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Baicalein and *Salvia officinalis* Extract Upregulate Transglutaminase 1 mRNA Expression via the Activation of Transient Receptor Potential Channel V4

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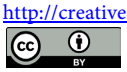

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Abstract

Background: It is important to maintain skin homeostasis for cosmetic and medical reasons. Many ceramide-related ingredients and cosmetics have been developed to improve the skin barrier function and skin hydration. Similar to extracellular lipids, the cornified envelope, which is a structure formed beneath the plasma membrane, contributes to the skin barrier function as a scaffold for extracellular lipids. Therefore, in this study, we focused on transglutaminase 1 (TGM1) which is the key enzyme for formation of the cornified envelope Objective: The objectives of this study were to identify compounds that could upregulate the expression of TGM1 and evaluate their underlying action mechanisms. Methods: Expression of the transient receptor potential channel vanilloid subfamily member 4 (TRPV4) at the mRNA and protein levels was estimated by PCR and western blotting. Effects of baicalein and *Salvia officinalis* (SO) extract on TGM1 mRNA expression were measured by PCR. The involvement of TRPV4 in TGM1 mRNA expression was evaluated by the inhibition and silencing of TRPV4. Results: TRPV4 was expressed in both basal cell-like HaCaT cells and suprabasal cell-like HaCaT cells. Baicalein and SO extract upregulated TGM1 mRNA expression in basal cell-like HaCaT cells. However, inhibition and silencing of TRPV4 abrogated the effects of baicalein and SO extract. Conclusion: Baicalein and SO extract upregulated the expression of TGM1 mRNA via the activation of TRPV4, suggesting that it may improve the skin barrier function by enhancing cornified envelope formation.

Keywords

Transient Receptor Potential Channels, Transglutaminase 1, *Salvia officinalis*,

Skin Homeostasis

1. Introduction

The 2021 Nobel Prize in Physiology or Medicine was awarded to Dr. Julius and Dr. Patapoutian for their discovery of receptors for temperature and touch. Julius *et al.* identified that the receptors belonging to the transient receptor potential (TRP) channels, are activated not only by heat but also by certain compounds [1] [2]. Subsequent studies demonstrated that TRP vanilloid subfamily members (TRPV1, TRPV3 and TRPV4) and melastatin subfamily member 8 (TRPM8) are expressed in skin [3] [4] and respond to temperatures above 43˚C [5], 36˚C - 38˚C [6], 32˚C - 39˚C [7] and 21˚C - 26˚C [8], respectively. Previously, Denda *et al.* reported that maintaining the skin surface temperature in the range of 36˚C - 40˚C and application of a TRPV4 activator accelerated the epidermal permeability barrier recovery, suggesting that TRPV4 plays a crucial role in the maintenance of skin homeostasis [9]. The cornified envelope, which is a structure formed beneath the plasma membrane and consists of a 10 nm thick layer of insoluble proteins cross-linked by transglutaminases (TGM), provides the firm scaffold to extracellular lipids in the stratum corneum [10]. Four TGM family members, TGM1, TGM2, TGM3, and TGM5, are expressed in the epidermis and catalyze the formation of isopeptide bonds to construct a cornified envelope [11]. A previous study demonstrated that the expression of TGM1 was enhanced by detergent-induced barrier disruption, suggesting that the enzyme is important for maintaining the physical barrier in the epidermis [12]. Another study showed that the application of retinol improved photo-aged skin conditions by upregulating TGM1 expression [13]. These results suggest that the upregulation of TGM1 expression can be a target to improve skin conditions. Therefore, in this study, we evaluated the effect of baicalein and SO extract on TGM1 expression. Baicalein and SO extract upregulated TGM1 expression in basal keratinocyte-like HaCaT cells, suggesting that it may improve the skin barrier function by enhancing cornified envelope formation.

