Pathogenic Role of Endoplasmic Reticulum Stress in Diabetic Corneal Endothelial Dysfunction

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Received: October 31, 2021
Accepted: February 14, 2022
Published: March 3, 2022

Citation: Chen C, Zhang B, Xue J, et al. Pathogenic role of endoplasmic reticulum stress in diabetic corneal endothelial dysfunction. *Invest Ophthalmol Vis Sci.* 2022;63(3):4. https://doi.org/10.1167/iovs.63.3.4

Purpose. Progressive corneal edema and endothelial cell loss represent the major corneal complications observed in diabetic patients after intraocular surgery. However, the underlying pathogenesis and potential treatment remain incompletely understood.

Methods. We used streptozotocin-induced type 1 diabetic mice and *db/db* type 2 diabetic mice as diabetic animal models. These mice were treated with the endoplasmic reticulum (ER) stress agonist thapsigargin; 60-mmHg intraocular pressure (IOP) with the ER stress antagonist 4-phenylbutyric acid (4-PBA); mitochondria-targeted antioxidant SkQ1; or reactive oxygen species scavenger *N*-acetyl-L-cysteine (NAC). Corneal thickness and endothelial cell density were measured before and after treatment. Human corneal endothelial cells were treated with high glucose with or without 4-PBA. The expression of corneal endothelial- and ER stress–related genes was detected by western blot and immunofluorescence staining. Mitochondrial bioenergetics were measured with an Agilent Seahorse XFp Analyzer.

Results. In diabetic mice, the appearance of ER stress preceded morphological changes in the corneal endothelium. The persistent ER stress directly caused corneal edema and endothelial cell loss in normal mice. Pharmacological inhibition of ER stress was sufficient to mitigate corneal edema and endothelial cell loss in both diabetic mice after high IOP treatment. Mechanistically, inhibiting ER stress ameliorated the hyperglycemia-induced mitochondrial bioenergetic deficits and improved the barrier and pump functional recovery of the corneal endothelium. When compared with NAC, 4-PBA and SkQ1 exhibited better improvement of corneal edema and endothelial cell loss in diabetic mice.

Conclusions. Hyperglycemia-induced ER stress contributes to the dysfunction of diabetic corneal endothelium, and inhibiting ER stress may offer therapeutic potential by improving mitochondrial bioenergetics.

Keywords: diabetes mellitus, corneal endothelial dysfunction, ER stress, 4-phenylbutyric acid, mitochondria

The corneal endothelium is a single layer of hexagonal cells that coats the posterior surface of the cornea, acting to maintain the dehydration and transparency of the cornea through the barrier properties of the tight junction protein zonula occludens protein 1 (ZO-1) and the pump properties of the ionic pump Na^+/K^+-ATPase.[1–3] Corneal endothelial cells exhibit limited regenerative capacity in adults.[4] When the endothelial cell density decreases below a critical threshold (400–500 cells/mm^2),[5] corneal endothelial dysfunction occurs and leads to corneal edema, pain, and even deterioration of vision.[6]

Corneal endothelial dysfunction can occur due to multiple causes, including diabetes[7,8] and other diseases.[5,9] Diabetes mellitus is a global health problem, and its incidence has tripled over the past 20 years; by 2045, the number of patients with diabetes worldwide is estimated to reach approximately 693 million.[10] Clinically, diabetic patients demonstrate more frequent endothelial cell loss and dysfunction than nondiabetic patients.[8] In addition, endothelial acute injuries such as acute glaucoma[11] and phacoemulsification cataract surgery[12,13] can often accelerate endothelial cell loss, decrease the density of the endothelium, and provoke endothelial dysfunction and corneal edema. However, the exact mechanisms and effective treatment options for corneal endothelial dysfunction in patients with diabetes require further investigation.

Endoplasmic reticulum (ER) stress is a vital regulatory mechanism that maintains intracellular homeostasis.[14,15] In the human body, ER stress can be mitigated by initiating the unfolded protein response (UPR).[16,17] Reports show that

ER stress accompanied by UPR activation is involved in the pathogenesis and treatment of diabetes.[18–22] ER stress can also cause mitochondrial dysfunction, oxidative stress, and cell death.[23,24] Overactivation of ER stress and dysfunction of mitochondria are predominant etiological components in the development of diabetes.[25] Our previous study showed that attenuating hyperglycemia-induced ER stress can promote diabetic corneal epithelial wound healing and nerve regeneration.[26] However, the role of ER stress in diabetic endothelial dysfunction remains unknown.

