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Glabridin blocks TRPV1 activation and inhibits skin inflammation

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1. Introduction

Sensitive skin has become a globally dermatological concern, particularly among Chinese female populations [1]. The SS is characterized by erythema, stinging, xerosis, and desquamation, reflect multifaceted pathophysiology involving neurovascular dysregulation, immune-inflammatory responses, and epidermal barrier compromise [2]. Transient receptor potential vanilloid 1 (TRPV1) is a calcium-permeable cation channel expressed in keratinocytes and mast cells [3]. TRPV1 activation triggers calcium ions influx, which will subsequently release of neurogenic inflammatory mediators, such as substance P and calcitonin gene-related peptide, and drive cutaneous hypersensitivity and inflammatory dermatoses [4]. Activation of TRPV1 is a common problem for a variety of cosmetic ingredient such as retinol [5]. Avoiding irritation caused by activation of TRPV1 in cosmetics has become a challenge for cosmetics producer. Usually, ingredients that specifically inhibit TRPV1 activation should be supplemented into cosmetics to alleviate the irritation. However, these ingredients usually either have bad side-effect or increase the cost of cosmetics.

Glabridin, an important cosmetic ingredient, was first discovered and isolated from the roots of *Glycyrrhiza glabra* L. in 1976 [6]. Due to its excellent anti-inflammatory, antioxidant, antibacterial, and whitening efficacy [7, 8], glabridin is now widely utilized in cosmetic formulations[9]. In practice, glabridin is also found to be capable to reduce the irritation from other cosmetic ingredients. It is supposed that glabridin can suppress the TRPV1-mediated skin sensitivity. However, data is still lacking to support this thesis.

In this study, we for the first time studied the regulatory effects of glabridin on capsaicin-activated TRPV1 signaling. The calcium ion inflow and gene expression of inflammatory factors were measured after treatment of glabridin.

2. Materials and Methods

2.1 Cell Culture

Human keratinocytes (HaCaT) were cultured in DMEM supplemented with 10% Fetal Bovine Serum and a mixture of antibiotics. HaCaT were cultured in a humidified incubator at 37 °C with 95% air-5% CO_2.

2.2 Cell Viability Test

The MTT assay was used to investigate the effects of glabridin on cell viability. HaCaT were seeded in a 96-well plate at a density of 1w cells/100 μL. After 36 h of incubation, different concentrations of glabridin were added to the wells and then incubated for another 24 h. At the end of the exposure, the supernatant was removed. The cells were treated with MTT (0.5 mg/mL) solution at 37 °C for 4 h, and formazan was dissolved with dimethyl sulfoxide (DMSO). The absorbance was measured at 490 nm using a plate reader, and the results were expressed as a percentage relative to the control.

2.3 Capsaicin Treatment

HaCaT were seeded in 6-well plates at a density of 11w cells/mL. After incubation for 36 h, when the cell density reached 50-60%, the cells were washed twice with phosphate-buffered saline (PBS) and incubated with fresh medium containing 50 μmol/L capsaicin with or without glabridin (3 μmol/L) for another 8 h before further experiments.

2.4 Ultraviolet B Irradiation

HaCaT were seeded in 6-well plates at a density of 11w cells/mL. After incubation for 36 h, when the cell density reached 50-60%, the cells were washed twice with phosphate-buffered saline (PBS), covered with a thin layer of PBS solution, and placed in a UV crosslinker for irradiation. Unirradiated cells were covered with foil to prevent exposure. The UVB dose was 5 mJ/cm^2. After irradiation, PBS was removed, and the cells were incubated in fresh medium with or without glabridin (5 μmol/L) for another 24 h before further experiments.