2. Material and Methods

2.1. Reagents

Baicalein was purchased from Sigma-Aldrich (St. Louis, MO, USA). HC-067047 was obtained from Selleck Chemicals (Houston, TX, USA). SO extract was provided by Maruzen Pharmaceuticals (Onomichi, Japan).

2.2. Cell Culture

To maintain HaCaT cells at a distinct stage of differentiation, the cells were cultured according to a previously reported method [14] [15]. Calcium in fetal bovine serum (FBS) was depleted by incubation with Chelex 100 resin (Bio-

Rad, Hercules, CA, USA) for 1 h at 4˚C. The resin was removed with a 0.22 μm filter. HaCaT cells were maintained in a Ca^{2+}-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine. 1 mM sodium pyruvate, 5% Ca^{2+}-depleted FBS, and 0.05 (LC) or 2.0 (HC) mM calcium chloride ($CaCl_2$).

2.3. Detection of TRPV4 Expression

LC- and HC-HaCaT cells were seeded into 6-well plates at a density of 3×10^5 cells/well and maintained in a 5% CO_2-humidified atmosphere at 37˚C until 80% confluence was reached. The cells were collected in RIPA buffer supplemented with protease inhibitors and a phosphatase inhibitor. Equal amounts of protein (10 μg) were loaded, resolved via SDS-PAGE and transferred to PVDF membrane, followed by immunoblotting with an anti-TRPV4 antibody (Thermo Fisher Scientific, Waltham, MA, USA). Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore, Bedford, MA, USA).

2.4. Treatment with Baicalein or SO Extract in the Absence or the Presence of HC-067047

LC-HaCaT cells were seeded into 24-well plates at a cell density of 1×10^5 cells/well and maintained in a 5% CO_2-humidified atmosphere at 37˚C. After cultivation for 24 h, the cells were treated with 100 μM baicalein or 2% SO extract in the absence or presence of 10 μM HC-067047, for further 24 hr.

2.5. Small Interfering RNA (siRNA) Transfection

LC-HaCaT cells were reverse-transfected with predesigned TRPV4 siRNA (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. Cells were seeded into 24-well plates at a cell density of 1×10^5 cells/well, and incubated in a humidified atmosphere of 5% CO_2 at 37˚C for 24 h, followed by treatment with baicalein or SO extract for 24 h. The harvested cells were then subjected to qPCR.

2.6. Real-Time PCR

Total RNA was extracted from LC-HaCaT cells with SV RNA isolation kit (Promega, Madison, WI, USA), according to the manufacturer's instruction and then reverse transcribed (37˚C 15 min, 95˚C 5 min) with ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan). PCR amplification and detection were performed on a CFX96 Touch (BioRad, Hercules, CA, USA) using the initial denaturation conditions of 95˚C for 3 min, followed by 40 cycles at 94˚C for 5 s, 60˚C for 30 s each with primers as described in Table 1. The expression of target mRNA was quantified using the comparative threshold cycle (Ct) method for relative quantification ($2^{-\Delta\Delta Ct}$), normalized to the geometric mean of the reference gene β-actin.

Table 1. Primer sequence.

ACTB	forward	GATGAGATTGGCATGGCTTT
	reverse	CACCTTCACCGTTCCAGTTT
TRPV4	forward	CCCCATCCTCAAAGTCTTCA
	reverse	ATGGCTCTCGAAACTCCTCA

3. Results

3.1. Difference in the Differentiation Stage between LC- and HC-HaCaT Cells

Morphological changes in LC-HaCaT and HC-HaCaT cells were observed. LC-HaCaT cells were less compact and spindle-shape with the absence of cell-to-cell tight junctions (**Figure 1(a)**). On the other hand, HC-HaCaT cells showed a more spread-out squamous shape with tight junctions among the cells (**Figure 1(b)**). The expression levels of integrin α6 (ITGA6) and integrin β1 (ITGB1) were downregulated in HC-HaCaT cells compared with that in LC-HaCaT cells, whereas the expression level of involucrin (IVL) was upregulated (**Figures 1(c)-1(e)**).

