. As shown in Fig. 1A, Rg3EKRG had a higher Rg3 content than KRG. The abilities of KRG and Rg3EKRG to inhibit CQ-induced responses were examined using calcium imaging. HEK293T cells were transiently transfected with *MrgprA3* and *TRPA1* cDNA ("MrgprA3/TRPA1"), and calcium imaging experiments were performed as illustrated in Fig. 1B. It was found that 100 μg/mL Rg3EKRG showed stronger inhibition of CQ-induced responses than the same dose of KRG (Fig. 1C). Compared with KRG, Rg3EKRG significantly suppressed the peak responses to CQ (Fig. 1D). Therefore, these data suggest that Rg3EKRG could be better than KRG in terms of inhibiting CQ-induced responses.

3.2. Rg3EKRG blocks CQ-induced calcium influx in cells expressing MrgprA3 and TRPA1

Next, we examined whether Rg3EKRG suppressed the CQ-induced responses in a dose-dependent manner. As a positive control, cells expressing MrgprA3/TRPA1 were treated with 1 mM CQ, which resulted in a strong increase in intracellular calcium levels (Fig. 2A). Next, the responses of cells pretreated with Rg3EKRG prior to CQ application were examined. When the cells were pretreated with 100 μg/mL Rg3EKRG prior to the application of 1 mM CQ, discernible reductions in calcium-dependent fluorescence intensities were observed (Fig. 2B). Furthermore, 10 μg/mL Rg3EKRG suppressed CQ-induced peak responses; however, this effect was not observed with 1 μg/mL Rg3EKRG (Fig. 2C and 2D). Therefore, Rg3EKRG inhibited CQ-induced intracellular calcium increase in MrgprA3/TRPA1-expressing cells in a dose-dependent manner.

3.3. CQ-induced excitation of sensory neurons is suppressed by Rg3EKRG in an MrgprA3-dependent manner

To further investigate the effect of Rg3EKRG on sensory neurons, primary cultures of mouse DRG neurons were performed, and calcium imaging experiments were conducted. As expected, 1 mM CQ induced an increase in intracellular calcium levels, which was significantly inhibited by pretreatment of the neurons with 100 μg/mL Rg3EKRG (Fig. 3A). The percentage of CQ-responsive DRG neurons was not altered by pretreatment with Rg3EKRG (Fig. 3B). Therefore, Rg3EKRG inhibited CQ-induced excitation of DRG neurons.

Because CQ-induced activation requires both MrgprA3 and TRPA1, it is unclear which molecule is the target of Rg3EKRG. To determine this, a specific TRPA1 agonist, AITC, was administered to HEK293T cells transiently expressing TRPA1 ("HEK293T-TRPA1"). However, AITC-induced responses in HEK293T-TRPA1 cells were not inhibited by pretreatment with 100 μg/mL Rg3EKRG (Fig. 3C and 3D), implying that Rg3EKRG did not inhibit the activity of TRPA1. To further verify this result, responses from AITC-sensitive mouse primary DRG neurons were compared with and without Rg3EKRG pretreatment. It was observed that AITC-induced responses in DRG neurons were not inhibited by pretreatment with 100 μg/mL Rg3EKRG (Fig. 3E). In addition, the percentage of AITC-responsive DRG neurons was not altered by Rg3EKRG pre-treatment (Fig. 3F). Overall, these data suggest that Rg3EKRG is unlikely to inhibit the activation of TRPA1, implying that MrgprA3 is a likely target for Rg3EKRG to exert its inhibitory effects.

