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磷酸化酪胺酸的訊息傳遞對角膜內皮細胞生理機制的影響(3/3)

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行政院國家科學委員會補助專題研究計畫 成果報告

磷酸化酪氨酸的訊息傳遞對眼角膜內皮細胞生理機制的影響
Phosphotyrosine signaling and the corneal endothelial cellular physiology

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中文摘要

眼角膜內皮細胞具有角膜基質水分調控 (stable corneal hydration status) 的功能，並且具有細胞不分裂 (non-proliferative status) 的特性，而細胞與細胞的交互作用 (Cell-cell interaction)、細胞間的通透性 (junctional permeability) 和細胞緊密接觸抑制細胞分裂 (contact inhibition) 等在此能與特性中扮演很重要的角色。蛋白質酪氨酸磷酸化之訊息傳遞在許多不同種類的細胞中，已經被證實在細胞增生、細胞與細胞間的接合、細胞移行與細胞骨骼的構成中，都扮演極為重要的角色，但目前僅有非常有限的研究著重於此訊息傳導對角膜內皮 (corneal endothelial cells) 細胞與細胞間的接合 (adherences junctions) 扮演之調控角色。我們推測酪氨酸磷酸酶 (protein tyrosine phsphatase, PTP) 在角膜內皮細胞與細胞間的接合扮演極重要的角色，一但使用酪氨酸磷酸酶抑制劑 (PTP inhibitor) 來抑制其功能，就會使細胞與細胞間的結合瓦解，並誘發許多下游的反應使得細胞進行分裂和改變其通透性。

實驗材料為初代培養之牛眼角膜內皮細胞，使用釩酸鹽鈉(sodium orthovanadate；SOV) 作為酪氨酸磷酸酶的抑制劑，以不同濃度 (25、50、100μM) 處理，在不同時間點 (12、24 小時) 觀察藥物對細胞的影響。我們在螢光染色後在共軛焦顯微鏡下觀察角膜內皮細胞與細胞間的接合結構 (p120、α-catenin、N-cadherin) 與細胞分裂標識 Ki67 的變化，以西方轉漬法 (Western blot analysis) 觀察膜內皮細胞的磷酸化、角膜內皮細胞間的接合蛋白 (p120、α-catenin、N-cadherin) 與細胞分裂相關蛋白質 (cell-cycle-associated proteins : PCNA, cyclin D1, cyclin E and cyclin A) 的變化，利用穿越上皮電阻 (trans-epithelial electric resistance，TEER) 的測量來觀察角膜內皮細胞通透性的變化。

本研究結果顯示，酪氨酸磷酸酶抑制劑會促使角膜細胞蛋白質的磷酸化，而且此現象隨著濃度與時間的增加而明顯增加；酪氨酸磷酸酶抑制劑也會明顯改變角膜內皮細胞的細胞型態、破壞細胞與細胞間的接合結構、增加細胞間的通透性，都具有濃度與時間效應，但是對細胞與細胞間的接合蛋白的表現毫無影響；在細胞分裂的觀察中，酪氨酸磷酸酶抑制劑並不會使 Ki67 陽性的細胞增加，而對於細胞分裂相關蛋白質的表現毫無影響。

本研究結論，酪氨酸磷酸酶抑制劑能促使細胞與細胞間繫緊密度降低，並增加其細胞間通透性，然而卻對細胞分裂無影響。

關鍵字

酪氨酸磷酸酶、釩酸鹽鈉、細胞緊密接觸抑制細胞分裂、穿越上皮電阻、眼角膜內皮細胞、細胞間的接合、細胞增生

English Abstract

Cell–cell interaction, junctional permeability and contact inhibition are important mechanisms that allow corneal endothelial cells to maintain stable corneal hydration status and also keep these cells in non-proliferative status. Protein tyrosine phosphatases (PTPs) are well known to play an important role in regulating cell–cell contacts, growth and differentiation. Inhibition of PTPs activity by a general PTP inhibitor has been proved to trigger post-confluent rat corneal endothelial cells to reenter cell cycles. In this study, we aimed to evaluate whether protein tyrosine phosphorylation is involved in cell–cell interactions, junctional permeability and cell cycle control in post-confluent, contact inhibited bovine corneal endothelial cells.

Confluent cultures of bovine corneal endothelial cells were treated with different concentrations of general phosphatase inhibitor, sodium orthovanadate (vanadate) for several different durations. Protein tyrosine phosphorylation was confirmed by Western blot analysis. Immunocytochemistry was used to evaluate the effect of vanadate on adherens-type junctional proteins by staining of p120, N-cadherin and α-catenin. Paracelluar permeability was evaluated by transepithelial electric resistance. The effect of vanadate on cell cycle progression was confirmed by immunostaining of Ki67. Western blot analysis was used to evaluate the expression level of cell-cycle-associated proteins, including PCNA, cyclin D1, cyclin E and cyclin A.

Time-dependent effects of vanadate on protein tyrosine phosphorylation were confirmed by Western blot analysis. ICC demonstrated the effect of vanadate on the disruption of p120, N-cadherin and α-catenin. Time- and dose-effects of vanadate on the severity of p120 disruption were also found. TER demonstrated the time- and dose-effect of vanadte on paracellular permeability. Although cell–cell junctions can be broken through by vanadate, no significant increase of Ki67 positive cells was found among the control group and all groups with different concentrations/durations of vanadate treatment. Western blot also showed no change of PCNA, cyclin D1, cyclin E and cyclin A after treatment with vanadate.

In conclusion, protein tyrosine phosphatase inhibition can induce time-dependent release of cell–cell contacts and increase transepithelial permeability in post-confluent cultures of bovine corneal endothelial cells. However, such phenomenon is not enough to promoted cell cycle progression as seen in rat corneal endothelial cells.

Keywords: protein tyrosine phsphatase, vanadate, contact inhibition, trans-epithelial electric resistance, corneal endothelial cells, adherences junctions, p120, proliferation

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1. Introduction

Corneal endothelium is a single layer of neural crestderived cells located on the posterior surface of the cornea. The endothelium helps maintain corneal transparency by regulating corneal hydration through its barrier (Iwamoto and Smelser, 1965; Kreutziger, 1976) and ionic pump functions (Maurice, 1972; Geroski and Edelhauser, 1984). Adult human corneal endothelial cells are known to be mitotically inactive, and several studies demonstrate that these cells are arrested at the G1 phase of the cell cycle. (Joyce et al., 1996; Senoo and Joyce, 2000; Senoo et al., 2000) Several mechanisms have been suggested to participate in the non-proliferative status of adult corneal endothelial cells. Among all, cellecell contact inhibition is an important one that has been widely studied (Joyce et al., 1998; Senoo and Joyce, 2000; Senoo et al., 2000; Joyce, 2003).

