Chronic stress weakens the skin barrier function owing to increased cortisol sensitivity through the imbalance in the expression of cortisol-metabolizing enzymes

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Abstract

Background: A temporary state of high cortisol concentration is caused by physical stress or diurnal variation. However, skin problems only occur under conditions of chronic high cortisol concentration. Thus, clarifying the effect of cortisol is essential to examining the influence of chronic stress conditions on the skin. Cortisol-metabolizing enzymes, such as 11β-hydroxysteroid dehydrogenases (11β HSDs), regulate intracellular cortisol concentrations. In this study, we focused on the relationship between cortisol and the metabolic capacity of 11β HSDs in keratinocytes and investigated the mechanism responsible for their adverse effects on the skin during chronic stress. Furthermore, we screened for natural extracts that improve chronic stress-induced skin problems.

Methods: Normal human epidermal keratinocytes (NHEKs) were treated with 20 μM cortisol once every three days or daily. The mRNA expression of 11β HSDs and tight-junction proteins (TJPs) were measured using RT-qPCR, and transepithelial electrical resistance was evaluated. The extracellular cortisol concentration when cortisone was added to NHEKs was measured using ELISA.

Results: The mRNA expression of 11β HSDs was differentially altered in NHEKs depending on the cortisol treatment method. Moreover, altered 11β HSD mRNA expression levels impaired the formation of the barrier function. Ganoderma Lucidum Stem Extract decreased the extracellular cortisol concentration in NHEKs and upregulated the mRNA expression of TJPs in NHEKs treated daily with 20 μM cortisol.

Conclusion: Chronic psychological stress may boost the influence of cortisol on the epidermis by altering 11β HSD expression levels. Moreover, GLSE may improve chronic stress-induced skin problems.

Keywords: cortisol; chronic stress; barrier function; Ganoderma Lucidum Stem Extract; 11β-hydroxysteroid dehydrogenases

Introduction.

Cortisol is a hormone released into the bloodstream during physical and psychological stress. It adversely affects the body when maintained at high levels for prolonged durations due to psychological stress. Cortisol reportedly causes skin problems, such as wrinkles [1], dry skin, and rough skin [2]. For example, in *in vitro* studies, cortisol reduces the expression levels of tight-junction protein (TJP)-encoding genes related to barrier function in epidermal keratinocytes [3].

Although a temporary state of high cortisol concentration is caused by physical stress or diurnal variation, skin problems only occur in conditions of chronic high cortisol concentration. Thus, clarifying the effect of high cortisol levels on epidermal keratinocytes is vital to examining the influence of chronic stress conditions on the skin.

Cortisol exerts various physiological effects by binding to glucocorticoid receptors on cells. The cortisol-metabolizing enzymes, 11β-hydroxysteroid dehydrogenases (11β HSDs), are present in cells and regulate intracellular cortisol concentrations. 11β HSD-1 converts inactive cortisone to active cortisol, and 11β HSD-2 converts cortisol to cortisone. Therefore, cortisol binding to glucocorticoid receptors is regulated by the cortisol metabolic balance, which depends on the expression levels of 11β HSDs. Moreover, cortisol reportedly increases the expression level of 11β HSD-1 [4], which also affects the metabolic balance. However, the relationship between stress and the ability to metabolize cortisol in the skin has not yet been completely elucidated.

In this study, we investigated the relationship between cortisol exposure and the metabolic capacity of 11β HSDs in keratinocytes and the mechanism responsible for their adverse effects on the skin under chronic stress conditions. Furthermore, we screened for natural extracts that improve skin problems caused by chronic stress.

Materials and Methods.

Plant extract

Freeze-dried Ganoderma Lucidum Stem Extract (GLSE) prepared by Maruzen Pharmaceuticals (Hiroshima, Japan), was stored in the dark at −30°C. GLSE was dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium to the appropriate concentrations, and the final DMSO concentration was adjusted to 0.1%.

