Nifedipine Prevents Sodium Caprate-Induced Barrier Dysfunction in Human Epidermal Keratinocyte Cultures

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Received December 17, 2014; accepted March 24, 2015

Tight junctions (TJs) of the epidermis play an important role in maintaining the epidermal barrier. TJ breakdown is associated with skin problems, such as wrinkles and transdermal water loss (TEWL). Clinical studies have reported that topical nifedipine is effective in reducing the depth of wrinkles and improving TEWL. However, it remains unknown whether nifedipine influences the TJ function in the epidermis. In the present study, we investigated the effect of nifedipine on epidermal barrier dysfunction in normal human epidermal keratinocytes (NHEKs) treated with sodium caprate (C10), a TJ inhibitor. Nifedipine reversed the C10-decreased transepithelial electrical resistance values as a measure of disruption of the epidermal barrier. Immunocytochemical observations revealed that nifedipine improved the C10-induced irregular arrangement of claudin-1, a key protein in TJs. Taken together, these findings suggest that nifedipine prevents epidermal barrier dysfunction, at least in part, by reconstituting the irregular claudin-1 localization at TJs in C10-treated NHEKs.

Key words epidermal barrier function; nifedipine; sodium caprate; transepithelial electrical resistance; normal human epidermal keratinocyte

Skin is the interface between air and water, and provides a barrier function that prevents invasion of toxic or infectious substances and escape of moisture.1,2) Disruption of the skin barrier results in not only several pathological conditions, such as atopic dermatitis, but also cosmetic problems, such as dry skin, aged skin, and acne.2,3) The most important skin barrier function is the physical barrier. This barrier is maintained by the lipid bilayer in the stratum corneum, the acidic pH of the epidermis, a calcium gradient that influences cellular turnover and differentiation of the epidermis, and tight junctions (TJs) formed by cells of the granular layer.1,2) Breakdown of each component produces hyperpermeability of the epidermis, leading to a variety of dermatological symptoms and signs.

Recently, clinical studies have reported the efficacy of topical nifedipine for the treatment of facial wrinkles.4,5) Specifically, topical application of nifedipine for 90 d was effective for reducing the depth of wrinkles and improving transdermal water loss (TEWL) as a measure of disruption of the epidermal barrier. In mice with epidermal barrier disruption induced by acetone, nifedipine accelerated the recovery of TEWL.6) These reports suggest that nifedipine is useful for facilitating epidermal barrier maintenance, although its mechanisms remain obscure.

Sodium caprate (C10), an epithelial transport enhancer, is known to disrupt not only the epithelial barrier, but also the epidermal barrier, resulting in hyperpermeability.7) C10 decreases the transepithelial electrical resistance (TER) values by disrupting the arrangement of TJ proteins, such as claudin-1 and occludin, in cultured keratinocytes.7) Considering that claudin-1-deficient mice show wrinkled skin and TEWL,8) C10-treated keratinocytes in culture are thought to provide a useful in vitro model for examining the effects of substances on and the mechanisms underlying wrinkled skin and TEWL. In the present study, we investigated the effect of nifedipine on the epidermal barrier function through TER measurements and claudin-1 expression using Western blot and immunocytochemical analyses in C10-treated normal human epidermal keratinocytes (NHEKs).

MATERIALS AND METHODS

Cells and Cell Culture NHEKs were purchased from Kurabo (Osaka, Japan). The cells were cultured in Humedia-KB2 (Kurabo) containing 0.15 mM Ca2+ supplemented with bovine pituitary extract (0.4% vol/vol), human recombinant epidermal growth factor (0.1 ng/mL), insulin (10 mg/mL), and hydrocortisol (0.5 mg/mL) at 37°C in a humidified atmosphere of 5% CO2–95% air.

