

High Glucose Suppresses Epidermal Growth Factor Receptor/Phosphatidylinositol 3-Kinase/Akt Signaling Pathway and Attenuates Corneal Epithelial Wound Healing

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OBJECTIVE—Patients with diabetes are at an increased risk for developing corneal complications and delayed wound healing. This study investigated the effects of high glucose on epidermal growth factor receptor (EGFR) signaling and on epithelial wound healing in the cornea.

RESEARCH DESIGN AND METHODS—Effects of high glucose on wound healing and on EGFR signaling were investigated in cultured porcine corneas, human corneal epithelial cells, and human corneas using Western blotting and immunofluorescence. Effects of high glucose on reactive oxygen species (ROS) and glutathione levels and on EGFR pathways were assessed in porcine and primary human corneal epithelial cells, respectively. The effects of EGFR ligands and antioxidants on high glucose–delayed epithelial wound healing were assessed in cultured porcine corneas.

RESULTS—High glucose impaired *ex vivo* epithelial wound healing and disturbed cell responses and EGFR signaling to wounding. High glucose suppressed Akt phosphorylation in an ROS-sensitive manner and decreased intracellular glutathione in cultured porcine corneas. Exposure to high glucose for 24 h resulted in an increase in ROS-positive cells in primary human corneal epithelial cells. Whereas heparin-binding EGF-like growth factor and antioxidant N-acetylcysteine had beneficial effects on epithelial wound closure, their combination significantly accelerated high glucose–delayed wound healing to a level similar to that seen in control subjects. Finally, Akt signaling pathway was perturbed in the epithelia of human diabetic corneas, but not in the corneas of nondiabetic, age-matched donors.

CONCLUSIONS—High glucose, likely through ROS, impairs the EGFR–phosphatidylinositol 3-kinase/Akt pathway, resulting in delayed corneal epithelial wound healing. Antioxidants in combination with EGFR ligands may be promising potential therapeutics for diabetic keratopathy. *Diabetes* 58:1077–1085, 2009

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With a rapid increase in the prevalence of diabetes, ocular complications have become a leading cause of blindness in the world (1). In addition to abnormalities of the retina (diabetic retinopathy) and the lens (cataracts), various types of corneal disorders are also relatively common in diabetes patients (2). Abnormalities of the cornea include alterations in the epithelial basement membrane, such as thickening (3,4), decreased number of hemidesmosomes (5), and deposition of advanced glycation end products (3,6). For the epithelium, hyperglycemia significantly alters its structure and function, resulting in basal cell degeneration (3,6–8), decreased (9) or increased (10) cell proliferation, superficial punctate keratitis (11), breakdown of barrier function (12), fragility (13), recurrent erosions, and persistent epithelial defects (14), depending on the duration of diabetes and on the serum concentration of A1C. The epithelial abnormalities, termed keratopathy/epitheliopathy, are resistant to conventional treatment regimens (15,16). Hence, a better understanding of the pathogenesis of diabetic keratopathy should lead to a better management of the disease.

Although the described keratopathy can be attributed in part to defects in epithelium–basement membrane adhesion and to corneal neuropathy (17,18), many epithelial abnormalities may also be related to alterations in cell signaling, particularly to epidermal growth factor (EGF) receptor (EGFR)-mediated cell responses to environmental challenges. EGFR signaling is needed for controlling the proliferation, differentiation, and survival of epithelial cells (19). Known ligands for EGFR include EGF, heparin-binding EGF-like growth factor (HB-EGF), and transforming growth factor- α . Analysis of EGFR-deficient mice revealed that the cell types most affected are epithelial and glial cells, the same cell types where EGFR is found to be overexpressed in human tumors (19,20). Interestingly, targeting EGFR with cetuximab (an EGFR monoclonal antibody) and Gefitinib/Iressa (an EGFR kinase inhibitor) for cancer treatments resulted in ocular abnormalities in some patients, including diffuse punctate keratitis and corneal erosion (21,22), which were also frequently observed in diabetic corneas. Thus, maintaining a proper level of EGFR signaling is critical for the physiological state of an epithelium in tissues, and long-term exposure to hyperglycemia may inevitably affect EGFR signaling apparatus, leading to cell dysfunction, including compromised barrier function and delayed wound healing.

In the last few years, oxidative stress, resulting from enhanced production of reactive oxygen species (ROS)

and impaired antioxidant defense capabilities in response to hyperglycemia, has been postulated as a unifying mechanism causing diabetic complications (23–25). In many tissues, including retina and kidney, increased ROS production is associated with the onset, progression, and pathological consequences of diabetes (26,27). However, whether hyperglycemia induces the generation of ROS and causes epithelial cell damage and dysfunction in the cornea has not been documented. The cornea's visual properties are exquisitely sensitive to the tissue damage caused by oxidative stress. Because the transparent cornea is frequently exposed to sunlight, including harmful ultraviolet (UV) light, there is a strong defense mechanism against UV-induced oxidative stress. However, severe tissue damage has been observed in the corneas of diabetic patients, especially those with diabetic retinopathy, which serves as an indication of long-term hyperglycemia (7,28). The characteristics of diabetic keratopathy implicate oxidative stress-induced cell injury and dysfunction. Hence, hyperglycemia may cause the oxidative stress, a result of the generation of ROS and impairment of the intracellular antioxidant defense system, in the cornea.

In this study, we tested the hypothesis that EGFR signaling pathways in diabetic corneal epithelial cells are impaired and that such alterations contribute to delayed epithelial wound healing. Our results suggest that therapeutics containing antioxidants and EGFR ligands may be effective in treating delayed wound healing in diabetic corneas.

RESEARCH DESIGN AND METHODS

Culture media were purchased from Invitrogen (Grand Island, NY). Human recombinant HB-EGF was obtained from R&D Systems (Minneapolis, MN). N-acetylcysteine (NAC), L-buthionine sulfoximine (BSO), and 2, 7-dichlorofluorescein (DCF) were from Sigma-Aldrich (St. Louis, MO). A glutathione assay kit was purchased from Cayman Chemical (Ann Arbor, MI). Rabbit anti-human EGFR, mouse anti-extracellular signal-regulated kinase 2 (ERK2) and phospho (p)-ERK1/2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Akt, phospho (p)-Akt (ser⁴⁷³), and phospho (p)-EGFR (Y⁸⁴⁵ and Y¹⁰⁶⁸) were obtained from Cell Signaling (Danvers, MA).