In the present study, we observed ER stress activation in diabetic mice and in diabetic human corneal endothelial cells, and this ER stress preceded the occurrence of morphological changes. Persistent ER stress activation can cause cell loss and corneal endothelial dysfunction. Pharmacological inhibition of ER stress can mitigate endothelial cell loss and corneal edema through the mitochondrial pathway in diabetes. To date, the main treatment strategy for corneal endothelial dysfunction has been endothelial keratoplasty or penetrating keratoplasty, which is limited by donor shortages worldwide. Our study provides a new strategy for the treatment of diabetic corneal endothelial dysfunction.

Materials and Methods

Animals

Male C57BL/6 mice (6–8 weeks old) were purchased from SPF Biotechnology (Beijing, China). DB/DB mice and db/+ mice (6–8 weeks old; weight, 20–24 g) were purchased from GemPharmatech (Nanjing, China). Type 1 diabetes was established by intraperitoneal injection of a low dose of streptozotocin (STZ) 50 mg/kg (Solarbio Science & Technology, Beijing, China) for 5 consecutive days. In the present study, diabetic mice with blood glucose levels above 16.7 mmol/L were used 4, 5, and 6 months after the final STZ injection. All of the animal experiments were approved by the ethics committee of Shandong Eye Institute and carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Culture and Treatment

The human corneal endothelial cell (HCEC) line B4G12 was cultured in human endothelial serum-free medium (Creative Bioarray, Shirley, NY, USA). We added 3% fetal bovine serum (FBS; Excell, Shanghai, China) and 1% penicillin–streptomycin solution (Corning Inc., Corning, NY, USA) to 50 mL of medium. To observe the changes in the HCECs, we incubated the cells with 30-mM glucose and 30-mM mannose as an osmotic control; the ER stress inducer thapsigargin (TG; 0.001 mM, T9033; Sigma-Aldrich, St. Louis, MO, USA) as a positive control or the classic ER stress inhibitor 4-phenylbutyric acid (4-PBA; 4 mmol/L, SML0309; Sigma-Aldrich) as a negative control.

Alizarin Red Staining and Immunohistochemical Staining

Mouse eyeballs were collected and fixed in a stroke-physiological saline solution (SPSS). Eye scissors were used to separate and flatmount the cornea by cutting it into four quadrants. To observe the changes in the corneal endothelial cells of mice, alizarin red stain was applied and incubated for 2 minutes, and the corneal samples were subsequently washed with SPSS three times. The samples were examined under an Olympus microscope (Tokyo, Japan). All of the experiments were repeated at least three times. Human corneas were collected, embedded in paraffin, and cut into 5-µm sections. Automated immunohistochemical staining for GRP78 (1:100; Proteintech Group, Rosemont, IL, USA) and CHOP (1:100; Proteintech Group) was performed on a Ventana Discovery XT immunostainer (Roche Diagnostics, Basel, Switzerland).

Administration of Medication

The ER stress inducer TG (0.001 mM) was injected into the anterior chamber of normal mice each day for 5 days. The mice were injected subconjunctivally with the U.S. Food and Drug Administration–approved drugs 4-PBA (20 mmol/L), plastoquinonyl-decyl-triphenylphosphonium bromide (SkQ1; 0.155 µg/mL in PBS, HY-100474; MedChemExpress, Monmouth Junction, NJ, USA), and *N*-acetyl-L-cysteine (NAC; 15 mM in PBS; Sigma-Aldrich) at 24 hours before and 0 hours after the injury, and the mice were administered 5 µL per injection. Then, eyedrops were administered three times a day. Eyedrops containing 4-PBA were administered three times each day starting 1 day before the injury and continuing every day after the injury. Irrigation solution containing 4-PBA (4 mmol/L) was used to perfuse the anterior chamber.

Acute Glaucoma Mouse Model

Diabetic mice and normal mice were randomly divided into the control group and the experimental group. Acute high intraocular pressure (IOP) has transient deleterious effects on corneal endothelial cells.[27] Before the high IOP model was established, the mice were anesthetized with 50 mg/kg body weight pentobarbital through intraperitoneal injection. After maintaining pupil dilation with tropicamide (Akorn Pharmaceuticals, Lake Forest, IL, USA) and achieving corneal analgesia using eyedrops containing 0.5% proparacaine hydrochloride ophthalmic solution (Alcaine; Alcon, Fort Worth, TX, USA), a 34-gauge microsyringe needle (JBP Korea, Seoul, Korea) was introduced into the anterior chamber through the corneal tunnel, avoiding contact with the corneal endothelium and the lens. The needle was connected to a 500-mL container of sodium chloride solution, and this solution was infused for 30 minutes.[27] Intraocular pressure was increased and maintained at 60 mmHg by elevating the solution container. At 0, 2, and 10 days after the termination of perfusion, all of the mice were examined. Ten days later they were euthanized and underwent a histological examination.

