Protection of Human Corneal Epithelial Cells From TNF-α–Induced Disruption of Barrier Function by Rebamipide

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PURPOSE. TNF-α disrupts the barrier function of cultured human corneal epithelial (HCE) cells. We investigated the effects of the cytoprotective drug rebamipide on this barrier disruption by TNF-α as well as on corneal epithelial damage in a rat model of dry eye.

METHODS. The barrier function of HCE cells was evaluated by measurement of transepithelial electrical resistance. The distribution of tight-junction (ZO-1, occludin) and adherens-junction (E-cadherin, β-catenin) proteins, and the p65 subunit of nuclear factor–κB (NF-κB) was determined by immunofluorescence microscopy. Expression of functional proteins as well as phosphorylation of the NF-κB inhibitor IκB-α and myosin light chain (MLC) were examined by immunoblot analysis. A rat model of dry eye was developed by surgical removal of exorbital lacrimal glands.

RESULTS. Rebamipide inhibited the disruption of barrier function as well as the downregulation of ZO-1 expression, and the disappearance of ZO-1 from the interfaces of neighboring HCE cells induced by TNF-α. It also inhibited the phosphorylation and downregulation of IκB-α, the translocation of p65 to the nucleus, the formation of actin stress fibers, and the phosphorylation of MLC induced by TNF-α in HCE cells. Treatment with rebamipide eyedrops promoted the healing of corneal epithelial defects as well as attenuated the loss of ZO-1 from the surface of corneal epithelial cells in rats.

CONCLUSIONS. Rebamipide protects corneal epithelial cells from the TNF-α–induced disruption of barrier function by maintaining the distribution and expression of ZO-1 as well as the organization of the actin cytoskeleton. Rebamipide is, thus, a potential drug for preventing or ameliorating the loss of corneal epithelial barrier function associated with ocular inflammation.

Keywords: cytokine, corneal epithelium, wound healing, barrier

The corneal epithelium serves as a barrier to protect the eye from external agents such as microbes and chemicals as well as to maintain corneal transparency.1–3 The formation of tight junctions (TJs) between adjacent epithelial cells at the apical plasma membrane4–5 gives rise to both a physical and functional barrier.6 Adherens junctions (AJs) between epithelial cells play an important role in the formation of TJs.7 The protein components of TJs include zonula occludens (ZO-1, occludin) and junctional adhesion molecules (JAMs),8,9 whereas AJs are composed of cadherins and catenins (α-catenin, β-catenin, p120 catenin).10,11 Both AJ and TJ components are tightly linked to the actin cytoskeleton.12,13 ZO-1, occludin, claudin, E-cadherin, and β-catenin have been detected in the corneal epithelium14–16 and are thought to determine its barrier function.17–22

The inflammatory response to a variety of insults such as injury and infection includes the production of various cytokines and chemokines. TNF-α, a proinflammatory cytokine,23 contributes to ocular inflammation such as that associated with infection,24 injury,25 and dry eye.26 Ocular inflammation associated with ocular surface diseases has been shown to affect corneal structure and function.27,28 The concentration of TNF-α in tear fluid has been found to be markedly increased in patients with dry eye.29 Moreover, the amount of TNF-α at the ocular surface is increased in a mouse model of dry eye.26,30,31

Rebamipide was selected from among more than 500 amino acid analogs of 2(1H)-quinolinone tested for the ability to prevent the development and to promote the healing of gastric ulcers by stimulating the production of prostaglandins by the gastric mucosa.32 It also exhibits anti-inflammatory activity in the esophagus and stomach as a result of its inhibition of the production of cytokines and chemokines by several cell types.33,34 Corneal damage including structural irregularities and haze induced by ultraviolet B radiation has also been found to be attenuated by rebamipide in mice.35

We have previously shown that TNF-α disrupts the barrier function of cultured human corneal epithelial (HCE) cells.21 In the present study, we examined the effect of rebamipide on the loss of transepithelial electrical resistance (TER) induced by TNF-α in these cells. We also investigated the effects of rebamipide on the expression and distribution of TJ and AJ proteins, on the nuclear factor (NF)-κB signaling pathway, and on the actin cytoskeleton in corneal epithelial cells exposed to TNF-α. Finally, we examined the effect of administration of rebamipide eyedrops on corneal epithelial damage in a rat model of dry eye induced by removal of lacrimal glands.
Rebamipide Protects the Barrier Disruption

