厚生労働科学研究費補助金難治性疾患克服研究事業
Fuchs 角膜内皮変性症および関連疾患に関する調査研究
分担研究報告書

Fuchs 角膜内皮変性症および関連疾患に対する角膜内皮移植の有効性の検討

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研究要旨：Fuchs 角膜内皮変性症の進行例は水疱性角膜症に至るが、本研究では京都府立医科大学における Fuchs 角膜内皮変性症に対する新しい治療方法である角膜内皮移植術(Descemet Stripping Automated Endothelial Keratoplasty:DSAEK)の有効性と治療予後について検討する。

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A．研究目的

Fuchs 角膜内皮変性症では病態の進行により角膜内皮細胞が減少し、水疱性角膜症に至り視機能障害に至る。従来は全層角膜移植が唯一の治療方法であったが、近年は新しい外科移植治療として角膜内皮移植術が導入され、視機能回復面からも有効性が期待されている。今回、我々は Fuchs 角膜内皮変性症および水疱性角膜症に対する角膜内皮移植術(DSAEK)の有効性および Fuchs 角膜内皮変性症と他疾患との治療比較を行った。また移植後の角膜内皮細胞数の推移について検討を行った。

B．研究方法

対象は、2007 年 8 月から 2010 年 7 月の間に京都府立医科大学および関連施設において Fuchs 角膜内皮変性症を含む水疱性角膜症に対して DSAEK を施行し、術後に拒絶反応や内眼手術の追加処置無く経過観察が可能であった 100 例 104 眼。方法はトロスペクティブに視機能回復、グラフト生着率、角膜内皮細胞数の推移などの臨床評価について検討した。角膜内皮細胞の変化は非接触型および接触型のスペキュラーマイクロスコープを用いて観察した。

（倫理面への配慮）

角膜内皮移植術および臨床評価に対する研究は京都府立医科大学倫理委員会での承認を得て実施した。

C．研究結果

Fuchs 角膜内皮変性症の角膜内皮移植に占める割合

全対象の 100 例 104 眼の性別は男性が 50 眼、女性が 54 眼であり、手術施行時の平均年齢は 72.9±10.6 歳であった。水疱性角膜症の第一原因はレーザー虹彩切開術後水疱性角膜症（LIBK）が 40 眼（39%)であり、Fuchs 角膜内皮変性症 14 眼（13%）であり、欧米とは異なる比率を示した。

角膜内皮移植後の内皮細胞数動態と Fuchs 角膜内皮変性症の関連性

角膜内皮移植術全体での角膜内皮細胞数の推移はプレカット前の平均ドナーECD は 2,946±313 cells/mm^{2}であり、プレカット処理による ECD 減少率は 5.1%であった。

また術後 6, 12, 24 ヵ月での ECD は、2,039 ±478 cells/mm²、1,919±550 cells /mm²、1,598±596 cells /mm²であり、減少率は 30.4%、34.6%、44.3%と良好であった。手術による ECD 減少率は、プレカット後と術後 1 ヵ月の間の減少率とし、平均減少率は 19.9% (96 眼)と良好であったが、グラフトの接着不良のため、9 眼 (9%) で初回手術以降に再度空気注入術を必要とし、これらの症例での ECD 減少率は 31.7%であり、再注入を行わなかった例と比べると有意に高かった (p=0.01) 。しかし接着不良のリスク因子としては角膜後面不正および眼内レンズ縫着であり Fuchs 角膜内皮変性症の原疾患はリスク因子とはならなかった。

原疾患と ECD 減少の関連性の検討では、術後 6 ヵ月での主要な原疾患別の ECD は、LIBK が 2,144±401 cells/mm² (37 眼) 、PBK が 1,911±508 cells/mm² (17 眼) 、多重緑内障手術後が 1,828±609 cells/mm² (12 眼) 、Fuchs 角膜内皮ジストロフィが 2,240±299 cells/mm² (10 眼) であり、他疾患と同等の内皮細胞生着が可能であった。唯一、多重緑内障手術後は他の群に比べて ECD が低い傾向にあったが統計学的な有意差は認めなかった。術後 12 ヵ月での原疾患別の ECD は LIBK で 2,098 ± 393 cells/mm² (24 眼) 、PBK が 1,898±659 cells/mm² (10 眼) 、多重緑内障手術後が 1,538±776 cells/mm² (8 眼) 、Fuchs 角膜内皮ジストロフィが 2,247±444 cells/mm² (10 眼) であり、同様の傾向であった

D．考察

今回、我々は新しい角膜内皮移植術の Fuchs 角膜内皮変性症に対する有効性について検討した。対象とした水疱性角膜症に占める Fuchs 角膜内皮変性症の割合は 13%と低く、欧米とは異なる原疾患内訳を示し、このことは疫学的背景および遺伝的背景の違いを反映していると考えられた。しかし日本での第一原因であるレーザー虹彩切開術後の症例には Fuchs 角膜内皮変性症が含まれている可能性があるため今後の検討が必要である。Fuchs 角膜内皮変性症の占める割合は欧米に比較して少ないものの、水疱性角膜症に至る症例や医原性水疱性角膜症の原因になることは明瞭であり、今後の進行予防的な治療法の開発が必要と考えられる。

本研究により、現在では角膜内皮移植術は Fuchs 角膜内皮変性症の外科的治療に安全であり、Fuchs 角膜内皮変性症においては良好なグラフト接着が可能であり、第一治療選択となりうることが裏付けられた。また術後の角膜内皮細胞数の推移は欧米の既報告とほぼ同等であり、本国にける新規治療方法として安全に導入されていることが証明された。また Fuchs 角膜内皮変性症に関しては多重緑内障手術眼以外の疾患と同等の内皮細胞数が維持できており、短期間の検討であるものの、その有効性が示された。今後は長期的な内皮細胞数の推移に Fuchs 角膜内皮変性症がどのように影響するかが検討課題の一つである。

E．結論

角膜内皮移植を必要とした水疱性角膜症に占める Fuchs 角膜内皮変性症の割合は 13%であった。また Fuchs 角膜内皮変性症に対する角膜内皮移植術(DSAEK)の手術成績は良好であり、他疾患と同様の角膜内皮細胞数の推移が期待でき、今後の Fuchs 角膜

内皮変性症の外科的治療の第一選択となると考えられる。

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G．健康危険情報

なし

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I．知的所有権の取得状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

Role of Insulin in Regulation of Na^+-/K^+-Dependent ATPase Activity and Pump Function in Corneal Endothelial Cells

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Purpose. The Na^+-/K^+-dependent ATPase (Na,K-ATPase) expressed in the basolateral membrane of corneal endothelial cells plays an important role in the pump function of the corneal endothelium. The role of insulin in the regulation of Na,K-ATPase activity and pump function in corneal endothelial cells was investigated.