Corneal Whole-Mount Staining

Mouse eyeballs were collected and fixed in 4% paraformaldehyde for 20 minutes at room temperature (RT). The cornea was then dissected around the scleral–limbal region, blocked with 5% bovine serum albumin (BSA) for 2 hours, and subsequently incubated with primary antibodies (listed in Supplementary Table S1) overnight at 4°C. The next day, the corneas were incubated with the corresponding secondary antibodies (listed in Supplementary Table S1) for 1 hour at RT. All of the samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Eye scissors were used to separate and cut the corneas into four quadrants;

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subsequently, the corneas were flatmounted on a glass-bottom cell culture dish. All of the samples were examined under a ZEISS confocal microscope (ZEISS, Jena, Germany), and all experiments were repeated at least three times.

Western Blot

Total protein was extracted from lysate samples of the mouse corneal endothelium (10 corneas were pooled per sample) or cultured HCECs and run on 12% SDS-PAGE gels before being transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The samples were blocked with 5% BSA (Solarbio Science & Technology), incubated with primary antibodies (listed in Supplementary Table S1) overnight at 4°C, and subsequently incubated with horseradish peroxidase–conjugated secondary antibodies (listed in Supplementary Table S1) for 1 hour at RT. Finally, the blots were visualized via enzyme-linked chemiluminescence by using an electrochemiluminescence kit (EMD Millipore) and ChemiDoc Touch (Bio-Rad, Hercules, CA, USA), and protein expression was quantified by using Image Lab software (Bio-Rad). All of the experiments were repeated at least three times.

Corneal Thickness Analysis

Transverse corneal images were obtained using OCT (RTVue-100; Optovue, Fremont, CA, USA) to assess the central thickness of the cornea. In summary, the eye was rinsed three times with sterile water and placed in front of an optical scanning probe to obtain an image. Corneal thickness was analyzed using a quartile scan.

Endothelial Cell Density Analysis

Cell counts were performed manually by two blinded observers using Premier Endothelial Analytics (Konan Medical, Irvine, CA, USA) to determine the cell density from the images of three mouse corneas stained with Alizarin Red or ZO-1 immunostaining. The cell density was normalized to the area of each image in square millimeters. For each image, an area containing at least 60 cells was selected.

Mitochondrial Respiration Analysis

The metabolic profiles of HCECs cultured for 72 hours were assessed using the Seahorse XFp Analyzer, Seahorse XF Cell Mito Stress Test Kit, Seahorse Real-Time ATP Rate Assay Kit, and Seahorse XF FluxPaks containing eight-well plates and cartridges (Agilent Technologies, Santa Clara, CA, USA). One day before the extracellular flux measurement, HCECs were seeded at a concentration of 25,000 cells per well in eight-well assay plates in complete growth medium. One hour prior to the experiment, the growth medium was removed, and the cells were washed two times and then incubated in low-buffered assay medium (Agilent Technologies) supplemented with 5-mM glucose and 1-mM sodium pyruvate (pH 7.4). The microplates were then assayed with the Seahorse XFp Analyzer. The mitochondrial membrane uncoupler FCCP was then injected at a concentration of 2 mM. A mixture of rotenone and antimycin A (1 µM) was injected. At the end of the experiments, 20 µL of cell lysis buffer was added per well, and the protein concentration was measured by a bicinchoninic acid assay (Beyotime Biotechnology, Beijing, China). The oxygen consumption rate (OCR) and extracellular acidification rate were normalized to the protein content, and the data are presented as pmolesO_2/min/µg protein and mpH/min/µg protein, respectively.

Statistical Analysis

Statistical analysis was performed using Prism 7 (GraphPad, San Diego, CA, USA). All of the data are expressed as the mean ± standard deviation (SD). Experiments with two treatments and/or conditions were analyzed using a two-tailed Student's *t*-test. Experiments with more than two groups were analyzed using one-way ANOVA. Differences were considered statistically significant at $P < 0.05$. The experiments were repeated at least three times to ensure reproducibility.