Porcine corneal epithelial wounding and organ culture. Porcine eyes were obtained from a local abattoir, transported to the laboratory on ice in a moist chamber, and processed for corneal culture the same day. An epithelial wound was made by demarcating an area on the central cornea with a 5-mm trephine and then removing the epithelium within the circle with a small scalpel, leaving an intact basement membrane (29). Corneal-scleral rims were excised and rinsed in sterilized PBS (pH 7.4). The excised corneas were placed epithelial side down into a sterile cup of silicon mold. The endothelial corneal cavity was then filled with minimum essential medium (MEM) containing 1% agarose and 1 mg/ml collagen maintained at 42°C. This mixture was allowed to gel. The cornea, along with its supporting gel, was inverted and transferred to a 35-mm dish. At that point, ~2 ml of MEM was added drop-wise to the surface of the central cornea until the limbal conjunctiva was covered, leaving the epithelium exposed to the air. The corneas were then cultured in a humidified 5% CO₂ incubator at 37°C in MEM containing 5 mmol/l (normal glucose), 25 mmol/l D-glucose (high glucose), or 5 mmol/l D-glucose plus 20 mmol/l mannitol (high mannitol). The wound was allowed to heal for 48 h in MEM with or without HB-EGF (50 ng/ml) or NAC (25 μmol/l). In our preliminary study, we found that high NAC concentrations (≥50 μmol/l) caused an increase in opacity of cultured corneas, and as a result, 25 μmol/l was chosen for the study. After 48 h culture, the corneas were stained with Richardson's staining solution to mark the remaining wound area, photographed, and quantified using Photoshop software. At least four corneas were used for each treatment, and two independent experiments were performed (eight or more corneas for each treatment).

Measurement of intracellular glutathione. Porcine corneal epithelial cells, collected 48 h postwounding, were lysed with radioimmunoprecipitation assay (RIPA) buffer with a proteinase inhibitor cocktail, sonicated, and used for determination of protein concentration by the micro-BCA (bicinchoninic acid) method. Cell lysates containing 10 μg of proteins were deproteinized with 10% meta-phosphoric acid, and glutathione was measured in the resultant

supernatant by the glutathione assay kit. The concentration of glutathione was obtained in reference to a standard curve.

Assessment of EGFR, ERK, and Akt phosphorylation ex vivo and in vitro. To assess effects of high glucose on cell signaling ex vivo, porcine corneal epithelial cells were isolated at the end of a 48-h organ culture, lysed in RIPA buffer, and processed for Western blotting.

Primary human corneal epithelial cells (29) and an SV40-immortalized/transformed human corneal epithelial cell line (THCE) (30) were cultured in defined keratinocyte serum-free medium containing normal or high glucose for 48 h and starved overnight in keratinocyte basal medium with the same glucose conditions. Cells on 100-mm dishes were wounded by multiple linear scratches using a cut of 48-well shark tooth comb for DNA sequencing gel (Bio-Rad, Hercules, CA) going from one side of the dish to the other. The dish was then rotated, and scrapes were made similarly to the original scrapes at 45°, 90°, and 135°. At the indicated times, cells were lysed with RIPA buffer and used for Western blotting. Cell lysate of 10–30 μg proteins was used to determine phosphatidylinositol (PI) 3-kinase and ERK activation using antibodies against p-Akt and p-ERK1/2, respectively, with Akt and ERK2 levels for equal protein loading. EGFR tyrosine phosphorylation in wounded THCE cells was analyzed using antibodies against p-EGFR (Y⁸⁴⁵ and Y¹⁰⁶⁸).

Immunohistochemical studies of human diabetic corneas. Normal and diseased human autopsy corneas obtained from the NDR1 (National Disease Research Interchange, Philadelphia, PA) were embedded in OCT (Ted Pella, Redding, CA) on arrival. The OCT blocks were immediately frozen in liquid nitrogen and stored at –80°C. The cryostat sections were used from corneas belonging to 10 diabetic patients (6 with type 1 diabetes, mean age 68.3 ± 4.8 years, and 4 with non-insulin-dependent type 2 diabetes, mean age 75.0 ± 7.5 years) and 4 patients without diabetes and eye disease (referred to as normal healthy; mean age 69.4 ± 4.6 years). The sections, usually three to four in each slide, were stained either with rabbit antibodies against p-Akt or with nonimmune rabbit IgG (negative control), and subsequently with goat anti-rabbit fluorescein-conjugated secondary antibody (Jackson ImmunoResearch Lab, West Grove, PA). The slides were then washed in PBS and mounted with Vectashield mounting medium containing 4',6'-diamidino-2-phenylindole (DAPI) nuclear staining and examined under a Carl Zeiss Axioplan 2 fluorescence microscope equipped with an ApoTome digital camera. Negative controls (rabbit IgG isotypes) exhibited no fluorescence (data not shown).

Measurement of intracellular ROS. The level of intracellular ROS was determined on the basis of the oxidative conversion of cell-permeable 2',7'-dichlorofluorescein diacetate (DCFH-DA) to fluorescent DCF on reaction with hydroxyl radical, hydrogen peroxide, or peroxynitrite. Primary human corneal epithelial cells at passage 3 were seeded onto 6-well plates. After cell attachment and growth for 1 day, the cells were treated with normal glucose, high mannitol, or high glucose in defined keratinocyte SFM for 24 h. Cells were then incubated with DCFH-DA (5 μmol/l) in Hank's balanced salt solution in 37°C for 45 min in the dark, detached from culture wells by 0.05% trypsin-EDTA, and washed twice with ice-cold PBS. Cells without DCFH-DA were used as the negative control; cells treated with BSO (250 μmol/l) to deplete intracellular glutathione were used as the positive control. Flow cytometric measurements (Becton Dickinson FACS DiVa cell sorter) were performed in triplicate for each treatment. The mean fluorescence intensity was quantified by CELLQuest software.