2.5 Reverse-Transcription PCR

Total mRNA was prepared from treated cells using RNA-easy Isolation Reagent. The quality and quantity of the isolated RNA samples were assessed by measuring the 260/280 nm absorbance ratio of RNA. The cDNA was used as a template for quantitative RT-PCR, and 40 cycles of PCR were performed. The denaturation, annealing, and extension conditions for each PCR cycle were 95 °C for 30 s, 95 °C for 5 s, and 60°C for 30 s, respectively. The primers used were as follows: IL-1α, 5'-CAATTGTATGGACTGCCCAAG-3', 5'-ATAGTTCTTAGTGCCGTGAGTT-3'; IL-6, 5'-GCAGAAAACAACCTGAACCTT-3', 5'-ACCTCAAACTCCAAAAGACCA-3'; IL-8, 5'-TCTGCAGCTCTGTGTGAAGG-3', 5'-TGGGGTGGAAAGGTTTGGAG-3'; COX2, 5'-CTGGCGCTCAGCCATACAG-3', 5'-ACACTCATACATACACCTCGGT-3'; TNF-α, 5' -GACAAGCCTGTAGCCCATGTTGTA-3', 5'-CAGCCTTGCCCCTTGAAGA-3' ; β-actin, 5'-TTCTACAATGAGCTGCGTGTGG-3', 5'-GTGTTGAAGGTCTCAAACATGAT-3'. The mRNA level of each target gene was normalized to the level of β-actin.

2.6 Fluorescent staining

HaCaT cells were seeded in a 12-well plate at a density of 120,000 cells/mL. After incubation for 36 h, the wells were incubated with DMEM containing 2 mmol/L calcium ions for 30 minutes, and the wells were washed with cold PBS containing 2 mmol/L calcium ions. Different concentrations of glabridin and capsaicin were added and incubated for 30 minutes, and the wells were washed again with cold PBS containing 2 mmol/L calcium ions. The probe was added and incubated for 30 minutes in dark. The cells were observed under a fluorescence microscope.

2.7 Flow Cytometry Analysis

HaCaT cells were seeded in 12-well plates at a density of 120,000 cells/mL. After 36 h of incubation, the wells were incubated with DMEM containing 2 mmol/L calcium for 30 min, and

the wells were washed with cold PBS containing 2 mmol/L calcium. Different concentrations of glabridin and capsaicin were added and the cells were incubated for 30 min, and the wells were washed again with cold PBS containing 2 mmol/L calcium. The treated cells were harvested, and the probe was added and incubated for 30 min in dark. The stained cells were analyzed by flow cytometry. A total of 10,000 events were collected.

2.8 Statistical Analysis

Data are expressed as average value ± S.D. T-test, one-way analysis of variance (ANOVA), post hoc test were used to analyze the differences among the three groups. $P < 0.05$ was considered statistically significant. Graphical representation of data was performed by GraphPad Prism.

3. Results

3.1 Cytotoxicity of glabridin on HaCaT cells

Glabridin did not affect cell viability compared to the control group when its concentrations were no more than 5 μmol/L (Figure 1). When the concentration of glabridin was 7 μmol/L, the cell viability was reduced by 19.77%. When the concentration of glabridin reached 9 μmol/L, the cell viability was reduced by 33%. These results show that glabridin has no cytotoxicity to HaCaT cells at a concentration of no more than 5 μmol/L. Therefore, the glabridin of no more than 5 μmol/L was selected for subsequent experiments using HaCaT cells.

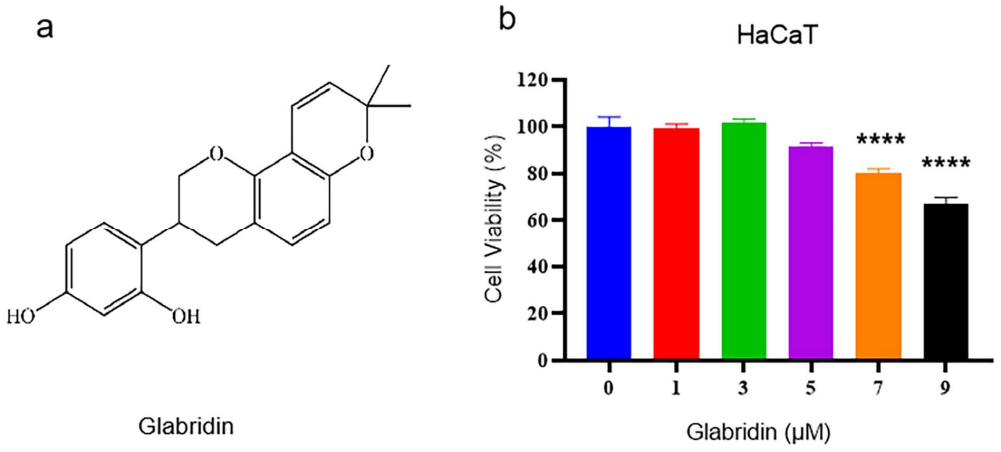

Figure 1. Cytotoxicity of glabridin on HaCaT cells. (A) The structural formula of glabridin. (B) Cell viability in the presence of different concentrations of glabridin. All data are presented as average value ± SD of triplicates (**** $P < 0.0001$).