Methods. Confluent monolayers of mouse corneal endothelial cells were exposed to insulin. ATPase activity was evaluated by spectrophotometric measurement of phosphate released from ATP with the use of ammonium molybdate; Na,K-ATPase activity was defined as the portion of total ATPase activity sensitive to ouabain. Pump function was measured with the use of a Ussing chamber; pump function attributable to Na,K-ATPase activity was defined as the portion of the total short-circuit current sensitive to ouabain. Western blot analysis and immunocytochemistry were performed to measure the expression of the Na,K-ATPase α_1-subunit.

Results. Insulin increased the Na,K-ATPase activity and pump function of cultured corneal endothelial cells. These effects were blocked by protein kinase C (PKC) inhibitors and protein phosphatases 1 and 2A inhibitor. Western blot analysis indicated that insulin decreased the ratio of the inactive Na,K-ATPase α_1-subunit. Immunocytochemistry indicated that insulin increased the cell surface expression of the Na,K-ATPase α_1-subunit.

Conclusions. These results suggest that insulin increases the Na,K-ATPase activity and pump function of cultured corneal endothelial cells. The effect of insulin is mediated by PKC and presumably results in the activation of PP1, 2A, or both, which are essential for activating Na,K-ATPase by α_1-subunit dephosphorylation. (*Invest Ophthalmol Vis Sci.* 2010;51: 3935–3942) DOI:10.1167/iovs.09-4027

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Clinical observations of a higher incidence of persistent corneal edema after vitrectomy and other surgical procedures for patients with diabetes mellitus have suggested that there is abnormal corneal endothelial function associated with diabetes mellitus.[1–6] Specular microscopic studies have shown morphologic abnormalities such as less endothelial cell density and increased endothelial pleomorphism in patients with type 1 and type 2 diabetes mellitus.[6–15] Some clinical studies have shown that patients with diabetes tend to have slightly thicker corneas and reduced recovery rates from hypoxia-induced corneal edema.[16–19]

The Na^+-/K^+-dependent ATPase (Na,K-ATPase), expressed in the basolateral membrane of corneal endothelial cells, is primarily responsible for the pump function of the corneal endothelium.[20] Herse and Adams[21,22] have shown that functional abnormalities, such as increased corneal thickness and decreased ability to recover from corneal edema in alloxan-induced diabetic rabbits, are associated with decreased Na,K-ATPase activity in the corneal endothelium. Whikehart et al.[23] have reported that elevated glucose levels reduce Na,K-ATPase activity in cultured bovine corneal endothelial cells. McNara et al.[24] have shown that acute hyperglycemia affects corneal hydration control in humans. These results suggest that a high glucose level itself is responsible for reduced Na,K-ATPase activity.

However, there might be another mechanism of reduced Na,K-ATPase activity in the corneal endothelia of persons with diabetes. It is well known that a lack of insulin plays a principal role in the pathogenesis of type 1 diabetes mellitus and that the intracellular insulin signal is reduced because of insulin resistance in type 2 diabetes mellitus.[25,26] Several studies have shown that insulin directly enhances Na,K-ATPase activity in skeletal muscle, liver, kidney, adipocytes, lymphocytes, avian salt glands, and many other cells and organs.[27–37] Insulin is present in the aqueous humor of rabbits at a concentration of approximately 3% of that in plasma, and the aqueous humor insulin concentration of alloxan-induced diabetic rabbits after feeding is lower than that of normal control animals.[38,39] Anderson and Fischberg[40] have reported that insulin has a significant effect on transendothelial fluid transport in rabbit cornea. Therefore, we hypothesized that a lack of insulin or a reduced level of intracellular insulin signaling may have a direct effect on the Na,K-ATPase activity of the corneal endothelial cells. Insulin and insulin-like growth factor-I (IGF-I) have been reported to stimulate DNA synthesis and cell proliferation in corneal endothelium by insulin receptor or IGF-I receptor,[41,42] whereas the effect of insulin on the Na,K-ATPase activity of corneal endothelial cells remains unknown.

Insulin and several other hormonal agents activate signaling pathways, including those mediated by protein kinases such as protein kinase C (PKC). A direct effect of PKC on Na,K-ATPase activity has been demonstrated in various tissues.[27,43] To date,

PKC is regarded to trigger the rapid action of insulin on the Na,K-ATPase and to be involved in the stimulation of the Na,K-ATPase by insulin in muscle cells.[27]

To investigate the role of insulin in the control of the Na,K-ATPase in corneal endothelial cells, we examined the effects of insulin activation on the enzymatic activity and pump function of Na,K-ATPase in cultured mouse corneal endothelial cells. We also examined whether PKC and its related enzymes, protein phosphatase 1 and 2A, might mediate the insulin activation of Na,K-ATPase.

METHODS

Chemicals

Ammonium molybdate reagent (Biomol Green) and phosphate standards were obtained from Biomol Research Laboratories (Plymouth, PA). Mammalian protein extraction reagent (M-PER) and BCA protein assay kit were obtained from Pierce Biotechnology (Rockford, IL). Anti-Na,K-ATPase α_1 antibody was obtained from Cosmo Bio (Tokyo, Japan). Anti-phospho-Na,K-ATPase α_1 (Ser18) antibody was obtained from Cell Signaling (Danvers, MA). Anti-β-actin antibody (AC-15) was obtained from Abcam (Cambridge, MA). ABC rabbit IgG kit (Vectastain Elite) was obtained from Funakoshi (Tokyo, Japan). Western blot analysis detection reagent (ECL Plus) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Reagent (ProLong Gold Antifade) with DAPI and signal enhancer (Image iT) were obtained from Life Technologies Japan (Tokyo, Japan). Insulin, ouabain, staurosporine, okadaic acid, and other chemicals were obtained from Sigma (St. Louis, MO). Water-insoluble compounds were dissolved in a minimal volume of methanol or dimethyl sulfoxide, with equal amounts of these solvents added to control solutions; the final concentration of methanol or dimethyl sulfoxide in incubations was <0.3% and was found to have no effect on adenosine triphosphate (ATP) hydrolysis or short-circuit current (data not shown).

