J. Soc. Cosmet. Sci. Korea
Vol. 46, No. 1, March 2020, 73-80

pISSN 1226-2587
eISSN 2288-9507
http://dx.doi.org/10.15230/SCSK.2020.46.1.73

밀착연접 조절을 통한 스트레스 호르몬 코티졸의 피부장벽 손상 연구

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(2020년 3월 12일 접수, 2020년 3월 27일 수정, 2020년 3월 30일 채택)

Stress Hormone Cortisol Damages the Skin Barrier by Regulating Tight Junctions

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(Received March 12, 2020; Revised March 27, 2020; Accepted March 30, 2020)

요 약: 심리적 스트레스는 피부의 생리적 상태에 영향을 미치고 다양한 피부 질환을 일으킬 수 있다. 스트레스 호르몬인 코티솔은 섬유질, 케라틴세포, 멜라노사이트와 같은 다양한 피부세포에 의해 분비된다. 밀착연접(tight junction, TJ) 은 포유류 피부의 과립층에서 장벽을 형성하는 세포 접합부위이다. TJ은 다른 피부 장벽기능에도 영향을 미칠 수 있으며 화학, 미생물 또는 면역학적 피부장벽에게 영향을 받는다. 스트레스로 인한 피부 장벽 손상에 관한 보고는 있지만 사람피부에서 코티솔이 TJ을 조절한다는 보고는 없다. 스트레스 호르몬 코티솔이 TJ을 조절하는 기능을 확인하기 위해 각질형성세포에 코티솔을 처리하였다. 코티솔은 TJ 구성 성분의 유전자 발현과 구조를 조절하여 피부 장벽 기능을 손상시켰다. 또한 코티솔은 인공피부 모델에서 과립층 형성을 억제하였다. 이러한 실험결과를 통해 스트레스 호르몬 코티솔이 TJ를 조절함으로써 피부 장벽 기능에 손상을 일으키는 것을 확인할 수 있었다.

Abstract: Psychological stress can affect the physiological condition of the skin and cause various cutaneous disorders. The stress hormone cortisol is secreted by various skin cells such as fibroblasts, keratinocytes, and melanocytes. Tight junctions (TJs) are cell–cell junctions that form a barrier in the stratum granulosum of mammalian skin. TJs can also affect other skin barriers and are affected by chemical, microbial, or immunological barriers. Stress can cause damage to the skin barrier. Interestingly, to our knowledge, there has not been any research demonstrating the involvement of TJs in this process. In this study, cortisol was used to treat keratinocytes to determine its role in regulating TJs. We found that cortisol damaged skin barrier function by regulating the gene expression and structure of TJ components. Cortisol also inhibited the development of the granular layer in a skin equivalent model. These results suggest that cortisol affects the skin barrier function by the regulation of TJs.

***Keywords*:** *cortisol, tight junction, skin barrier*

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1. Introduction

Psychological stress has been reported to damage skin barrier function[1-5]. Increased glucocorticoid levels caused by stress are a major factor in barrier function damage [6, 7]. The stress hormone cortisol is secreted by various cells and is produced by keratinocytes and melanocytes in skin cells[8-10]. Human skin expresses proteins involved in the hypothalamo-pituitary-adrenal axis, including proopiomelanocortin, corticotropin-releasing hormone (CRH), corticotropin-releasing hormone receptor-1, which are the main enzymes involved in corticosteroid and glucocorticoid synthesis. These proteins comprise functional, cell type-specific regulatory loops which mimic the signaling structural class of the hypothalamo-pituitary-adrenal axis[11]. UVB and UVC irradiation also stimulate *de novo* cortisol synthesis and its activation by 11 β-hydroxysteroid dehydrogenase type 1, which converts inactive cortisone to active cortisol[12-14].

Tight junctions (TJs) have been shown to be responsible for skin barrier functions[15]. TJs are cell–cell junctions that form paired strands which seal the space between neighboring cells and control the paracellular passage of small molecules[16]. In addition to forming barriers, TJs influence and are influenced by other skin barriers such as chemical, microbiome, or immunological barriers and likely by the basement membrane[17]. Chronic stress and increased glucocorticoid hormones are known to cause intestinal barrier dysfunction[18-20]. However, TJ regulation under stress conditions or in the presence of glucocorticoid hormone in the skin is not well understood.