Drug Treatment NHEKs were seeded on the insides of Transwell™ inserts (6.5-mm diameter; 0.4-µm pore size; Corning, Midland, MI, U.S.A.) coated with collagen type IV, and then placed into 24-well plates. When the cells reached confluence (day 0), they were transferred to Humedia-KB2 containing 1.8 mM Ca2+ (differentiation medium) for induction of differentiation. On the following day, the cells were transferred to differentiation medium containing drugs, which was replaced every 2–3 d until measurement of the TER. Nifedipine (Sigma-Aldrich, St. Louis, MO, U.S.A.) was dissolved in dimethyl sulfoxide. Sodium caprate (C10; Sigma-Aldrich) (100 mM) was dissolved in distilled water and diluted to the required concentrations.

Measurement of TER The TER of NHEK sheets grown on the insert membranes was measured with an EVOM resistance meter in an EndOhm tissue resistance measurement chamber (World Precision Instruments, Sarasota, FL, U.S.A.). The values are presented as Ω×cm2 culture insert. The TER of cell-free inserts was subtracted from the obtained values.

Immunostaining NHEKs on Transwell inserts were in-

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cubated in ethanol for 30 min at −20°C, and then incubated in ice-cold acetone for 3 min at room temperature. After three washes with phosphate buffered saline (PBS), the cells were treated with 0.2% Triton X-100 in Blocking One (Nacalai Tesque Inc., Kyoto, Japan) for 15 min at room temperature. Subsequently, the cells were incubated with a primary antibody against claudin-1 (Invitrogen, Carlsbad, CA, U.S.A.) for 24 h at 4°C. After rinsing with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit immunoglobulin G (IgG) (Jackson Immunoresearch, West Grove, PA, U.S.A.) for 1 h at room temperature. The stained cells were examined using a fluorescence microscope (BZ-X710; Keyence, Osaka, Japan).

Western Blot Analysis Cells were scraped and lysed in protein lysis buffer (10 mM Tris–HCl pH 6.8, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 2 mM Na3VO4, 50 mM NaF, 20 mM sodium pyrophosphate decahydrate, 50 mg/mL phenylmethylsulfonyl fluoride (PMSF)) containing 1% protease inhibitor cocktail (Sigma). The total protein concentrations in the cell lysates were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, U.S.A.). Equivalent amounts of protein from each sample were separated by electrophoresis in SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The membranes were blocked with Blocking One (Nacalai Tesque). Claudin-1 and β-actin were detected with primary antibodies against claudin-1 (Invitrogen) and β-actin (Abcam, Cambridge, MA, U.S.A.). The immunoreactive bands were visualized using an Immunostar Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Images of the bands were digitally captured with a FluorChem SP Imaging System (Alpha Innotech, San Leandro, CA, U.S.A.).

Statistical Analysis Values are expressed as means ± standard error of the mean (S.E.M.). One-way or two-way ANOVA followed by Dunnett’s test was applied to multiple comparisons. Differences between mean values were considered to be significant for values of p < 0.05. All analyses were performed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, U.S.A.).

RESULTS

Changes in NHEK Barrier Function after Nifedipine Treatment To determine the effect of nifedipine on the NHEK barrier function by measuring TER, we treated NHEK cultures with 0.2 or 1 µM nifedipine for 10 d. Following transfer of the cells to differentiation medium, TER was increased in all groups and the maximum values were observed on day 7 in cultures with vehicle (control: 1226.4 ± 241.1 Ω·cm2) or nifedipine (0.2 µM: 1290.0 ± 133.2 Ω·cm2; 1 µM: 1748.0 ± 115.3 Ω·cm2) (Fig. 1). Nifedipine at 0.2 µM was almost the maximal concentration showing no effect on the normal NHEK barrier function. Therefore, this concentration of nifedipine was employed for the following experiments.

C10-Induced Barrier Impairments in NHEK Cultures Previous studies demonstrated that C10 increases the paracellular permeability to FITC-dextran or mannitol and decreases TER in different types of cultured cells.6-9 To examine the effect of C10 on the NHEK barrier function, NHEK cultures were incubated with C10 (1 or 10 µM) for 10 d. As shown in Fig. 2, C10 decreased TER in the NHEK cultures in a concentration-dependent manner. When cells were exposed to 1 and 10 µM C10, TER was significantly decreased to 73.3% and 59.2% of the control value (884.3 ± 98.5 Ω·cm2), respectively (p < 0.05). C10 at concentrations below 15 µM had no effect on NHEK viability, as determined by WST assays (data not shown).