Cell Culture

A simian virus 40 (SV40)-transformed mouse corneal endothelial cell line (C3H derived) was the kind contribution of the late J. Wayne Streilein (Harvard Medical School). The cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were passaged at a split ratio of 1:2 to 1:4, at which they become confluent in 4 to 7 days.

Experiments examining PKC activity or Na,K-ATPase activity were performed with cells cultured in 24-well culture plates (Nunc, Roskilde, Denmark). Ussing chamber experiments were performed with confluent monolayers of cells cultured on six-well polycarbonate inserts (Snapwell; Corning, Acton, MA) with a membrane pore size of 0.4 μm. The insert membrane growth area was 4.67 cm^2. All experiments were performed with cell monolayers within 1 day of the cells reaching confluence and with the cells maintained in the culture incubator at 37°C.

Measurement of Na,K-ATPase Activity

The culture medium was removed from cell monolayers, ultrapure distilled water (150 μL) was added to each well, and the culture plate was then placed in liquid nitrogen for 10 seconds before the addition to each well at room temperature of 150 μL solution containing 80 mM histidine, 20 mM KCl, 6 mM $MgCl_2$, 2 mM EGTA, alamethicin (2 μg/mL), 30 μM digitonin, and 200 mM NaCl at pH 7.4.[44] To duplicate wells, 10 μL of 30 mM ouabain (final concentration, 1 mM) or vehicle were added, and the plate was incubated for 30 minutes at 37°C. After the further addition of 10 μL of 300 mM ATP (final concentration, 10 mM), the reaction mixtures were incubated for an additional 30 minutes at 37°C. The ATP hydrolysis reaction was terminated by the addition of 75 μL of 50% trichloroacetic acid to each well. The contents of each well were then centrifuged at 3000 rpm for 10 minutes at room temperature.

The resultant supernatants were diluted 50-fold with ultrapure distilled water, and portions (50 μL) of the diluted samples were added to tubes containing 100 μL ammonium molybdate reagent (Biomol Green; Biomol Research Laboratories) for determination of phosphate content by measurement of absorbance at 640 nm. Phosphate solutions of 0 to 40 μM were used as standards. The Na,K-ATPase activity was calculated as the difference in ATPase activity between cells exposed to ouabain and those not exposed and was expressed as millimoles of ATP hydrolyzed per milligram of protein per hour. The protein concentration of the reaction mixtures was determined by the method of Lowry et al.,[45] with bovine serum albumin as the standard.

Measurement of Pump Function

The pump function of confluent monolayers of corneal endothelial cells was measured with the use of a Ussing chamber basically as described previously.[46,47] The cells cultured on inserts (Snapwell; Corning) were placed in a Ussing chamber (EM-CSYS-2; Physiologic Instruments, San Diego, CA). The endothelial cell surface side was in contact with one chamber, and the insert membrane side was in contact with another chamber. The chambers were carefully filled with Krebs-Ringer bicarbonate (120.7 mM NaCl, 24 mM $NaHCO_3$, 4.6 mM KCl, 0.5 mM $MgCl_2$, 0.7 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, and 10 mM glucose bubbled with a mixture of 5% CO_2, 7% O_2, and 88% N_2 to pH 7.4). The chambers were maintained at 37°C by an attached heater. The short-circuit current was sensed by narrow polyethylene tubes positioned close to either side of the insert, filled with 3 M KCl and 4% agar gel, and connected to silver electrodes. These electrodes were connected to a computer through a Ussing system (VCC-MC2; Physiologic Instruments) and research grade recorder (iWorx 118; iWorx Systems, Dover, NH), and the short-circuit current was recorded (Labscribe 2 Software for Research; iWorx Systems). After the short-circuit current had reached steady state, ouabain (final concentration, 1 mM) was added to the chamber, and the short-circuit current was measured again. The pump function attributable to Na,K-ATPase activity was calculated as the difference in short-circuit current measured before and after the addition of ouabain.

Measurement of PKC Activity

After removal of the culture medium, cells were washed with phosphate-buffered saline and lysed in 1 mL solution containing 20 mM MOPS (pH 7.4), 50 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium vanadate, 5 mM EGTA, 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/mL), and aprotinin (10 μg/mL). The lysates were centrifuged at 13,000 rpm for 15 minutes at 4°C, and the resultant supernatants were assayed for PKC kinase activity with the use of a PKC kinase activity assay kit (Assay Designs, Ann Arbor, MI) based on a solid-phase enzyme-linked immunosorbent assay that recognizes the phosphorylated form of the substrate. PKC activity was expressed as nanograms of phosphorylated substrate formed per milligram of protein. The protein concentration of lysate supernatants was assayed by the method of Lowry et al.,[45] with bovine serum albumin as the standard.

Western Blot Analysis of Na,K-ATPase α_1-Subunit

The culture medium was removed from cell monolayers and then lysed with M-PER. The supernatant was collected, and total protein in each sample was measured with the use of a protein assay reagent (Advanced; Cytoskeleton, Inc., Denver, CO) BCA protein assay kit. Approximately 1 μg of each sample was separated on a 7.5% polyacrylamide gel containing sodium dodecyl sulfate and then was transferred to polyvinylidene membranes. After blocking with 1% normal goat or bovine serum and 0.1% Tween-20 in TBS, the membrane was incubated with anti-Na,K-ATPase α_1 antibody (1:5000 dilution with TBS), anti-phospho-Na,K-ATPase α_1 (Ser18) antibody (1:2000 dilution with TBS), or anti β-actin (AC-15) antibody (1:2000 dilution with TBS)

overnight at 4°C. Incubation with biotinylated secondary antibodies was followed with the use of elite reagent (Vectastain ABC; Vector Laboratories) for 30 minutes at room temperature. Positive immunoreactions were made visible by an enhanced chemiluminescence Western blot analysis reagent detection system (ECL Plus; Amersham Pharmacia Biotech). A densitometer (ChemiDoc XRS; Bio-Rad, Hercules, CA) was used for quantization of band intensities.