In most epithelial cells of various tissues, the cellecell contact or so called cellular barrier is provided by a combination of the impermeability of the cellular plasma membrane and the intercellular tight junction found towards the apical surface of the cell (Gumbiner, 1987; Anderson et al., 1993; Citi, 1993). The cellular barrier also creates a boundary between the apical and basolateral domains of the plasma membrane and is important in the maintenance of cell surface polarity (Rodriguez-Boulan and Nelson, 1989). Corneal endothelial cells are different from most epithelial cells in the body because of their ‘‘leaky’’ tight junctions. Focal, rather than belt-like, tight junctions are located toward the apical aspect of the lateral membranes of corneal endothelial cells (Hirsch et al., 1977; Montcourrier and Hirsch, 1985; Stiemke et al, 1991). These leaky tight junctions are important because they permit diffusion of nutrients from the anterior chamber to the avascular corneal stroma (Mandell et al., 2006). So far, corneal endothelial cells are found to have the following junctional proteins: (1) tight junction-associated proteins, which included occludin (Stevenson et al., 1986; Furuse et al., 1993; McCarthy et al., 1996), and ZO-1 (Stevenson et al., 1986; Barry et al., 1995; Joyce et al., 1998; Petroll et al., 1999). (2) gap junctional protein, which include connexin-43 (Jongen et al., 1991). These proteins are found between cells and located on the lateral plasma membranes anterior to the tight junctions

(Iwamoto and Smelser, 1965; Leuenberger, 1973; Kreutziger, 1976; Raviola et al., 1980). These junctions are sites of dye and electrical coupling between neighboring cells (Rae et al., 1989). (3) adherens-type junctions which include N- (neuronal) cadherin (Beebe and Coats, 2000; Ickes et al., 2002), E-(epithelial) cadherin (Ickes et al., 2002), and a-, b-, and g-catenin (plakoglobin) (Petroll et al., 1999). These adhesion junction-associated proteins mediate close contact between plasma membranes of adjacent cells and the underlying actin filament network, thereby strengthening cellecell associations.

Although the anatomic structure of corneal endothelial junctions has been studied extensively, there are only limited studies on the regulation of these junctional molecules. The control of corneal endothelial junctions is clinically important for several reasons. First of all, it may control barrier functions and maintain stable corneal hydration status. In addition, it takes part in controlling the diffusion of nutrients from anterior chamber into avascular corneal stroma. Finally, it is involved in maintaining the non-proliferative status of confluent corneal endothelial cells due to the mechanism of contact inhibition. Because the adult human corneal endothelial cells are nonproliferative, marked decrease in corneal endothelial cellular counts due to aging, injury or diseases may lead to corneal endothelial decompensation and eventually corneal transplantation. Triggering adult corneal endothelial cells to reenter cell cycle by breaking through cellecell contacts or other mechanism is thus clinically important because it may have the clinical applicability to avoid corneal transplantations.

Recently, it has been proposed that in certain endothelial and epithelial cells, the ability of cellular junctions to restrict paracellular flux is not immutable. Rather, a dynamic regulatory mechanism is found in controlling the gate function of cellular junctions (Madara, 1988; Rubin, 1992). Protein kinase C (Citi, 1993), cyclic AMP (Stelzner et al., 1989; Langeler and van Hinsbergh, 1991), extracellular calcium level (Martinez-Palomo et al., 1980; Gumbiner and Simons, 1986) and protein tyrosine phosphorylation (Matsuyoshi et al., 1992; Behrens et al., 1993; Young et al., 2003) were all proven to be involve in dynamic changes of tight junctions and adherens-type junctions. Among all, studies on extracellular calcium levels (Senoo and

Joyce, 2000; Senoo et al., 2000) and protein tyrosine phosphorylation (Chen et al., 2005) have been applied to enhance corneal endothelial proliferation under the mechanism of disrupting cellecell contact. Several adherens/gap/tight junction associated proteins that help maintain corneal endothelial cells in a non-reproliferative status are calcium sensitive. These calcium-dependent junctional proteins include the cadherins (adherens-type junction proteins) (Nagar et al., 1996; Pertz et al., 1999), occludin (tight-junction protein) (McCarthy et al., 1996), ZO-1 (tight junction-associated protein) (Siliciano and Goodenough, 1988), and connexin-43 (gap junction protein) (Jongen et al., 1991). Exposure of corneal endothelial cells to calcium-free medium was found to cause disruption of apical junctions, increase transendothelial perfusion and corneal edema (Kaye et al., 1968; Stern et al., 1981). Senoo et al. (2000) also demonstrated that treating corneal endothelial cells with ethylenediamine tetra-acetic acid (EDTA), a calcium chelator that may interfere with calcium sensitive junctional proteins, will release cellecell contacts and trigger cell cycle entry in organ-cultured human donor corneas.

Protein tyrosine phosphorylation and dephosphorylation are important physiological mechanisms that control various cellular behaviors, such as proliferation, adhesion, and migration (Balsamo et al., 1998; Brady-Kalnay et al., 1993; Ostman et al., 1994; Pallen and Tong, 1991). Dephosphorylation events are mediated by a diverse group of enzymes, including protein tyrosine phosphatases (PTPs) (Barford et al., 1998). A number of studies suggest that PTPs are important in regulating the integrity of cellecell contacts, including the assembly state of adherens-type junctions (Volberg et al., 1992; Mu¨ller et al., 1999; Zondag et al., 2000) and tight junctions. This role for PTPs is suggested by the finding that PTP activity is significantly higher in confluent than in subconfluent cells (Pallen and Tong, 1991; Gebbink et al., 1995). Volberg et al. showed that pervanadate, a general PTP inhibitor, elicited tyrosine phosphorylation of adherens-type junction in MDCK cells (Volberg et al., 1992). Young et al. (1998, 2003) showed that tyrosine phosphatase inhibition increased albumin flux across postconfluent bovine pulmonary artery endothelial cells. Staddon et al. (1995) found that tyrosine phosphorylation causes a dramatic and rapid increase in the ionic permeability of tight junctions in MDCK cells,

and an increase in the tyrosine phosphorylation of b-catenin was involved. Chen et al. (2005) demonstrated that treating the confluent-cultured rat corneal endothelial cells with sodium orthovanadate, a generalized protein tyrosine phosphatase inhibitor, can break through tight-junctional protein ZO-1 and trigger confluent corneal endothelial cells to reenter cell cycles. All these data indicate that cellecell contact and junctional permeability may be regulated via mechanisms involving tyrosine phosphorylation of adherens-type junction and tight junction proteins.