Effect of Nifedipine on the C10-Induced Barrier Impairments in NHEK Cultures We investigated whether nifedipine affects the C10-decreased TER in NHEK cultures. Without changing the normal barrier function (as shown in Fig. 1), 0.2 µM nifedipine significantly reversed the TER decreased by 10 µM C10 to 93.3 ± 6.0% of the control value in NHEK cultures (Fig. 3A). Next, we examined the changes in the localization and expression of claudin-1 after treatment with C10 or C10 plus nifedipine. Immunostaining for claudin-1 revealed regular and linear distributions at the borders between cells in some regions. When NHEKs were exposed to 10 µM C10, claudin-1 immunoreactivities at regions of cell–cell contact became less apparent and displayed irregular distributions. This irregular staining pattern of claudin-1 was restored to regular lines at the borders between cells by treatment with 0.2 µM nifedipine (Fig. 3B). Western blot analysis of claudin-1 showed no changes in NHEKs exposed to 10 µM C10 alone or 10 µM C10 plus 0.2 µM nifedipine (Fig. 3C).
DISCUSSION

In the present study, we have demonstrated that nifedipine reversed the decreased TER and irregular distribution of claudin-1 immunoreactivities induced by C10 in NHEK cultures. These findings suggest that nifedipine improves the epidermal barrier dysfunction induced by C10.

C10 was reported to induce barrier dysfunction and hyperpermeability via impaired TJs in several cell types, such as epithelial and epidermal cells. Consistent with these reports, our present study demonstrated that C10 induced barrier dysfunction and irregular distribution of claudin-1 in NHEKs. Nifedipine protected NHEKs against the C10-induced functional and morphological impairments of the TJ barrier. These results suggest that nifedipine reconstitutes the C10-induced irregular conformation of TJ-associated proteins such as claudin-1, leading to improvement of the barrier dysfunction. Two possible mechanisms, regulation of intracellular Ca\(^{2+}\) mobilization and lamellar granule (LG) secretion, are considered to be involved in this protective action of nifedipine. C10 is known to increase the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), which is associated with alterations in the actin cytoskeleton and TJ conformation. It is therefore conceivable that nifedipine, a voltage-gated Ca\(^{2+}\) channel blocker, inhibits the C10-induced increase in [Ca\(^{2+}\)]\(_i\), thereby producing a protective action on NHEK barrier dysfunction. Kuroda et al. demonstrated that C10 perturbs LG secretion, which is associated with TJ function. If nifedipine can prevent the C10-induced abnormal LG secretion, this mechanism may underlie a protective action of nifedipine on the C10-induced NHEK barrier dysfunction. Further experiments are required to clarify these underlying mechanisms.

On the other hand, differentiation of cultured NHEKs was induced by switching the extracellular Ca\(^{2+}\) concentration from low (0.15 mM) to high (1.8 mM), suggesting that high Ca\(^{2+}\) concentrations play a key role in the formation and establishment of the NHEK barrier function. Therefore, a discrepancy
is likely to exist in the role of the intracellular Ca\textsuperscript{2+} dynamics for NHEK barrier maintenance. Further experiments are required to clarify this point.

In light of the present findings, we tentatively conclude that nifedipine improves the C10-induced NHEK barrier disruption by reconstituting the irregular claudin-1 localization at TJs. This in vitro study provides the first experimental evidence supporting the clinical efficacy of topical nifedipine for the treatment of facial wrinkles and TEWL.

**Acknowledgments** The authors thank Dr. Shinya Dohgu, Dr. Fuyuko Takata, Dr. Mitsuhisa Koga, and Mr. Takashi Machida for constructive discussions.

**Conflict of Interest** The authors declare no conflict of interest.

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