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"Immediate osmotic stress triggered by skin dryness: A key factor in the persistence of dry skin"

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1. Introduction

Have you ever thought about what cosmetics were originally used for? They were originally used to prevent or treat skin dryness by providing water to the stratum corneum (SC), a process known as moisturization. Skin dryness can cause a variety of problems, both functional and cosmetic. For instance, skin dryness can lead to cracking, itching, inflammation, and fine wrinkling. We believe that an adequate water content in the SC is essential to maintain healthy and beautiful skin, as it helps to protect the skin from dryness. However, despite the importance of maintaining sufficient moisture in the SC, the mechanisms underlying the progression of skin dryness remain largely unknown and no one has clarified those to date. We believe that elucidating that mechanism will lead to the discovery of new treatments for skin dryness, in addition to the use of moisturizers.

When does skin dryness occur? In Japan, it often occurs during the winter when humidity levels are low. Simply thinking about the changes in the skin when exposed to low-humidity, lwater evaporation from the surface of the skin is enhanced, which results in a decreased water content in the SC. That water loss leads to an increased concentration of solutes in the SC, which subsequently causes hyperosmotic stress (HOS) in granular keratinocytes. In a previous study, we reported that exposure to low-humidity reduced the expression of epidermal differentiation markers, particularly tight junction (TJ)-related proteins, in a reconstructed human epidermal model (RHEM), with significant decreases observed within just a few hours [1]. Thus, we hypothesized that HOS acts as an initial trigger of skin dryness and may also function as an important developmental factor by impairing the barrier function of the skin through reductions in TJ-related proteins.

In response to HOS, it is known that cells activate stress-adaptive pathways involving the nuclear translocation of nuclear factor of activated T cells 5 (NFAT5) that regulates the expression of osmo-protective genes [2]. Therefore, in this study, we first confirmed whether HOS induces similar decreases in TJ-related proteins in RHEMs under low-humidity conditions. In

this study, we explored the molecular mechanism underlying the progression of skin dryness, with particular focus on NFAT5 behavior in response to HOS.

2. Materials and Methods

2.1. RHEMs

RHEMs (LabCyte EPI-MODEL 12D, J-TEC, Japan) were cultured in assay-specific medium (J-TEC).

2.2. Trans-Epithelial Electrical Resistance (TEER)

RHEMs were exposed to dry air or 1 M sorbitol (an inducer of HOS) [3] for 1 h. TEER values were measured using an EVOM™ system (World Precision Instruments, USA) immediately after exposure and at multiple time points up to 48 h to assess temporal changes.

2.3. Lucifer Yellow (LY) Penetration

RHEMs were placed in wells filled with 1 mL PBS without Mg^{2+} and Ca^{2+} (PBS (−)) as the reservoir solution. A 2 mM Lucifer Yellow CH dipotassium salt (FUJIFILM Wako Pure Chemical Corporation, Japan) aqueous solution was applied onto the SC of each RHEM. The fluorescence intensity of LY that penetrated through the RHEM into the reservoir solution was measured at each time point (excitation/emission: 465/535 nm) using a Spark 10M microplate reader (Tecan, Switzerland). Quantification of LY in the reservoir solution was performed using calibration curves prepared simultaneously with the measurements.

2.4. Immunostaining

Frozen thin sections of RHEMs were fixed with 4% paraformaldehyde and then blocked with 1% IgG-free bovine serum albumin (BSA). Sections were incubated with the following primary antibodies, followed by incubation with corresponding AlexaFluor™ 488-conjugated secondary antibodies (Invitrogen, USA): NFAT5 (Santa Cruz Biotechnology, USA), claudin-1 (CLDN1; Abcam, UK), ZO-1 (Proteintech, USA). After staining the nuclei with Hoechst 33342 (Invitrogen), images were obtained using a BZ-X800 fluorescence microscope (Keyence, Japan).

2.5. Western Blotting

Proteins secreted into the culture medium from RHEMs were separated by SDS-PAGE, and then transferred onto PVDF membranes (ATTO, Japan). The membranes were then incubated with an anti-TNF-α antibody (Proteintech, USA), and the bands were visualized using Ez-WestBlue (ATTO).