In this study, we continue with Chen et al.'s study (2005), and aimed to identify the role of PTP in regulating the adherens type-junctional proteins and paracellular permeability in postconfluent bovine corneal endothelial cells. We also aimed to evaluate the possibility of cell-cycle reentry after disrupting the contact inhibition by PTP inhibition by vanadate.

2. Materials and methods

2.1. Antibodies and reagents

Polyclonal rabbit-anti-human p120, polyclonal rabbitanti-human a catenin antibody, polyclonal rabbit-anti-human cyclin A, cyclin E, Cyclin D1 and proliferating cell nuclear antigen (PCNA) antibodies, mouse-anti-human Ki67 antibody, monoclonal mouse-anti-human N-cadherin antibody, fluorescein isothiocynante (FITC)-conjugated goat-ant-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-b-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal mouse anti-phosphotyrosine antibody (clone 4G10) was purchased from Upstate Biotechnology, Inc (Lake Placid, NY).

Sodium orthovanadate (vanadate) was purchased from Sigma-Aldrich (St. Louis, MO). Mounting medium containing propidium iodide (PI) was purchased from Vector Laboratories (Burlingame, CA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fungizone, trypsin-EDTA and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY).

2.2. Culture of bovine corneal endothelial cells

Adult bovine corneal buttons were obtained from a local slaughterhouse within 3 h after enucleation. Endothelial cells were grown in single cells with the method modified from previous study (Chen et al., 2006). Briefly, the corneal endothelial layer was immersed with typsin-EDTA solution and subsequently scraped. The endothelial cell suspension was cultured in DMEM with 10% fetal bovine serum, 100 IU/ml penicillin, 100 mg/ml streptomycin and 1.5 mg/ml fungizone. The culture medium was changed every 2 days. Second passage post-confluent cells were used for experiments. In addition, cells cultured in subconfluent condition (50% and 80% confluency) and post-confluent cells at 12 h after wound scraping were used as positive control for cellular proliferation.

2.3. Immunocytochemical localization

For immunocytochemical (ICC) analysis of cultured cells, cells were seeded onto chamber slides and grown to confluence. After washing 3 times with phosphate-buffered saline (PBS), cells were fixed in 4% paraformaldehyde at room temperature for 10 min followed by a further wash with PBS. The fixed cells were then permeabilized with 0.4% Triton X-100 in PBS for 10 min. After a PBS wash, cells were pre-incubated for 10 min in 2% bovine serum albumin (BSA) in PBS (5% donkey serum when goat antibody was used) to block nonspecific binding and then incubated with primary antibody at room temperature for 2 h. Concentrations of primary antibodies were as follows: anti-p120 = 1:100; anti-a catenin = 1:100; anti-N-cadherin = 1:100; anti-Ki67 = 1:100. According to the suppliers, all primary antibodies used in these studies were cross-reactive with the appropriate bovine antigen. Following primary antibody incubation, cells were washed with PBS, incubated for 10 min with blocking buffer and then incubated for 1 h with a 1:100 dilution of the appropriate FITC-conjugated secondary antibody. To assess specificity of the staining, cells were processed without primary antibody in the control group. Cells were then washed with PBS and mounted in medium containing propidium iodide for visualization of nuclei. All experiments were repeated 3 times to ensure consistent results. Positive staining was visualized using a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) or conventional fluorescence microscopy using an Eclipse E800 Nikon Microscope with a VFM Epi-Fluorescence Attachment (Nikon, Melville, NY) equipped with a Spot Digital Camera and Spot version 1.1 CE software (Diagnostic Instruments, Sterling Heights, MI).

2.4. Protein extraction and immunoblotting

Cultured cells grown in T75 flasks were trypsinized, suspended in buffer containing 1% Triton X-100, 250 mM NaCl, 2 mM EDTA, 50 mM TriseHCl, 10 mg/ml leupeptin, and 1 mm phenylmethylsulfonyl fluoride (all from Sigma), and homogenized. Equal amounts of extracted protein were loaded on 4e12% polyacrylamide gels (Invitrogen, Carlsbad, CA) for SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp, Bedford, MA). Non-specific binding was blocked by incubation overnight at 4

_C with 5% non-fat dry milk in PBS. Membranes were then incubated at room temperature for 2 h with anti-phosphotyrosine antibody at the dilution of 1:5000, anti-p120, anti-a catenin or anti-N-cadherin primary antibodies at the same dilutions indicated for ICC, and anti-cyclin E, anti-cyclin A, anti-cyclin D1 and anti-PCNA at the dilution of 1:1000. Anti-β-actin was used as a internal control for the immunoblot. Membranes were washed 3 times with the blocking buffer and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody at a final dilution of 1:1000. After final washes with 0.1% Triton X-100 in PBS, signal was detected by enhanced chemiluminescence following manufacturer's instructions (Pierce, Rockford, IL) and exposed to autoradiographic film. All experiments were repeated 3 times to ensure consistent results.

2.5. Measurement of TER

Bovine corneal endothelial cells were seeded in the upper chamber of a Costar transwell (1.12 cm^2 diameter, 0.4 mmpore size) and allowed to reach confluency. Transepithelial/ endothelial 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 calculations. Cells were used for further study only if the TER value was more than 30 Ω cm^2. TER was measured at 0, 12 and 24 h 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 6 times to ensure consistent results.

2.6. Statistical analysis

Experimental data were analyzed using one way analysis of variance (ANOVA), Dunnett's multiple comparison test, and Student's paired t-test. The results are expressed as the mean ± standard error of the mean (SEM). A p value <0.05 was considered to be statistically significant.