Results

ER Stress Precedes Morphological Changes in Diabetic Corneal Endothelium

To demonstrate the morphological and functional changes in the corneal endothelium as the duration of diabetes increases, we established type 1 diabetic mice (DM) and followed them for 4 to 6 months after the final STZ injection. The mice were confirmed to have high levels of blood glucose and glycosylated hemoglobin (Supplementary Figs. S1A, S1B). We found that the diabetic mice exhibited corneal edema, increased corneal thickness (Fig. 1A), and reduced endothelial cell density (1292.3 ± 127 vs. 804.3 ± 107.5 cells/mm^2) until the 6-month time point (Figs. 1B, 1C). However, there was no significant difference between the coefficient of variation and hexagonality (Supplementary Figs. S1C, S1D). We further evaluated multiple time points of diabetes to determine the threshold of downregulation of protein expression. We found that the expression of ZO-1 was upregulated, whereas that of aquaporin 1 (AQP1) was downregulated in the corneal epithelium of diabetic mice at 4 months (Fig. 1D). The expression level of these proteins at 5 months was similar to that at 6 months (Supplementary Fig. S2). Consistently, the expression of the corneal endothelial markers Na^+/K^+-ATPase (ATP1a1), *N*-cadherin, AQP1, and ZO-1 was significantly downregulated at 6 months in diabetic mice, which was further confirmed by corneal whole-mount staining (Supplementary Fig. S2). These data suggest that long-term hyperglycemia causes morphological and functional impairment of the mouse corneal endothelium.

More importantly, the expression of the ER stress and UPR-related proteins calreticulin (CALR), glucose-regulated protein 8 (GRP78), C/EBP homologous protein (CHOP), and activating transcription factor 6 (ATF6) was significantly increased at 4 months in the diabetic mice compared with the age-matched control mice (Fig. 1E). These findings were also demonstrated by corneal whole-mount staining (Figs. 1F, 1G). In addition, we further confirmed that the immunohistochemical staining of the ER stress markers GRP78 and CHOP was stronger in the diabetic human corneal endothelium than in the normal human corneal endothelium (Fig. 1H). Therefore, ER stress in the corneal endothelium occurs in the early stage of diabetes mellitus, whereas there is no significant morphological or functional impairment.

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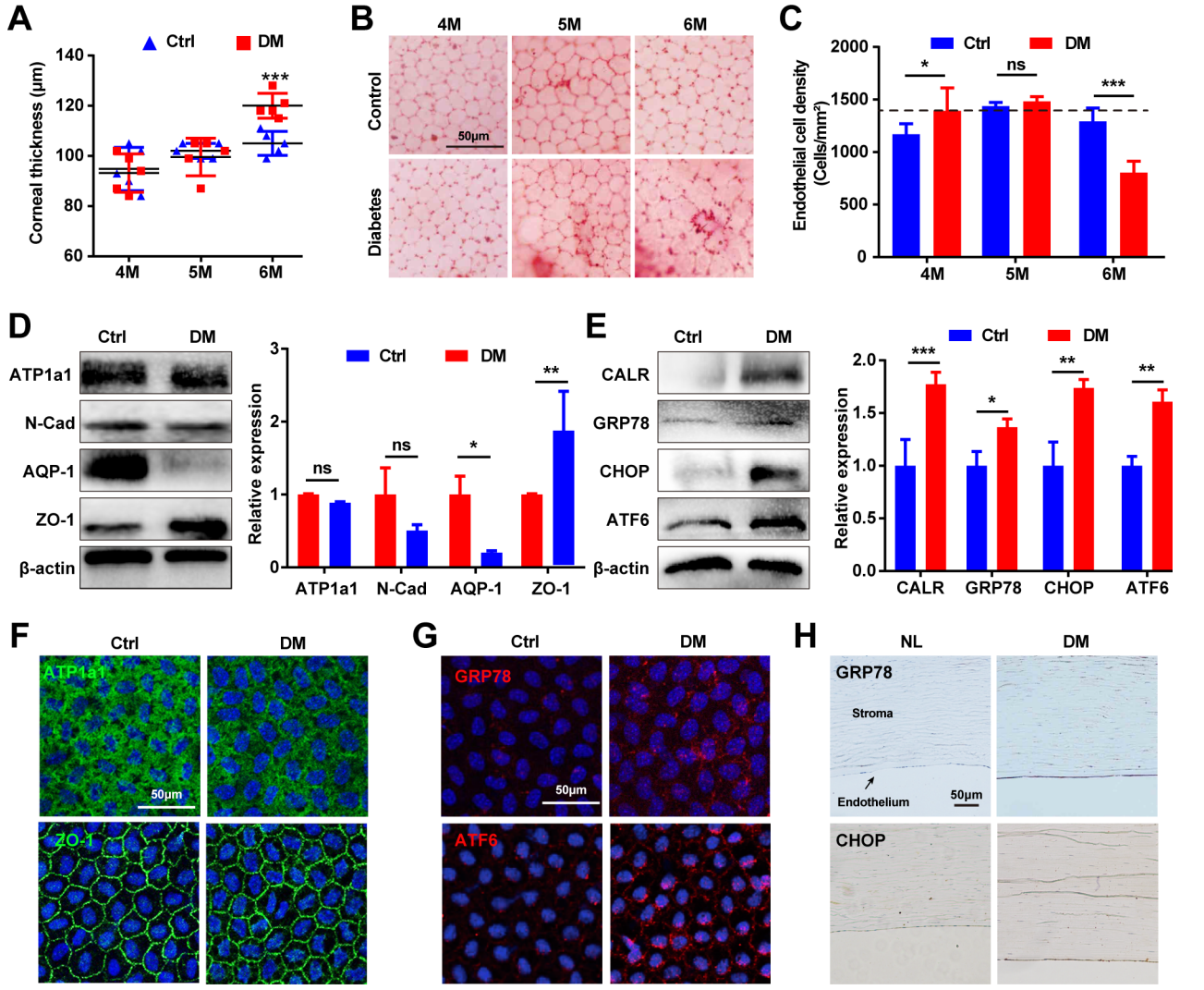

