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Regulation of tight junction permeability by sodium caprate in human keratinocytes and reconstructed epidermis

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ABSTRACT

Tight junctions (TJs) restrict paracellular flux of water and solutes in epithelia and endothelia. In epidermis, the physiological role of TJs is not fully understood. In this study, sodium caprate (C10), which dilates intestinal TJs, was applied to cultured human epidermal keratinocytes and reconstructed human epidermis to investigate the effects of C10 on epidermal TJs. C10 treatment decreased transepithelial electrical resistance and increased paracellular permeability, although Western blots showed that the expression of TJ-related transmembrane proteins was not decreased. The effects of C10 were reversible. Immunofluorescence microscopy and immuno-replica electron microscopy showed that the localization of TJ strands were disintegrated, concomitant with the dispersion and/or disappearance of TJ-related molecules from the cell surface. These findings suggest that C10 impairs barrier function by physically disrupting TJ conformation in the epidermis. Furthermore, these results also show that proper localization of the molecules on the cellular membrane is important for TJ barrier function.

Introduction

It is well known that tight junctions (TJs), which are found in simple epithelia, regulate the paracellular flux of water and solutes. Recently, it has been suggested that epidermal TJs act as a skin barrier [1–3] as well. Furuse et al. reported that mice with a knock out mutation of claudin-1, one of the TJ-related molecules, exhibited abnormally high transepidermal water loss and lethality [1]. A study from this laboratory revealed that UV-induced epidermal barrier perturbation was associated with TJ barrier impairment [2]. We also reported that there is a relationship between the expression of TJ-related molecules and the function of the epidermal barrier using RNA interference against claudin-1 or occludin in cultured human epidermal keratinocytes (HEKs) [3]. Although these studies have largely contributed to our understanding of the role of TJs concerning epidermal barrier function, in order to thoroughly clarify TJ barrier function, it is critically important to be able to investigate the physicochemical properties of TJ-related molecules without using genetic methods that are limited in terms of TJ knock-down efficiency in cultured cells. Therefore, a more specific and preferably reversible method for disrupting TJs is needed for the further study of epidermal TJ function.

In the study of drug delivery systems, paracellular transport of compounds through intestinal TJs is of great interest. Various kinds of transport enhancers have been reported such as medium chain fatty acids, surfactants, Ca^{2+} chelators, glycerides, acylcarnitine, alkenoyl cholines, N-acetylated α-amino acids, N-acetylated non-α-amino acids, chitosans, mucoadhesive polymers, and phospholipids [4–15]. Among these drug transport enhancers, many act as surfactants to increase transcellular transport by fenestrating the lipid bilayer and rendering the cell membrane more permeable. Others act as Ca^{2+} chelators that disrupt intercellular adhesions including TJs. However, drug transport enhancers that deplete Ca^{2+} can lack specificity for TJs and induce universal fatal changes in the epidermal keratinocytes.

Some transport enhancers such as sodium caprate (C10) and long chain acylcarnitines have been shown to improve drug absorption without obvious harmful effects in intestinal mucosa [16]. Specifically, C10 has been shown to dilate TJs, increase the permeability of fluorescein isothiocyanate (FITC)-dextran or [^{14}C]mannitol, and decrease transepithelial electrical resistance (TER) in intestine-derived cell line monolayers [14,17–21] and rat and human colon [10]. The mechanism of these effects was stated to be via phospholipase C activation and upregulation of intracellular Ca^{2+}, which would lead to calmodulin dependent contraction of actin–myosin filaments attached to the intracellular domain of TJs

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[14,19]. This series of events results in dilatation of the TJ paracellular route without considerable cytotoxicity up to 13 mM in Caco-2 cells [20].

In this study, we have chosen C10 as an epidermal TJ barrier regulator for its specificity and mechanism of action. Using HEKs and reconstructed human epidermis (RHE), we successfully investigated epidermal barrier function, TJ-related molecule expression/localization, and TJ ultrastructure. The results show that C10 acts as reversible epidermal TJ barrier disruptor by altering TJ structure and the localization of TJ-related proteins on the cell surface, which indicates that C10 might be a useful tool for further investigation of TJ function in the epidermis. We believe that the results of this study provide important information on the biochemical and/or physicochemical nature of epidermal TJ barrier function.

Materials and methods

Cells and cell culture. HEKs (Invitrogen, CA, USA) were maintained in complete HuMedia-KG2 culture medium (Invitrogen) and induced to differentiate by high calcium-switching, which has been described previously [3]. Briefly, HEKs were first cultured in HuMedia-KG2 culture medium containing 0.15 mM calcium (low-calcium medium), and then switched into HuMedia-KG2 culture medium supplemented with 1.45 mM $CaCl_2$ (high-calcium medium) after 3 days. Reconstructed human epidermis EpiDerm™ (EPI-200, MatTek, MA, USA) was pre-incubated in assay medium EPI-100 (MatTek) for 5 h before use.

TJ barrier disruption. Culture medium containing 1 mM C10 (Sigma–Aldrich Corp., MO, USA) was used for application of C10 to the cells and tissues. One day after differentiation, HEKs were incubated in the presence of C10 for 24 h, and then incubated in the presence (C10 group) or in the absence (C10E group) of C10 for an additional 2 h. For freeze fracture electron microscopy, cells 3 days post-differentiation were treated with or without C10 for 4 h and sampled, or sampled after an additional hour of incubation in fresh culture medium. For RHE, cells were incubated with or without C10 for 18 h, or incubated with C10 for 12 h followed by incubation without C10 for an additional 6 h. Vehicle (purified water) treated cells were prepared and used as an intact control in all experiments.