3. Results

3.1. Barrier Function of RHEMs Exposed to Low-humidity Air or Sorbitol

To verify whether HOS occurs in RHEMs exposed to low-humidity air, similarities in TEER values and LY penetration were evaluated between RHEMs subjected to low-humidity air and those treated with sorbitol, which is a known inducer of HOS [3]. RHEMs exposed to low-

humidity air had significantly decreased TEER values and increased LY penetration compared to the control RHEMs. Treatment with sorbitol had similar effects to low-humidity air exposure on TEER values and LY penetration (Figure 1). Interestingly, TEER values were lower immediately after exposure to low-humidity air or sorbitol, which indicates that TJs were opened by both stimuli. Moreover, despite being exposed to those stimuli only once, the changes in TEER values and LY penetration persisted for up to 48 h (Figure 1).

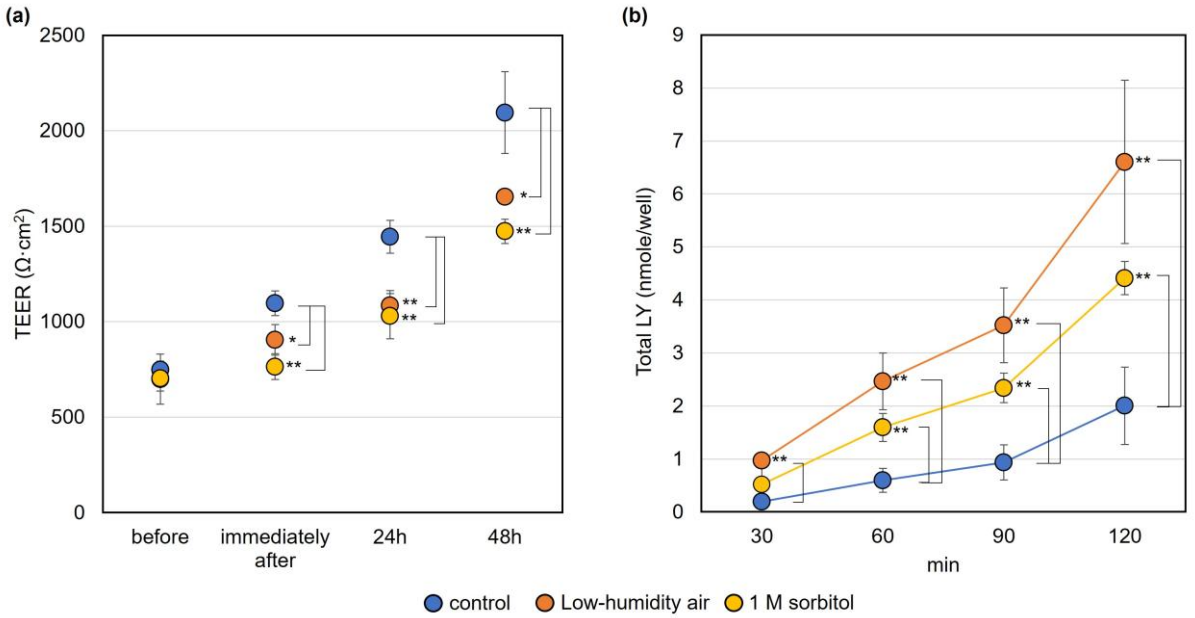

Figure 1. TEER values and permeability immediately after exposure to low-humidity air or sorbitol. RHEMs were exposed to low-humidity air or 1 M sorbitol on the SC. **(a)** Time-dependent changes of TEER values after exposure to low-humidity air or 1 M sorbitol for 1 h. **(b)** Penetration of LY applied on the SC at a concentration of 2 mM immediately after exposure to dry air or 1 M sorbitol. Significance * $p < 0.05$, ** $p < 0.01$ (Dunnett's test)

To confirm the cause of the persistent barrier dysfunction, we examined the status of TJ-related proteins in RHEMs at 24 h after exposure to low-humidity air or sorbitol. Immunostaining confirmed that the levels of CLDN1 and ZO-1 in RHEMs were decreased 24 h after exposure to low-humidity air or sorbitol (Figure 2).