The intracellular accumulation of ROS was also detected in THCE cells plated on eight chamber slides cultured in normal glucose, high mannitol, or high glucose with or without NAC for 24 h by the addition of DCFH-DA to the culture. The distribution of fluorescent DCF on the cell monolayer was visualized under a fluorescence microscope, and the pictures were taken using a Spot digital camera.

Statistical analysis. Data were presented as the means ± SD. Statistical differences among three or more groups were identified using one-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

RESULTS

High glucose significantly attenuates porcine corneal epithelial wound healing. We previously used cultured porcine corneas as an ex vivo model to study epithelial wound healing, and this air/liquid corneal organ culture model has been shown to preserve the structural integrity and the responsiveness of epithelial cells to wounding and growth factor stimulation (29,31,32). To assess whether high glucose influences corneal epithelial wound healing, we cultured porcine corneas in high glucose, using normal glucose or high mannitol as controls. The epithelial wounds

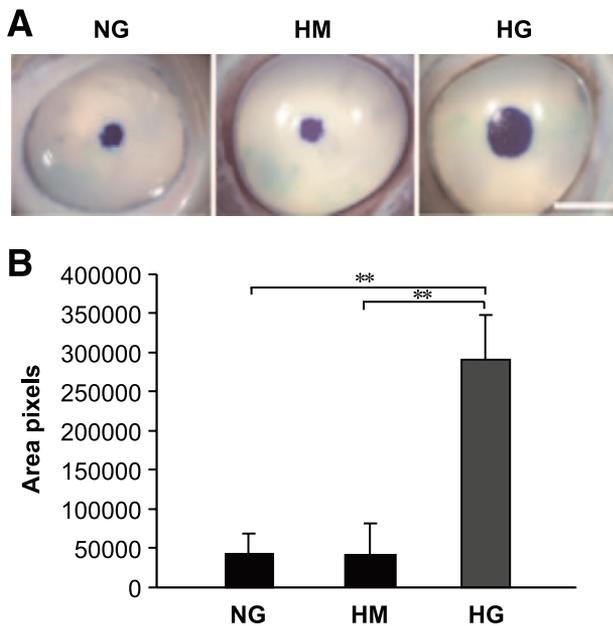


FIG. 1. High glucose delays epithelial wound closure in cultured porcine corneas. A corneal epithelial wound of 5-mm diameter was made and allowed to heal for 48 h in MEM containing normal glucose (NG; 5 mmol/l D-glucose), glucose plus mannitol (HM; 5 mmol/l D-glucose plus 20 mmol/l mannitol), and high glucose (HG; 25 mmol/l D-glucose). Wounded corneas were stained with Richardson solution to show the remaining wound area at the end of the culture. *A*: These figures are representatives of four corneas per condition from five independent experiments. Scale bar: 5 mm. *B*: Remaining wound area after 48 hours. Changes in the mean of the remaining wound areas in pixels were calculated by Adobe Photoshop software. *****P* < 0.01.** (A high-quality digital representation of this figure is available in the online issue.)

were created by epithelial debridement at the center of the cornea and allowed to heal for 48 h. As shown in Fig. 1, a 5-mm epithelial wound was ~75% covered in the corneas cultured in normal glucose. Interestingly, high glucose significantly impaired epithelial wound closure in cultured porcine corneas (only 29% of the wound was covered) in a manner similar to that observed in cultured human diabetic corneas (8). Importantly, high glucose-induced delay in epithelial wound healing was independent of osmotic changes because 20 mmol/l D-mannitol had minimal effects on the process. We concluded that high glucose impairs corneal epithelial wound closure in cultured corneas.

High glucose decreases antioxidant abilities of corneal epithelial cells in cultured porcine corneas.

Glutathione is a major intracellular free radical scavenger, and its intracellular levels have been used as a parameter to measure cellular antioxidant ability (33). We measured the levels of intracellular glutathione in collected porcine corneal epithelial cells. Cells cultured in high glucose for 48 h had significantly reduced cellular glutathione concentration (Fig. 2) compared with those cultured in normal glucose or high mannitol.

High glucose targets PI 3-kinase/Akt pathways in cultured porcine corneas.

We previously showed that wounding elicits EGFR-mediated ERK and PI 3-kinase/Akt activation in corneal epithelial cells (29,34). To elucidate the mechanism underlying high glucose-induced impairment in wound closure, we next investigated the effects of high glucose on EGFR signaling (Fig. 3). In the corneas cultured in both normal and high glucose, wounding stimulated the phosphorylation of ERK1/2. On the other hand, whereas the level of p-Akt in the wounded corneas

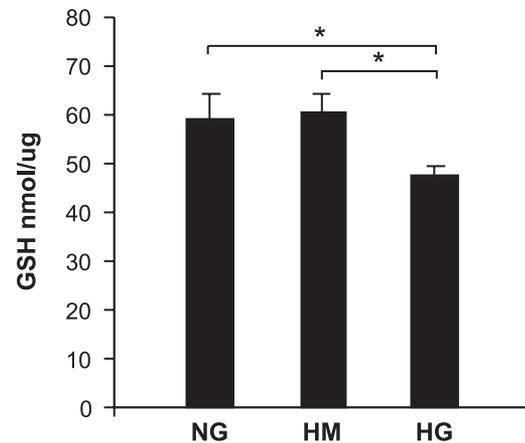


FIG. 2. High glucose decreases intracellular levels of reduced glutathione (GSH) in porcine corneal epithelial cells. Porcine corneas with epithelial debridement wounds were cultured as described in Fig. 1. At the end of the culture, corneal epithelium was scraped off, lysed in RIPA buffer, and sonicated. The glutathione was measured in cellular supernatant using a glutathione assay kit, and the results were presented as nanomoles of total glutathione per microgram of cell extract after normalization of glutathione reading with the total protein content of each sample. Values are the means \pm SD of six corneas in each group. ****P* < 0.05.** HG, high glucose; HM, high mannitol; NG, normal glucose.

cultured in normal glucose appeared to be higher than that of the unwounded controls, the level of p-Akt, clearly detectable, in the porcine corneal epithelial cells cultured in high glucose with or without wounding was much lower than in those cultured in normal glucose. The cellular protein levels of Akt and ERK2 were largely the same in all samples tested. Thus, high glucose appears to specifically target PI 3-kinase/Akt in corneal epithelial cells.