Figure 1. Hyperglycemia impairs corneal endothelial function in diabetic mice. Mouse model of type I diabetes established by STZ injection. (**A**) Evaluation of corneal thickness by OCT ($n = 5$). (**B**) Alizarin Red staining revealed the morphology of the corneal endothelium in diabetic mice 4, 5, and 6 months after STZ injection and in control mice ($n = 3$). (**C**) Alizarin Red staining–based analysis of cell density ($n = 3$). (**D**, **E**) Western blot bands showing ATP1a1, *N*-cadherin, AQP1, ZO-1, CALR, GRP78, CHOP, and ATF6 expression in endothelial cells from normal mice and mice 4 months after diabetes establishment. *β*-Actin served as a normalizing control. Densitometry analyses of the enzyme levels are indicated on the *right* as *bar graphs* ($n = 3$). (**F**, **G**) Corneal whole-mount staining shows the expression of ZO-1, ATP1a1, GRP78, and ATF6 in mice 4 months after diabetes establishment and normal mice ($n = 3$). DAPI was used to stain the nuclei (*blue*). *Scale bar:* 50 μm. (**H**) Immunohistochemical staining shows the expression of GRP78 and CHOP in the corneal endothelium of normal and diabetic patients. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Activation of ER Stress Causes Corneal Endothelial Impairment in Normal Mice

To determine the effect of ER stress activation on the corneal endothelium, we injected the ER stress inducer TG (0.001 mM) into the anterior chamber of normal mice for 5 days. Compared with the controls, the mice stimulated with TG exhibited apparent corneal edema (Fig. 2A) with increased corneal thickness (Fig. 2B). Moreover, corneal endothelial cell density was decreased significantly after TG stimulation (909.67 ± 85.11 vs. 1542.33 ± 92.14 cells/mm^2) (Fig. 2C). Further corneal whole-mount staining revealed inconsistent staining for the tight junction marker ZO-1 and ionic pump marker ATP1a1 in the TG-stimulated corneal endothelium, whereas these staining patterns were normal in the control mice (Fig. 2D). Consistently, western blot confirmed that TG stimulation significantly increased the expression of ER stress indicators, including CALR, CHOP, and GRP78, and decreased the protein expression of ZO-1 and ATP1a1 in the corneal endothelium (Figs. 2E, 2F). These results suggest that the activation of ER stress directly causes corneal cell loss and endothelial dysfunction.