3. Results

3.1. Time-dependent effect of PTP inhibition on tyrosine phosphorylation of corneal endothelial proteins

PTP inhibition with sodium orthovanate (vanadate complexed with H_2O_2) has previously been demonstrated to induce rapid and dramatic increases in the tyrosine phosphorylation of protein localized to the intercellular boundaries (Staddon et al., 1995; Ayalon and Geiger, 1997; Young et al., 2003). Time- and dose-dependent effects have been demonstrated in these studies. We therefore asked whether vanadate induced the similar tyrosine phosphorylation of corneal endothelial proteins. Accordingly, corneal endothelial cells exposed at 50 μM vanadate at different durations were processed for phosphotyrosine immunoblotting (Fig. 1). PTP inhibition induced time-dependent increase of tyrosine phosphorylation of a number of corneal endothelial proteins, and exposure time as brief as 15 min was associated with an increase in phosphotyrosine signal compared with the control group without

adding vanadate.

3.2. PTP inhibition disrupts adherence type junctional proteins in postconfluent bovine corneal endothelial cells

Cells were grown to confluence and maintained for an additional week to ensure the formation of a mature, contactinhibited monolayer. To investigate dose- and time-dependent effects, confluent cultures were incubated in 50 μM and 100 μM vanadate added in media for 12 and 24 h. The cells were then fixed and immunostained with p120, which in control confluent cultures appears as a single, discontinuous linear pattern at cell borders (Fig. 2A), indicating intact cellecell contacts. Treatment of confluent cultures with dimethyl sulfoxide (DMSO) (Fig. 2D) did not produce any changes. Treatment of confluent cells with vanadate induced a time- and dose-dependent release of cellecell contacts, resulting in the appearance of gaps between cells and a gradual loss of monolayer integrity (Fig. 2B,C,E,F). The immunostaining pattern of N-cadherin and a-catenin in cells incubated with 50 mm vanadate for 24 h showed similar result (Fig. 3).

3.3. PTP inhibition increased dose- and time-dependent trans-epithelial electric resistance of postconfluent bovine corneal endothelial cells

When cells were grown to confluency and maintained for an additional week to ensure mature cellecell contact, TER was measured to investigate the time- and dose-dependent effects of vanadate on intercellular permeability. At 12 h after vanadate treatment, 100 μM of vanadate treatment induced significant decrease of TER compared with the control group (Fig. 4). Twenty-four hours later, both 50 μM and 100 μM of vanadate treatment induced significant decrease of TER (Fig. 4). Time- and dose-depended increasing effect of vanadate on intercellular permeability was found, which was consistent with the disrupting effects of vanadate on adherenstype junctional proteins evaluated by ICC.

3.4. PTP inhibition did not promote cell cycle entry in post-confluent bovine corneal endothelial cells

Because Chen et al. (2005) have demonstrated that PTP inhibition with sodium orthovanate can promote cell-cycle progression in postconfluent cultures of rat corneal endothelial cells, we next decided to test whether such proliferationpromoting effect can be found in bovine corneal endothelial cells. For this purpose, fully confluent cultures were treated and incubated for different concentrations (0, 25, 50, 100 μM) and durations (0, 12, 24 h) of vanadate, then fixed and immunostained for Ki67, a marker of actively cycling cells that is expressed from mid-G1 phase through mitosis (Gerdes, 1991). Cells cultured in a subconfluent condition (50% confluency) (Fig. 5B) and confluent cells at 12 h after wound scraping (Fig. 5C) were found to have positive Ki67 nucleus, while less than 1% of postconfluent cells were found to reveal positive staining for Ki67 (Figs. 5A and 6A). In Fig. 6, no significant increase of Ki67 positive cells can be found in all vanadate-treated groups (Fig. 6B,C,GeI). That lack of an increase of positive Ki67 positive cells suggested that phosphotase inhibition by itself, even in medium with serum, can not promote cells to enter into the mid-G1 phase, not to mention S-phase.

3.5. PTP inhibition did not change the expression of cell-cycle associated proteins, such as

cyclin D1, cyclin E, cyclin A and PCNA

Ki67-positive cells did not increase after the treatment with vanadate in this study as postconfluent rat corneal endothelial cells (Chen et al., 2005). However Suzuki et al. (2000) demonstrated that in postconflunet human umbilical vascular endothelial cells (HUVECs), treatment with vanadate can stimulate the expression of cyclin D1, cyclin E, and cyclin A. We next examined the possibility of increased expression of earlier cell-cycle-associated proteins by vanadate, even the cells didn't show positive Ki67 results. Our results demonstrate the confluency dependent expression levels of cell-cycle regulatory factors (Fig. 7A), which were consistent with the positive Ki 67 staining result in subconfluent cells (Fig. 5). However, no change of expression levels of cyclin D1, cyclin E, cyclin A and PCNA could be found among all vanadate treated groups (Fig. 7B).

4. Discussion

In this study, we demonstrated that non-selective PTP inhibition with vanadate promotes time-dependent increase in protein tyrosine phosphorylation that precedes cell--cell junctional disruption in postconfluent bovine corneal endothelial cells. Adherens-type junction associated proteins, including p120, N-cadherin and a-catenin were found to be disrupted from their original places after treatment. Vanadate treatment also increased TER in a time- and dose-dependent manner, which confirmed the disruption of cellular junctions. However, such cellecell junctional disruption did not trigger the postconfluent bovine corneal endothelial cells to reenter cellular cycles as seen in rat corneal endothelial cells by either Ki 67 staining (Chen et al., 2005), or an increase in protein expression levels of PCNA, cyclin D1, cyclin E and cyclin A as HUVEC (Suzuki et al., 2000). These data suggested that PTPs regulate the corneal endothelial adherens-type junctional proteins and paracellular permeability. However, species- and cell-type related differences of proliferative capacity of post-confluent cultured cells under the same treatment should be noticed.