Immunocytochemistry

The Na,K-ATPase α_1-subunit was studied by indirect immunocytochemistry. Corneal endothelial cells cultured on four-well chamber slides were fixed at room temperature for 15 minutes in 4% formaldehyde in PBS. After three PBS washes, the specimens were incubated for 30 minutes in signal enhancer (Image iT; Life Technologies Japan). After two PBS washes, the specimens were incubated for 30 minutes in 10% normal goat serum to block nonspecific binding. This was followed by overnight incubation at 4°C with 1:500-diluted rabbit anti-Na,K-ATPase α_1 antibody (final concentration, 2 μg/mL) and three washes in PBS. Corneal endothelial cells were then incubated for 1 hour in a 1:500 dilution of FITC-conjugated goat anti-rabbit IgG antibody and again washed three times in the dark. Specimens were mounted on glass slides with anti-fading mounting medium containing 4′,6-diamino-2-phenylindole (ProLong Gold Antifade Reagent with DAPI; Life Technologies Japan), and the slides were inspected with a confocal microscope (Radiance 2100; Bio-Rad).

Statistical Analysis

Data are presented as mean ± SD and were compared by Student's *t*-test with the use of spreadsheet software (Excel 2003; Microsoft, Redmond, WA). $P < 0.05$ was considered statistically significant.

Results

Effect of Insulin on Na,K-ATPase Activity

To determine whether insulin affects Na,K-ATPase activity in corneal endothelial cells, we exposed the cells to 0.1 μM insulin for various times, and ouabain-sensitive ATP hydrolysis was measured. Insulin had a transient, stimulatory effect on Na,K-ATPase activity that was significant at 6 hours and 12 hours; after that, Na,K-ATPase activity returned to the baseline (Fig. 1A). The stimulatory effect of insulin on Na,K-ATPase activity was also concentration dependent and was apparent at 0.01 to 10 μM (Fig. 1B).

Effect of Insulin on Pump Function

We next examined whether insulin affects the pump function of corneal endothelial cells. Tracings of short-circuit current obtained with a Ussing chamber revealed that insulin at 0.1 μM increased the ouabain-sensitive pump function of the cells compared with that observed for control cells (Fig. 2A). This effect of insulin was statistically significant at 6 hours, similar to the results obtained from Na,K-ATPase activity measurements (Fig. 2B). The stimulatory effect of insulin on pump function was concentration dependent and was apparent at 0.01 to 10 μM (Fig. 2C).

Activation of PKC by Insulin in Corneal Endothelial Cells

To examine whether insulin activates PKC in cultured mouse corneal endothelial cells, we measured PKC activity in cell extracts after treatment of the cells with various concentrations of insulin for 30 minutes Insulin indeed increased PKC activity in a concentration-dependent manner; this effect was significant at concentrations of 0.1 to 10 μM (Fig. 3).

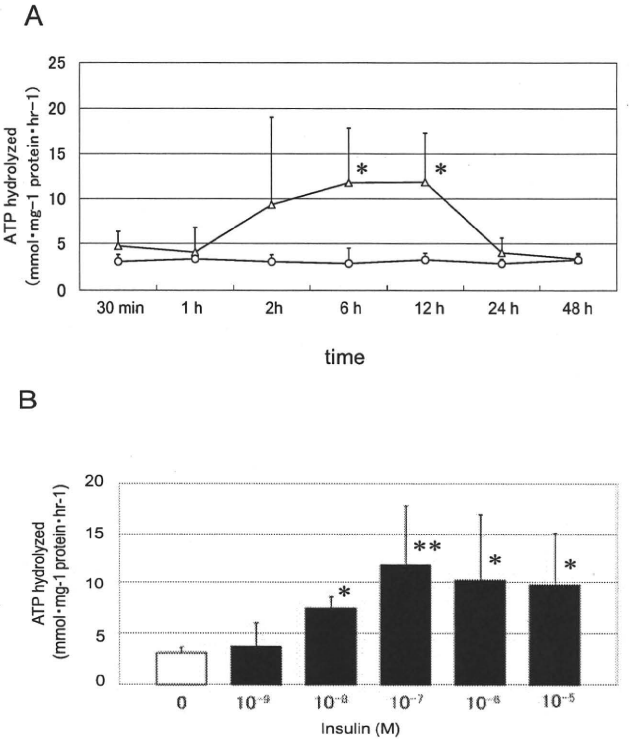

Figure 1. Effect of insulin on Na,K-ATPase activity in cultured mouse corneal endothelial cells. (**A**) Cells were incubated in the absence (*open circles*) or presence (*open triangles*) of 0.1 μM insulin for the indicated times and then assayed for Na,K-ATPase activity. Data are mean ± SD of values of four replicates from a representative experiment. $^*P < 0.05$ compared with the corresponding value for cells incubated without insulin (Student's *t*-test). (**B**) Cells were incubated with the indicated concentrations of insulin for 6 hours and then assayed for Na,K-ATPase activity. Data are mean ± SD of values of four replicates from four representative experiments. $^*P < 0.05$, $^{**}P < 0.01$ for the indicated comparisons (Student's *t*-test).