Inhibiting ER Stress Mitigates Diabetic Corneal Edema and Endothelial Cell Loss

To clarify the pathogenic role of ER stress in diabetic corneal endothelial dysfunction, we established mouse models of acute glaucoma, which is one major trigger of corneal endothelial dysfunction in the context of diabetes mellitus. These animals were injected subconjunctivally with

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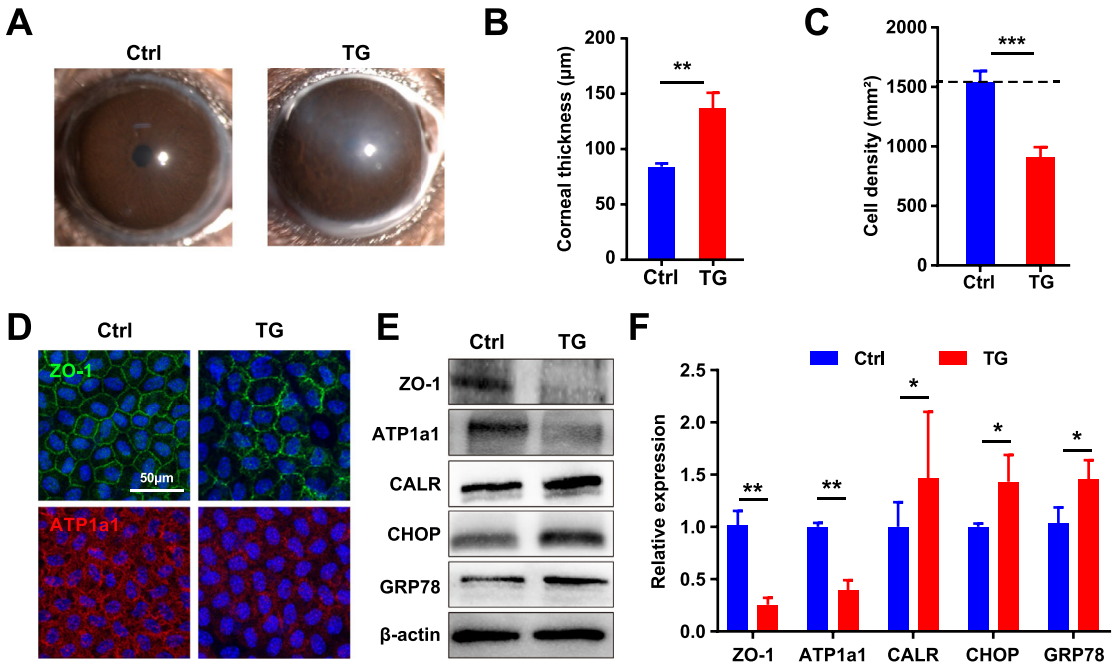

Figure 2. ER stress promotes corneal endothelium dysfunction in normal mice. The ER stress inducer TG was injected into the anterior chamber of normal mice for 5 days. (**A**) Slit-lamp microscopy images show the corneal changes in PBS-injected mice (Ctrl) and TG-injected mice (TG) ($n = 3$). (**B**) Evaluation of corneal thickness by OCT in PBS- or TG-treated mice ($n = 3$). (**C**) ZO-1 immunostaining-based analysis of cell density ($n = 3$). (**D**) Corneal whole-mount staining shows the expression of ZO-1 and ATP1a1 in PBS-injected mice and TG-injected mice ($n = 3$). DAPI was used to stain the nuclei (*blue*). *Scale bar:* 50 μm. (**E**) Western blot analysis of the levels of ZO-1, ATP1a1, CALR, CHOP, and GRP78 in mouse corneal endothelium ($n = 3$). (**F**) β-Actin served as a normalizing control. Densitometry analysis of the enzyme levels is indicated on the *right* as *bar graphs*. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

4-PBA (20 mmol/L).[26] Consistent with clinical observations,[5] acute glaucoma caused persistent corneal edema with increased corneal thickness and reduced cell density in the diabetic mice compared with the control mice. However, treatment with 4-PBA improved the corneal edema, decreased endothelial cell density, and diminished the extensive decrease in the expression of the corneal endothelial proteins ZO-1 and ATP1a1 in both mice with type 1 diabetes (Figs. 3A–3D, Supplementary Figs. S3A–S3C) and type 2 diabetes (Figs. 3E–3H, Supplementary Figs. S3D–S3F). In addition, we further evaluated multiple methods of 4-PBA administration. The results showed that anterior chamber perfusion with 4 mmol/L 4-PBA into the anterior chamber was sufficient to rapidly restore corneal transparency and thickness within 10 days, achieving an endothelial cell density similar to that observed in the group that received subconjunctival 4-PBA injection (Supplementary Fig. S4).