3.2. TRPV4 Expression in LC- and HC-HaCaT

To compare the expression levels of TRPV4 in LC- and HC-HaCaT cells, qPCR and western blotting were performed. The expression levels of TRPV4 mRNA in HC-HaCaT cells were significantly downregulated, compared to those in LC-HaCaT cells (**Figure 2(a)**). Similar to mRNA expression, the protein expression levels of TRPV4 in HC-HaCaT cells were lower than those in LC-HaCaT cells (**Figure 2(b)**).

3.3. Upregulation of TGM1 Expression by Baicalein and SO Extract

To evaluate the effects of baicalein and SO extract on TGM1 mRNA expression, the levels of TGM1 mRNA expression in baicalein- and SO extract-treated LC-HaCaT cells were measured. Baicalein (100 μM) significantly upregulated TGM1 mRNA expression to 1.48 ± 0.29 fold of the expression in untreated LC-HaCaT cells (**Figure 2(a)**). The SO extract (2%) also significantly enhanced the expression level to 1.60 ± 0.20 fold of the expression in untreated LC-HaCaT cells (**Figure 2(b)**).

3.4. TRPV4 Is Involved in Baicalein- and SO Extract-Induced Upregulation of TGM1

To evaluate the involvement of TRPV4 in baicalein- and SO extract-enhanced TGM1 expression, TRPV4 inhibition and TRPV4 knockdown experiments were performed. The addition of HC-067047, aTRPV4 antagonist, abolished baicalein- and SO extract-enhanced TGM1 expression (**Figure 4**). While TRPV4 expression was suppressed to 0.47 ± 0.05 by TRPV4 knockdown (**Figure 5(a)**),

both baicalein-enhanced and SO extract-enhanced TGM1 expression were significantly reduced to the level of the control (Figure 5(b)).

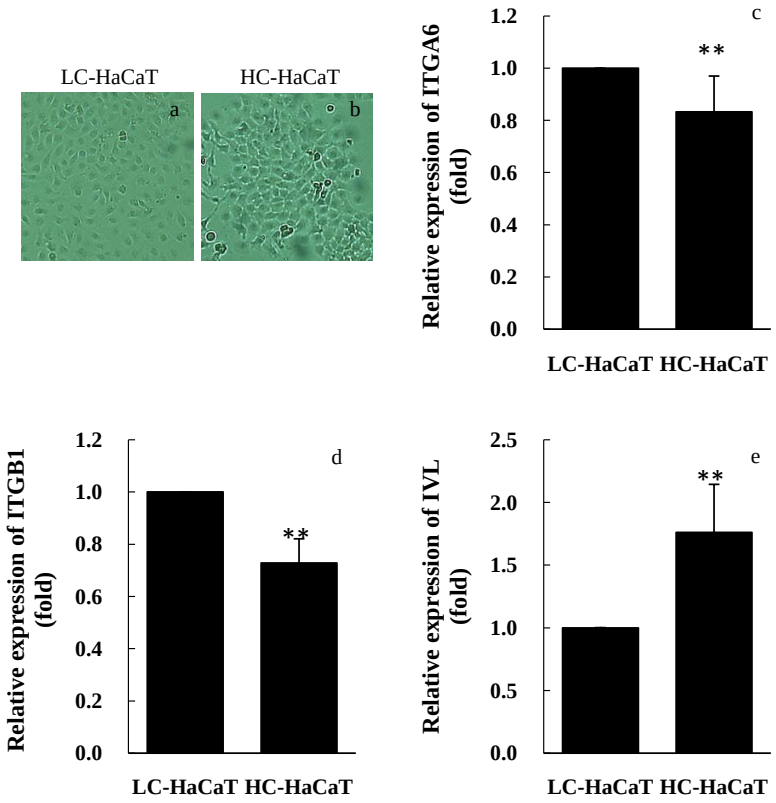

Figure 1. The difference in morphology and differentiation marker expression in LC- and HC-HaCaT cells. (a) LC-HaCaT cells showed less compacted and spindle shape. (b) HC-HaCaT cells showed a more spread-out squamous shape with tight junction. (c) ITGA6 expression was significantly reduced in HC-HaCaT. (d) ITGB1 expression was significantly reduced in HC-HaCaT. (e) IVL expression was upregulated in HC-HaCaT.