Effect of Insulin on Na,K-ATPase α_1-Subunit Phosphorylation

To determine whether insulin affects Na,K-ATPase expression in corneal endothelial cells, we exposed the cells to 0.1 μM insulin for 6 hours and then measured expression of the total Na,K-ATPase α_1-subunit and the phospho-Na,K-ATPase α_1-subunit by Western blot analysis (Fig. 4A). We used 0.1 μM phorbol 12,13-dibutyrate (PDBu), which is an established specific and strong activator for PKC and immediately phosphorylates Ser18 of Na,K-ATPase α_1-subunit, for 30 minutes as a positive control to confirm that the phospho-Ser18 antibody works. The phosphorylation of Na,K-ATPase α_1-subunit by PDBu was decreased at 6 hours. Ser18 phosphorylation triggers the endocytosis of the Na,K-ATPase α_1-subunit and results in the inhibition of Na,K-ATPase activity.[48,49] Thus, the phospho-Na,K-ATPase α_1-subunit is considered to be the inactive state of the Na,K-ATPase α_1-subunit. Expression of the Na,K-ATPase α_1-subunit and phospho-Na,K-ATPase α_1-subunit were measured as the ratio of the signal intensity to β-actin. Although there were no statistically significant differences in the expression of total Na,K-ATPase α_1-subunit (Fig. 4B), insulin significantly decreased the ratio of phospho-Na,K-ATPase α_1-subunit expression to the total Na,K-ATPase α_1-subunit (Fig. 4C). These results indicate that insulin decreases the ratio of the inactive state (i.e., increases the ratio of the active state of the Na,K-ATPase α_1-subunit). In the presence of staurosporine and GF109203X, an established PKC inhibitor, and okadaic acid, an

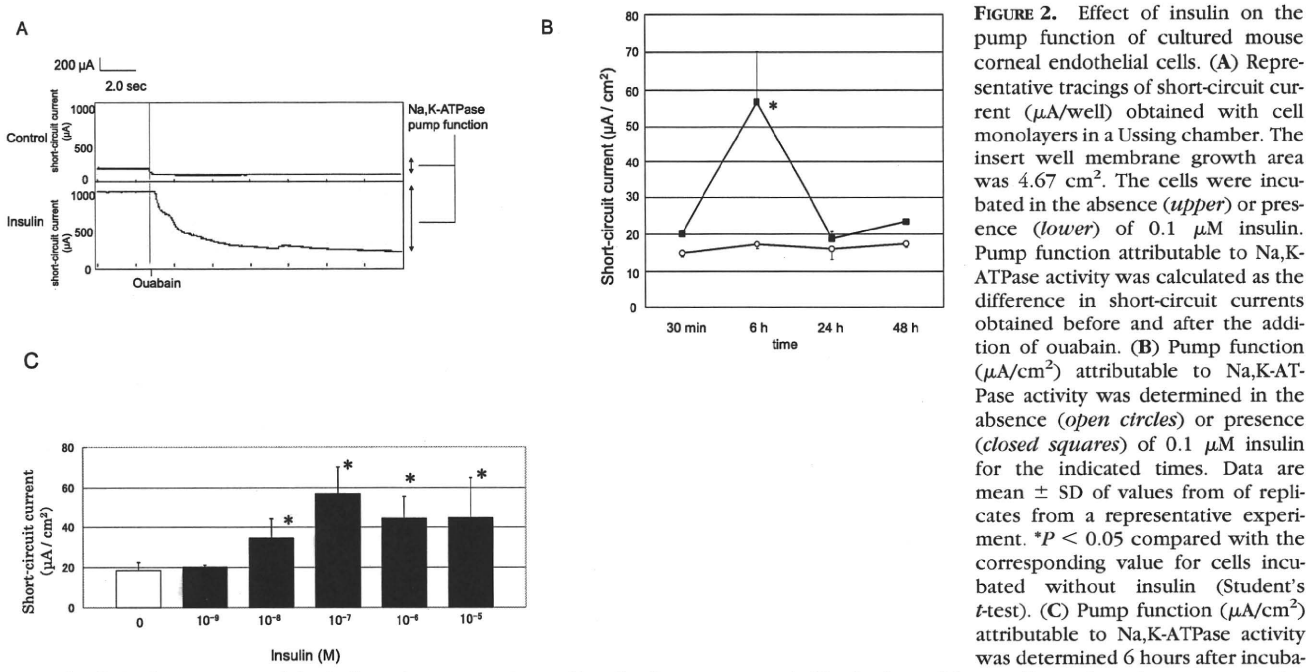

FIGURE 2. Effect of insulin on the pump function of cultured mouse corneal endothelial cells. (**A**) Representative tracings of short-circuit current (μA/well) obtained with cell monolayers in a Ussing chamber. The insert well membrane growth area was 4.67 cm^2. The cells were incubated in the absence (*upper*) or presence (*lower*) of 0.1 μM insulin. Pump function attributable to Na,K-ATPase activity was calculated as the difference in short-circuit currents obtained before and after the addition of ouabain. (**B**) Pump function (μA/cm^2) attributable to Na,K-ATPase activity was determined in the absence (*open circles*) or presence (*closed squares*) of 0.1 μM insulin for the indicated times. Data are mean ± SD of values from of replicates from a representative experiment. *$P < 0.05$ compared with the corresponding value for cells incubated without insulin (Student's *t*-test). (**C**) Pump function (μA/cm^2) attributable to Na,K-ATPase activity was determined 6 hours after incubation of cells in the presence of the indicated concentrations of insulin. Data are mean ± SD of values of four replicates from four representative experiments. *$P < 0.05$ for the indicated comparisons (Student's *t*-test).

inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), expression of the total Na,K-ATPase α_1-subunit did not change (Fig. 4B), and the insulin-induced dephosphorylation of Na,K-ATPase α_1-subunit was diminished (Fig. 4C).

Effect of Staurosporine, GF109203X, and Okadaic Acid on Insulin-Induced Na,K-ATPase Activation

To test whether the stimulatory effect of insulin on Na,K-ATPase activity was mediated by PKC, we examined the effects of staurosporine and GF109203X. The increase in Na,K-ATPase activity induced by insulin was significantly inhibited by staurosporine and GF109203X (Fig. 5). These results indicated that the increase in Na,K-ATPase activity induced by insulin at a concentration of 0.1 μM was mediated by PKC.

We next examined whether okadaic acid might affect the Na,K-ATPase activation induced by insulin. The activity of Na,K-ATPase at 0.1 μM insulin was significantly reduced in the presence of 1 μM okadaic acid (Fig. 5). These results suggest that the activity of PP1, PP2A, or both is essential to insulin-induced Na,K-ATPase activation.