METHODS

Materials

Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM/F-12), PBS, fetal bovine serum, trypsin-EDTA, and gentamicin were all obtained from Invitrogen-Gibco (Carlsbad, CA). Bovine serum albumin (BSA), recombinant bovine insulin, cholera toxin, recombinant human epidermal growth factor, a mouse monoclonal antibody to myosin light chain (MLC), and a protease inhibitor cocktail were all obtained from Sigma-Aldrich (St. Louis, MO). Six- or 24-well transwell plates as well as 24-well culture plates were obtained from Corning (Corning, NY). Rabbit polyclonal antibodies to the p65 subunit of NF-κB and mouse monoclonal antibodies to IκB-α or to phosphorylated IκB-α were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to phosphorylated MLC were from Cell Signaling (Danvers, MA), whereas those to ZO-1 or to occludin were from Zymed (San Francisco, CA). Mouse monoclonal antibodies to E-cadherin or to β-catenin were obtained from BD Biosciences (Carlsbad, CA). Cyto59, horseradish peroxidase–conjugated, or AlexaFluor 488–labeled goat antibodies to mouse or rabbit immunoglobulin G, and rhodamine-phalloidin were from Invitrogen-Gibco. ECL Plus reagents were obtained from GE Healthcare (Little Chalfont, UK). 2-(4-Chlorobenzamido)-3-[2(1H)-quinoxalin-4-yl]propionic acid (rebamipide) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Rebamipide ophthalmic suspension (2%) and vehicle (the same solution without rebamipide) were formulated at Otsuka Pharmaceutical Co., Ltd. (Ako, Hyogo, Japan).

Cells and Cell Culture

Simian virus 40–immortalized HCE cells were obtained from RIKEN Biosource Center (Tokyo, Japan). They were passaged in supplemented hormonal epithelial medium (SHEM), which comprises DMEM/F-12 supplemented with 15% heat-inactivated fetal bovine serum, bovine insulin (5 μg/mL), cholera toxin (0.1 μg/mL), human epidermal growth factor (10 ng/mL), and gentamicin (40 μg/mL). For experiments, HCE cells were plated at a density of 1 × 10⁶ or 5 × 10⁵ cells per well in 24- or 6-well transwell plates (filter pore size of 0.4 μm), respectively, or at 1 × 10⁵ cells per well in 24-well culture plates. They were then cultured for 4 days in SHEM and for 24 hours in unsupplemented DMEM/F-12 before incubation first for 12 hours with or without rebamipide and then for the indicated times in the additional absence or presence of TNF-α (10 ng/mL) in unsupplemented DMEM/F-12. Rebamipide was dissolved in unsupplemented DMEM/F-12 at a concentration of 2 mM (stock solution) for HCE cell experiments.

Measurement of TER

The TER of HCE cells cultured in 24-well transwell plates was measured with the use of an Epithelial Volt-ohm meter (EVIOM; World Precision Instruments, Sarasota, FL). TER (ohms × centimeter squared) was calculated by multiplying the measured resistance by the area of the transwell filter. The background resistance due to the filter alone (~130 ohms × cm²) was measured before cell plating and was subtracted from the experimental values.

Immunofluorescence Analysis

HCE cells were cultured in 24-well culture plates. For ZO-1, occludin, E-cadherin, and β-catenin staining, the cells were fixed with 100% methanol for 20 minutes at room temperature. For staining of the p65 subunit of NF-κB, the cells were fixed with 4% paraformaldehyde in PBS, washed with PBS, and permeabilized with 100% methanol for 5 minutes at −20°C. All cells were then washed with PBS and incubated at room temperature for 1 hour with 1% BSA in PBS and then for 1 hour with antibodies to ZO-1, to occludin, to E-cadherin, to β-catenin, or to p65, each at a 1:100 dilution in PBS containing 1% BSA. After washing with PBS, the cells were incubated at room temperature for 1 hour with AlexaFluor 488–labeled secondary antibodies at a 1:1000 dilution in PBS containing 1% BSA, and then for 10 minutes with Cyto59 for staining of nuclei. For staining of the actin cytoskeleton, cells fixed and permeabilized as for staining of p65 were incubated for 1 hour at room temperature with rhodamine-phalloidin at a 1:100 dilution in PBS containing 1% BSA. All cells were then examined with a laser confocal microscope (LSM5; Zeiss, Oberkochen, Germany). The intensity of ZO-1 immunofluorescence was measured with the use of the National Institutes of Health ImageJ software (version 1.46r; Bethesda, MD).