Cell culture

Normal human epidermal keratinocytes (NHEKs) were purchased from Kurabo Industries (Osaka, Japan). NHEKs were cultured in serum-free keratinocyte growth medium (KGM; keratinocyte basal medium [KBM] supplemented with human epidermal growth factor, insulin, hydrocortisone [cortisol], gentamycin/amphotericin B, and bovine pituitary extract; Kurabo Industries) at 37°C and 5% CO_2. After culture, the cells were washed twice with phosphate-buffered saline (PBS), and KGM without hydrocortisone (0 μM cortisol) or KGM without hydrocortisone, but with 20 μM cortisol added. NHEKs were untreated (non-stressed cells; NSCs) or treated with 20 μM cortisol once every three days (temporarily stressed cells; TSCs) or daily (chronically stressed cells; CSCs).

Reverse transcription-quantitative polymerase chain reaction

Total RNA was isolated from NHEKs using ISOGEN II (NIPPON GENE, Toyama, Japan) according to the manufacturer's instructions, and cDNA was synthesized using PrimeScript RT Master Mix TaKaRa PCR Thermal Cycler Dice Touch (TAKARA BIO, Shiga, Japan). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using TB Green Fast qPCR Mix using a Thermal Cycler Dice Real-Time System III (TAKARA BIO) for 11β HSD-1 (*HSD11B1*), 11β HSD-2 (*HSD11B2*), and other relevant genes. The primer sets for 11β HSD-2 were synthesized as previously described [5], and the primer sets for the other genes were purchased from TAKARA BIO. 11β HSD-1, 11β HSD-2, and TJP mRNA expression levels were normalized to that of *GAPDH*.

Barrier function evaluation

NHEKs were seeded onto 12-well polyester membranes (Transwell Clear Inserts, Corning Inc, Corning, NY, USA) and cultured in the presence of 0 or 20 μM cortisol. After three days, the medium was changed to differentiation medium (KGM with 1.5 mM $CaCl_2$) conta

containing 0 or 20 μM cortisol. Changes in transepithelial electrical resistance (TEER) were monitored using a Millicell Electrical Resistance System (Millipore, Burlington, MA, USA) to evaluate the formation of the barrier function every 24 h for three days. The flux of fluorescein isothiocyanate (FITC)-conjugated dextran (4 kDa; Sigma Aldrich, St Louis, MO, USA) across the NHEK monolayers was used to determine paracellular permeability. NHEK monolayers were gently washed with Hank's balanced salt solution (HBSS) and transferred to 1 mL of HBSS in the basal chamber. The media in the apical chamber was replaced with 500 μL of 1 mg/mL FITC-dextran in HBSS. After incubation for 2 h, 100 μL of HBSS was taken from the basal chamber, and the fluorescence was determined using a fluorescent microplate reader (Varioskan Flash; Thermo Fisher Scientific, Waltham, MA, USA) with an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

Cortisone to cortisol conversion assay

NHEKs were seeded onto 24-well collagen-coated plates. After one day of incubation, the cells were washed twice with PBS, and KBM containing 1 μM cortisone was added for 24 h. The supernatants of cultured medium were then collected, and cortisol concentrations were measured using a Cortisol Parameter Assay Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Cortisol concentrations were normalized to the number of live cells. The number of live cells was measured using a cell counting assay.

Cell counting assay

After the cells were cultured, they were fixed in 10% formalin and incubated for 30 min with Hoechst 33342 (DOJINDO, Kumamoto, Japan) to stain the nuclei. Images were captured under a fluorescence microscope (Keyence, Osaka, Japan), and the number of cells was calculated from the number of nuclear staining using image analysis software (Keyence).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Comparisons between two groups were performed using a Student's two-tailed unpaired *t*-test. Comparisons between more than two groups were performed using Dunnett's or Tukey's test, and statistical significance was set at $p < 0.05$.

Results.

Cortisol affected 11β HSD-1 and 11β HSD-2 mRNA expression levels.