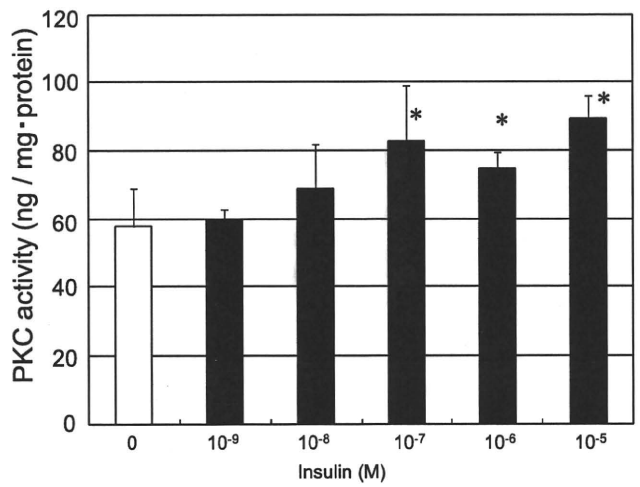

FIGURE 3. Effect of insulin concentration on PKC activity in cultured mouse corneal endothelial cells. Cells were incubated with the indicated concentrations of insulin for 30 minutes, after which the activity of PKC was measured in cell extracts. Data are mean ± SD of values of four replicates from four representative experiments. *$P < 0.05$ versus the value for cells incubated without insulin (Student's *t*-test).

Effect of Insulin on Na,K-ATPase α_1-Subunit Cell Surface Expression

To determine whether the effect of insulin changes the cell surface expression of the Na,K-ATPase α_1-subunit, we examined the immunocytochemistry of the Na,K-ATPase α1-subunit after insulin treatment in the presence and absence of the inhibitors staurosporine, GF109203X, and okadaic acid. The staining was performed without permeabilization and the majority of observed staining was on the cell surface; thus, inactive Na,K-ATPase was not detected. Insulin-treated corneal endothelial cells expressed the Na,K-ATPase α1-subunit at their lateral cell membranes more than did control cells (Figs. 6A, 6B). In the presence of inhibitors, Na,K-ATPase α1-subunit expression of insulin-treated corneal endothelial cells was weakened at their lateral cell membranes (Figs. 6C–E).

DISCUSSION

In the present study, we show that insulin increases Na,K-ATPase activity and its related pump function in cultured corneal endothelial cells. Changes in Na,K-ATPase activity and pump function under various experimental conditions were well correlated. Our results support the notion that Na,K-ATPase activity is an important determinant of the ability of corneal endothelial cells to maintain the water content of the corneal stroma.[50] Our results suggest that the observed effect of insulin on Na,K-ATPase activity in corneal endothelial cells is transient. A chronic lack of insulin in type 1 diabetes mellitus or a chronic reduced level of insulin signaling by insulin resis-

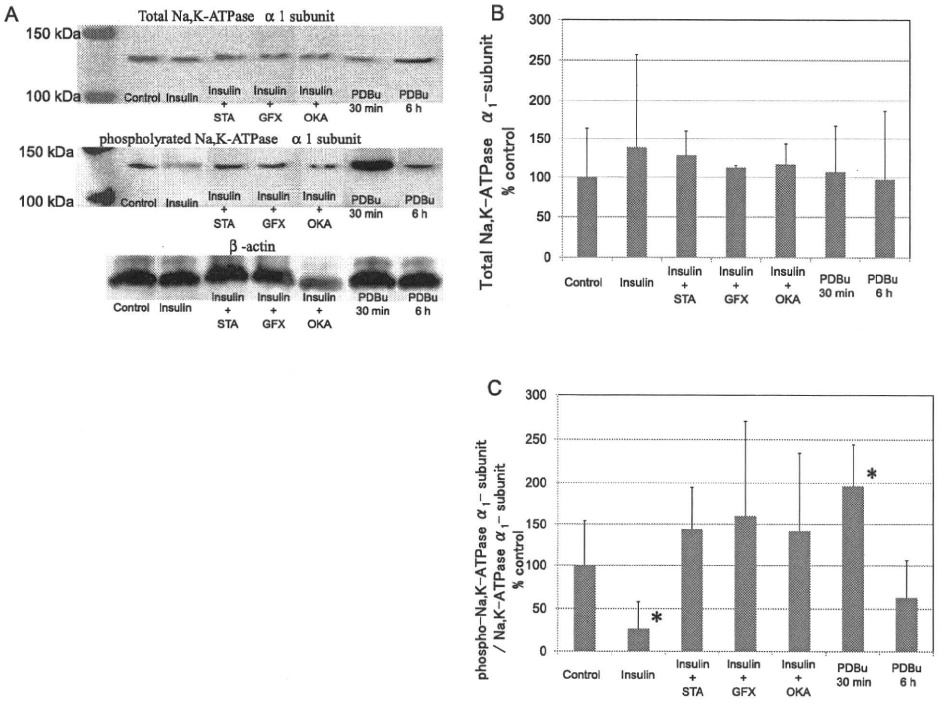

FIGURE 4. Western blot analysis of Na,K-ATPase α_1-subunit and phospho-Na,K-ATPase α_1-subunit expression. (**A**) Representative signals of expression. *Top*: Na,K-ATPase α_1-subunit. *Middle*: phospho-Na,K-ATPase α_1-subunit. *Bottom*: β-Actin. For the following, the relative intensity of each band to β-actin was measured by a densitometer as the expression of Na,K-ATPase α_1 or phospho-Na,K-ATPase α_1-subunit. (**B**) Cells were incubated in the absence (control) or presence of 0.1 μM insulin for 6 hours, 0.1 μM insulin for 6 hours with 30 minutes preincubation of 1 μM staurosporine (insulin+STA), 0.1 μM GF109203X (insulin+GFX), or 1 μM okadaic acid (insulin+OKA), 0.1 μM PDBu for 30 minutes as a positive control, and 0.1 μM PDBu for 6 hours and were then assayed for the expression of Na,K-ATPase α_1-subunit. Data are mean ± SD from five experiments, expressed as a percentage of control. (**C**) The rate of inactive state of Na,K-ATPase α_1-subunit with insulin, insulin+STA, insulin+GFX, insulin+OKA, and PDBu for 30 minutes and 6 hours. Values represent the ratio of phospho-Na,K-ATPase α_1-subunit expression to Na,K-ATPase α_1-subunit expression. Data are mean ± SD of values from five experiments. $^*P < 0.05$ versus the value for cells incubated without insulin (Student's *t*-test).

tance in type 2 diabetes mellitus is essential in the pathogenesis of corneal abnormalities in diabetes.