Altered p-Akt staining in diabetic human corneal epithelium.

Having shown that the EGFR-mediated PI 3-kinase/Akt signaling pathway was altered in porcine corneal epithelial cells cultured in high glucose, we then investigated whether this pathway was also affected by hyperglycemia in the corneas of patients with diabetes. Figure 4 shows representative staining of p-Akt in each diabetes category, within which a similar staining pattern was observed: normal (four subjects), type 1 diabetic (six

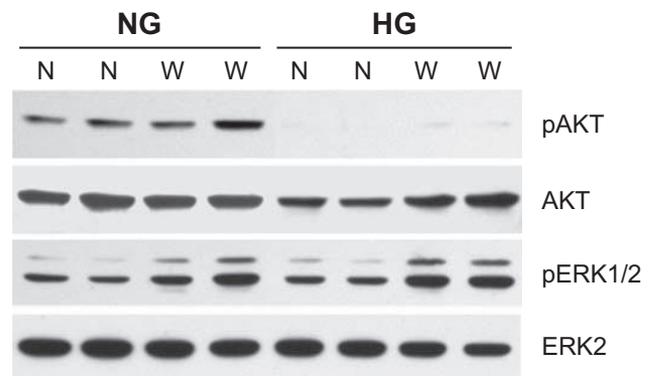


FIG. 3. Downregulation of PI 3-kinase/Akt pathways in porcine corneas cultured in high glucose. Porcine corneas with (W) or without (N) epithelial debridement wounds were cultured in normal glucose (NG; 5 mmol/l D-glucose) and high glucose (HG; 25 mmol/l D-glucose) and allowed to heal for 48 h. At the end of the culture, corneal epithelium was scraped off, lysed in RIPA buffer, and sonicated. Equal amounts of cell lysates were immunoblotted with antibodies against p-Akt or p-ERK1/2. The immunoreactivities were stripped off, and the same membranes were reprobed with antibodies against cellular Akt or ERK2, respectively, for proper protein loading.

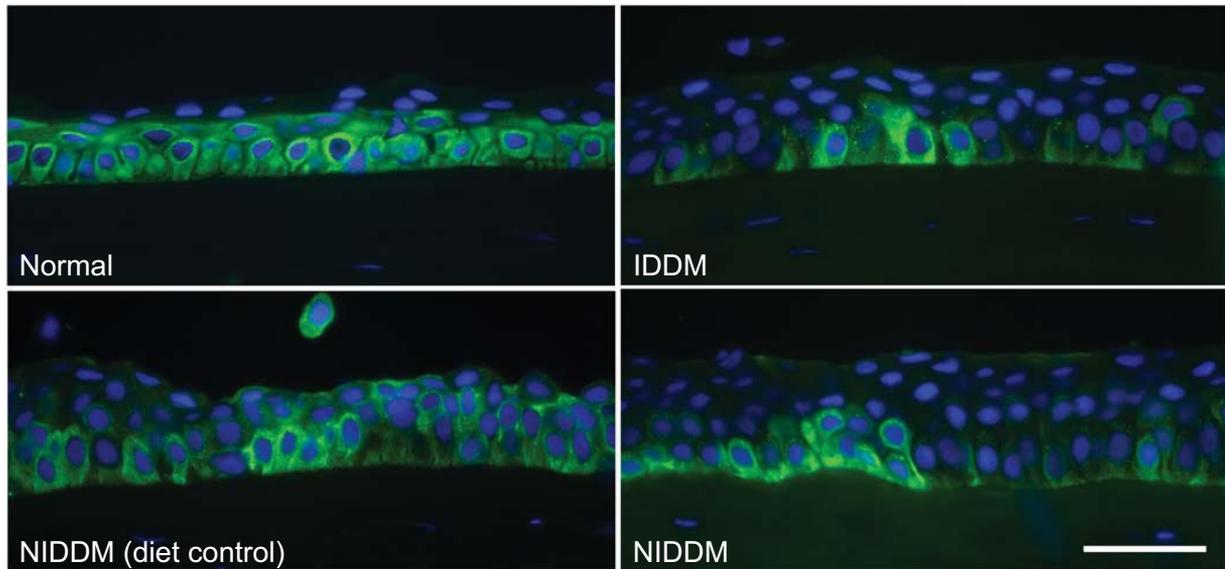


FIG. 4. Altered pAkt staining pattern in diabetic human corneal epithelium. Human corneal frozen sections from patients with type 1 diabetes (IDDM) and non-insulin-dependent type 2 diabetes (NIDDM), with normal subjects and diet-controlled type 2 diabetic patients as the control subjects, were stained by immunofluorescence with antibody against pAkt. Photos show merged images of immunoreactivity of pAkt and nuclear staining of DAPI. Scale bar = 50 μ m. (A high-quality digital representation of this figure is available in the online issue.)

subjects), type 2 diabetic (three subjects), and type 2 diabetic (diet controlled, one subject). In the basal layer of the corneal epithelium of a normal subject (74 years old), p-Akt staining was strong and continuous, consistent with the metabolically active and proliferative characteristics of the basal layer. A similar staining pattern of p-Akt was noticed in the cornea of a patient with non-insulin-dependent, diet-controlled diabetes (64 years old). However, in a patient with insulin-dependent type 1 diabetes (63 years old with 54 years of diabetes), a few cells at the basal layer were p-Akt-positive, and the staining pattern was patchy and discontinuous. A similar pattern of p-Akt staining was also observed in the cornea of a patient with non-insulin-dependent type 2 diabetes (78 years old with 8 years of diabetes).