11β HSD-1 mRNA expression levels were not different between compared TSCs and NSCs, whereas 11β HSD-2 mRNA expression levels clearly increased after 72 h of cortisol treatment (Fig. 1A). In contrast, 11β HSD-1 mRNA expression levels increased in CSCs compared to that in NSCs, whereas 11β HSD-2 mRNA expression levels did not (Fig. 1B).

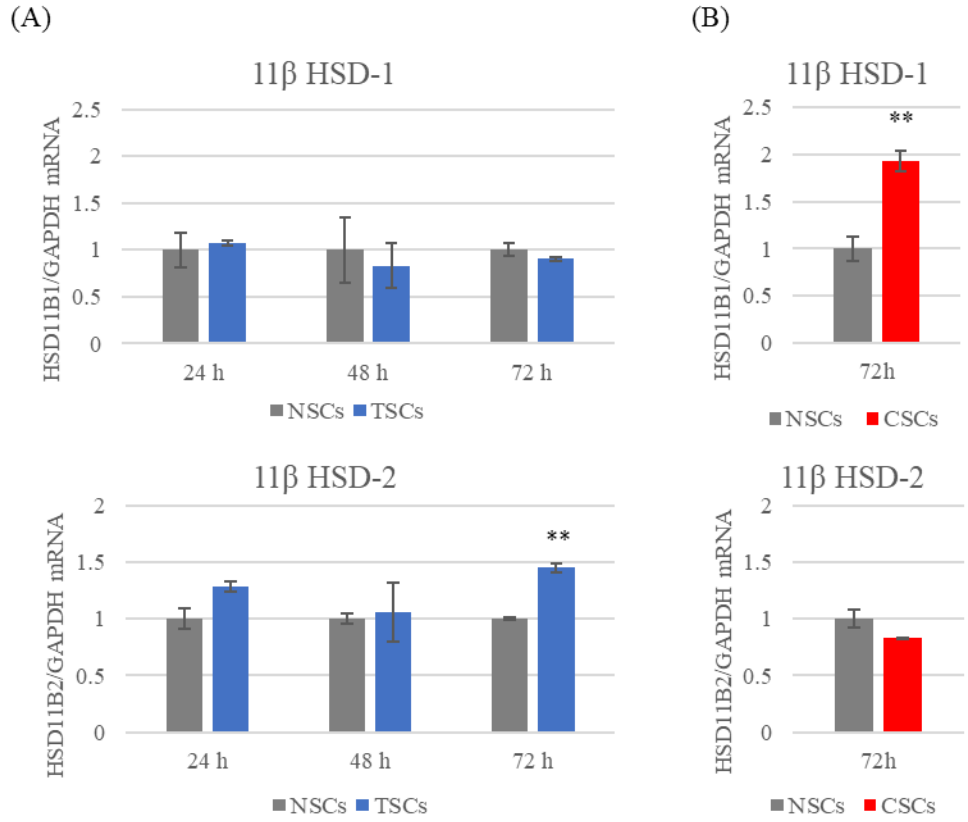

Fig. 1 Effect of cortisol on 11β HSD-1 and 11β HSD-2 mRNA expression levels in NHEKs under different cortisol treatment conditions

NHEKs in KGM were seeded onto 24-well plates. After one day of incubation, the cells were washed twice with PBS then treated with 0 or 20 µM cortisol. Cortisol treatment was applied once or every 24 h. The cells were incubated for 24–72 h. After each hour of incubation, 11β HSD-1 and 11β HSD-2 mRNA expression levels were analyzed by RT-qPCR. Data are expressed relative to the expression levels in untreated NHEKs (NSCs). (A) NHEKs treated with 20 µM cortisol once (TSCs) were cultured for 24, 48, or 72 h. (B) NHEKs treated with 20 µM cortisol every 24 h (CSCs) were cultured for 72 h. Each bar indicates the mean ± SEM, n = 3, ** $p < 0.01$, using a Student's *t*-test.