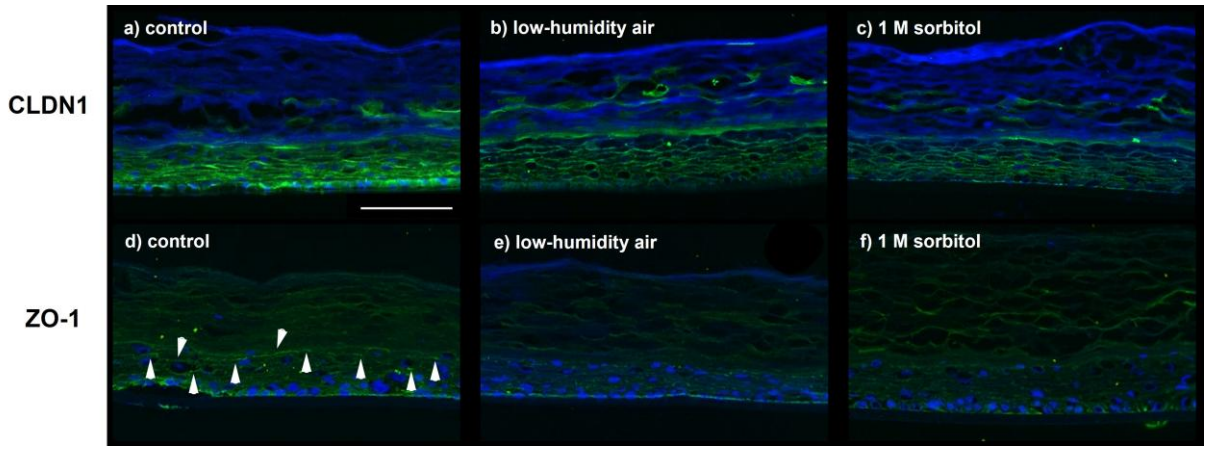

Figure 2. Expression of TJ-related proteins in RHEMs exposed to low-humidity air or sorbitol. Representative images of RHEMs exposed for 1 h to dry air or 1 M sorbitol after 24 h. **(a, b and c)** CLDN1 was visualized by immunostaining with an anti-CLDN1 antibody (1:100), **(d, e and f)** ZO-1 was visualized by immunostaining with an anti-ZO-1 antibody (1:100) and anti-rabbit IgG Alexa FluorTM 488 (1:1000) (green). Nuclei are stained with Hoechst33342. Scale bar: 100 μm.

3.2. NFAT5 in RHEMs exposed to low-humidity air or sorbitol

To further confirm the occurrence of HOS in RHEMs due to exposure to low-humidity air, we examined the expression of NFAT5 and its localization in the keratinocytes of RHEMs. As shown in Figure 3, sorbitol induced the expression and nuclear translocation of NFAT5 in RHEMs after 24 h. Exposure to low-humidity air had similar effects on NFAT5 expression and localization. Nuclear translocation of NFAT5 in RHEMs was observed within 6 h after exposure to low-humidity air or sorbitol, and it persisted for up to 48 h (data not shown). Furthermore, protein expression levels of TNF-α, which is synthesized upon activation of NFAT5 [4], were increased in RHEMs exposed to low-humidity air or sorbitol (Figure 4).