PTP inhibition itself increased tyrosine phosphorylation of proteins almost exclusively restricted to cellecell boundaries. Several studies showed that it decreased both cellecell tightand adherens-type junctional integrity (Volberg et al., 1992; Staddon et al., 1995). Tyrosine phosphorylation of adherenstype junctional proteins by PTP inhibition has been proved to reduce cellecell junctional integrity and open the vascular endothelial paracellular pathway in various studies (Bannerman and Goldblum, 1997; Esser et al., 1998; Young et al., 1998; Goldblum et al., 1999). Although most studies on the regulation of this tyrosine phosphorylation-responsive paracellular pathway have mainly emphasized in protein tyrosine kinases (PTK) driven events (Hamaguchi et al., 1993; Behrens et al., 1993; Bannerman and Goldblum, 1997; Esser et al., 1998), evidences also exist for its counter-regulation by PTPs (Volberg et al., 1992; Staddon et al., 1995). As cells become confluent with the formation and stabilization of intercellular junctions, PTP activity increases (Ostman et al., 1994; Gaits et al., 1995). Whereas adherens-type junctional protein tyrosine phosphorylation decreases (Ayalon and Geiger, 1997; Lampugnani et

al., 1997). Several PTPs, such as PTP-m, PTP-k, LAR-PTP, SHP-2, PTP-1B and PTP-l have been proved to involve in this process (Brady-Kalnay et al., 1993, 1995; Gebbink et al., 1995; Fuchs et al., 1996; Kypta et al., 1996; Cheng et al., 1997; Balsamo et al., 1998; Brady-Kalnay et al., 1998; Mu¨ller et al., 1999; Ukropec et al., 2000; Zondag et al., 2000). In addition, the disruption of cellular junctions by PTP inhibition were found to decrease transcellular electric resistance and increase paracellular permeability (Volberg et al., 1992; Staddon et al., 1995). With these combined data in mind, we first asked whether PTPs also participate in the regulation of adherens-type junctional protein in corneal endothelial cells as other cell types.

In our study, non-selective PTP inhibition with vanadateinduced time-dependent increase of protein tyrosine phosphorylation in corneal endothelial proteins. We then evaluated the cellular distribution pattern of several adherens-type junctions- associated proteins such as p120, N-cadherin and a-catenin after PTP inhibition. The confocal microscopy results demonstrated time- and dose-dependent disruption of these proteins after vanadate treatment. The disrupted proteins were demonstrated from single-line staining by immunostaining into double-lines formation at lower doses of vanadate (Fig. 2C). When the cells were treated with 100 mM of vanadate for 24 h, p120 seems to translocate from cellecell interface to the cytoplasm (Fig. 2F). Because the protein expression levels of p120, N-cadherin and a-catenin evaluated byWestern blot analysis in different vanadate treatment groups did not change (data not shown), and several studies demonstrated that the adheres-type junctional proteins translocated from cellecell interface to the cytoplasm after PTP inhibition (Kim and Lee, 2001; Young et al., 2003), we supposed that p120, N-cadherin and a-catenin became tyrosine phosphorylated after PTP inhibition, and translocated from cellular border into the cytoplasm without altered total protein expression level. Such phenomenon can be further confirmed by TER. In our study, TER also show time- and dose-dependent decrease of electric resistance after vanadate treatment, which is consistent with the staining pattern of adherens-type junctional proteins by confocal microscopy. These data indicate that non-selective PTP inhibition disrupt corneal endothelial adherens-type junctional protein, and such phenomenon is consistent with the other studies (Young et al., 2003). However, because the total protein expression level of p120, a-catenin and N-cadherin did not

change, protein translocation instead of expression level change was involved in this process.

Previously, Chen et al. has demonstrated that phosphatases are involved in regulation of tight junctional integrity and cell proliferation in postconfluent rat corneal endothelial cells (Chen et al., 2005). PTP-m, PTP1B, SHP-1, SHP-2, and PTEN were expressed in rat corneal endothelium and may be involved in regulation of contact inhibition in these normally non-proliferating cells. In this study, we found that PTP inhibition did promote dose- and time-dependent disruption of bovine corneal endothelial cellular junctions as rat corneal endothelial cells. Interestingly, those bovine corneal endothelial cells do not proliferate after treating with vanadate as rat cells. In Chen et al.'s study, the Ki67 positive cells after vanadate treatment could be found even in the absence of serum. In this study, the positive Ki67 cells did not increase after vanadate treatment either in the presence or absence of serum. We therefore tried to investigate whether the cell-cycle entry did exist, but the cells just enter the very beginning phases of cell cycles. Since Suzuki et al. (2000) has found the change of cell-cycle-associated proteins, such as cyclin D1, cyclin E and cyclin A after vanadate treatment in HUVEC, we evaluated the protein expression levels of similar cell cycle associated proteins and found no changes. The reason for the nonresponsiveness of postconfluent bovine corneal endothelial cells to enter cell-cycle compared with rat corneal endothelial cells and HUVEC needed to be clarified. Mechanisms other than contact inhibition still exist and control the cell cycle reentry in certain species and certain types of postconfluent contact-inhibited cells. Species-related differences in corneal endothelial proliferative capacity have been reported before. In vivo and ex vivo observations, as well as tissue culture studies, suggest that the relative proliferative capacity of corneal endothelial cells may differ among species. For example, bovine (Savion et al., 1982), rabbit (Van Horn et al., 1977) and rat (Chen et al., 1999) endothelial cells grow easily in culture. On the other hand, cat (Van Horn et al., 1977), monkey (Matsubara and Tanishima, 1983) and human cells (Baum et al., 1979) do not culture as readily. The reason of this speciesrelated difference in endothelial proliferative activity has not been adequately determined. One possible explanation is that most laboratory animals are quite young and the relative proliferative activity of their endothelial cells may be closer to that of human neonates

than of adults. Because the bovine corneal endothelial cells in this study are from adult animals, this can partially explain the non-responsiveness of vanadate in the proliferating capacity. In addition, other methods of disrupting cell junctions, such as calcium depletion (Senoo et al., 2000), SiRNA knockdown (Yanagiya et al., 2007; Akoyev and Takemoto, 2007), or functional-blocking antibodies (Mandell et al., 2006; West et al., 2002) should be used to examine how loss of cell contacts affects corneal endothelial proliferations.

To the best of our knowledge, this is the first study regarding phosphatase function, cellecell adheres-type junctional proteins and permeability in corneal endothelium. In addition, this is the first study that demonstrated that phosphatase inhibition can not trigger the similar corneal endothelial cell-cycle reentry in bovine corneal endothelial cells as rat cells. Although underling mechanisms for this phenomenon need to be clarified, and rat/bovine cells may be quite different from human ones, the results of this study suggest that phosphatases may play an important role in regulating the integrity of cell--cell junctions, and the clinical applicability may not be so optimistic due to the non-responsiveness of cell-cycle reentry. This study has opened a avenue for future investigation and may lead to a greater understanding of the specific mechanisms underlying regulation of contact-dependent inhibition in corneal endothelium.