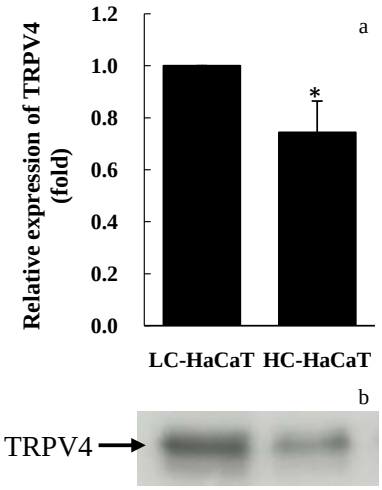

Figure 2. The TRPV4 expression. (a) The expression of TRPV4 mRNA in HC-HaCaT cells was significantly downregulated. (b) The protein levels of TRPV4 is also reduced in HC-HaCaT.

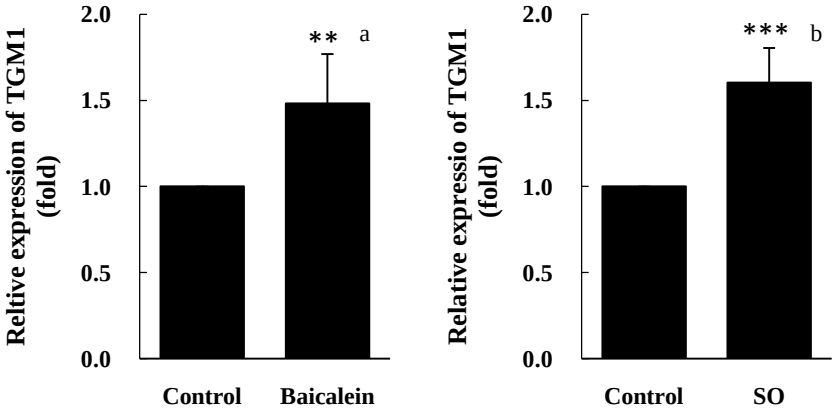

Figure 3. Baicalein and SO extract enhanced TGM1 mRNA expression. (a) The treatment with baicalein significantly enhanced TGM1 mRNA expression. (b) The treatment with SO extract significantly enhanced TGM1 mRNA expression as well.

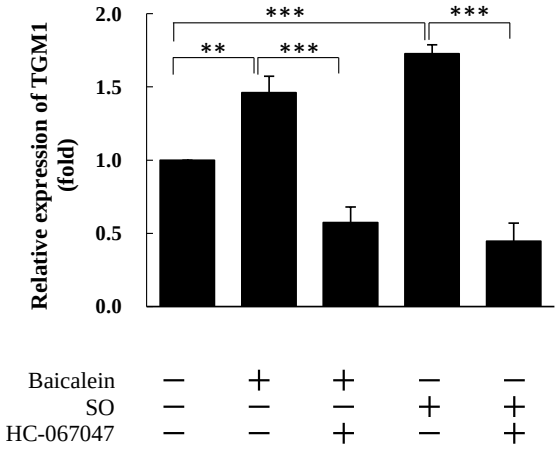

Figure 4. Effect of TRPV4 inhibition on TGM1 expression. The inhibition of TRPV4 by HC-067047 abrogated baicalein- and SO extract-enhanced TGM1 mRNA expression.