In the present study, we examined the effect of cortisol on TJ function in human epidermal keratinocytes and found that cortisol damaged skin barrier function by regulating the gene expression and structures of TJ components.

2. Material and Methods

2.1. Cell Culture

Human neonatal epidermal keratinocytes were purchased from Invitrogen (USA). The cells were maintained in keratinocyte growth medium (KGM Gold) with BulletKit (Lonza, Switzerland). The cells were serially passaged at 70–80% confluence, and experiments were carried out using subconfluent cells at passage two, at which time the cells were proliferating actively. Incubation was performed at 37 ℃ in a humidified atmosphere containing 5% CO_2.

2.2. Cell Cytotoxicity Assay

Cell viability was analyzed using a CCK-8 (Cell Counting Kit-8) according to the manufacturer's instructions (CK04-05, Dojindo, Japan).

2.3. Transepithelial Electrical Resistance (TEER)

Cells were plated onto 12-mm membrane culture inserts (Corning Costar, USA). The medium was changed to keratinocyte growth medium without cortisol on the next day, and the cells were cultured for an additional 3 days. The medium was changed to keratinocyte growth medium with 1.2 mM $CaCl_2$ and 1 μM cortisol. The TEER was measured using a Millicell-ERS epithelial voltohmmeter (Millicell ERS-2, Millipore, USA) as described previously[21]. All experiments were repeated at least three times in triplicate wells within individual experiments.

2.4. Paracellular Permeability Assay

Dextran permeability was measured as described previously [22]. In this assay, 1.25 mg/mL fluorescein isothiocyanate (FITC)-dextran (average molecular weight 70,000, Sigma, USA) in phosphate-buffered saline (PBS) was added to the upper wells, whereas PBS alone was added to the lower wells. Samples were collected from the lower well after 1 h. FITC-dextran levels were measured with a fluorometer (excitation, 485 nm, emission, 528 nm).

2.5. Analysis of mRNA Expression by RT–PCR

To determine the relative mRNA expression of selected genes, total RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions, and 4 μg RNA

was reverse-transcribed into cDNA using SuperScriptIII reverse transcriptase (Invitrogen). Aliquots were stored at -20 ℃. Quantitative PCR was performed using an ABI 7500 Fast Real-Time PCR System (ABI 7500, Thermo Fisher, USA) with commercially available TaqMan site-specific primers and probes (Applied Biosystems, USA). The cDNA samples were analyzed for claudin-1 (Hs00221623_m1), claudin-2 (Hs00252666_s1), claudin-3 (Hs00265816_s1), claudin-4 (Hs00976831_s1), claudin-7 (Hs00600772_m1), occludin (Hs00170162_m1), and zona occludins (ZO)-1 (Hs01551861_m1). The results were normalized to the level of RPL13A (Hs04194366_g1).

2.6. Immunofluorescence

Immunofluorescence microscopy was carried out using the following antibodies: claudin-1 (51-9000), claudin-2 (51-6100), claudin-3 (34-1700), claudin-4 (32-9400), claudin-7 (34-9100), occludin (OC-3F10), and ZO-1 (ZO1-1A12, 33-9100) from Thermo Fisher Scientific (USA). Immunostaining was performed on keratinocytes grown on glass Lab Tek II chamber slides (Nunc, Denmark). The cells were fixed with 4% formaldehyde for 20 min. After fixation, the cells were washed twice with wash buffer (0.05% Tween-20 in PBS), followed by permeabilization with 0.1% Triton X-100 for 10 min. The cells were washed again, and then exposed to blocking solution consisting of 1% bovine serum albumin in PBS for 60 min. Next, the cells were treated with antibodies in blocking solution for 2 h. After three subsequent washing steps (10 min each), each sample was treated with Alexa Fluor 488-conjugated secondary antibody and Texas Red-conjugated second antibody in blocking solution and incubated for 1 h. Microscopy was performed using an inverted Axiovert 200 Zeiss LSM 510 Meta confocal laser scanning microscope (LSM 510, Zeiss, Germany) with a water immersion objective lens.