3.2 Influx of calcium ions in HaCaT cells induced by capsaicin

Activation of TRPV1 by capsaicin was reported to result in an influx of calcium ions, which was shown as an increase of calcium concentration inside cells. We measured the concentration of calcium inside HaCaT cells to monitor activation of TRPV1 (Figure 2). The degree of calcium influx in cells increased with concentration of capsaicin. With the presence of 50 μmol/L of capsaicin, the concentration of calcium inside HaCat cell was increased by ca. 3 times. Consequently, we used 50 μmol/L of capsaicin to test the inhibition effect of glabridin on the capsaicin-induced TRPV1 activation.

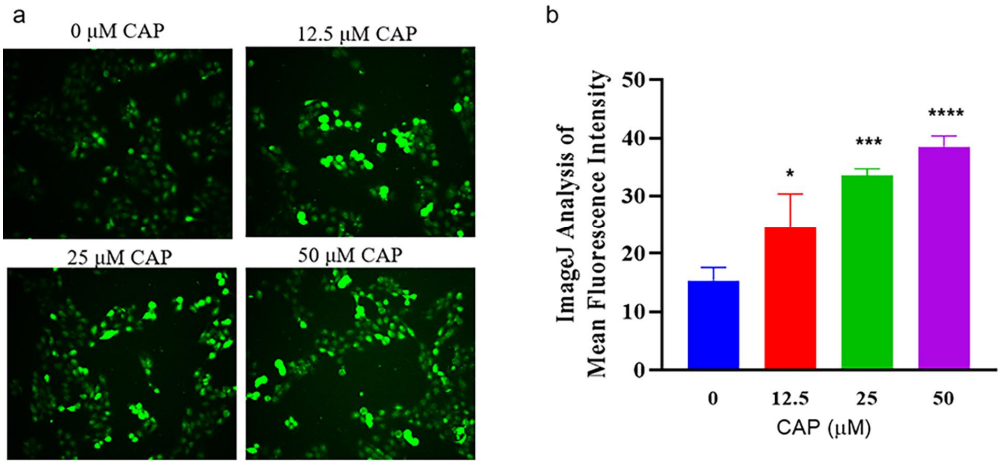

Figure 2. Capsaicin-induced calcium influx in HaCaT cells. (A) Fluorescence imaging of intracellular calcium flow after capsaicin treatment. (B) Quantitative calcium inflow caused by capsaicin of different concentrations. All data are presented as average value ± SD of triplicates (* P < 0.05, **P < 0.01, *** P < 0.001, **** P < 0.0001). CAP : capsaicin.

3.3 Influx of calcium ions in HaCaT cells induced by capsaicin

After treatment with different concentrations of glabridin, an inhibitory effect on calcium influx caused by capsaicin stimulation was observed (Figure 3). 50 μmol/L of capsaicin caused an increase in the florescence signal in HaCaT cells, indicating an increase in the concentration of calcium (Figure 3a). The calcium came from the culture medium through activated TRPV1. After treatment with 5 μmol/L glabridin, the fluorescence intensity decreased by 80% compared to capsaicin treated cells (Figure 3b). The calcium influx inhibitory effect of glabridin was quantitatively confirmed by flow cytometry (Figure 3c). The results showed that glabridin could effectively alleviate the activation of TRPV1 by capsaicin and had a good soothing effect.