3.4. CQ-induced acute scratching behavior is inhibited by Rg3EKRG

To further confirm the effect of Rg3EKRG *in vivo*, Rg3EKRG (25 mg/kg and 50 mg/kg) was orally administered to mice 1 h before CQ injection into their nape, and scratching be-

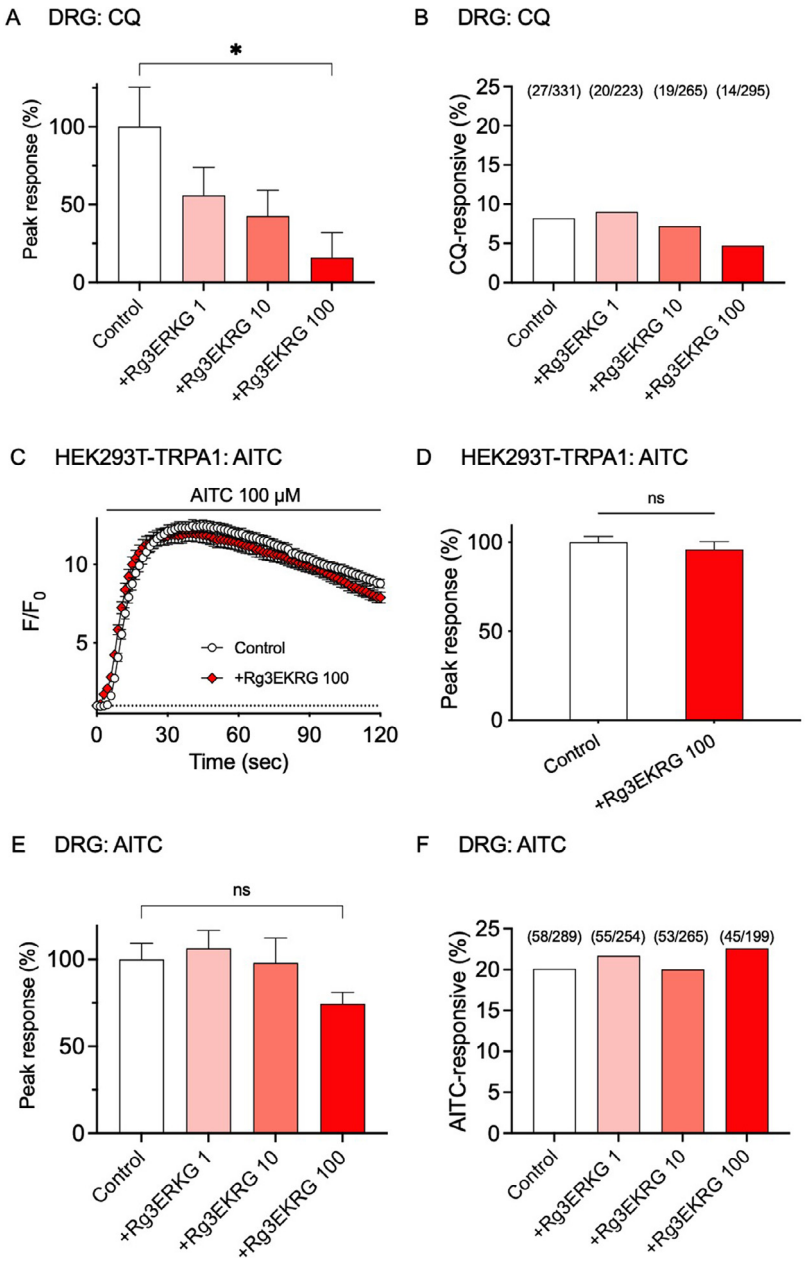

Fig. 3. Rg3EKRG inhibition of intracellular calcium changes depends on MrgprA3 but not TRPA1. (A) 1 mM CQ was applied on the primary culture of mouse dorsal root ganglia (DRG) neurons pretreated with Rg3EKRG. The Rg3EKRG-pretreated groups showed a trend of reduction in CQ-induced responses. Significant change was observed when DRG neurons were pretreated with 100 μg/mL Rg3EKRG. (B) Comparison of CQ-responsive DRG neurons with or without pretreatment with 1, 10, or 100 μg/mL Rg3EKRG. The numbers above the column bar indicate counts of *responsive cells* divided by *total cells*. (C) Time course graphs of responses induced by 100 μM AITC in HEK293T cells transiently expressing TRPA1 ("HEK293T-TRPA1"). Notice the lack of difference in calcium responses between the control group and the group pretreated with 100 μg/mL Rg3EKRG. (D) Comparison of AITC-induced peak responses between control (n = 914) and 100 μg/mL Rg3EKRG-pretreated (n = 863) HEK293T-TRPA1 cells . (E) 100 μM AITC was applied in the primary cultures of mouse DRG neurons following pretreatment with Rg3EKRG. Note that none of the groups shows changes in peak responses. (F) Comparison of AITC-responsive DRG neurons with or without pretreatment with 1, 10, or 100 μg/mL Rg3EKRG. The numbers above the column bar indicate counts of *responsive cells* divided by *total cells*. * $p < 0.05$, ns: not significant.