Insulin has been shown to stimulate the electrogenic sodium transport in a variety of cells.[27–37] In most cases, the increase in Na^+ transport is thought to be a result of the stimulation of the Na,K-ATPase. Various mechanisms of insulin action have been advocated, including changes of the kinetic properties of the enzyme,[28,29] an increase in the intracellular Na concentration, which in turn leads to a subsequent pump stimulation,[30–34] and an increase in the pump concentration at the cell surface by serum and glucocorticoid-dependent kinase (SGK).[35–37] Regardless whether insulin stimulates pump activity by a previous increase in cytosolic Na^+, in its affinity for Na^+, or in pump availability at the cell surface, the insulin receptor signaling cascades must be involved.[27] The signaling cascades include those mediated by protein kinases such as PKC. To date, PKC is regarded to trigger the rapid action of insulin on the Na,K-ATPase and to be involved in the stimulation of the Na,K-ATPase by insulin in muscle cells.[27] Our results suggest that the regulation of Na,K-ATPase activity by insulin in corneal endothelial cells is associated with the active state of the Na,K-ATPase α_1-subunit, and Na,K-ATPase activation by insulin appears to be mediated by PKC and PP1 or PP2A.

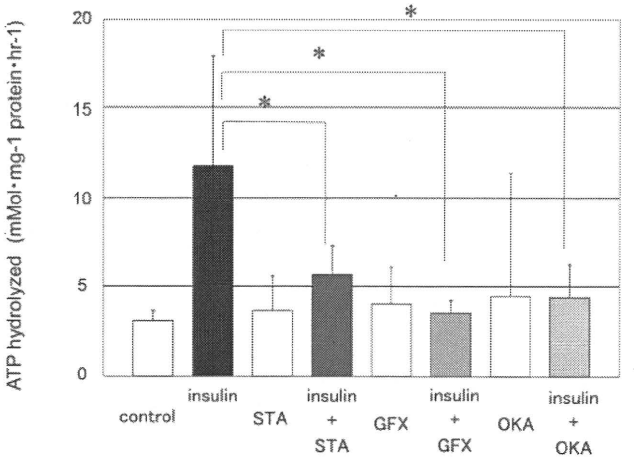

FIGURE 5. Effect of staurosporine (STA), GF109203X (GFX), and okadaic acid (OKA) on insulin-induced Na,K-ATPase activity in cultured mouse corneal endothelial cells. Cells were incubated first for 30 minutes in the absence or presence of 1 μM staurosporine, 0.1 μM GF109203X, or 1 μM okadaic acid and then for an additional 6 hours in the additional presence of 0.1 μM insulin before measurement of Na,K-ATPase activity. Data are mean ± SD of values of four replicates from four representative experiments. $^*P < 0.01$ versus the value for cells incubated with insulin alone (Student's *t*-test). Na,K-ATPase activity did not significantly increase in the presence of staurosporine + insulin, GF109203X + insulin, or okadaic acid + insulin compared with control.

Na,K-ATPase is the largest protein complex in the family of P-type cation pumps, and its minimum functional unit is a heterodimer of the α- and β-subunits.[51] In the case of Na,K-ATPase α-subunits, four isoforms (α_1, α_2, α_3, α_4) are present in mammalian cells.[52] The α_2 isoform appears to be involved in regulating Ca^{2+} transients involved in muscle contraction, whereas the α_1 isoform probably plays a more generalized role.[52] Huang et al.[53] reported that both the α_1 and the α_3 isoforms are expressed in human corneal endothelial cells. We examined Na,K-ATPase α_1-subunit expression in corneal endothelial cells because of its generality. It remains to be investigated whether other isoforms play any role in corneal endothelial cells.

The anti-phospho-Na/K ATPase α_1 antibody we used in the present study recognizes the Na,K-ATPase α_1-subunit only when phosphorylated at Ser18. This phosphorylation triggers endocytosis of the Na,K-ATPase α_1-subunit and results in inhibition of the Na,K-ATPase activity.[48,49] The phospho-Na,K-ATPase α_1-subunit (Ser18) could be regarded as an inactive state of the Na,K-ATPase α_1-subunit. Ser18 itself may be phos-