Immunoblot Analysis

HCE cells cultured in 6-well transwell plates were lysed in 300 μL of a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM NaF, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4, and 1% protease inhibitor cocktail. The lysates were centrifuged at 15,000 g for 10 minutes at 4°C, and the resulting supernatants were subjected to SDS-PAGE on a 10% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then incubated at room temperature first for 1 hour with blocking buffer (20 mM Tris-HCl [pH 7.4], 5% dried skim milk, 0.1% Tween 20) and then for 2 hours with primary antibodies, each at a 1:1000 dilution in blocking buffer. The membrane was washed with washing buffer (20 mM Tris-HCl [pH 7.4], 0.1% Tween 20), incubated for 1 hour at room temperature with horseradish peroxidase–conjugated goat secondary antibodies (1:1000 dilution in washing buffer), washed again, incubated with ECL Plus detection reagents for 5 minutes, and then exposed to film.

Rat Model of Dry Eye and Evaluation of Corneal Epithelial Damage

Male 4-week-old Sprague-Dawley rats were obtained from SLC Japan (Shizuoka, Japan). All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the animal experimentation committee of Yamaguchi University Graduate School of Medicine. A rat dry eye model was prepared. In brief, rats at 5 weeks of age were anesthetized by intraperitoneal injection of pentobarbital sodium for bilateral removal of the exorbital lacrimal glands. Dry eye was evaluated 5 weeks after surgery by staining of the cornea with 1 μL of 1% fluorescein in saline. Excess fluorescein was washed out from the eye with saline, and corneal staining was photographed with the use of a confocal laser-scanning ophthalmoscope (F-10; NIDEK, Aichi, Japan). The rats (n = 8 per group) were then treated four times daily for 4 weeks in each eye with 5 μL of 2% rebamipide ophthalmic suspension or corresponding vehicle. The extent of corneal epithelial damage was evaluated by fluorescein staining at 4, 7, 14, 21, and 28 days after the onset of treatment. For evaluation of staining, photographs of the cornea were divided transversely into three sections and each section was scored from 0 to 5 (0, no staining; 1, scattered staining [punctate or dispersed]; 2, moderate staining; 3, pronounced staining) by an observer not aware of whether the eye received rebamipide or vehicle.
The scores for the three sections were summed to give the total score for the cornea (maximum score of 9).

After the final evaluation of corneal epithelial damage, rats were killed with a lethal dose of pentobarbital sodium, the eyes were enucleated, and each cornea was isolated, and cut into four tissue blocks. The tissue was fixed with acetone for 15 minutes at room temperature, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and then incubated with PBS containing 1% BSA for 1 hour to block nonspecific binding of antibodies. The tissue was then incubated overnight at 4°C with antibodies to ZO-1 (1:100 dilution), washed with PBS, incubated for 1 hour at room temperature with Alexa Fluor 488–conjugated secondary antibodies (1:200 dilution), washed with PBS, and stained with 4′,6-diamidino-2-phenylindole. The tissue blocks were then examined with a laser confocal microscope (LSM5; Zeiss) as described previously for immunofluorescence analysis of HCE cells. The ZO-1 signal was binarized and the defective area of ZO-1 staining was measured with ImageJ software (version 1.46r; National Institutes of Health).

Statistical Analysis

Quantitative data are presented as means ± SEM. Differences between two groups were analyzed with Student’s nonparametric t-test, and those among multiple groups were evaluated with ANOVA followed by Dunnett’s multiple-comparison test. A P value of less than 0.05 was considered statistically significant.

RESULTS

We have previously shown that TNF-α downregulates the barrier function of cultured HCE cells.21 To examine the effect of rebamipide on the TNF-α–induced disruption of barrier function in these cells, we measured TER. A confluent monolayer of HCE cells was cultured in serum-free medium for 24 hours and then exposed to various concentrations of rebamipide (0–0.3 mM) for 12 hours before incubation in the additional presence of TNF-α for 24 hours. Whereas TNF-α reduced the TER of HCE cells, this effect was inhibited by rebamipide in a concentration-dependent manner, with the action of rebamipide being significant at 0.1 and 0.3 mM (Fig. 1).