havior was recorded (Fig. 4A). As expected, CQ injection resulted in markedly elevated scratching bouts in control mice (Fig. 4B), which is consistent with a previous report.[4] In contrast, Rg3EKRG-administered mice showed significantly repressed scratching behavior (Fig. 4B). Rg3EKRG administration consistently inhibited CQ-induced scratching episodes throughout the study period (Fig. 4C). Therefore, these data suggest that Rg3EKRG has the potential to inhibit CQ-induced acute scratching behavior *in vivo*.

3.5. Scratching behavior of chronic dry skin mouse can be suppressed by Rg3EKRG administration

Finally, it was determined whether Rg3EKRG is also effective in a chronic itch mouse model. Based on a previous report of increased innervation by MrgprA3^{+} itch-sensory neurons in dry skin conditions[11], an animal model treated with acetone/ether/water (AEW) was used to mimic chronic dry skin conditions. Thus, we investigated whether AEW-treated mice had an altered expression

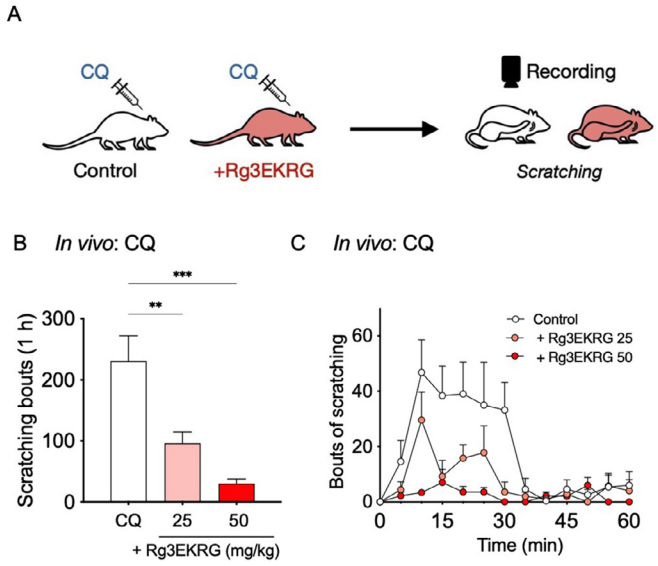

Fig. 4. Rg3EKRG inhibits CQ-induced acute scratching behavior in mice. (A) Schematic illustration of the scratching behavior test. Mice in the control group were subcutaneously injected with 200 μg CQ, whereas mice in the other groups were given oral Rg3EKRG (50 mg/kg) 1 h before CQ injection. (B) CQ-evoked increased bouts of scratching in the control (n = 4), which was significantly suppressed in the Rg3EKRG-administered group (n = 4, "+Rg3EKRG"). (C) Time course profile with 5 min intervals of scratching bouts between the two groups. Notice that the Rg3EKRG-administered groups showed consistent suppression of scratching behaviors. ** $p < 0.01$

of *MrgprA3* and other itch-related genes in sensory neurons. To achieve this, RNA-seq was performed using total RNAs obtained from DRGs of control and AEW-treated mice. The levels of various gene transcripts were found to be significantly altered. Among the selected itch-related ion channels and receptors, MrgprA3 levels were noticeably increased in DRG neurons obtained from AEW-treated mice (Fig. 5A). Real-time PCR results confirmed that DRG neurons from AEW-treated mice showed increased mRNA levels of *MrgprA3*. In contrast, neither *Trpa1* nor *Trpv1* showed any visible changes at the transcriptional level (Fig. 5B).