Cortisol treatment before inducing differentiation reduced the mRNA expression of TJPs.

The mRNA expression levels of claudin 4 (*CLDN4*), claudin 1 (*CLDN1*), and occludin (*OCLN*) were suppressed in NSCs cultured with 20 μM cortisol during differentiation (Fig. 2A). When differentiation was induced under the presence of 20 μM cortisol, the expression levels of *CLDN4*, *CLDN1*, and *OCLN* were substantial lower in TSCs and CSCs than in NSCs, and significantly lower in CSCs than in TSCs (Fig. 2B).

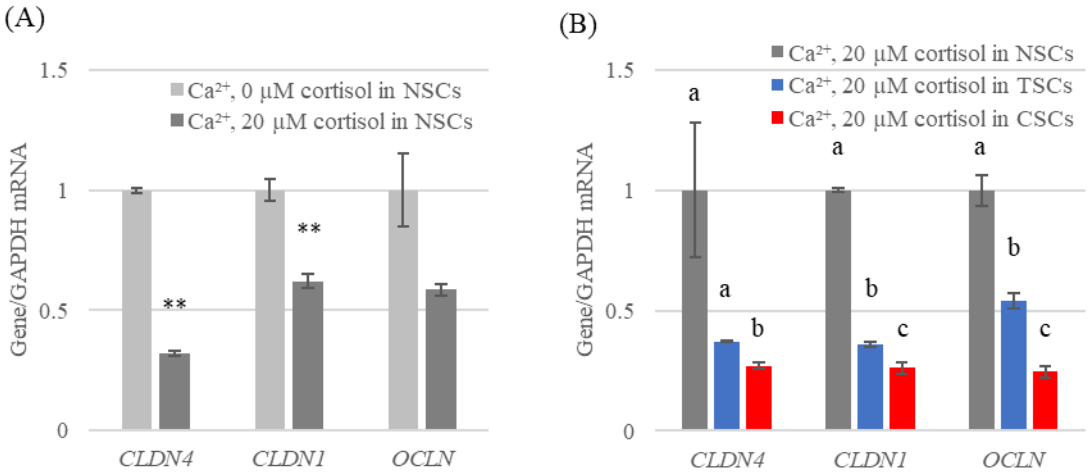

Fig. 2 Comparison of TJP mRNA expression levels under different cortisol treatment conditions

NHEKs were seeded onto 24-well plates in KGM. After one day of incubation, the cells were washed twice with PBS and then treated with 0 or 20 μM cortisol. Cortisol treatment was applied once or every 24 h. After three days, the medium was changed to differentiation medium with 0 or 20 μM cortisol. The mRNA expression levels of TJPs were analyzed by RT-qPCR 72 h after differentiation induction. (A) NSCs were induced to differentiate with the absence (Ca^{2+}, 0 μM cortisol) or presence of cortisol (Ca^{2+}, 20 μM cortisol). The data are expressed relative to the expression levels in NSCs treated with Ca^{2+} and 0 μM cortisol. Each bar indicates the mean ± SEM, n = 3, ** $p < 0.01$, using the Student's t-test. (B) TSCs and CSCs were induced to differentiate with 20 μM cortisol (Ca^{2+}, 20 μM cortisol). The data are expressed relative to the expression levels in NSCs treated with Ca^{2+} and 20 μM cortisol. Each bar indicates the mean ± SEM, n = 3; different letters (a, b, or c) indicate significant differences ($p < 0.05$) using Tukey's test.

Cortisol treatment before inducing differentiation affected the mRNA expression of TJPs.

Compared to the results in untreated NSCs, treatment with 20 μM cortisol during the differentiation of NSCs diminished the increase in TEER and increased flux through the FITC-dextran monolayer (Fig. 3). NSCs and TSCs treated with 20 μM cortisol during

differentiation showed no differences, except for an increase in TEER in TSCs at 72 h (Fig. 3). However, a comparison of TSCs and CSCs treated with 20 μM cortisol during differentiation suggested that the increase in TEER was diminished for 24–72 h, and FITC-dextran permeation was significantly increased in CSCs (Fig. 3).