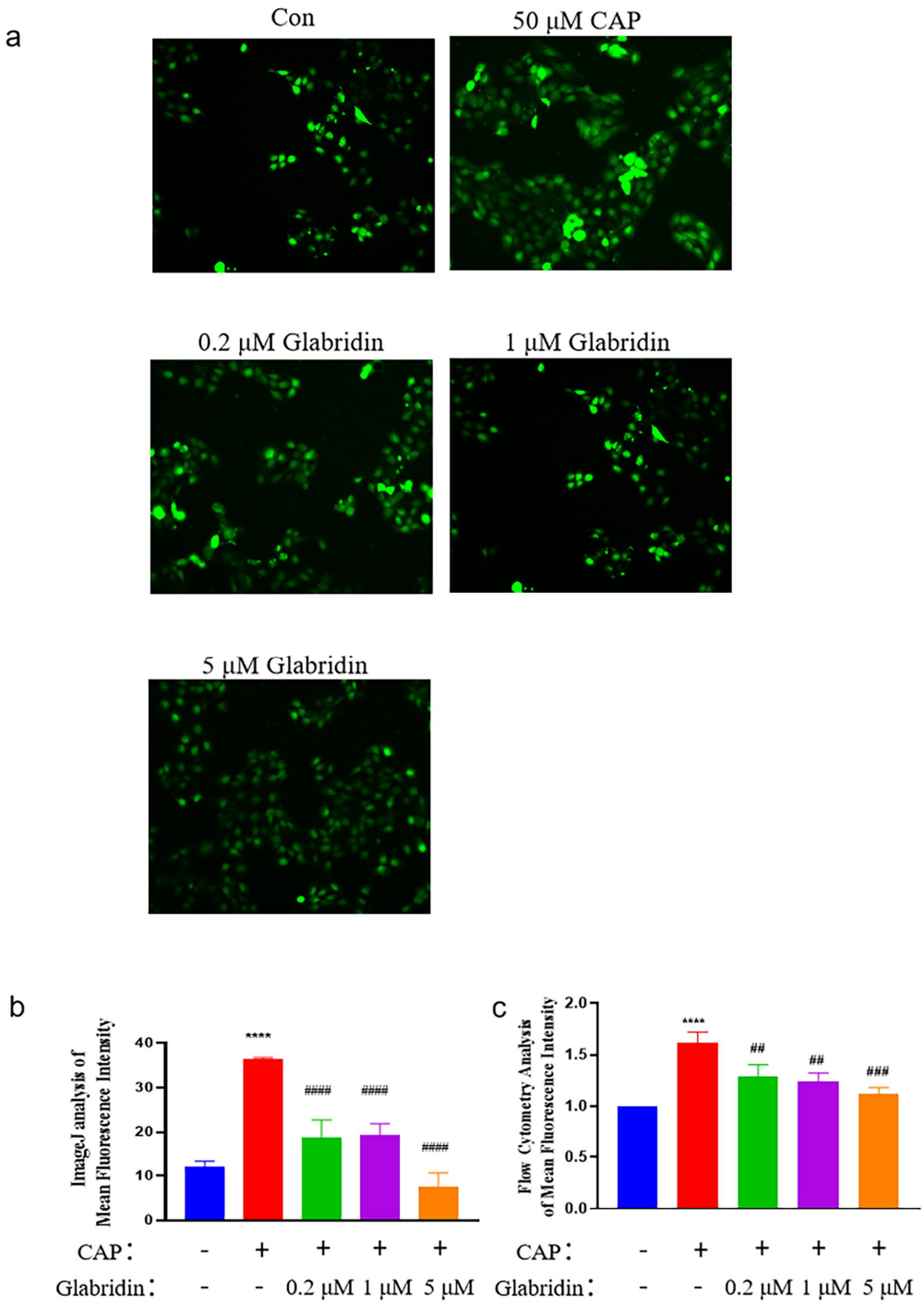

Figure 3. Glabridin inhibits capsaicin-induced calcium influx in HaCaT cells. (A) Fluorescence imaging demonstrating glabridin inhibited capsaicin-induced HaCaT calcium influx. (B) The effects of glabridin on capsaicin-induced calcium inflow were quantitatively measured by ImageJ analysis. (C) The effect of glabridin on calcium influx induced by capsaicin was detected by flow cytometry. All data are presented as average value ± SD of triplicates (* P <

0.05, **P < 0.01, *** P < 0.001, **** P < 0.0001 vs. the first column; # P < 0.05, ## P < 0.01, ### P < 0.001, #### P < 0.0001 vs. the second column). CAP: capsaicin; Con: control.

3.4 Effect of glabridin on secretion of pro-inflammatory cytokines in HaCaT cells

We found that some types of pro-inflammatory cytokines could be induced by TRPV1 activation, such as IL-8 and COX-2 (Figure 4a and 4b). After capsaicin treatment, the gene expression of IL-8 and COX-2 increased by ca. twice. However, addition of glabridin significantly decreased the gene expression of IL-8 and COX-2. The result indicated that glabridin can alleviate pro-inflammatory cytokines through inhibiting the activation of TRPV1.