As MrgprA3 is overexpressed in dry skin conditions, we further investigated whether Rg3EKRG is beneficial for alleviating symptoms related to dry skin. To achieve dry skin conditions, AEW treatment was performed twice per day for five consecutive days, and 50 mg/kg Rg3EKRG was orally administered throughout the study period. After the completion of AEW treatment, control mice developed noticeable skin lesions (Fig. 5C). However, the skin condition did not deteriorate when Rg3EKRG was administered orally during AEW treatment (Fig. 5D), demonstrating the beneficial effects of Rg3EKRG on dry skin conditions. In terms of scratching behavior, AEW-treated mice exhibited a significant increase in spontaneous scratching bouts compared with non-AEW-treated mice (Fig. 5E). The increased spontaneous scratching behavior was significantly decreased even when KRG was administered orally during AEW treatment (Fig. 5E). Most importantly, AEW-treated mice that were administered Rg3EKRG showed scratching bouts that were similar to those of non-AEW-treated mice (Fig. 5E). To further investigate the underlying mechanisms, the CQ-induced responses of DRG neurons cultured from AEW-treated mice were investigated. As shown in Fig. 5F, responses to CQ were increased in DRG neurons from AEW-treated mice compared with those from non-AEW-treated mice. However, the responses to CQ was significantly lower in mice administered Rg3EKRG during AEW treatment than in AEW-treated control mice. Taken together, these data suggest that Rg3EKRG can inhibit chronic itching induced by dry skin in an MrgprA3-dependent manner.

