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Original article

The role of protein tyrosine phosphorylation in the cell–cell junctions and intercellular permeability of post-confluent bovine corneal epithelial cells

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ABSTRACT

Purpose: To evaluate the role of protein tyrosine phosphatase (PTP) in controlling the integrity of cell–cell junction and intercellular permeability in postconfluent bovine corneal epithelial cells.
Methods: Confluent cultures of bovine corneal epithelial cells were treated with different concentrations of general phosphate inhibitors andsodium orthovanadate for varying periods. An MTS assay was used to confirm no cellular death under the treatment profile. Immunocytochemical (ICC) analysis was performed to demonstrate protein tyrosine phosphorylation after treatment with sodium orthovanadate, and the effect of sodium orthovanadate on junctional proteins such as p120, α-catenin, occludin, ZO-1, and ZO-2. Western blot analysis was used to analyze the changes in p120, α-catenin, occludin, ZO-1, and ZO-2 after treatment. Paracellular permeability was evaluated by transepithelial electrical resistance (TER).
Results: During the whole course of treatment, no significant cellular death was noticed. Dose- and time-dependent effects of sodium orthovanadate on protein tyrosine phosphorylation were confirmed by ICC. ICC also demonstrated the dose- and time-dependent effect of sodium orthovanadate on the disruption of p120, α-catenin, occludin, ZO-1, and ZO-2. However, results of Western blot analysis showed no change in the expression levels of p120, α-catenin, occludin, ZO-1, and ZO-2. Dose- and time-dependent increase of paracellular permeability was evaluated by TER.
Conclusion: Inhibition of protein tyrosine phosphatase activity can increase protein tyrosine phosphorylation. A dose- and time-dependent release of cell–cell contacts and increased transepithelial permeability were found in postconfluent culture of bovine corneal epithelial cells.

1. Introduction

Corneal epithelium, a multilayered stratified squamous epithelial sheet, provides the first-line defense against invasion of allergens, toxins, and pathogens.[1–3] In addition, the barrier function of corneal epithelium also controls the movement of fluid into corneal stroma and is associated with corneal transparency.[4–6] Hypoxia, chemical damage, and dryness disrupt corneal epithelial barrier function, resulting in corneal edema and impaired vision.[7–9]

The barrier function of corneal epithelium is owing to the presence of intracellular junctional proteins between adjacent epithelial cells, such as tight junction and adherens junction proteins.[7,10] Tight junctions, located on the apical-most lateral membranes, contains transmembrane proteins, occludin and claudin, and associated proteins, such as ZO-1, ZO-2, and ZO-3. These tight junction proteins contribute to major intracellular connecting force. Adherens proteins include transmembrane proteins and cadherin, which connect with actin cytoskeleton through associated proteins, such as α, β, and γ-catenin. Adherens proteins not only connect with adjacent epithelial cells, but also play an important role in maintaining the stability of cell skeleton and normal epithelial morphology.[10]

Protein tyrosine phosphatases (PTPs) are enzymes that catalyze the dephosphorylation of tyrosine-phosphorylated proteins.[11–14]

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PTPs can function as either negative or positive regulators of signaling triggered by receptor tyrosine kinase. It is known that the PTPs constitute a very large family of phosphatases, which can be broadly classified into transmembrane, receptor-like, and nontransmembrane, or nonreceptor PTPs. They are differentiated by their noncatalytic segments, which are important for their cellular targeting. The effects of phosphorylation on regulating cellular proliferation, migration, differentiation, and junctional integrity of corneal endothelial cells have been reported.[15–17] However, the studies on corneal epithelial cells are limited. Previously, we have showed that protein tyrosine phosphorylation of intracellular junctional proteins plays an important role in the barrier function of bovine corneal endothelial cells, as well as controlling corneal endothelial proliferation.[16,17] However, little information is available about the effect of protein tyrosine phosphorylation in controlling the integrity of cell junction in corneal epithelial cells. Therefore, in this study, we aimed at investigating the role of protein tyrosine phosphorylation in controlling the integrity of cell junction and intracellular permeability in bovine epithelial cells. Sodium orthovanadate was used to induce general inhibition of PTPs in this study.