Interestingly, we found that the expression of some other types of pro-inflammatory cytokines was not influenced significantly by capsaicin treatment, such as IL-1α, IL-6 and TNF-α). However, they could be triggered by UV irradiation. The mRNA expression of IL-1α, IL-6, and TNF-α increased significantly after HaCaT cells were irradiated with UV. The gene expression of IL-1α was upregulated by ca. 3.5 times, IL-6 by ca. 5.5 times, and TNF-α by ca. 3 times, respectively. Glabridin treatment significantly deceased the gene expression of IL-1α, IL-6 and TNF-α after UV irradiation. IL-1α was downregulated by 49.6%, IL-6 was downregulated by 36.2%, and TNF-α was downregulated by 22.3%. These results indicate that glabridin can prevent the pro-inflammatory effects in UV-irradiated HaCaT cells.

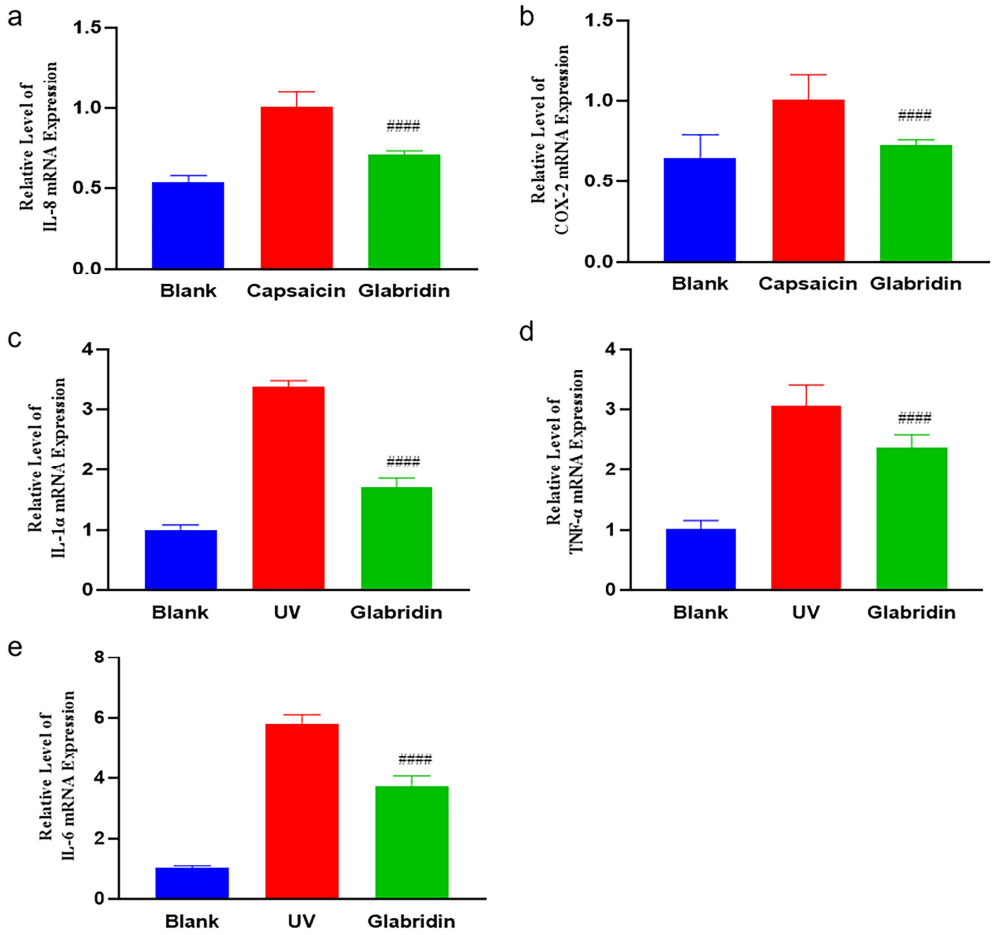

Figure 4. Glabridin inhibits pro-inflammatory cytokine gene expression in HaCaT cells. (a) Down-regulated gene expression of IL-8 by glabridin after capsaicin treatment. (b) Down-regulated gene expression of COX-2 by glabridin after capsaicin treatment. (c)

Down-regulated gene expression of IL-1α by glabridin after UV treatment. (d) Down-regulated gene expression of TNF-α by glabridin after UV treatment. (e) Down-regulated gene expression of IL-6 by glabridin after UV treatment. All data are presented as average value ± SD of triplicates ($^{\#}$ P < 0.05, $^{\#\#}$ P < 0.01, $^{\#\#\#}$ P < 0.001, $^{\#\#\#\#}$ P < 0.0001 vs. the second column).