Activation of ER Stress Causes Mitochondrial Bioenergy Deficiency in Cultured Cells

To explore the potential mechanisms by which ER stress affects diabetic corneal endothelial dysfunction, we treated HCECs with the ER stress inducer TG (0.001 mM) for 72 hours. We confirmed that TG increased the expression of the ER stress indicators GPR78 and CHOP and reduced the expression of the corneal endothelial markers ZO-1 and ATP1a1 (Figs. 4A, 4B). Due to the importance of mitochondrial function in the corneal endothelium, we further evaluated mitochondrial bioenergetic changes using a Seahorse XFp Analyzer. The experiments revealed mitochondrial impairment in TG-stimulated HCECs, including impairments in basal respiration, ATP-linked respiration, and proton leakage, as well as an approximately 50% reduction in maximal respiration (Figs. 4C–4H). These results suggest that ER stress activation can lead to mitochondrial bioenergy deficiency, which may be the potential mechanism that leads to endothelial dysfunction.

Inhibiting ER Stress Ameliorates Hyperglycemia-Induced Mitochondrial Bioenergy Deficiency in Cultured Cells

To investigate the mechanism by which ER stress inhibitors work in diabetes, we treated HCECs with 30-mM mannose as an osmotic control and with 30-mM high glucose (HG) containing 4 mmol/L 4-PBA as a negative control for 72 hours. We revealed that HG treatment increased the expression of GPR78, CHOP, and the mitochondrial dynamics protein mitofusin 1 (MFN1) and reduced the expression of ZO-1 and ATP1a1 by western blot analysis. However, 4-PBA reversed these changes in the expression of proteins related to ER stress and endothelial and mitochondrial dynamics induced by hyperglycemia (Figs. 5A, 5B). HG led to mitochondrial bioenergetic deficits (Supplementary Fig. S5A), and the production of ATP shifted from the oxidative phosphorylation pathway to glycolysis (Fig. 5C); however, mannitol had no significant effect on the mitochondria of HCECs according to the results obtained with a Seahorse XFp Analyzer (Supplementary Fig. S5B). Moreover, the administration of 4-PBA reduced the mitochondrial damage and

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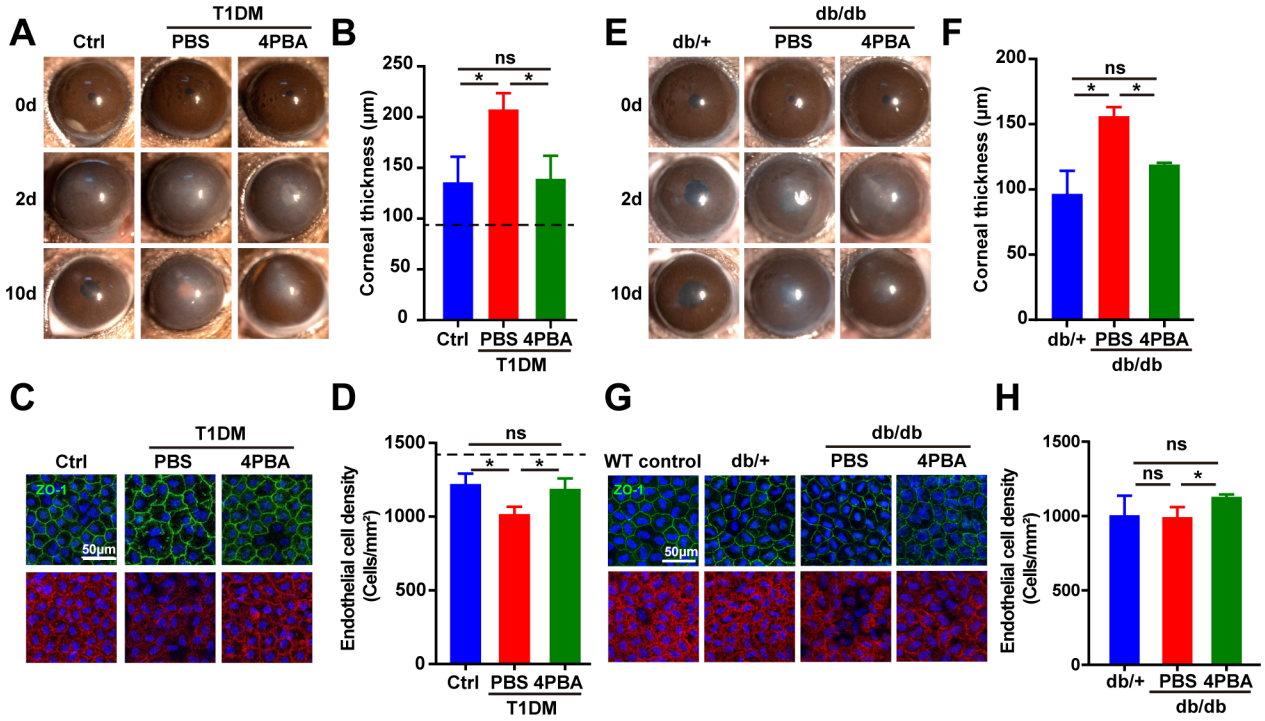