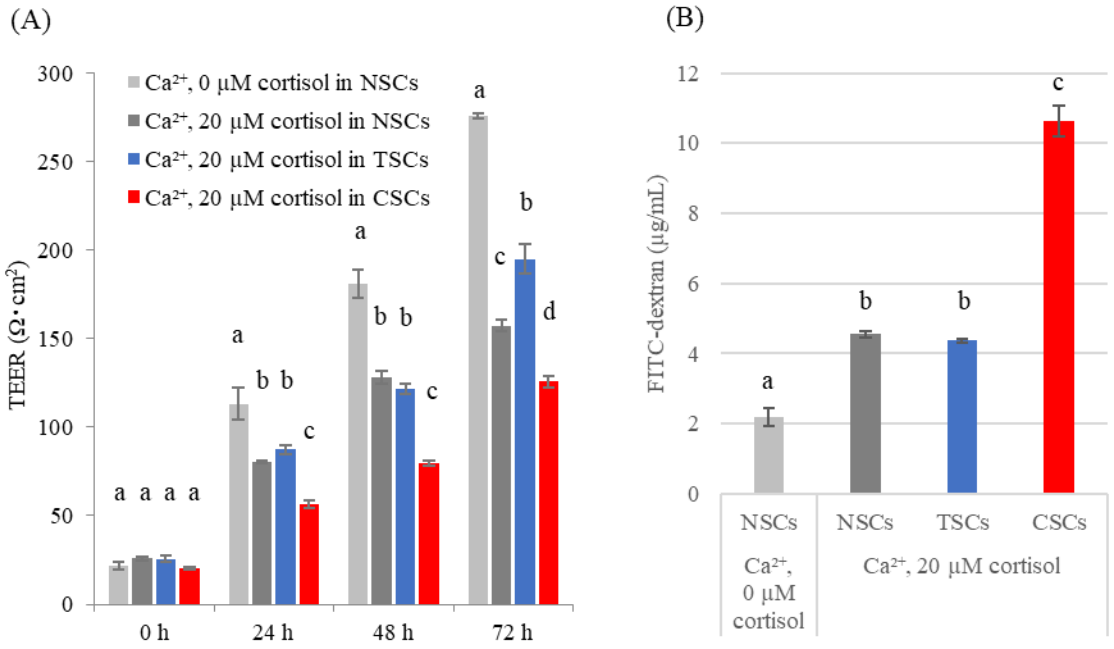

Fig. 3 Evaluation of barrier function under different cortisol treatment conditions

Differentiation was induced by 1.5 mM $CaCl_2$ with 0 or 20 μM cortisol in NSCs, TSCs, and CSCs. (A) TEER was measured up to 72 h. (B) The concentration of FITC-dextran in basal chamber media was determined at 72 h. Each bar indicates the mean ± SEM, n = 3; different letters (a, b, c, or d) indicate significant differences ($p < 0.05$) at each given time point using Tukey's test.

<u>Effect of cortisol during differentiation induction was enhanced by a 11β HSD-2 inhibitor in TSCs.</u>

A 11β HSD-1 inhibitor (PF915275; PF) [6] did not increase the mRNA expression levels of TJPs in CSCs differentiated in the presence of cortisol (Fig. 4A). In contrast, a 11β HSD-2 inhibitor (Dibutyl phthalate; DBP) [7] decreased the mRNA expression levels of TJPs in TSCs differentiated in the presence of cortisol (Fig. 4B).