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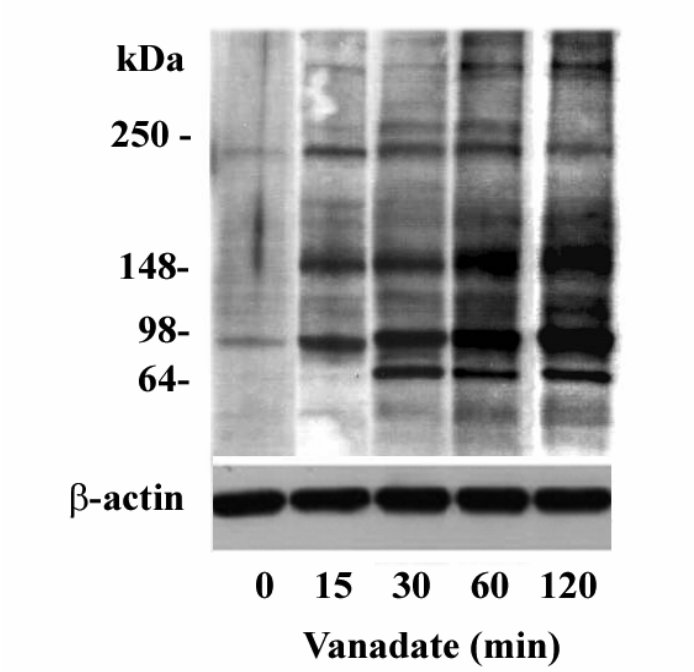

Figure 1. Effect of PTP inhibition with vanadate on protein tyrosine phosphorylation in corneal endothelial cells. Time-dependent effect of protein phosphorylation was found. Corneal endothelial cells were exposed at 50μM of vanadate for 15, 30, 60 and 120 minutes. Cell lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes, and the blots were probed with anti-phosphotyrosine antibody. To confirm equivalent protein loading and transfer, we stripped and reprobed each blot with anti β̃actin antibody. Molecular masses in kDa are indicated at *left*. Each blot is representative of 3 experiments.

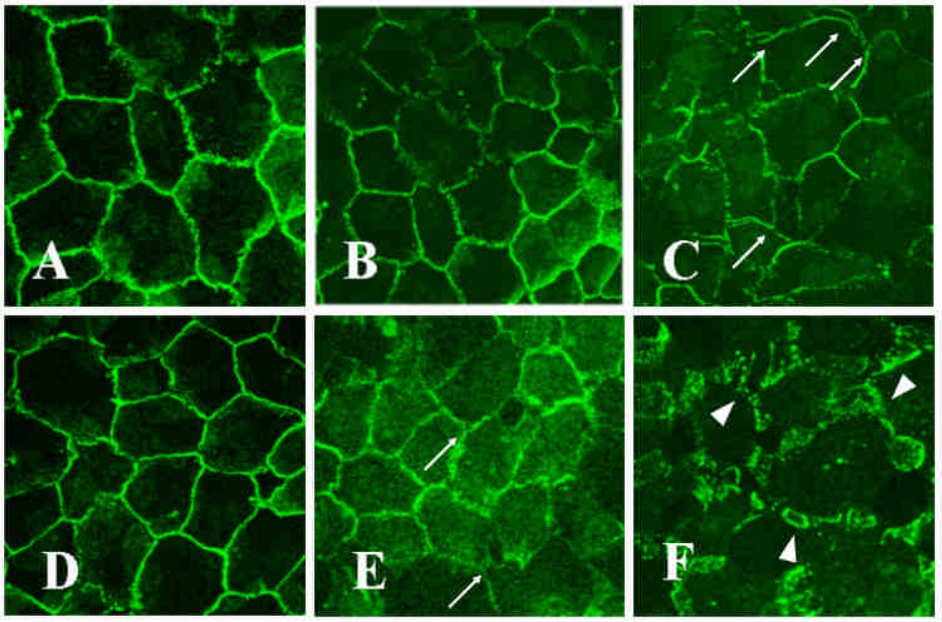

Figure 2. Representative images of immunolocalization of p120 in post-confluent cultures of bovine corneal endothelial cells. Control cultures were maintained in medium (A), and medium added with DMSO (D). Other cultures were incubated in 50μM vanadate for 12 hrs (B) and 24 hrs (C) and 100μM vanadate for 12 hrs (E) and 24 hrs (F) prior to immunostaining. Arrows in C and E indicate double lines or intercellular break in the staining pattern of p120. Arrow heads in F indicate severe disruption of p120 staining pattern with fragmentations of staining protein. Original magnification=400×. Confocal microscopy.

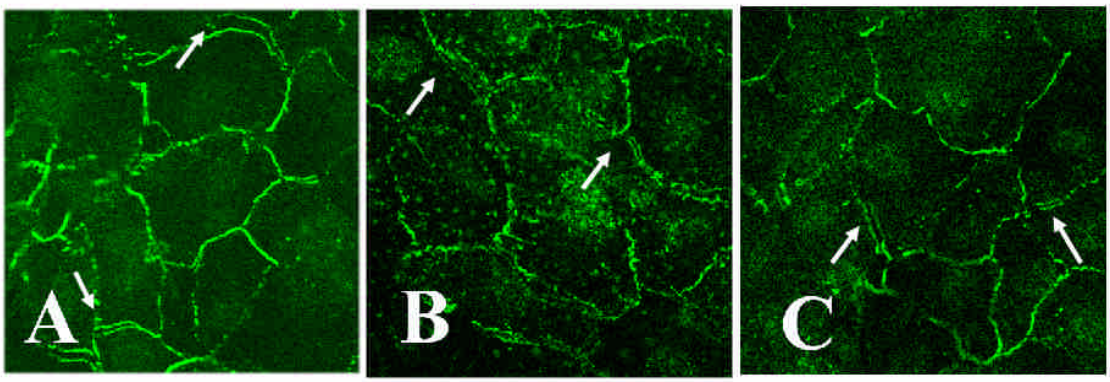

Figure 3. Representative images of immunolocalization of (A) p120, (B) N-cadherin and (C)α-catenin in post-confluent cultures of bovine corneal endothelial cells. Post-confluent cells were incubated in 50μM vanadate for 24 hrs prior to immunostaining. Arrows indicate double lines or intercellular breaks in the staining pattern of p120, N-cadherin andα-catenin. Original magnification=400×. Confocal microscopy