2.7. Western Blotting

The cells were lysed with RIPA lysis buffer (Millipore) containing protease inhibitors (Millipore). The protein concentration was determined by BCA assay, and cell lysates were resolved by SDS-PAGE on 4 – 12% gradient Bis-Tris gels (Thermo Fisher Scientific, USA), transferred to nitrocellulose membranes (Thermo Fisher Scientific, USA), and probed with antibodies against claudin-1 (51-9000), claudin-2 (51-6100), claudin-3 (34-1700), claudin-4 (32-9400), claudin-7 (34-9100), occludin (OC-3F10), ZO-1 (ZO1-1A12, 33-9100) from Thermo Fisher Scientific and horseradish peroxidase-conjugated secondary anti-rabbit IgG (Santa Cruz Biotechnology, USA). GAPDH (14C10, Cell Signaling Technology, USA) was used to normalize the levels of protein expression. Western blotting electrochemiluminescence reagent (RPN 2232, GE Healthcare, UK) was used for signal development.

2.8. Production of Skin Equivalents

Skin equivalents (SEs) were prepared as previously described [23]. The dermal layer was prepared by mixing type I collagen (Advanced Biomatrix, USA) with human dermal fibroblasts (6.0×10^4cells/well)in media cocktail [DMEM, F12, $NaHCO_3$, and NaOH]. The mixture was added to each insert of 12 mm Snapwell cell culture inserts (CorningCostar) and incubated for 2 h at 37 ℃ to allow polymerization. The dermal layer was then cultured in 106 media (Cascade Biologics, USA) and allowed to contract for 7 days at 37 ℃ and 5% CO_2. Human epidermal neonatal keratinocytes (2.0×10^5 cells/well) were seeded onto the dermal layer. SEs were cultured for 1 day in EpiLife media (Thermo Fisher Scientific, USA) and 1 day in 3D culture media (CnT-PR-3D, CELLnTEC, Switzerland), after which the SEs were fed strictly from the bottom in 3D culture media with the surfaces exposed to air for 10 days to promote epidermal differentiation. SEs were fed media with or without cortisol for 4 days. For analysis, SEs were embedded in formalin for hematoxylin and eosin staining.

2.9. Statistical Analysis

Data are expressed as the mean ± standard deviation (SD), and statistical significance was determined by using the student's t-test. A p-value < 0.05 was considered as statistically significant.

3. Results

3.1. Cortisol Decreased TEER and Increased Paracellular Permeability

The keratinocytes were treated with cortisol to determine the effect of cortisol on skin barrier function. We initially investigated whether cortisol treatment affected cell viability. Keratinocytes were cultured in media containing cortisol and then cytotoxicity was evaluated. No cytotoxicity was observed when keratinocytes were treated with up to 10 μM cortisol (Figure 1A). The cells were treated with 1 μM cortisol in the following experiments. TEER was measured across confluent monolayers of cells every 24 h. The TEER values of cortisol-treated cells were lower than those of control cells (Figure 1B). FITC-dextran measurement showed that the intensity of FITC-dextran was increased in the lower well of the transwell cell cultures compared to controls (Figure 1C).

3.2. Cortisol Induced Alterations in TJ Components

To investigate whether cortisol treatment affects the regulation of TJs, we performed quantitative PCR and western blotting. As shown in Figure 2A, cortisol treatment significantly decreased the mRNA levels of TJ components such as claudin-1, claudin-2, claudin-3, claudin-4, claudin-7, occludin, and ZO-1. Furthermore, western blot analysis showed that cortisol treatment induced decreases in claudin-1, claudin-2, claudin-3, occludin, and ZO-1 but not