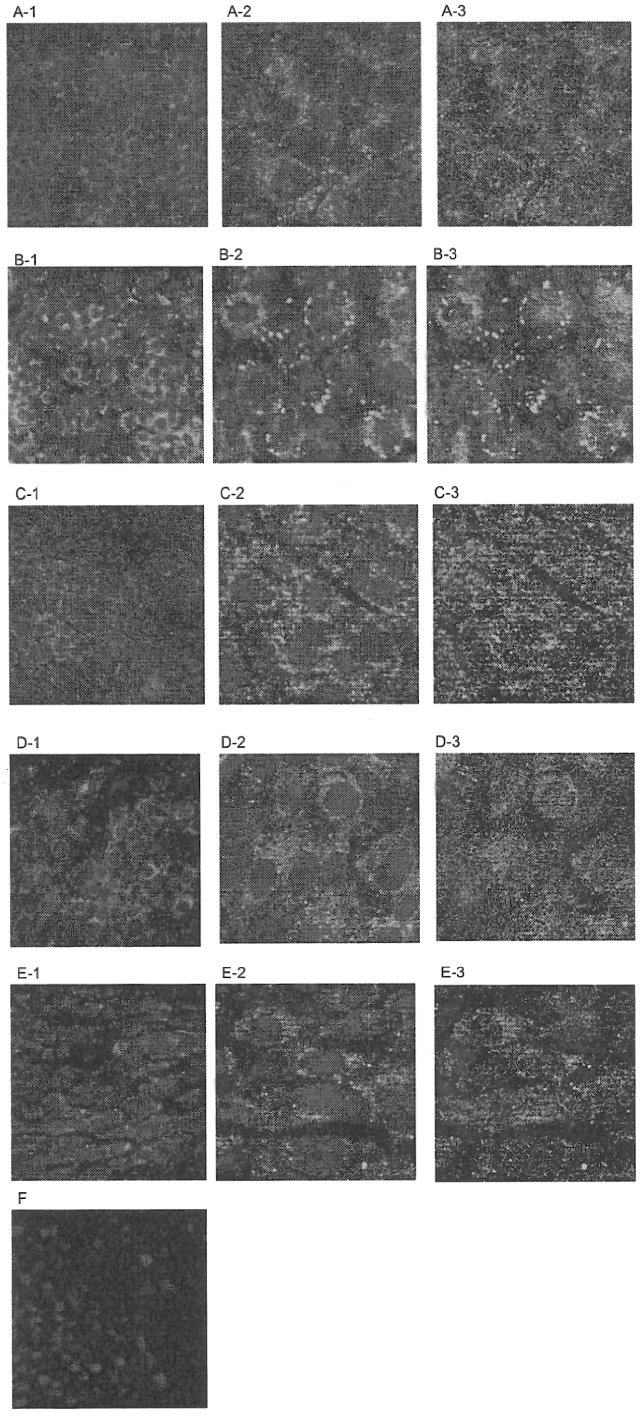

FIGURE 6. Effect of insulin on Na,K ATPase α_1-subunit cell surface expression. Cells were incubated in the absence of insulin (**A**), presence of 0.1 μM insulin for 6 hours (**B**), 0.1 μM insulin for 6 hours with 30 minutes preincubation of 1 μM staurosporine (**C**), 0.1 μM GF109203X (**D**), or 1 μM okadaic acid (**E**) and then were assayed for the cell surface expression of Na,K-ATPase α_1-subunit by immunocytochemistry. (**A-1-E-1**) Low magnification. (**A-2-E-2**) High magnification. (**A-3-E-3**) Without nuclear staining. (**F**) Negative control by using goat anti rabbit IgG (final concentration 2 μg/mL) as a primary antibody.

phorylated directly by PKC.[54-56] In our study, although insulin increased PKC activity, insulin decreased the ratio of phospho-Na,K-ATPase α_1-subunit expression to total Na,K-ATPase α_1-subunit expression. As we previously reported, PKC exerts bidirectional (stimulatory and inhibitory) regulation of Na,K-ATPase activity in mouse corneal endothelial cells, and PKC stimulates Na,K-ATPase activity by activating PP1, PP2A, or both, which dephosphorylates the Na,K-ATPase α_1-subunit in corneal endothelial cells.[57] We also reported that PKC has an inhibitory effect on Na,K-ATPase activity,[57] and this effect may be attributed to Ser18 direct phosphorylation by PKC. In the present study, PDBu phosphorylated the Na,K-ATPase α_1-subunit at 30 minutes; phosphorylation was decreased at 6 hours. The time-response curve of Na,K-ATPase activity by insulin (Fig. 1A) seemed to rise at 2 hours, and the effect became significant at 6 hours and 12 hours. There appears to be a time lag between PKC activation and Na,K-ATPase activation. Some time may be required for subsequent dephosphorylation and cell surface expression of Na,K-ATPase, and it may support our idea that PP1 or PP2A is subsequently activated by insulin-induced PKC. In addition, PP1- and PP2A-induced dephosphorylation of Na,K-ATPase may overcome direct phosphorylation by PKC in corneal endothelial cells. Previous reports also have shown that insulin activates phosphatidylinositol 3-kinase (PI-3 kinase) by insulin/IGF-I receptor, and that PI-3 kinase, presumably acting through PKC, subsequently activates PP1, PP2A, or both in porcine endometrial epithelial cells,[29] rat skeletal muscle cells,[58-60] and frog skin.[61] PP1 or PP2A subsequently dephosphorylates the α-subunit of Na,K-ATPase and stimulates its enzymatic activity.[29,58-60] Ser18 is one of the phosphorylation sites of Na,K-ATPase. Other phosphorylation mechanisms, such as Ser11 dephosphorylation and Tyr10 phosphorylation, may also play roles in Na,K-ATPase activation.[62-64] We selected Ser18 dephosphorylation to prove that dephosphorylation by protein phosphatase 1 or 2A affects Na,K-ATPase activity. Although we did not examine the effect of protein phosphatases on Tyr10 phosphorylation, activation phosphatases should be synergistic and may not prevent the increase in activity by insulin. In the immunocytochemistry phase, insulin increased cell surface expression of the Na,K-ATPase α_1-subunit, and the presence of inhibitors such as staurosporine, okadaic acid, and GF109203X decreased its expression. These results support our conclusions.

Although we did not measure the activity of other kinases, such as SGK or AKT/protein kinase B (PKB), in corneal endothelial cells, recent studies have reported that SGK also activates Na,K-ATPase by increasing the availability of the enzyme at the basolateral membrane and that SGK is under the control of insulin.[35-37] AKT/PKB has been reported to be activated by insulin-induced PI-3 kinase phosphorylation.[65] The ouabain-induced PI-3 kinase-AKT/PKB signaling pathway has been reported to upregulate Na,K-ATPase expression in rat cardiac myocytes[66] and pig kidney epithelial cells,[67] but whether the insulin-induced PI-3 kinase-AKT/PKB pathway activates Na,K-ATPase in corneal endothelium remains unknown. In our study, PKC inhibitors and the PP1/PP2A inhibitor significantly reduced the insulin-induced activation of Na,K-ATPase. This result suggests insulin-induced PKC and PP1/PP2A activation has a significant effect on Na,K-ATPase activation in corneal endothelial cells. However, a slight but insignificant difference existed between the inhibitory effects of PKC inhibitors and the PP1/PP2A inhibitor. In addition, for significant increases, differences in concentrations were seen between PKC activation and Na,K-ATPase activation by insulin, although each similarly reached a plateau at >0.1 μM insulin concentration. In corneal endothelial cells, PP1, PP2A, or both may be activated primarily by insulin-induced PKC activation, whereas the existence of other insulin-induced kinases such as SGK and AKT/PKB must be clarified in further studies. Thus, the mechanism of insulin action is complex, and further studies are necessary to elucidate the pathways by which the effect of insulin on corneal endothelial cells is mediated.

In conclusion, we have shown that insulin increases Na,K-ATPase activity and pump function in corneal endothelial cells. Furthermore, our results support a model in which PKC and PP1 or PP2A mediates the activation of Na,K-ATPase by insulin in corneal endothelial cells. A lack of insulin in type 1 diabetes mellitus or a reduced level of insulin signaling by insulin resistance in type 2 diabetes mellitus may play a role in the pathogenesis of corneal abnormalities in diabetes.

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