High glucose influences EGFR signaling in response to wounding in THCE cells. To understand the effects of elevated glucose on cell response to wounding, we investigated EGFR signaling in THCE cells cultured in high glucose. In cells cultured in both normal and high glucose, wounding resulted in a rapid increase in the phosphorylation of ERK1/2 and Akt (Fig. 5). However, the intensity of such activation in cells cultured in high glucose was much reduced compared with cells cultured in normal glucose. Furthermore, the activation of ERK and Akt in high glucose was more transient, as indicated by phosphorylation levels that declined to a level similar to those of the unwounded cells at 30 min postwounding versus 2 h postwounding in cells cultured in normal glucose. The low levels of ERK and PI 3-kinase/Akt activation in cells cultured in high glucose appeared to be related to the wound-induced EGFR activation because site-specific phosphorylation of EGFR at Y¹⁰⁶⁸ and Y⁸⁴⁵ declined more quickly in cells cultured in high glucose than in normal glucose. These results suggest that chronic exposure to high glucose could blunt EGFR signaling and its downstream Akt and ERK activation in corneal epithelial cells.

High glucose increases the generation of ROS in primary human corneal epithelial cells. To examine whether high-glucose treatment induces ROS production,

we measured intracellular ROS levels in primary human corneal epithelial cells using the redox-sensitive fluorescent dye DCFH-DA and flow cytometry. Figure 6A shows that high glucose increased the number of cells with high intensity of fluorescence compared with cells cultured in normal glucose or high mannitol. High glucose caused a 40.1% increase in fluorescence intensity over that in normal glucose, indicating oxidative stress on high-glucose treatment (Fig. 6B). High-glucose-induced generation of ROS in cultured human corneal epithelial cells was confirmed by fluorescent microscopy. Although only a few cells were fluorescent in normal-glucose and high-manni-

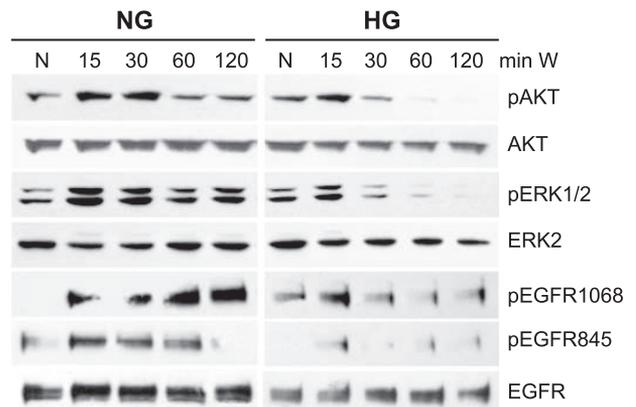


FIG. 5. Weakening of wound induced EGFR signaling pathways in human corneal epithelial cells cultured in high glucose. THCE cells were cultured in normal glucose (NG; 5 mmol/l D-glucose) and high glucose (HG; 25 mmol/l D-glucose) for 48 h and starved overnight in keratinocyte basal medium with the same glucose conditions as before. Cells were wounded extensively by sequence comb scratching (W) at the indicated times (N, without wound). Cells were then lysed, and equal amounts of proteins of 10–30 μ g were subjected to Western blot using antibodies against phosphorylated proteins pAkt, pERK1/2, or pEGFR (Y⁸⁴⁵, -Y^{1,068}). After stripping off the immunoreactivities, the membranes were reprobbed with antibody against Akt, ERK2, or EGFR, respectively, for proper protein loading. As each condition was processed separately for Western blotting, each comparison between the control and treated samples were made only within each group.