TER measurement. A Millicell-ERS volt ohmmeter (Millipore Corp., MA, USA) was used to determine the TER value as described previously [22]. For RHE, 0.3 ml of EPI-100 assay medium was added to the apical side before measurement. All TER values were calculated by subtracting the resistance measured in a blank insert from the resistance measured in an insert containing cell layer, and then multiplying by the surface area of the membrane.

Paracellular tracer flux assay. Paracellular flux was assessed by transport of FITC-dextran (Sigma–Aldrich, average MW 4400) in the HEKs from the basolateral to the apical chamber as described previously [3]. After each C10 treatment period, phosphate-buffered saline (PBS) containing 1.45 mM $CaCl_2$ and 10 mM glucose, with or without 1 mg/ml FITC-dextran was applied to the basolateral and apical sides, respectively. After 3 h, the FITC-dextran concentration on the apical side was quantified using an ARVO SX Multilabel Counter (PerkinElmer life science, MA, USA). Data presented are the means of three separate experiments.

The paracellular flux assay in RHEs was modified from the protocol described previously [2]. In brief, freshly prepared 10 mg/ml EZ-Link™ sulfo-NHS-LC-biotin (Pierce, IL, USA) was applied to the basolateral side of the RHEs. After 1 h, samples were washed and frozen. Frozen sections were stained with rabbit anti-human occludin IgG (Invitrogen, CA, USA) and a mixture of FITC-conjugated donkey anti-rabbit antibody (ICN Pharmaceuticals, OH, USA) and streptavidin-Texas red (Oncogene, CA, USA).

Western blot analysis. Protein was extracted and resolved by gel electrophoresis. After transblotting and blocking, the membranes were incubated with rabbit anti-human claudin-1 IgG, rabbit anti-human occludin IgG (both Invitrogen), or rabbit anti-human beta actin IgG (Abcam, Cambridge, UK). Immunoreactive bands were detected using an ECL Plus Kit and horseradish peroxidase-conjugated goat anti-rabbit IgG (both GE Healthcare Bio-Sciences Corp.).

Immunofluorescence microscopy. For immunofluorescence microscopy, HEKs were sequentially fixed with ice-cold ethanol and acetone for 10 min, washed with PBS, and soaked in 1% BSA in PBS. Samples were incubated with rabbit anti-human claudin-1 IgG or rabbit anti-human occludin IgG (both Invitrogen) and then incubated with FITC-conjugated donkey anti-rabbit antibody (ICN Pharmaceuticals). The samples were observed and photographed with an Axiovert microscope (Carl Zeiss, Overkochen, Germany).

Freeze-fracture and immuno-replica electron microscopy. HEKs and RHEs were fixed in 0.1 M (PB, pH 7.3) containing 1% paraformaldehyde at 4 °C, washed with 0.1 M PB at 4 °C, immersed in 0.1 M phosphate buffer containing 30% glycerol at 4 °C for 12 h, and then frozen in liquid nitrogen. Frozen samples were fractured at −100 °C and platinum-shadowed unidirectionally at an angle of 45 °C in a Freeze Etching System (BAF060; Bal-Tek, Hudson, NH, USA). The HEKs were processed for immuno-replica electron microscopy as described [23]. The replicas were incubated with mouse anti-human claudin-1 IgG (Invitrogen) and rabbit anti-human occludin IgG, then incubated with 10 nm gold-conjugated goat anti-mouse antibody (EY Laboratories, CA, USA) and 25 nm gold-conjugated goat anti-rabbit IgG (Aurion, Wageningen, Netherlands). Replicas of RHEs and immuno-reacted replicas of HEKs were then observed with an H-7500 electron microscope (Hitachi, Tokyo, Japan) at 100 kV.

Statistics. Dunnett's multiple comparison analysis was performed using JMP 6.0 software (SAS Institute, Cary, NC, USA).

Results and discussion

Barrier disruption by C10 treatment

For HEKs, a long treatment period was used with the intent of observing a large effect of treatment. As shown in Fig. 1A, TER increased in HEKs that were not treated with C10 (intact control), whereas TER was suppressed during C10 treatment. However, 2 h after C10 elimination, TER increased to levels as high as those observed in intact cells. In RHE, C10 treatment also suppressed TER compared to the intact control and removal of C10 resulted in the recovery of TER (Fig. 1B) in a similar fashion as in the HEKs.

In order to assess paracellular permeability in HEKs, flux of FITC-dextran under each experimental condition was examined (Fig. 2A). Paracellular permeability was significantly higher in C10 treated cells than in control cells, whereas permeability recovered in the C10E group and was not significantly different from control. In the RHEs, the paracellular barrier of the TJs was visually assessed using double stained section imaging (Fig. 2B). Immunodetected occludin was used as an indicator of TJ position and sulfo-NHS-LC-biotin was used as a diffusion tracer. Images of control RHE indicated that the diffusion tracer was restricted at the occludin-positive sites, while the tracer permeated through the occludin-positive sites in C10 treated RHE. In C10E RHE, diffusion was restricted at the occludin-positive sites.

In this study, we successfully investigated the effects of C10 on TJs in HEKs and RHEs for the first time. Furthermore, the alterations of epidermal TJ caused by C10 treatment were examined physiologically and morphologically. To evaluate TJ barrier function, TER or paracellular permeability measurement is rou-