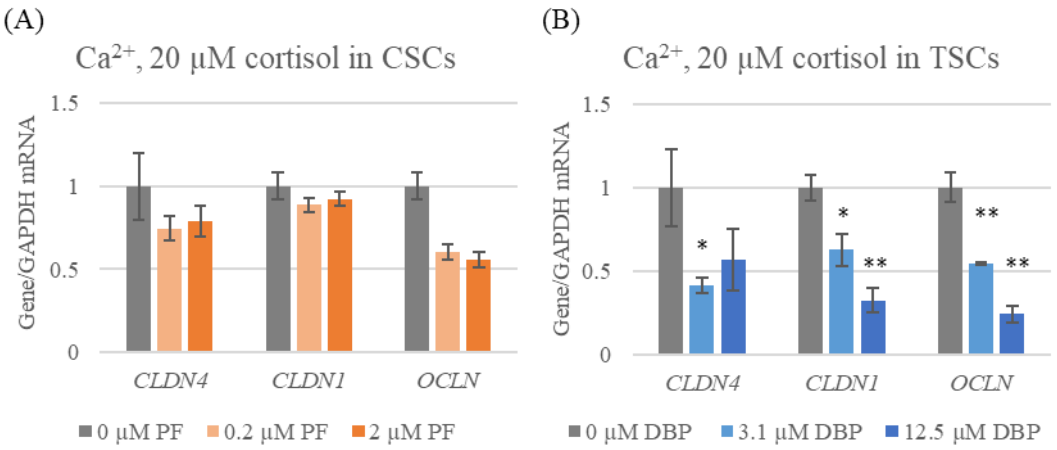

Fig. 4 Effect of 11β HSD-1 and 11β HSD-2 inhibitors on differentiation under cortisol

NHEKs were seeded onto 24-well plates in KGM. After one day of incubation, the cells were washed twice with PBS and treated with 20 μM cortisol. Cortisol treatment was applied once or every 24 h. After three days, the medium was changed to treat cells with 1.5 mM $CaCl_2$ and 20 μM cortisol. The mRNA expression levels of TJPs were analyzed by RT-qPCR 72 h after differentiation induction. (A) CSCs were treated with 0.2–2 μM 11β HSD-1 inhibitor (PF) while inducing differentiation. The data are expressed relative to the expression levels in 0 μM PF. Each bar indicates the mean ± SEM, n = 3. (B) TSCs were treated with 3.1–12.5 μM of 11β HSD-2 inhibitor (DBP) during differentiation induction. The data are expressed relative to the expression levels in 0 μM DBP. Each bar indicates the mean ± SEM, n = 3, * $p < 0.05$, ** $p < 0.01$, vs. 0 μM DBP using Dunnett's test.

<u>GLSE inhibited the conversion of cortisone to cortisol in NHEKs.</u>

GLSE decreased the cortisol concentration in the supernatant of NHEKs treated with 1 μM cortisone (Fig. 5).

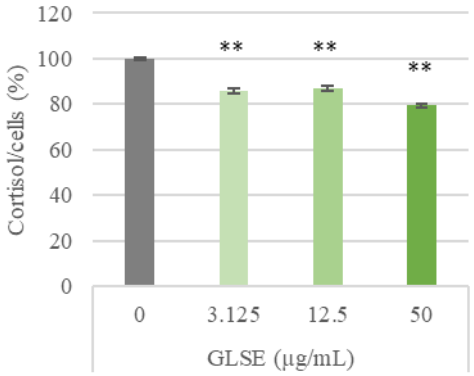

Fig. 5 Effect of GLSE on the conversion of cortisone to cortisol

NHEKs were treated with 1 μM cortisone and GLSE. The amount of cortisol in the supernatant of cultured medium was determined using ELISA after 24 h. Each bar indicates the mean ± SEM, n = 3, ** $p < 0.01$, vs. 0 μg/mL GLSE using Dunnett's test.

GLSE inhibited the effect of cortisol during differentiation induction in CSCs.

GLSE significantly upregulated the mRNA expression level of *CLDN4* at 72 h in CSCs differentiated in the presence of cortisol (Fig. 6).