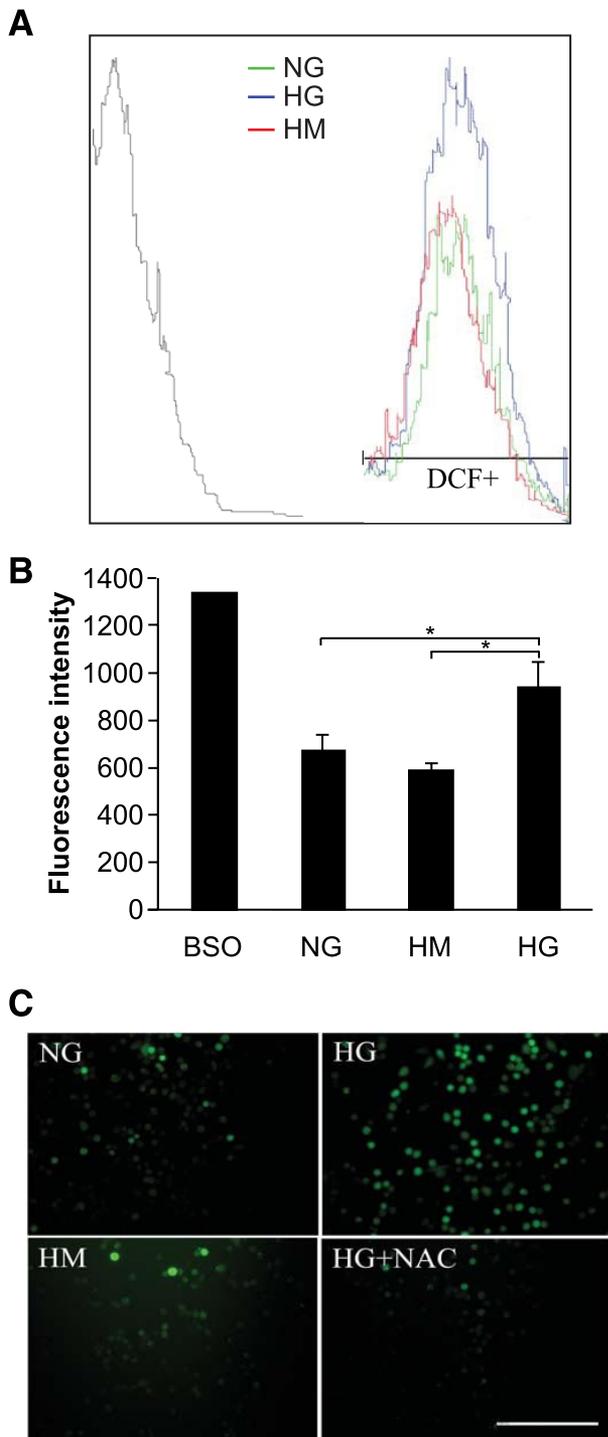


FIG. 6. Overproduction of ROS in cultured primary human corneal epithelial cells. Primary human corneal epithelial cells were cultured in medium containing normal glucose (NG), high glucose (HG), or high mannitol (HM) or medium with BSO (250 $\mu\text{mol/l}$) as positive control for 24 h and then incubated with cell-permeable DCFH-DA (5 $\mu\text{mol/l}$) for 45 min. **A:** Cells were detached by trypsin digestion and washed with PBS. Fluorescence of oxidized DCFH-DA by intracellular ROS was detected by flow cytometry for quantitative measurement. Fluorescence peaks in normal glucose, high mannitol, or high glucose were compared in terms of DCF-positive (DCF+) cells relative to the cells with nonfluorescence peak on the left. **B:** The mean fluorescence intensity of flow cytometry was quantified as the means \pm SD. * $P < 0.05$. **C:** THCE cells were grown in normal glucose, high mannitol, or high glucose with or without NAC for 24 h and incubated with DCFH-DA as mentioned above. ROS generation was visualized under a fluorescent microscope. Scale bar = 50 μm . (A high-quality digital representation of this figure is available in the online issue.)

tol cultures, high glucose greatly increased in the number of fluorescent cells after 24 h culture. Furthermore, the production of ROS induced by high glucose was sensitive to antioxidant present in the culture medium because NAC, a nonspecific ROS scavenger, attenuated ROS generation in THCE cells cultured in high glucose for 24 h (Fig. 6C). HB-EGF, on the other hand, exhibited no effects on fluorescence-positive cells in high-glucose-cultured cells with or without NAC (data not shown).

Antioxidants in combination with an EGFR ligand enhanced ex vivo corneal epithelial wound closure. Having demonstrated that the antioxidant NAC suppresses high-glucose-induced ROS generation in human corneal epithelial cells, we next investigated whether high-glucose-delayed epithelial wound closure could be reversed by antioxidants. We previously showed that HB-EGF accelerates epithelial wound healing in normal corneas (29). Hence, we first tested the effects of HB-EGF and NAC on epithelial wound closure in cultured porcine corneas. Unlike HB-EGF, NAC alone had no effect on wound healing of corneas cultured in either normal or high glucose (data not shown). Although both HB-EGF and NAC noticeably accelerated epithelial wound closure, the rate of closure in high-glucose-cultured corneas in the presence of HB-EGF or NAC was not significantly higher than that in the absence of HB-EGF or NAC (Fig. 7). Remarkably, the presence of both HB-EGF (50 ng/ml) and the antioxidant NAC (25 $\mu\text{mol/l}$) resulted in significantly accelerated wound healing with a rate similar to that observed in corneas cultured in normal glucose ($P < 0.01$) (Fig. 7B). This accelerated wound healing was sensitive to the presence of BSO, which inhibits the generation of intracellular glutathione. Hence, we conclude that EGFR activation alone may not be sufficient for restoring corneal epithelial wound healing impaired by high glucose, and antioxidants may serve as adjunctive therapy to EGFR ligands for treating diabetic keratopathy.

Effects of antioxidants on EGFR, ERK, and PI 3-kinase/Akt phosphorylation in porcine corneas cultured in high glucose. The effects of high glucose on ex vivo corneal epithelial wound healing were dramatic and highly reproducible. To elucidate the underlying mechanisms for antioxidants to affect epithelial wound closure, we assessed the signaling pathways in healing epithelial cells of porcine corneas cultured in normal and high glucose with or without antioxidants. Figure 8 shows that whereas high glucose appeared to increase ERK activation, phosphorylation of Akt was reduced in high-glucose-cultured cells. Addition of HB-EGF or NAC in high-glucose culture media restored partially high-glucose-impaired Akt phosphorylation. Consistent with greater acceleration of high-glucose-impaired wound closure, a combination of HB-EGF and NAC in high-glucose-cultured porcine corneas also enhanced wound-induced Akt phosphorylation to a larger extent than either HB-EGF or NAC acting individually. NAC appeared to have some effects on ERK phosphorylation as well because NAC treatment increased the levels of p-ERK in migrating cells in cultured pig corneas. Taken together, our results suggest that high glucose may affect EGFR signaling, particularly the PI 3-kinase/Akt pathway, through elevated ROS, a process that can be reversed by the antioxidant NAC (35,36).