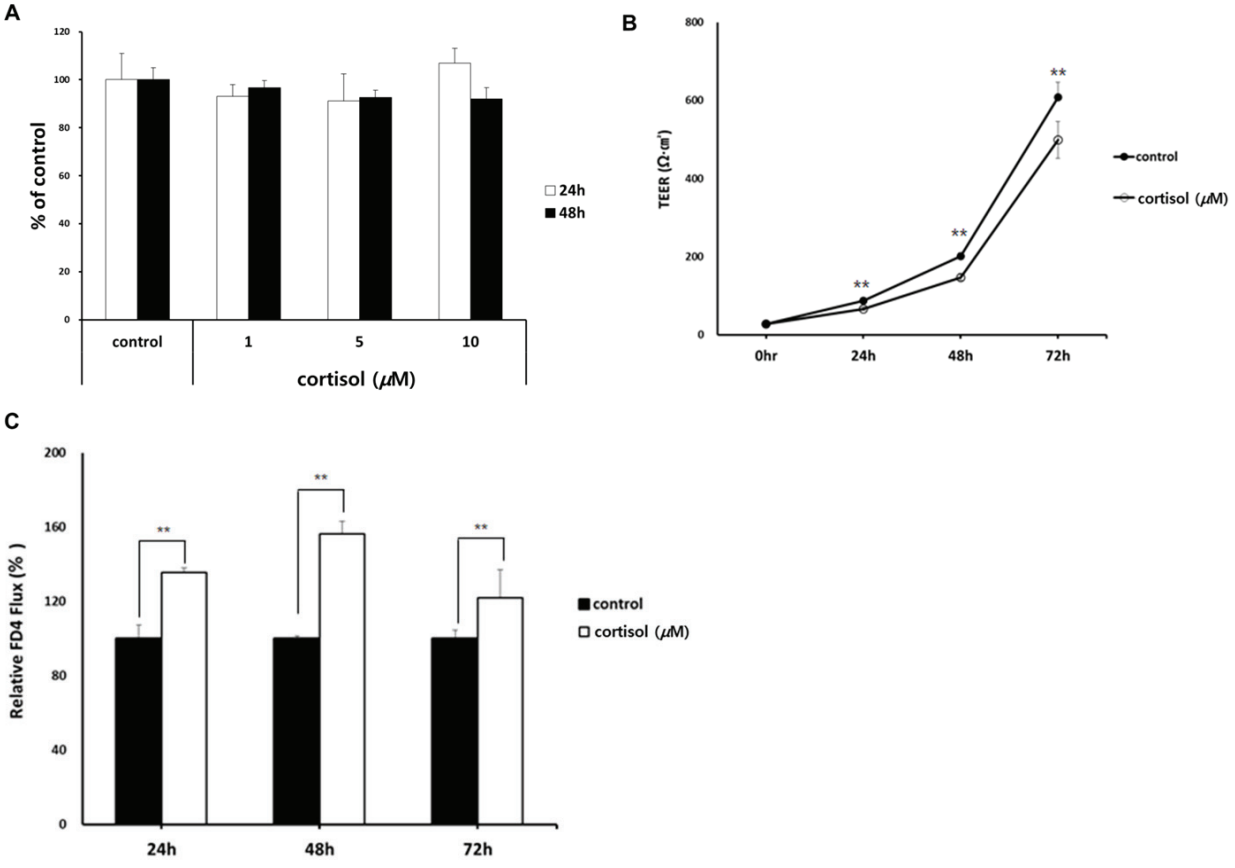

Figure 1. Induction of skin barrier dysfunction by cortisol treatment. (A) Keratinocytes were cultured in media with the indicated concentration of cortisol and then cytotoxicity was evaluated at the indicated times. (B) The TEER of the monolayers of keratinocytes was monitored over time in control, untreated monolayers (filled circle), and cortisol treated monolayers (open circle). (C) Flux of FITC-dextran was measured over time in control, untreated monolayers (filled square), and cortisol treated monolayers (open square). The TEER and flux of FITC-dextran is presented as the mean ± SD of the experiment (N = 3) ($^{**}p < 0.01$).