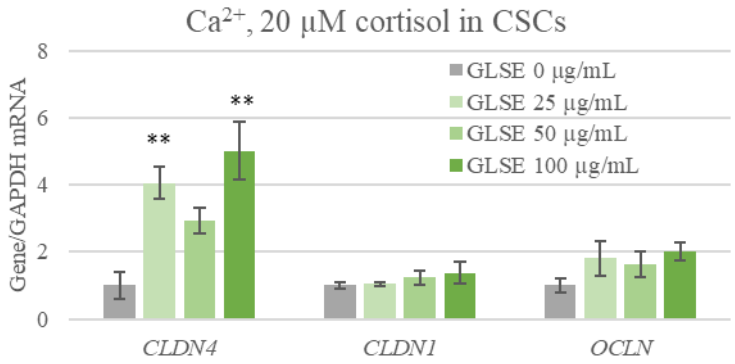

Fig. 6 Effects of GLSE on the mRNA expression levels of TJPs in CSCs induced to differentiate in the presence of cortisol

NHEKs were seeded onto 24-well plates in KGM. After one day of incubation, the cells were washed twice with PBS and treated with 20 μM cortisol. Cortisol treatment was performed every 24 h. After three days, the medium was changed to treat cells with 1.5 mM $CaCl_2$, 20 μM cortisol, and 0–100 μg/mL GLSE. The mRNA expression levels of TJPs were analyzed by RT-qPCR 72 h after differentiation induction. The data are expressed relative to the expression levels in 0 μg/mL GLSE. Each bar indicates the mean ± SEM, n =3, ** $p < 0.01$, vs. 0 μg/mL GLSE using Dunnett's test.

Discussion.

In this study, we examined whether cortisol, a biochemical marker of psychological stress, modulates the effects of itself by altering the expression levels of 11β HSDs, which are enzymes involved in cortisol metabolism.

Cortisol is responsible for the body's regulatory actions, and its blood levels fluctuate widely during daily activities. Prolonged high levels of cortisol in the blood due to chronic psychological stress cause various ailments. High levels of cortisol lead to dryness [2], itching [8], and decreased blood flow [9] in the skin, resulting in decreased quality of life. 11β HSDs are involved in various phenotypes due to differences in glucocorticoid receptor activation, which is temporarily or continuously induced by high levels of cortisol [10] [11] [12]. To understand the mechanisms involved in regulating cortisol and the factors that lead to stress in the skin, we devised two models, a temporary and chronic stress model, using

epidermal keratinocytes with different cortisol treatment conditions. We examined the putative differences in 11β HSD gene expression levels between the stress responses of the two models. Specifically, the condition in which NHEKs were treated with cortisol once every three days was used as the temporary stress model, and the condition in which NHEKs were treated with cortisol every 24 h was used as the chronic stress model. As a result, the 11β HSD-2 mRNA expression level increased in TSCs 72 h after cortisol treatment (Fig. 1A). These results are consistent with those of previous studies demonstrating that a single treatment with dexamethasone, a synthetic glucocorticoid, increases the mRNA expression level of 11β HSD-2 in epidermal keratinocytes [10]. Increasing expression level of 11β HSD-2 mRNA leads to decrease intracellular cortisol levels. Thus, one of feedback mechanisms which is temporary cortisol-induced increase in 11β HSD-2 expression is speculated to reduce the adverse effects of cortisol. In contrast, in CSCs, 11β HSD-1 mRNA expression level clearly increased, whereas 11β HSD-2 mRNA expression level did not increase at 72 h (Fig. 1B). These results are consistent with those of previous studies showing that the continuous application of corticosterone, which is equivalent of human cortisol, increases the mRNA expression level of 11β HSD-1 in mice [11]. It was presumed that the continuous treatment of NHEKs with cortisol for more than three days corresponded to a chronic stress state, which is usually accompanied by an increase in 11β HSD-1 level. It is reported that cortisol is a factor involved in barrier function impairment [3].

Therefore, the effects of cortisol on the mRNA expression levels of TJPs in epidermal keratinocytes were examined in each model. The results showed that treatment with cortisol before the induction of differentiation also affected the expression levels of TJPs during differentiation induction under cortisol treatment, with the expression levels decreasing in the order NSCs > TSCs > CSCs (Fig. 2B).