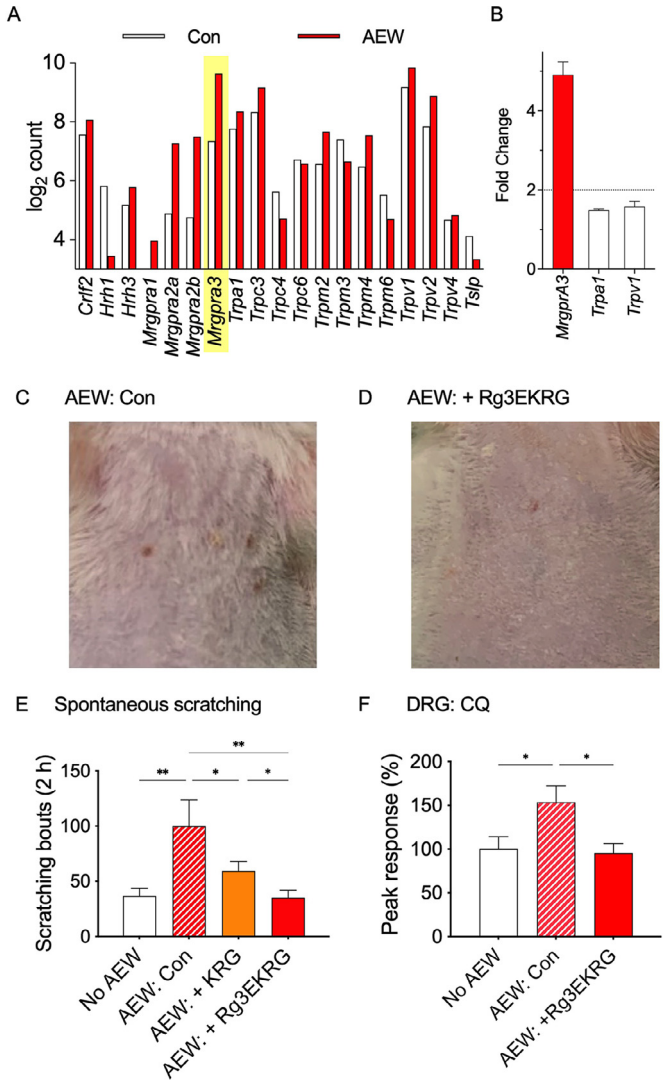

Fig. 5. Rg3EKRG alleviates scratching behavior in chronic dry skin mice. (A) Changes in mRNA levels of some itch-related receptor and ion channel genes in dorsal root ganglia (DRG) neurons between the two groups (control and AEW-treated dry skin mice) by RNA-seq. (B) Real-time PCR results showed that DRG neurons of AEW-treated mice had elevated levels of *MrgprA3* mRNA but not of *Trpa1* or *Trpv1* mRNA. Representative skin pictures of control (C) and "+Rg3EKRG" mice (D), which were administered 50 mg/kg of Rg3EKRG daily orally throughout AEW treatments. (E) AEW-treated mice showed significantly increased spontaneous scratching bouts (n = 6, "AEW: Con") than normal mice (n = 6, "No AEW"). The increased spontaneous scratching behavior $\varsigma\alpha\sigma$ markedly reduced when 50 mg/kg of Rg3EKRG was administered (n = 6, "AEW: +Rg3EKRG"). (F) Primary culture of DRG neurons from these mice showed similar response patterns to 1 mM CQ in calcium imaging experiments. DRG neurons from "AEW: Con" showed significantly higher peak responses (n = 32) compared to those of the "No AEW" (n = 29) group. Treatment with 100 μg/mL Rg3EKRG remarkably decreased peak responses in "AEW: +Rg3EKRG" (n = 30). * $p < 0.05$

4. Discussion

The present study identified the advantages of Rg3EKRG over KRG in terms of CQ-related pruritic conditions. The improved potency of Rg3EKRG is possibly due to the high amount of Rg3, because Rg3 has an inhibitory effect on CQ-induced itch.[21] Therefore, the enrichment of Rg3 in KRG is advantageous for enhancing antipruritic effects. Furthermore, as Rg3 can reduce skin lesions in mouse models of DNFB-induced atopic dermatitis[27], it is expected that Rg3EKRG can be applied to other pruritic conditions such as atopic dermatitis.

In this study, we used 100 μg/ml for the *in vitro* experiments, which was estimated by referring to the Rg3 pharmacokinetic profile *in vivo*. When 50 mg/kg of Rg3 was administered orally to rats, its C_{max} value was 81.6 ± 24.6 μg/mL, probably due to its relatively low bioavailability.[28] Because the molecular weight of Rg3 was 785.025, the expected maximum concentration was 104.46 μM. In other words, oral administration of 50 mg/kg Rg3 is equivalent to 100 μM Rg3, when absorbed. Importantly, the antipruritic effect of Rg3 was observed at 100 μM, which was the concentration used in this study. Furthermore, the dose we have used in our *in vivo* experiments is not extremely low, supporting our hypothesis that the observed *in vivo* anti-pruritic effects are likely to be a result of the actions of Rg3.

Although our previous study reported that KRG and Rg3 can inhibit CQ-induced activation of MrgprA3/TRPA1 [21], it was not clear whether MrgprA3 or TRPA1 is the molecular target for the inhibitory action of Rg3EKRG. The present study showed that Rg3EKRG preferentially inhibits MrgprA3-dependent responses rather than TRPA1 activation (Fig. 3). Thus, it appears that certain ginsenosides present in Rg3EKRG, most likely Rg3, possess inhibitory effect against MrgprA3. This finding is of substantial importance given that there are currently no MrgprA3 inhibitors available. Therefore, the present study not only demonstrated the potent antipruritic activity of Rg3EKRG but also suggested a novel approach to inhibit MrgprA3-dependent activity in future studies.

TRPA1 is a nonselective, polymodally activated cation channel predominantly expressed in sensory neurons that can sense various stimuli such as noxious coldness and oxidative stress.[29] TRPA1 also plays an essential role in the transmission of acute and chronic itch in a histamine-independent manner.[12,30,31] Thus, it was hypothesized that inhibition of TRPA1 could be a promising antipruritic strategy. However, due to limited bioavailability[32], the development of TRPA1 antagonists is not feasible. Furthermore, activation of TRPA1 supports skin barrier repair and epidermal permeability homeostasis[33], implying that inhibition of TRPA1 may not be beneficial for maintaining skin barrier function. In this context, the current finding that Rg3EKRG does not inhibit TRPA1 (Fig. 3C-3F) further implies that Rg3EKRG is an attractive and promising antipruritic agent that does not affect the activity of TRPA1.