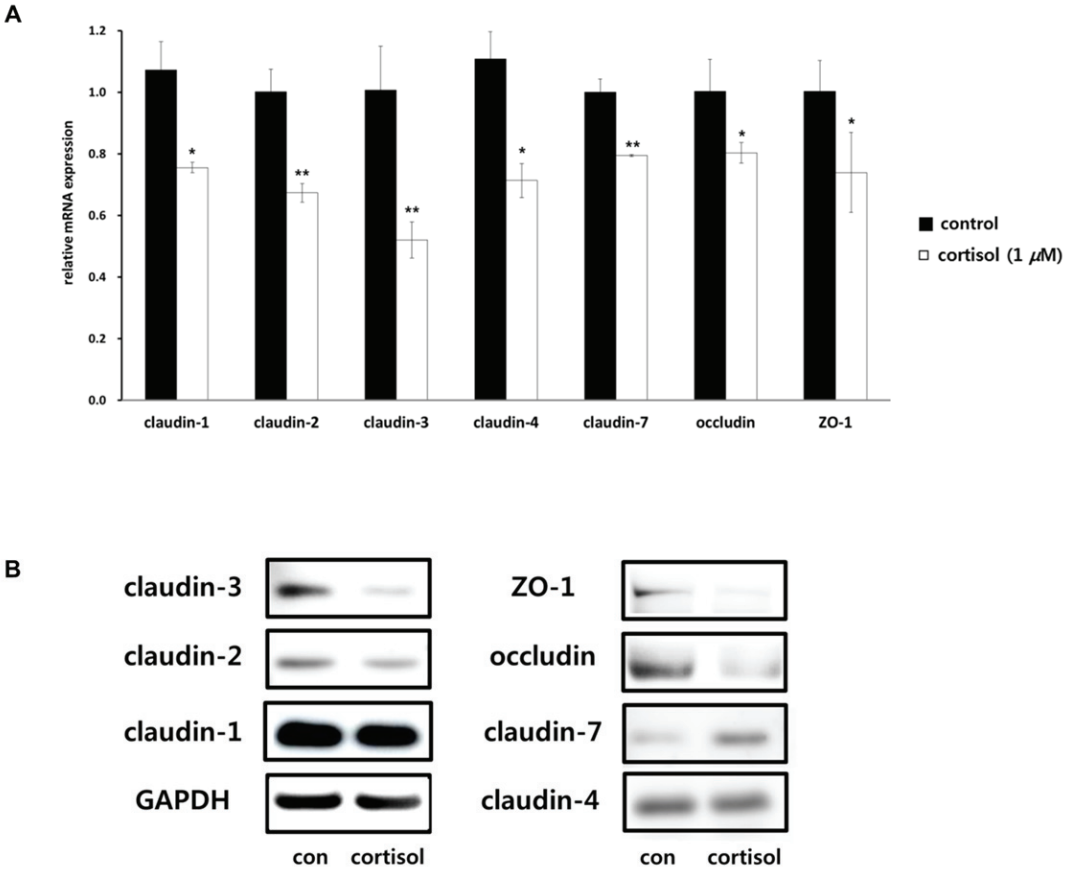

Figure 2. Alterations in epidermal tight junction by cortisol treatment. (A) Total RNA of keratinocytes was subjected to qRT-PCR to evaluate the mRNA levels of claudin-1, claudin-2, claudin-3, claudin-4, claudin-7, occludin, and ZO-1. The result is presented as the mean ± SD of the experiment (N = 3). (B) Protein expression of TJ components was measured after 1 μM cortisol treatment. ($^{*}p < 0.05$, $^{**}p < 0.01$).

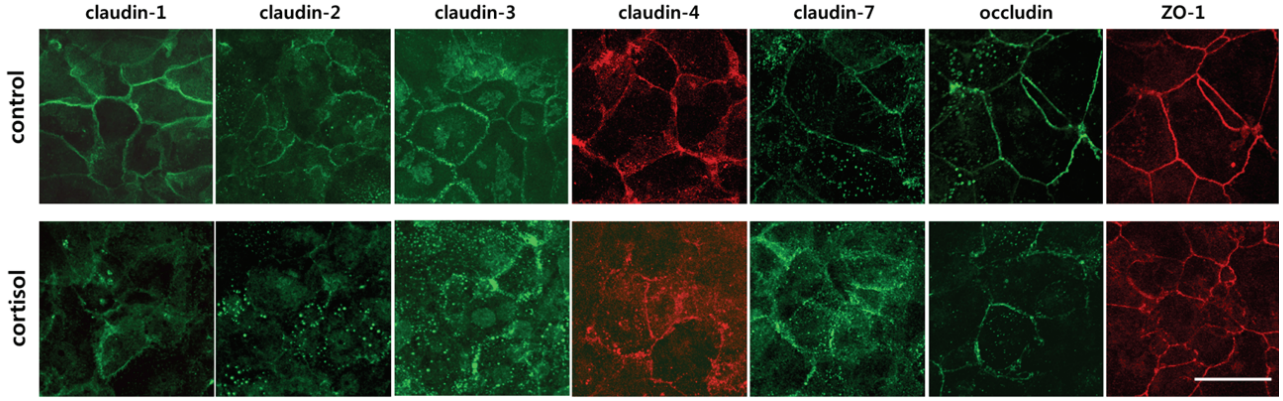

Figure 3. Alterations in the organization of epidermal tight junction proteins in normal human keratinocytes following cortisol treatment. Keratinocytes were cultured for 3 days. The medium was changed to keratinocyte growth medium containing 1.2 mM $CaCl_2$ and 1 μM cortisol and cultured for 72 h to investigate whether cortisol impairs the formation of mature TJs. Immunofluorescence microscopy was carried out to investigate the organization of TJ proteins (scalebar, 50 μm).