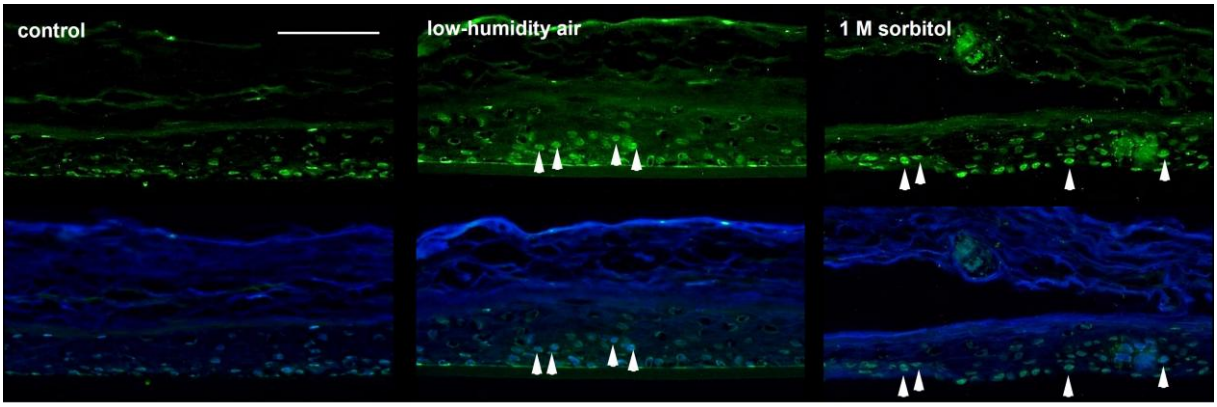

Figure 3. NFAT5 nuclear translocation induced by exposure to low-humidity air or sorbitol. Representative images of RHEMs exposed for 1 h to dry air or 1 M sorbitol after 24 h. NFAT5 was visualized by immunostaining with an anti-NFAT5 antibody (1:50) and anti-mouse IgG Alexa FluorTM 488 (1:200) (green). Translocation of NFAT5 into nuclei is indicated by the white arrows. Nuclei are stained with Hoechst33342. Scale bar: 100 μm.

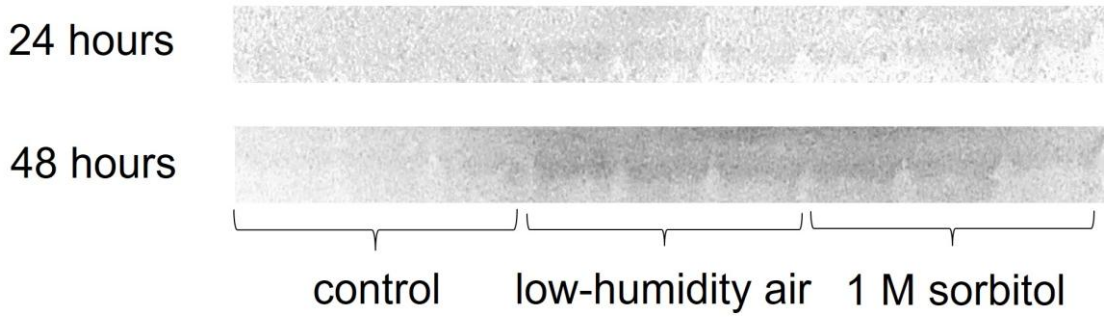

Figure 4. TNF-α secretion in RHEMs exposed to low-humidity air or sorbitol. Protein levels of TNF-α in the culture medium of RHEMs 24 or 48 h after treatment with or without low-humidity air or 1 M sorbitol for 1 h.

3.3. TJ-related proteins in RHEMs exposed to TNF-α

To explore the role of NFAT5 in the barrier dysfunction of RHEMs exposed to low-humidity air, we investigated the effect of TNF-α, which is synthesized through NFAT5 signaling, on TEER values and on the expression of CLDN1 and ZO-1 in RHEMs. TNF-α significantly reduced the TEER value of RHEMs at 24 h after treatment, and furthermore, the levels of CLDN1 and ZO-1 were decreased (Figure 5).