4. Discussion

Sensitive skin is a complex pathological condition driven by dysregulated neurovascular signaling, immune-inflammatory cascades, and impaired epidermal barrier function [10]. Transient receptor potential vanilloid 1 (TRPV1) is a multimodal ion channel that plays a central role in mediating skin hypersensitivity, inflammation, and pain [3]. Current treatment strategies for SS are still limited and rely mostly on synthetic anti-inflammatory drugs, but their potential side effects cannot be ignored [11].

This study fills a key gap by revealing the multidimensional efficacy of glabridin in the management of sensitive skin. The experimental results show that glabridin can not only inhibit the UV-triggered inflammatory pathway, but also directly inhibit TRPV1 activation, thus providing dual therapeutic functions. Activation of TRPV1 will induce influx of calcium ions, which will cause the over expression of a series of proinflammatory factors. The accumulation of these factors will lead to an amplification of activation of TRPV1. Herein with the activation of TRPV1, a self-amplification system is initiated, which will lead to the inflammation of sensitive skin. Glabridin on one hand inhibits the activation of TRPV1 by directly binding to it; one another hand, down-regulates the gene expression of proinflammatory cytokines. The two mechanisms jointly alleviate the inflammation of skin (Figure 5).This finding makes glabridin an ideal candidate molecule to simultaneously address environmental (such as UV) and neurogenic (such as TRPV1 activation) triggers of SS.

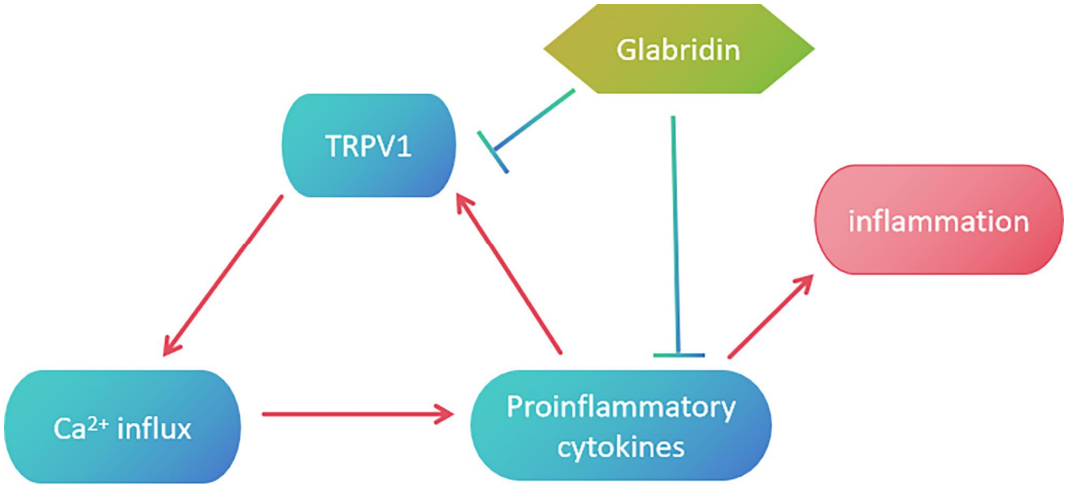

Figure 5. Schematic diagram of the anti-inflammatory and soothing mechanism of glabridin.

Although this study highlights the soothing potential of glabridin in cosmetics, its limitations still need to be addressed. Data obtained from *In vitro* experiments cannot fully simulate the in vivo complexity of sensitive skin. Future studies focused on the bioavailability, long-term safety and synergistic effects of glabridin with other bioactive ingredients are still needed.

5. Conclusion

In this study, we for the first time reported that glabridin can inhibit TRPV1 activation, a novel mechanism for its anti-irritation function. Glabridin significantly reduced capsaicin-induced calcium influx and inhibited TRPV1 activation. Moreover, glabridin significantly down-regulated inflammatory factor production in HaCaT, such as IL-1α, IL-6, IL-8 and TNF-α. Our study demonstrates that glabridin is a potent TRPV1 antagonist, exhibiting substantial potential for application in skincare for sensitive skin.

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