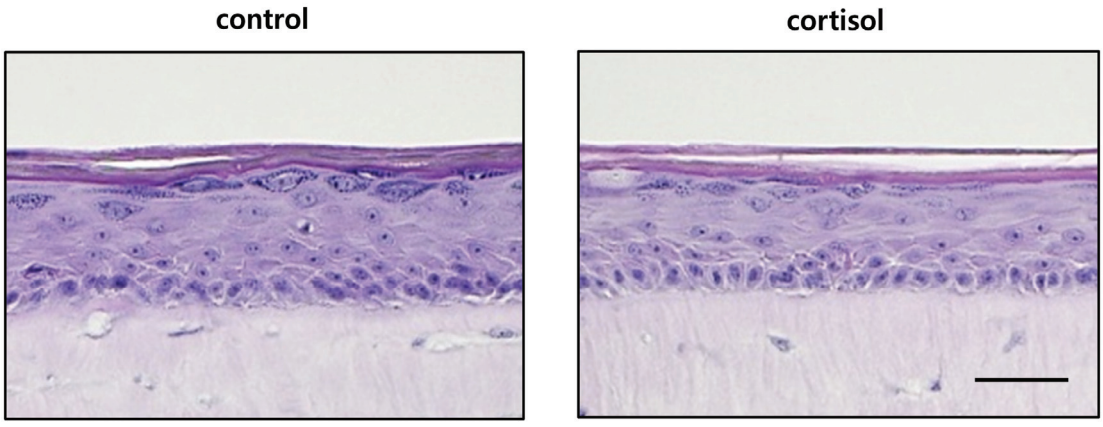

Figure 4. Effect of cortisol on the development of the skin equivalent model. After 10 days of air-liquid interface culture, SEs were fed media with or without cortisol for 4 days. Hematoxylin and eosin staining was used to analyze histological changes in the skin equivalent model following cortisol treatment (scale bar, 50 μm).

in claudin-4 and claudin-7 compared to in controls (Figure 2B). To investigate whether cortisol impairs the ability to form mature TJs, keratinocytes were treated with cortisol for 72 h after Ca^{2+}-induced differentiation. A continuous network of TJs formed along the plasma membrane in non-treated keratinocytes. In contrast, cortisol-treated keratinocytes exhibited dispersed, fragmented TJs (Figure 3).

3.3. Cortisol Disturbed the Epidermal Development of SEs

To investigate the effect of cortisol treatment on epidermal development, SEs were treated with cortisol for the last 4 days of the air-liquid interface culture period. Treatment with cortisol inhibited development of the granular layer in SEs. As shown in Figure 4, cortisol induced weak staining density of the granular layers.

4. Discussion

Psychological stress is associated with skin barrier damage [1-5]. Glucocorticoid caused by stress is known to be a major factor in functional damage to the skin barrier [6, 7]. Many studies have shown that cortisol, a glucocorticoid hormone, induces a decrease in the intestinal epithelial TJ protein levels, and disrupts the intestinal barrier permeability [18-20]. However, TJ regulation under stress conditions and in the presence of glucocorticoid hormones in the skin is not well understood. We examined the effects of cortisol on TJ function in epidermal keratinocytes and found that cortisol damaged the skin barrier function by regulating the gene expression and structure of TJ components.

Modulation of myosin light chain kinase (MLCK) is known to enhance paracellular permeability [24] and is required for TJ barrier regulation in response to Na^{+}-nutrient cotransport, inflammatory cytokines, or pathogenic bacteria [25]. Shen *et al.* reported that MLCK-dependent myosin II regulatory light chain (MLC) is sufficient to trigger TJ regulation in mature, assembled epithelial monolayers [26]. Therefore, cortisol may be involved in regulating MLCK and MLC to modulate TJs.

In conclusion, this study demonstrated that cortisol damages the skin barrier by regulating TJs in human epidermal keratinocytes. Further studies are needed to determine the underlying molecular mechanisms involved in this process.

Acknowledgments

This research was funded by the Amorepacific R&D Center.

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