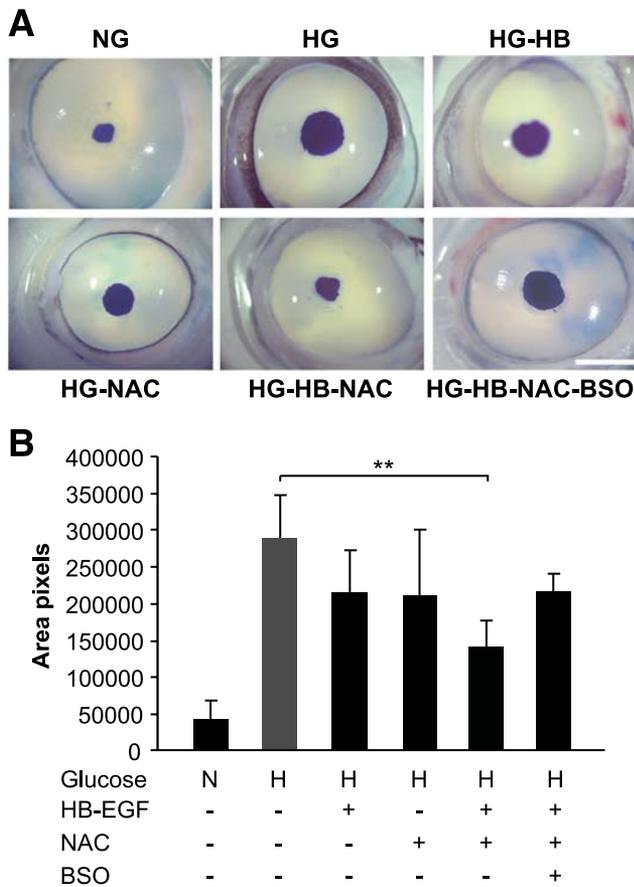


FIG. 7. Effects of HB-EGF and NAC on corneal epithelial wound healing in high-glucose conditions. **A:** Representative epithelial wound closure in cultured porcine corneas. A corneal epithelial wound of 5-mm diameter was made and allowed to heal in MEM containing normal glucose (NG; 5 mmol/l D-glucose) and in high glucose (HG; 25 mmol/l D-glucose) alone or in the presence of HB-EGF (HG-HB; 50 ng/ml), with (HG-HB-NAC) or without NAC (HG-NAC; 25 μ mol/l) and BSO (HG-HB-NAC-BSO; 250 μ mol/l) for 48 h, followed by Richardson staining to show the remaining wound. **B:** Remaining wound area after 48 hours. Changes in the means of the remaining wound area in pixels were analyzed by ANOVA. Data are the means \pm SD of at least six corneas from three independent experiments. $**P < 0.01$. Scale bar = 5 mm. H, high; N, normal. (A high-quality digital representation of this figure is available in the online issue.)

DISCUSSION

In this study, we investigated the effects of high glucose on corneal epithelial wound healing. We showed that high glucose delayed epithelial wound closure in cultured porcine corneas and impaired both basal and wound-induced PI 3-kinase/Akt signaling pathway. Hyperglycemia-induced impairment of the PI 3-kinase/Akt pathway was also apparent in the corneal epithelium of diabetic patients, but not in the age-matched nondiabetic control subjects. When human corneal epithelial cells were exposed to high glucose for 48 h, their response to wounding was blunted in terms of the duration and intensity of EGFR phosphorylation and of Akt and ERK activation. The downregulated cell signaling was related to oxidative stress because antioxidant NAC prevented high-glucose-targeted impairment of PI 3-kinase/Akt signaling in cultured corneas. Furthermore, the addition of HB-EGF was unable to overcome the inhibitory effects of high glucose on corneal epithelial wound healing, whereas the presence of both the antioxidant and EGFR ligand greatly accelerated wound healing in cultured porcine corneas. Taken together, we conclude that oxidative stress induced by high

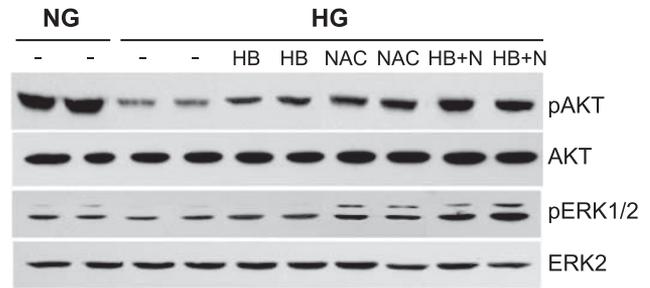


FIG. 8. Antioxidant NAC restored high-glucose-impaired PI 3-kinase/Akt pathway in cultured porcine corneas. Porcine corneas with 5-mm diameter epithelial wounds were cultured in MEM containing normal glucose (NG) and in high glucose (HG) in the presence of HB-EGF (HB) with (HB+N) or without NAC for 48 h. Epithelial cells from two representative corneas per group ($n = 6$) were collected by scraping cells off of the cultured corneas and lysed at the end of the culture. Equal amounts of cell lysate were immunoblotted with antibodies against tyrosine-phosphorylated Akt or ERK1/2. The same membranes were stripped off the immunoreactivities and reprobed with Akt or ERK2, respectively, for equal protein loading.

glucose weakens EGFR signaling and suppresses the PI 3-kinase/Akt pathway, resulting in the impairment of epithelial wound healing in diabetic corneas.

In the literature, both in vitro and animal models of type 1 and type 2 diabetes have been used to study diabetic corneal complications and wound healing, and a wealth of information has been obtained from these studies (7,9,25,37–42). For example, hyperglycemia was found to decrease cell proliferation but have no effects on apoptosis and necrosis (43) in the corneal epithelium of streptozotocin-induced diabetic rats, leading to decreased cell density and increased intercellular spaces (44), characteristics of diabetic keratopathy. However, the limitation in tissue size of diseased animals such as rats has hampered the biochemical analysis of hyperglycemia-induced alterations in the cornea. As for the in vitro studies, discrepant results have been reported regarding the effects of high glucose on human corneal epithelial cell proliferation; whereas McDermott et al. (40) reported that high glucose (17.5 mmol/l) increases cell proliferation, Fujita et al. (39) showed a decrease in cell proliferation in the presence of high glucose. Organ culture represents a more relevant experimental approach compared with cultured cells (45–47). Using human corneas, Kabosova et al. (8) showed that organ-cultured human corneas from diabetic retinopathy patients exhibited much-delayed epithelial wound healing, in complete accordance with clinical data in diabetic patients. Moreover, the corneas of patients in organ culture preserved the same marked abnormalities and altered expression of proteinase and growth factors as in vivo corneas. We previously used cultured porcine corneas as an ex vivo model to study epithelial wound healing and showed that healing an epithelial wound requires EGFR and its downstream signaling pathways, notably ERK and PI 3-kinase/Akt (29). Interestingly, high glucose induced a highly reproducible and marked delay in epithelial wound healing in cultured porcine corneas, similar to that observed in patients and in cultured human corneas (8), validating the porcine corneal organ culture system as a valuable ex vivo model to study the effects of high glucose on corneal wound healing. It should be mentioned that Zagon et al. (48) reported that there was no abnormality in corneal reepithelialization after epithelial debridement within the first 2 weeks of streptozotocin-induced diabetes in rats. The reason for the discrepancy in the results of in

vivo (rats) (9) and our ex vivo studies (cultured porcine corneas) is not clear and may be related to the lack of neuronal nourishment in cultured corneas. Indeed, the combination of substance P and IGF-I has been shown to promote delayed corneal epithelial wound healing in diabetic rats (49). The neurological protection against corneal diabetic complications is of great interest and should be further explored in ex vivo studies and in animal models. Whereas the described keratopathy/epitheliopathy can be attributed in part to defects in epithelium–basement membrane adhesion and to diabetic corneal neuropathy (17,18), we propose that many epithelial defects and abnormalities may also be related to alterations in EGFR signaling in the epithelial cells themselves.