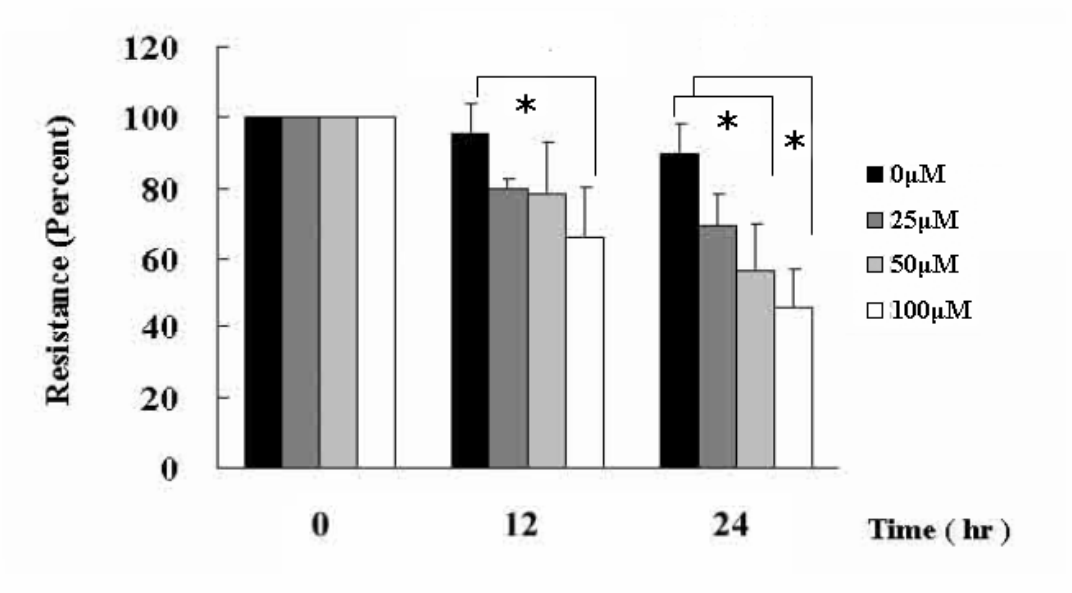

Figure 4. The effects of vanadate on trans-epithelial electric resistance of post-confluent bovine corneal endothelial cells. Post-confluent corneal endothelial cells were incubated in the absence (■) or presence of (■) 25μM, (■) 50μM, and (□)100μM of vanadate for 12 and 24 hours. At the indicated time points, the TER was measured and transformed to percentage compared to the hour 0. *P<0.05 tested by Dunnett's test.

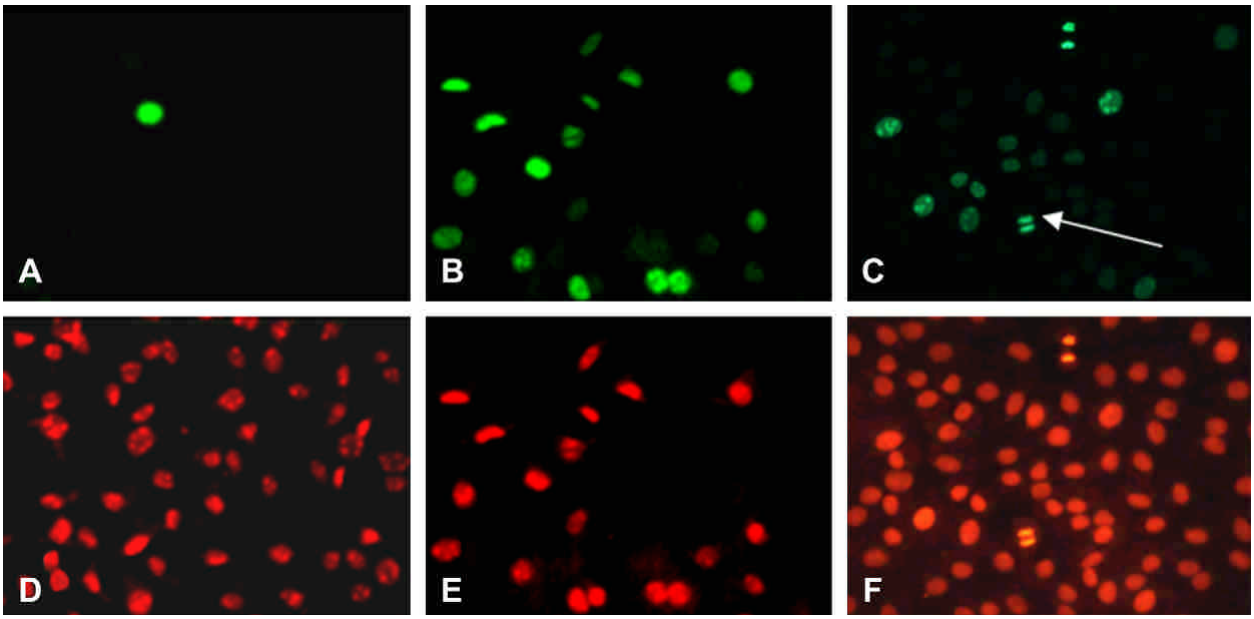

Figure 5. Ki 67 positive staining can be seen in bovine corneal endothelial cells under proliferative conditions. (A, D) Post-confluent bovine corneal endothelial cells. Less than 1% of nuclei showed positive Ki 67 staining; (B, E) Subconfluent bovine corneal endothelial cells. Nearly 100% of nuclei showed positive Ki67 staining; (C, F) Bovine corneal endothelial cells close to scrape wound. Positive Ki67 nuclei with dividing nuclei (whit arrow) can be seen. Green: Ki67; Red: PI. Original magnification 400X.