We next examined by immunofluorescence analysis the effects of rebamipide on the distribution of TJ (ZO-1 and occludin) and AJ (E-cadherin and β-catenin) proteins in HCE cells exposed to TNF-α. We previously showed that TNF-α induced the disappearance of ZO-1 from the cell border,21 and this effect was significantly inhibited by rebamipide at 0.3 mM (Fig. 2). TNF-α did not affect the localization of occludin, E-cadherin, or β-catenin at the borders of adjacent cells, and rebamipide also had no effect on the localization of these proteins in the presence of TNF-α (Fig. 2).

We previously showed that TNF-α downregulated the amount of ZO-1 in HCE cells.21 Immunoblot analysis revealed that rebamipide significantly inhibited this effect of TNF-α (Fig. 3). TNF-α did not affect the abundance of occludin, E-cadherin, or β-catenin, and rebamipide also had no effect on the expression of these proteins in the presence of TNF-α (Fig. 5).

NF-κB activation is required for the TNF-α–induced disruption of barrier function in HCE cells;21 therefore, we next examined the effect of rebamipide on NF-κB activation induced by TNF-α in these cells. Incubation of the cells with rebamipide for 12 hours before exposure to TNF-α markedly inhibited both the phosphorylation and degradation of the endogenous NF-κB inhibitor IκB-α (Fig. 4A) as well as the translocation of the p65 subunit of NF-κB to the nucleus (Fig. 4B) induced by TNF-α.

We next examined the effect of rebamipide on the reorganization of the actin cytoskeleton induced by TNF-α. TNF-α induced the formation of actin stress fibers in HCE cells, and this effect was inhibited by rebamipide (Fig. 5A). TNF-α also induced the phosphorylation of MLC in HCE cells in a manner sensitive to inhibition by rebamipide (Fig. 5B).

Finally, we examined the effect of eyedrops containing rebamipide on corneal epithelial damage in a rat model of dry eye induced by bilateral removal of the exorbital lacrimal glands. Eyedrops containing rebamipide (2%) were adminis-
tered at 6 hour intervals for 28 days. Fluorescein staining revealed that rebamipide induced significant resurfacing of the corneal epithelial defects in this model compared with eyes treated with vehicle (Fig. 6). We also examined the distribution of ZO-1 in the corneal epithelium at the end of the treatment period. Immunofluorescence analysis revealed that, whereas ZO-1 was localized at the interfaces of adjacent corneal epithelial cells in the healthy eye, removal of the lacrimal glands resulted in a loss of ZO-1 immunoreactivity from the borders of superficial epithelial cells in defective areas and that this effect was greatly attenuated by rebamipide treatment (Fig. 7).

**DISCUSSION**

We have shown that rebamipide inhibited the TNF-α-induced decrease in TER of HCE cells in a concentration-dependent manner. This effect of rebamipide was associated with inhibition of the TNF-α-induced disappearance of ZO-1 from the borders of adjacent HCE cells as well as of the TNF-α-induced downregulation of ZO-1 expression. Rebamipide did not affect the distribution or abundance of occludin, E-cadherin, or β-catenin in HCE cells exposed to TNF-α. The activation of NF-κB signaling, formation of actin stress fibers, and phosphorylation of MLC induced by TNF-α were also attenuated by rebamipide. These results, thus, suggest that rebamipide inhibits the TNF-α-induced disruption of barrier function in cultured corneal epithelial cells by maintaining the expression and distribution of ZO-1. Furthermore, rebamipide ameliorated the corneal epithelial damage as well as inhibited the loss of ZO-1 from the borders of corneal epithelial cells induced by removal of lacrimal glands in a rat model of dry eye. Rebamipide, thus, also appears to protect the corneal epithelium from disruption of barrier function in vivo.

Rebamipide inhibits the disruption of barrier function in gastric and intestinal epithelial cells as well as in other epithelial cell types. We now show that rebamipide protects against barrier disruption associated with the downregulation of ZO-1 expression and the loss of ZO-1 from cell–cell borders induced by TNF-α in HCE cells, and that it exerts similar effects in a rat model of dry eye. These results suggest that rebamipide may regulate barrier function by modulating the expression and localization of the TJ protein ZO-1 in corneal epithelial cells.