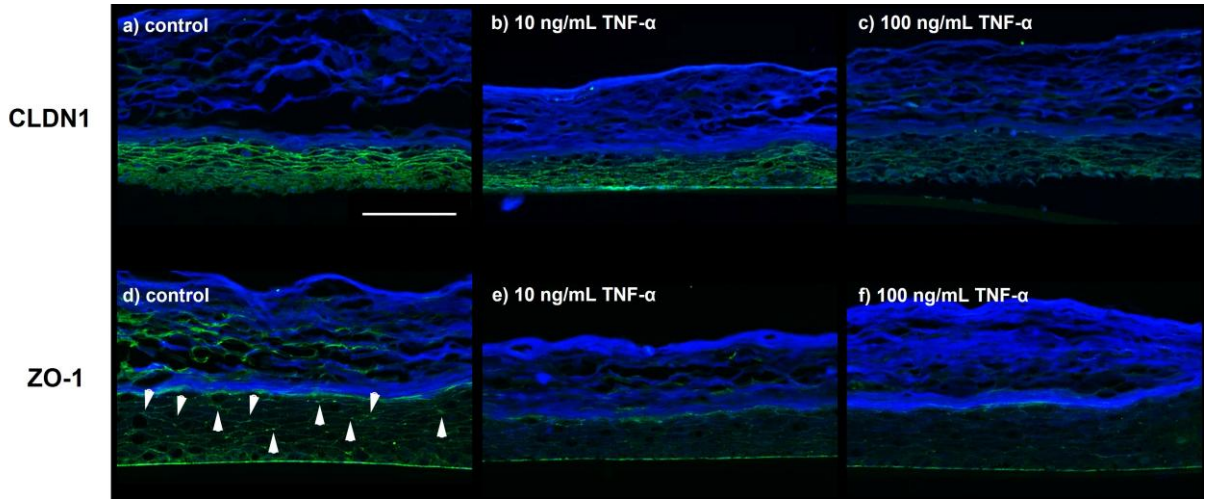

Figure 5. Expression of TJ-related proteins in RHEMs exposed to TNF-α. Representative images of RHEMs exposed TNF-α for 48 h**. (a, b and c)** CLDN1 was visualized by immunostaining with an anti-CLDN1 antibody (1:100), **(d, e and f)** ZO-1 was visualized by immunostaining with an anti-ZO-1 antibody (1:100) and anti-rabbit IgG Alexa FluorTM 488 (1:1000) (green). Nuclei are stained with Hoechst33342. Scale bar: 100 μm.

4. Discussion

This study was conducted to clarify the triggers and promotion factors of dry skin in a low-humidity environment. To determine the contributing factors, we focused on the involvement of HOS. From a physical perspective, a low-humidity environment promotes water evaporation

from the skin, particularly from the SC, which leads to the concentration of solutes within the SC resulting in a state of high osmolarity.

First, we confirmed whether HOS occurs in skin exposed to low-humidity air using RHEMs and comparing that response to RHEMs treated with sorbitol, a known inducer of HOS. As expected, the decrease in barrier function of RHEMs caused by exposure to low-humidity air was reproduced in RHEMs treated with sorbitol (Figure 1). Furthermore, the expression and localization of NFAT5, a transcription factor responsive to HOS, showed similar patterns in RHEMs exposed to low-humidity air or sorbitol (Figure 3). Taken together, these results indicate that a low-humidity environment induces HOS within the epidermis by enhancing water evaporation from the surface of the skin. Therefore, our hypothesis was validated, and the downstream events of HOS could serve as the triggering and progression factors of skin dryness.

When considering the barrier dysfunction caused by low-humidity, we should recognize that it occurs in two phases. As shown in Figure 1, TEER values in RHEMs exposed to low-humidity air or sorbitol were lower than in the control immediately after exposure. It is likely that the barrier dysfunction of the skin observed at 24 and 48 h after exposure can be explained by analyzing the quantity of barrier-related molecules. However, the dysfunction that occurs immediately after exposure cannot be attributed to quantitative changes in those molecules. In general, HOS is the shrinkage of cell size due to the withdrawal of cytosolic water [3]. Presumably, the barrier dysfunction of the skin observed immediately after exposure could be attributed to the physical shrinkage of granular keratinocytes.