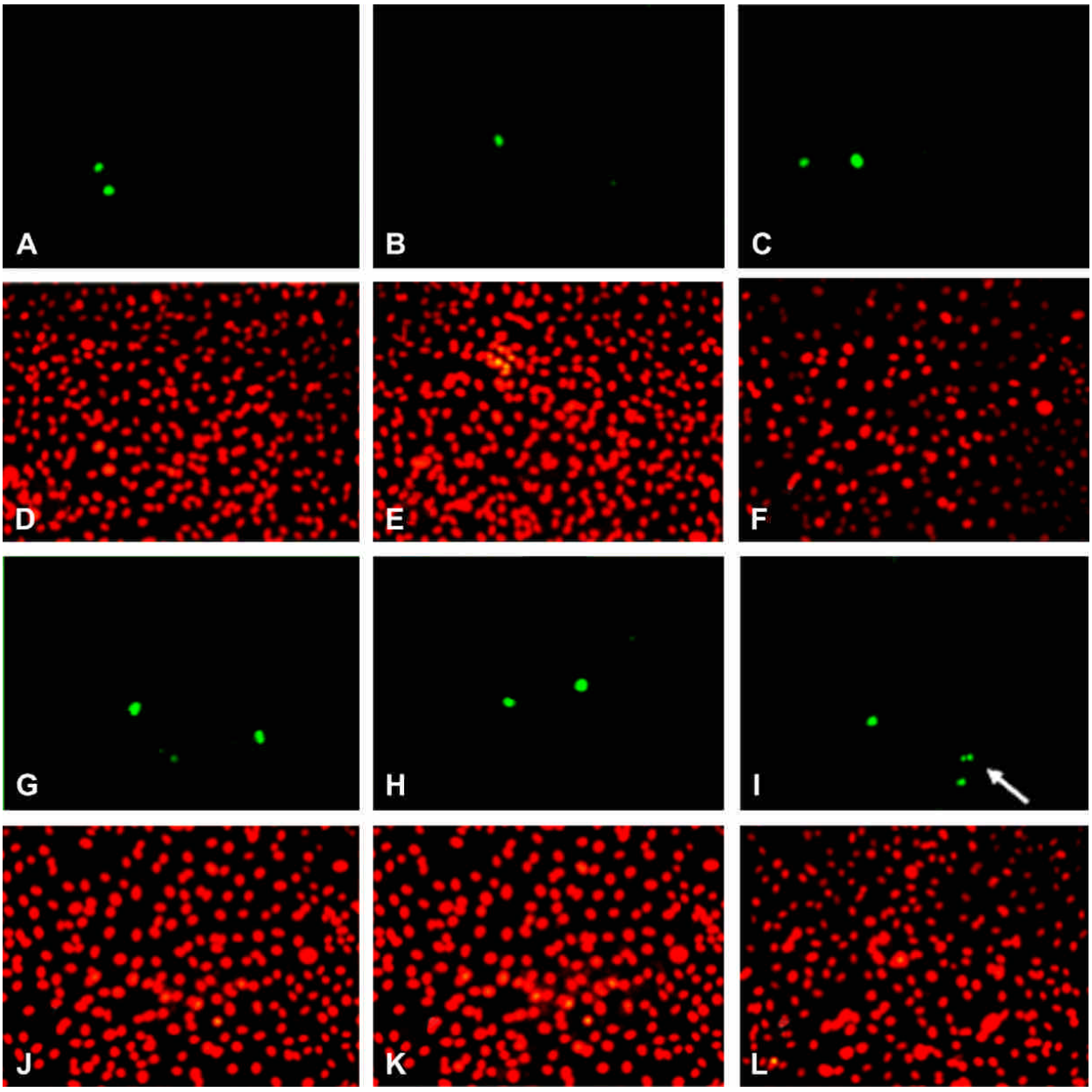

Figure 6. The effects of PTP inhibition on Ki67 staining pattern in post-confluent, contact inhibited bovine corneal endothelial cells incubated with serum. (A, D) without vanadate; (B, E) in 50 μM vanadate for 12 h (C, F) in 50 μM vanadate for 24 h; (G, J) in 100 mM vanadate for 6 h, (E, K) 1 in 100 μM vanadate for 12 h (F, L) in 100 μM of vanadate for 24 h. In all conditions, less than 1% of nuclei showed positive Ki67 staining. There was no significant difference among all groups. White arrow indicates dividing cells occasionally found in all groups. Green: Ki67; Red: PI. Original magnification: 200X.

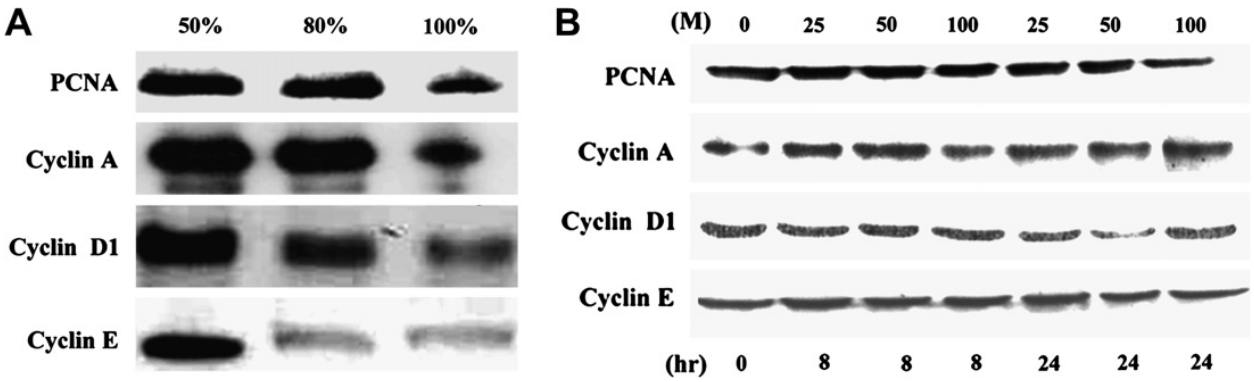

Figure 7. Effect of PTP inhibition with vanadate on expression level of cell cycle regulatory factors in post-confluent bovine corneal endothelial cells. (A) Corneal endothelial cells showed confluency dependent expression levels of cell cycle regulatory factors. 50%: Cells cultured to 50% subconfluent condition; 80%: Cell cultured to 80 % subconfluent condition; 100%: Post-confluent corneal endothelial cells. (B) Post-confluent, contact inhibited cells were stimulated with medium in the presence of different concentrations and durations of vanadate. Seventy μg of each protein extract was immunoblotted with the indicated antibodies. The same protein exacts were used for all of the immunoblots. To confirm equivalent protein loading and transfer, we stripped and reprobed each blot with anti β̃-actin antibody. Each blot is representative of 3 experiments.

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Dear Dr Hu,

I am pleased to tell you that your work has now been accepted for publication in Experimental Eye Research.

You will shortly receive an acknowledgement letter from the Production Log In Department with further administrative instructions.

Thank you for submitting your work to Experimental Eye Research. I look forward to seeing your work published in our journal.

Yours sincerely,

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