TJPs play an essential role in the formation of the barrier function via their expression at the mRNA and protein levels and their correct localization [13]. Therefore, to confirm that cortisol affects both the mRNA expression levels of TJPs, and the formation of the barrier function, TEER, and FITC-dextran permeability were measured. The results confirmed that cortisol suppressed both the mRNA expression levels of TJPs and the formation of the barrier function. However, a further reduction in barrier function when cortisol was administered prior to the induction of differentiation was only observed in CSCs, and not in TSCs (Fig. 3

A, B). Collectively, we suggest that chronic and temporary stress reactions are affected differently by freshly supplied cortisol.

Next, to clarify the relationship between changes in the expression levels of 11β HSDs in each stress model and the decrease in barrier function, the effect of cortisol on barrier function during differentiation induction was confirmed in each stress model using 11β HSD inhibitors. Based on the results shown in Fig. 1, the effects of 11β HSD-1 and 11β HSD-2 inhibitors on differentiation in the presence of cortisol were examined in CSCs and TSCs, respectively. The results showed that the 11β HSD-1 inhibitor did not increase the mRNA expression levels of TJPs in CSCs (Fig. 4A), whereas the 11β HSD-2 inhibitor decreased their expression levels in TSCs (Fig. 4B). These results suggested that intracellular cortisol levels were not altered by 11β HSD-1 inhibitors, and the effects of cortisol during differentiation induction were not mitigated because the substrate for 11β HSD-1, cortisone, is present in low quantities under the presence of 20 μM cortisol. In contrast, temporary stress may reduce the effects of cortisol during differentiation induction by decreasing the intracellular levels of cortisol through increased 11β HSD-2 expression. Moreover, these results suggested that the intracellular levels of cortisol was not reduced by the 11β HSD-2 inhibitor and that the mRNA expression levels of TJPs were reduced due to increased susceptibility to cortisol. Thus, 11β HSD-2 is more critical than 11β HSD-1 in the formation of the barrier function during differentiation in the presence of cortisol. In other words, temporary stress was presumed to reduce the effects of cortisol by increasing 11β HSD-2 expression level; however, under chronic stress conditions, the metabolic balance predominantly favored 11β HSD-1, resulting in increased susceptibility to cortisol. Therefore, intracellular cortisol levels should be reduced to ameliorate the effects of chronic stress on the skin.

Thus, we searched for natural extracts that inhibited the conversion of cortisone to cortisol in NHEKs. We confirmed that GLSE reduced the cortisol concentration in the supernatant of NHEKs (Fig. 5). Next, we evaluated the mRNA expression levels of TJPs to determine whether GLSE reduced the effects of cortisol durting differentiation induction in CSCs. GLSE significantly increased the mRNA expression level of *CLDN4* in CSCs (Fig. 6). Moreover, we confirmed that GLSE did not increase the mRNA expression level of *CLDN4*

during differentiation in the absence of cortisol (data not shown). This suggests that GLSE reduced the effects of cortisol in CSCs.

In this study, we exhibited that the altered expression levels of the cortisol metabolic enzymes, 11β HSD-1 and 11β HSD-2, may be involved in the differential effects of temporary and chronic stress. In addition, we confirmed that the effects of cortisol during differentiation induction under conditions of chronic stress might be suppressed by GLSE, which can inhibit its conversion cortisone to cortisol.

Conclusion.

The maintenance of high cortisol levels due to chronic psychological stress may increase the sensitivity to cortisol by changing the expression levels of 11β HSD-1 and 11β HSD-2, thus enhancing the effect of cortisol on the epidermis and excessively attenuating barrier function. Moreover, GLSE, which regulates the metabolic activity of 11β HSDs, is expected to prevent or ameliorate skin problems caused by chronic stress.

Conflict of Interest Statement.

None.

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