TJ and AJ proteins are associated with perijunctional actin filaments, which modulate barrier function. The contraction and rearrangement of the actin cytoskeleton, thus, contribute to barrier disruption. The phosphorylation of MLC induces the reorganization of perijunctional actin and disrupts TJs. We have now shown that rebamipide inhibited TNF-α-induced MLC phosphorylation and rearrangement of the actin cytoskeleton in HCE cells, suggesting that rebamipide modulates the linkage between junctional complexes and the...
FIGURE 3. Effect of rebamipide on the expression of junctional proteins in HCE cells exposed to TNF-α. (A) Cells were incubated in the absence or presence of rebamipide (0.3 mM) for 12 hours and then in the additional absence or presence of TNF-α for 24 hours, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to ZO-1, to occludin, to E-cadherin, to β-catenin, or to β-actin (loading control). (B) Immunoblots similar to those in (A) were subjected to densitometric analysis for determination of the abundance of each junctional protein normalized by that of β-actin. Data are expressed relative to the corresponding value for control cells and are means ± SEM from three independent experiments. *P < 0.05 (ANOVA followed by Dunnett’s test).

FIGURE 4. Inhibition by rebamipide of the TNF-α–induced activation of the NF-κB signaling pathway in HCE cells. (A) Cells were incubated for 12 hours in the absence or presence of rebamipide (0.3 mM) and then for 30 minutes in the additional absence or presence of TNF-α. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to IκB-α or to phosphorylated IκB-α (left panel). The amount of phosphorylated IκB-α was determined by densitometric scanning of immunoblots and normalized by the corresponding amount of β-actin (right panel); data are expressed relative to the normalized value for cells treated with TNF-α alone and are means ± SEM from three independent experiments. *P < 0.05 (ANOVA followed by Dunnett’s test). (B) Cells were incubated for 12 hours in the absence or presence of rebamipide (0.3 mM) and then for 30 minutes in the additional absence or presence of TNF-α. They were then fixed, permeabilized, and subjected to immunofluorescence analysis with antibodies to the p65 subunit of NF-κB (green signal). Nuclei were detected by staining with Cyto59 (blue signal). Scale bar: 10 μm.
actin cytoskeleton in these cells, and that such modulation may contribute to the protection of barrier function.

Rebamipide reduced the concentrations of TNF-α and IL-1β in serum as well as suppressed gastric inflammation in mice infected with Helicobacter pylori. It also inhibited the expression of IL-8 induced by TNF-α in human umbilical vein endothelial cells. Corneal epithelial disorders are associated with various ocular surface diseases involving inflammation. We have now shown that rebamipide attenuated TNF-α-induced barrier disruption in HCE cells as well as ameliorated corneal epithelial damage in a rat model of dry eye. The levels of IL-1β and TNF-α at the ocular surface were previously found...
to be increased in a dry eye model. These various observations suggest that rebamipide may suppress ocular inflammation, and thereby help to maintain the integrity of the corneal epithelium. Indeed, rebamipide has recently been applied clinically to the treatment of patients with dry eye symptoms in Japan. Further investigations are warranted to determine the clinical efficacy of rebamipide for other ocular inflammatory disorders.

TNF-α induces the expression of matrix metalloproteinases (MMPs) in certain cell types. Activation of MMP-9 at the ocular surface in experimental dry eye has been shown to promote disruption of corneal epithelial barrier function as a result of the loss of TJs from superficial corneal epithelial cells. Signaling by NF-κB contributes to the expression of MMPs. Rebamipide reduced the expression of MMP-13 in human chondrocytes. We have now shown that rebamipide inhibited the TNF-α–induced activation of NF-κB and downregulation of ZO-1 in HCE cells. These results suggest that rebamipide may suppress TNF-α–induced expression of MMPs through inhibition of NF-κB activation, thereby, attenuating the proteolysis of ZO-1 in HCE cells. TNF-α also promotes the release of intracellular free radicals, which have been found to contribute to disruption of endothelial barrier function, and rebamipide is thought to act as a radical scavenger.

Ocular inflammation can lead to increased corneal epithelial permeability as a result of disruption of TJ structure. TNF-α plays an important role in corneal epithelial diseases associated with ocular inflammation. Inhibition of the adverse effects of TNF-α on corneal epithelial barrier function by rebamipide is, thus, a potential new approach to the treatment of corneal epithelial disorders associated with ocular inflammation.

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