2. Materials and methods

2.1. Antibodies and reagents

Polyclonal rabbit-anti-human p120, polyclonal rabbit anti-human α-catenin antibody, fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit and goat-anti-mouse antibodies, horseradish peroxidase-conjugated goat-anti-rabbit antibody, and goat anti-mouse antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal mouse anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-ZO-1, anti-ZO-2, anti-occludin, and anti-phosphotyrosine antibodies were purchased from Zymed Laboratories (San Francisco, CA). Sodium orthovanadate was purchased from Sigma-Aldrich (St. Louis, MO) and was prepared as a 0.1M stock solution (pH 7). Mounting medium containing propidium iodide was purchased from Vector Laboratories (Burlingame, CA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, Fungizone, trypsin–ethylenediaminetetraacetic acid, and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY).

2.2. Culture of bovine corneal epithelial cells

Bovine corneal epithelial cells were prepared as described previously.[18] In brief, bovine cornea was isolated from the eyeball and immersed with 1.2 U/mL dispase II at 37 °C for 1 hour, following which the epithelial layer was gently isolated. The suspended cells were cultured with DMEM-F12 containing 1% insulin–transferrin–selenium supplement, 10% FBS, penicillin, streptomycin, and amphotericin B. Cultured epithelium cells with two passages were used for the experiments. Confluent cells were treated with 0, 25, 50, and 100 μM sodium orthovanadate for different periods of time.

2.3. MTS assay

Corneal epithelial cells were cultured at a density of 1×10^4 cells in 96-well plates and allowed to reach confluence. Corneal epithelial cells were treated with 0, 25, 50, and 100 μM vanadate for 24 and 48 hours. At the end of experiments, cell viability was evaluated using CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI) according to the manufacturer's instructions. In brief, the medium was removed, and MTS/phenazine methosulfate solution was added. After incubating at 37 °C for 2 hours, cell viability was determined by measuring the absorbance at 490 nm using an enzyme-linked immunosorbent assay reader. All experiments were repeated six times to ensure consistent results. In all experiments, only vehicle without adding sodium orthovanadate was used as control.

2.4. Immunocytochemical localization

Confluent cells treated with different concentrations of sodium orthovanadate were fixed in 10% natural formalin followed by permeabilization with 0.4% Triton X-100. Subsequently, cells were blocked by 1% FBS in phosphate-buffered saline (PBS) at 37 °C for 1 hour followed by incubation with primary antibodies at 4 °C overnight. Concentrations of primary antibodies were all 1:100. Subsequently, a 1:100 diluted solution of the appropriate FITC-conjugated secondary antibodies was added. All the experiments were repeated three times to ensure consistent results. Positive staining was visualized using a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany). In all experiments, only vehicle without adding sodium orthovanadate was used as control.

2.5. Protein extraction and immunoblotting

The cultured cells grown were trypsinized, suspended in buffer containing 1% Triton X-100, 250 mM NaCl, 2 mM EDTA, 50 mM Tris–HCl, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (all from Sigma), and homogenized. Equal amounts of extracted protein were loaded on 4–12% polyacrylamide gels (Invitrogen, Carlsbad, CA) for sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA), and then incubated at room temperature for 2 hours with antibodies against p120, α-catenin, ZO-1, ZO-2, and occludin. Anti-β-actin was used as an internal control. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody at a final dilution of 1:1000. After a final washing with 0.1% Triton X-100 in PBS, fluorescent signals were detected by enhanced chemiluminescence following the manufacturer's instructions (Pierce, Rockford, IL, USA) and exposed to autoradiographic film. All experiments were repeated three times to ensure consistent results. In all experiments, only vehicle without adding sodium orthovanadate was used as a control.

2.6. Measurement of transepithelial electrical resistance

Bovine corneal epithelial cells were seeded in the upper chamber of Costar Transwell (1.12 cm^2 diameter, 0.4 μm pore size) and allowed to reach confluency. Transepithelial electrical resistance (TER) was measured using a Millicell-ERS electrical resistance system (Millipore, Bedford, MA) and calculated as Ωcm^2 by multiplying it with the surface area of the monolayer (1.12 cm^2). The resistance of the supporting membrane in Transwell filters is subtracted from all readings before performing the calculations. Cells were used for further study only if the TER value was more than 30 Ωcm^2. TER was measured at 0, 24, and 48 hours after adding vanadate. At the indicated time points, the TER value was measured and transformed to a percentage compared with the value at hour 0. All experiments were repeated six times to ensure consistent results. In all experiments, only vehicle without adding sodium orthovanadate was used as control.