Unlike cancerous epithelial cells, there are very few reports regarding EGFR signaling in diabetic tissues/cells. Although a dramatic decrease in hepatic EGFR transcripts was observed during the progression of diabetes (50), type 2 diabetic mice were shown to have upregulated EGFR and elevated EGFR phosphorylation, which were suspected to play a role in kidney enlargement in diabetes (51). In diabetic glomeruli and in mesangial cells treated with high glucose, elevated Akt phosphorylation, through the EGFR/PI 3-kinase pathway, was shown to mediate high-glucose–induced collagen I upregulation (52). On the other hand, high glucose specifically downregulates Akt activities in endothelial cells (53,54) and endothelial progenitor cells (35,36), and disruption of Akt pathways has been linked to endothelial dysfunction and diabetic complications, both macro- and microvascular (55). Thus, the effects of hyperglycemia on the PI 3-kinase/Akt pathway may be tissue-/cell type–specific.

Our study was the first to show that high glucose is specifically targeting PI 3-kinase in corneal epithelial cells. Interestingly, 48-h exposure to high glucose did not significantly affect another major EGFR-mediated signaling pathway, the ERK pathway, in either normal unwounded controls or in healing corneal epithelial cells. Our previous studies revealed that the basal and wound-induced ERK and Akt phosphorylation/activation are EGFR dependent, and those pathways are required for epithelial wound healing in corneal epithelial cells (34). Hence, high-glucose–mediated impairment of the PI 3-kinase/Akt pathway may contribute to delayed wound healing in porcine corneas cultured in high glucose. Most strikingly, we observed a severely altered staining pattern of p-Akt in the corneas of both insulin-dependent and insulin-independent diabetic patients compared with that of age-matched normal subjects. Our data suggest a direct correlation between hyperglycemia and p-Akt expression/distribution in the human corneas. Because PI 3-kinase and Akt have been shown to control diverse cellular activities, including cell survival, growth, proliferation, metabolism, and migration (56), low Akt activity induced by hyperglycemia may be a contributing factor to abnormalities such as basal cell degeneration (6), decreased cell proliferation (9), and, more importantly, the delayed epithelial wound healing (8,9,57) observed in diabetic corneas.

Oxidative stress caused by hyperglycemia is important for the development of diabetic abnormalities (23,24). In addition to being an avascular and transparent tissue, the cornea is unique in that it is constantly exposed to and absorbs most of the harmful UV light. Therefore, a strong, intrinsic defense mechanism against oxidative stress must exist in corneal epithelial cells. Two enzymes, aldehyde dehydrogenase 3 (58,59) and transketolase (60,61), abundantly expressed in corneal epithelial cells have been

suggested to function as “corneal crystallins.” They play a protective role against UV-induced oxidative stress through the generation of antioxidant NADPH, the scavenging of ROS, and/or the possession of chaperone-like activity (62). Nevertheless, severe abnormalities, including epithelial cell damage in the corneas of diabetic patients (7,8), suggest the dysfunction of intracellular antioxidant apparatus induced by hyperglycemia. To that end, we used DCFH-DA as a probe for intracellular oxidative stress and showed that high glucose increased levels of DCFH in cultured primary human corneal epithelial cells and in the number of fluorescent cells in THCE cell culture. The notion that high glucose increases in oxidative stress was further affirmed by the detection of a decrease in cellular glutathione levels, an indicator of cellular antioxidant capacity, in porcine corneal epithelial cells cultured in high glucose and by the observation that L-buthionine sulfoximine, an inhibitor of glutathione synthesis, reversed the effects of NAC on restoring high-glucose–delayed epithelial wound healing. The latter observation also suggests that depletion of the intracellular glutathione contents may be an underlying mechanism for hyperglycemia-induced oxidative stress in diabetic cornea. Taken together, our data suggest that oxidative stress may also be a cause of diabetic keratopathy and contribute to the delay of epithelial wound healing in diabetic corneas.

The observation that the antioxidant NAC partially reversed high-glucose–induced suppression of Akt phosphorylation suggests that the generated ROS is a contributing factor for high-glucose–induced disruption of the PI 3-kinase/Akt signaling pathway. As such, it is possible that the Akt pathway may be particularly sensitive to oxidative stress, as shown in culture bovine retinal (63) and human umbilical vein (53) endothelial cells. In the literature, oxidative stress has been shown to target p85 of PI 3-kinase (63), tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) (53), or inositol 5-phosphatase SHIP2 (SH2-containing inositol 5'-phosphatase 2) (64), all of which influence Akt activation and signaling. Which molecule(s) in the PI 3-kinase/Akt pathway might be the target of high-glucose–induced oxidative stress in the diabetic cornea is under investigation in our laboratories.

Because delays in epithelial wound healing may result in sight-threatening complications and increase the possibility of microbial infections in diabetic patients with an already weakened immune system (2,57), efforts have been made to identify reagents that accelerate epithelial wound healing in diabetic corneas (57). Growth factors such as EGF (65,66), insulin (9), and antioxidants such as trolox (25) have been tested in animal models and were shown to have limited success in managing diabetic wound healing. Using corneal organ culture, we showed that HB-EGF or NAC alone does not effectively restore the high-glucose–delayed epithelial wound healing. However, treatment of high-glucose–cultured corneas with a combination of antioxidant and HB-EGF restored the rate of wound closure to a level close to that of corneas cultured in normal glucose. Hence, HB-EGF elevating EGFR signaling and antioxidants such as NAC reducing oxidative stress may have synergistic effects on epithelial cells and therefore may prevent or reverse epithelial defects, including compromised barrier function and delayed wound healing, in diabetic corneas. The unique feature of treating diabetic keratopathy is with the use of topical application

of therapeutics. Hence, further studies aimed at understanding how aberrant EGFR signaling, including PI 3-kinase/Akt pathways and enhanced production of ROS by hyperglycemia, results in diabetic corneal defects and complications should provide the molecular basis for developing better combinational therapies to treat diabetic corneal complications, including delayed epithelial wound healing.

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