Dry skin is an abnormal condition in which the skin becomes rough and scaly, with pruritus. Recent studies have revealed several molecular mechanisms underlying dry skin pruritus. For example, it was found that there are complex cutaneous neuroimmune interactions between TSLP cytokines and TRPV4 ion channel.[34] More importantly, dry skin induces overexpression of MrgprA3 as well as an increase in the percentage of MrgprA3$^+$ sensory neurons.[11] We also observed that *MrgprA3* is the most abundantly expressed itch-related gene in DRG neurons of mice treated with AEW. Furthermore, Rg3EKRG significantly suppressed CQ-induced scratching behavior and decreased the responses to CQ in DRG calcium imaging experiments. These findings confirmed the possibility that the use of Rg3EKRG can be expanded to dry skin conditions. Indeed, we confirmed that spontaneous scratching behaviors increased in AEW-treated mice, implying an expected antipruritic effect of Rg3EKRG in dry skin conditions in humans.

Interestingly, other genes related to itch, such as *Trpc3* and *Trpv1,* also showed elevated levels, although these genes were already prominently expressed in the controls. Although *Trpa1* expression was also increased in AEW-treated mice, the difference in *Trpa1* levels between control and AEW-treated mice was smaller than that of *MrgprA3*. Interestingly, certain transcripts, such as *Crlf2 (PAR2), Trpc3, Trpm2, and Trpv2,* exhibited unexpected upregulation in DRG neurons treated with AEW. Therefore, the present study also suggests other molecular candidates that may be involved in dry skin pruritus.

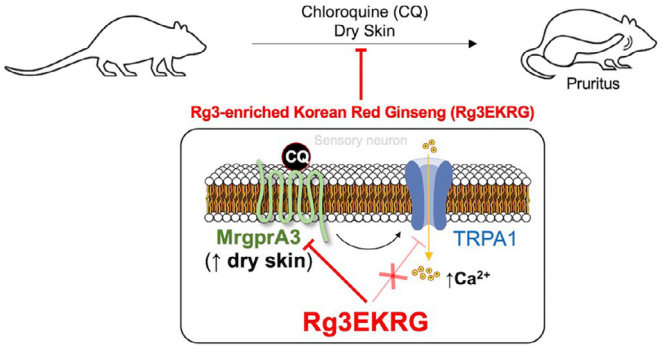

Fig. 6. Summary of the present study. An illustration summarizing the current findings. Pruritus evoked by CQ and dry skin can be suppressed by Rg3EKRG. The mechanisms underlying these effects are expected to involve the inhibition of the activity of CQ-sensitive MrgprA3 GPCR rather than the calcium-permeable TRPA1 ion channel.

However, the present study has some limitations. One example is that the MRGPR genes in mice are not well conserved in humans. In particular, the human gene most functionally similar to MrgprA3 is MRGPRX1 because it is also sensitive to CQ.[5] However, the sequence of MRGPRX1 is most similar to that of MrgprC11, which is another MRGPR member activated by the distinct pruritogen BAM8-22, but not by CQ.[5] Therefore, human MRGPRX1 is believed to be a combined counterpart gene of MrgprA3 and MrgprC11 in mice. This may complicate the interpretation of the present study because human MRGPRX1 is the target molecule under dry skin conditions. Further studies using human MRGPRX1 are needed to clarify this issue.

In conclusion, the present study showed that Rg3EKRG has a stronger suppressive effect than KRG on acute CQ-induced and chronic dry skin pruritus. Furthermore, Rg3EKRG inhibited CQ- and dry skin-induced pruritus in an MrgprA3-dependent manner, but not TRPA1 (Fig. 6). Therefore, these data strongly suggest that Rg3EKRG is a potential anti-pruritic agent that can be used to alleviate dry skin-induced pruritus in humans, although future studies are warranted.

Author contributions

WJL and WSS conceptualized the study; WJL performed the experiments; WJL and WSS wrote the manuscript; and WSS supervised the study.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Ethical statement

This study was approved by the Institutional Animal Care and Use Committee of Gachon University (GIACUC-R2020002) and performed in accordance with the guidelines for the Care and Use of Laboratory Animals issued by Gachon University.

Data availability

All the data analyzed in this study were included in this article. Further inquiries can be directed to the corresponding authors.

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