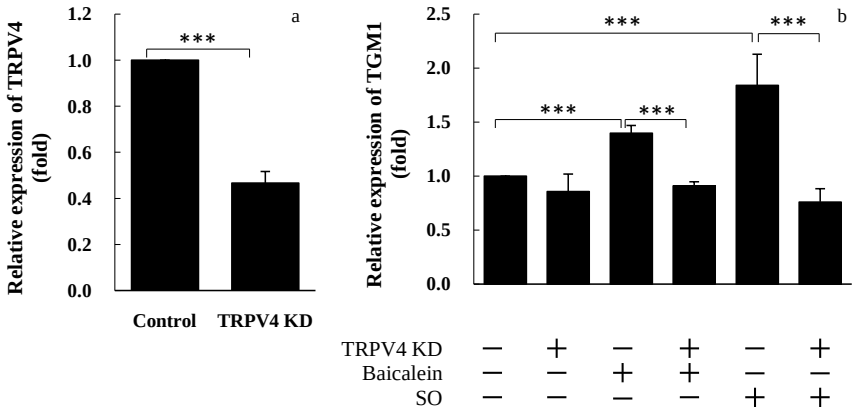

Figure 5. Effect of TRPV4 silencing of TGM1 expression. (a) TRPV4 expression was significantly reduced in LC-HaCaT transfected predesigned siRNA. (b) Baicalein- and SO extract-enhanced TGM1 expression was significantly demished by silencing of TRPV4.

4. Discussion

TRPV4, a member of the TRP channel, is broadly expressed and evoked by moderately high temperature (32˚C - 39˚C) and chemicals. Previous studies have shown that TRPV4 is expressed in the basal and suprabasal keratinocytes of the skin [16] [17] [18]. We confirmed that TRPV4 is expressed in both LC-HaCaT cells, supposed to be basal-like keratinocytes, and HC-HaCaT cells, supposed to be suprabasal-like keratinocytes (**Figure 1**). Moreover, we showed that the expression levels of TRPV4 in HC-HaCaT cells were lower than those in LC-HaCaT, suggesting that TRPV4 expression is suppressed along with keratinocyte differentiation. As previously reported, TRPV4 antagonism has therapeutic potential in edema, pain, gastrointestinal disorders, and lung diseases [19]. In contrast, TRPV4 agonism is expected to maintain epidermal permeability barrier function, while TRPV1 activation delays the recovery of the epidermal barrier function [9]. Two barrier layers are present in the epidermis. The tight junction, which is the intercellular junction in the upper layer of the stratum granulosum, serves as a barrier to prevent extensive transepidermal water loss. Previous studies have demonstrated that the activation of TRPV4 by high temperature or chemicals accelerates the tight junction formation [20] [21] [22]. Another layer in the stratum corneum is the extracellular lipid lamellar layer, which contributes to the permeability barrier function and skin hydration. The cornified envelope, which consists of insoluble proteins cross-linked by TGM, is considered to be important to provide the firm scaffold maintaining the structure of the extracellular lipid layer [10]. Thus we searched for compounds that upregulate the expression of TGM1 which is a key enzyme in the development of the cornified envelope. We demonstrated here that baicalein and SO extract enhanced the mRNA expression of TGM1 (**Figure 3**) and that the inhibition and silencing of TRPV4 diminished the upregulation of TGM1 mRNA (**Figure 4** and **Figure 5(b)**). These results suggest that TRPV4 is involved in the upregulation of TGM1 mRNA. Studies have previously reported that baicalein increased keratin 1 and 10 expression levels in HaCaT cells via TRPV4 activation, followed by the phosphorylation of the extracellular signal-regulated kinase (ERK) [23] and that the UVB-stimulated expression of TGM1 is mediated predominantly via the nuclear factor (NF)-κB pathway [24]. Accordingly, the ERK/NF κB signaling pathway may be the underlying mechanism associated with the baicalein- and SO extract-induced upregulation of TGM1 mRNA expression. Collectively, these results suggest that baicalein and SO extract may improve the skin barrier function by enhancing the cornified envelope formation via TRPV4 activation, however, their underlying action mechanisms should be explored further in future studies.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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