The next challenge is why the barrier dysfunction persists up to 48 h after exposure. To resolve that challenge, we considered the involvement of NFAT5 signaling. NFAT5 is widely known to cooperate with NF-κB in the induction of inflammation [5]. Among the potential cytokines and growth factors that could be involved, we focused on TNF-α because it has been reported to impair TJs in vascular endothelial cells and hepatocytes [6, 7]. In fact, TNF-α suppressed the expression of CLDN1 and ZO-1 in RHEMs and impaired barrier function (Figure 4). Thus, under low-humidity conditions, the epidermis continuously suffers from HOS, and persistent HOS is considered to impair barrier function due to continuously lowered levels of TJ-related proteins.

To summarize these results, the process by which skin dryness develops in a low-humidity environment can be explained as follows:

- Step 1: HOS occurs due to the low-humidity environment, physically opening TJs.
- Step 2: NFAT5 signaling is activated by HOS, leading to the synthesis of inflammatory cytokines such as TNF-α.
- Step 3: These cytokines suppress the synthesis of TJ-related proteins.

As these steps are repeated, the skin gradually loses its barrier function, which ultimately results in skin dryness.

So, the next question is how does TNF-α suppress the synthesis of TJ-related proteins. In a previous study, we reported that exposure to low-humidity air increases the level of reactive oxygen species (ROS) in RHEMs, and that elevation could be detected immediately after

exposure [8]. On the other hand, HOS also elevates intracellular ROS by enhancing Ca^{2+} influx [9]. In fact, in a preliminary experiment, we detected an increase in cytosolic ROS following sorbitol treatment, which was associated with an increase in Ca^{2+} flux (data not shown). Furthermore, it is known that TNF-α increases intracellular ROS [10]. These facts indicate that most of the events triggered by low-humidity are associated with enhanced oxidative stress. In addition, it has been reported that ROS, specifically H_2O_2, reduce the synthesis of TJ-related proteins [11]. We also found that keratinocytes treated with buthionine sulfoximine, an inhibitor of glutathione [12], had a reduced antioxidant capacity and a lowered synthesis of CLDN1 and ZO-1 (data not shown). These findings suggested that ROS, generated by HOS and by TNF-α synthesized through NFAT5 signaling activated by HOS, are responsible for skin dryness as both a triggering and a promotion factor.

5. Conclusion

The results of this study demonstrate that skin dryness occurring under low-humidity conditions is triggered by HOS and progresses into true skin dryness due to a sustained decrease in TJ-related proteins, which is caused by NFAT5 signaling activated by repetitive HOS exposure. Furthermore, these facts suggest that intracellular ROS are responsible for the progression of skin dryness (shown schematically in Figure 6). Thus, we propose using moisturizers that have antioxidant functions to prevent skin dryness in low-humidity environments.

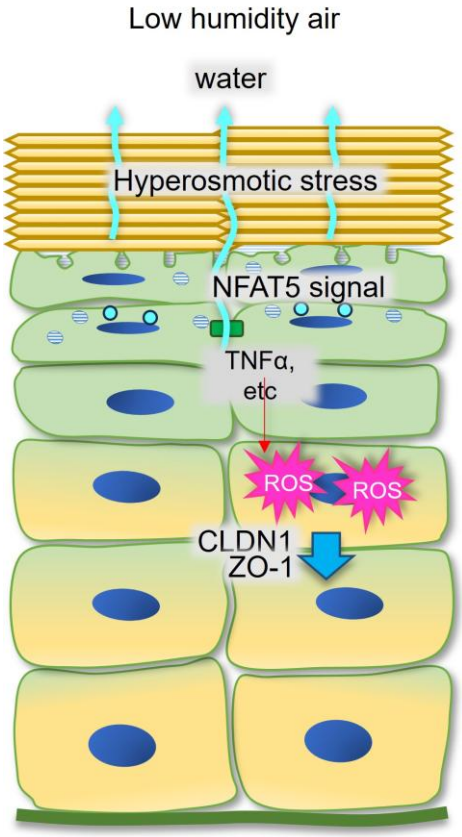

Figure 6. Mechanism of skin dryness progression via osmotic stress-induced ROS under a low-humidity environment.

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