Figure 3. Inhibition of ER stress protects the corneal endothelium in diabetic mice. A model of acute glaucoma was established by sodium chloride perfusion. Type 1 diabetic (T1DM) and age-matched control mice (Ctrl) were used 4 months after intraperitoneal STZ injection. Mice were subconjunctivally injected with PBS (as the control) or 4-PBA 24 hours before irrigation and 0 hours postperfusion. (**A**) Slit-lamp images of mice at 2 days and 10 days postperfusion ($n = 5$). (**B**) Evaluation of corneal thickness by OCT ($n = 3$). (**C**) Corneal whole-mount staining shows the expression of ZO-1 and ATP1a1 after 10 days of perfusion in T1DM mice. DAPI was used to stain the nuclei (*blue*). *Scale bar:* 50 μm ($n = 3$). (**D**) ZO-1 immunostaining-based analysis of cell density ($n = 3$). Type II diabetic mice (db/db) and age-matched control mice (db/+) were used to establish acute glaucoma models. (**E**) Slit-lamp images of mice at 2 days and 10 days postperfusion ($n = 5$). (**F**) Evaluation of corneal thickness by OCT ($n = 3$). (**G**) Corneal whole-mount staining shows the expression of ZO-1 and ATP1a1 after 10 days of perfusion in type 2 diabetic mice compared with wild-type (WT) control. DAPI was used to stain the nuclei (*blue*). *Scale bar:* 50 μm ($n = 5$). (**H**) ZO-1 immunostaining-based analysis of cell density ($n = 3$). The dotted line represents the normal value. *$P < 0.05$.

corneal endothelial functional impairment caused by hyperglycemia and increased basal respiration, adenosine triphosphate (ATP)-linked respiration, proton leak, and the production of ATP from the glycolysis pathway to oxidative phosphorylation (Figs. 5C, 5D). Collectively, inhibition of ER stress can protect endothelial function through the mitochondrial pathway.

Mitochondria-Targeting Antioxidants Mitigate Diabetic Corneal Edema and Endothelial Cell Loss

To investigate the role of mitochondrial dysfunction in diabetic corneal endothelial dysfunction, we further compared the efficacy of the mitochondrial-targeted antioxidant SkQ1 (0.155 μg/mL in PBS) and the ER stress inhibitor 4-PBA in a diabetic mouse acute glaucoma model. PBS and the reactive oxygen species (ROS) scavenger NAC (15 mM in PBS) were used as controls. After continuous observation for 10 days, the mice treated with NAC showed no improvement in corneal edema or thickness, indicating that diabetic corneal endothelial dysfunction may not be caused by oxidative stress. However, the mice treated with SkQ1 exhibited improvements in corneal edema and corneal thickness that were similar to those treated with 4-PBA (Figs. 6A, 6B). Further corneal whole-mount staining confirmed that ZO-1 and ATP1a1 expression and endothelial cell density were maintained in the corneal endothelium of the mice treated with 4-PBA or SkQ1 (Figs. 6C, 6D). Therefore, the mitochondria-targeted antioxidant SkQ1 provided protective effects on the corneal endothelium that were similar to those exerted by ER stress inhibition.

Discussion

Diabetic corneal endothelial dysfunction is an underestimated complication of diabetes that increases the risk for corneal decompensation. Patients with endothelial dysfunction experience corneal edema, endothelial cell loss, vision decline, pain, and discomfort on the ocular surface.[6] The common triggers of endothelial dysfunction in the clinic include acute glaucoma and cataract phacoemulsification.[5,28] In this study, we first confirmed that ER stress was a significant pathological mechanism underlying diabetic corneal endothelial dysfunction that preceded the development of morphological changes. Inhibition of ER stress can obviously mitigate endothelial dysfunction in diabetes. We further demonstrated that ER stress can induce mitochondrial bioenergy deficiency, whereas inhibition of ER stress can protect the function of mitochondria during hyperglycemia, and further protection of mitochondria by SkQ1 can ameliorate corneal edema and thickening. Overall, we provide mechanistic insights into the role of ER stress in diabetic corneal endothelial dysfunction and present

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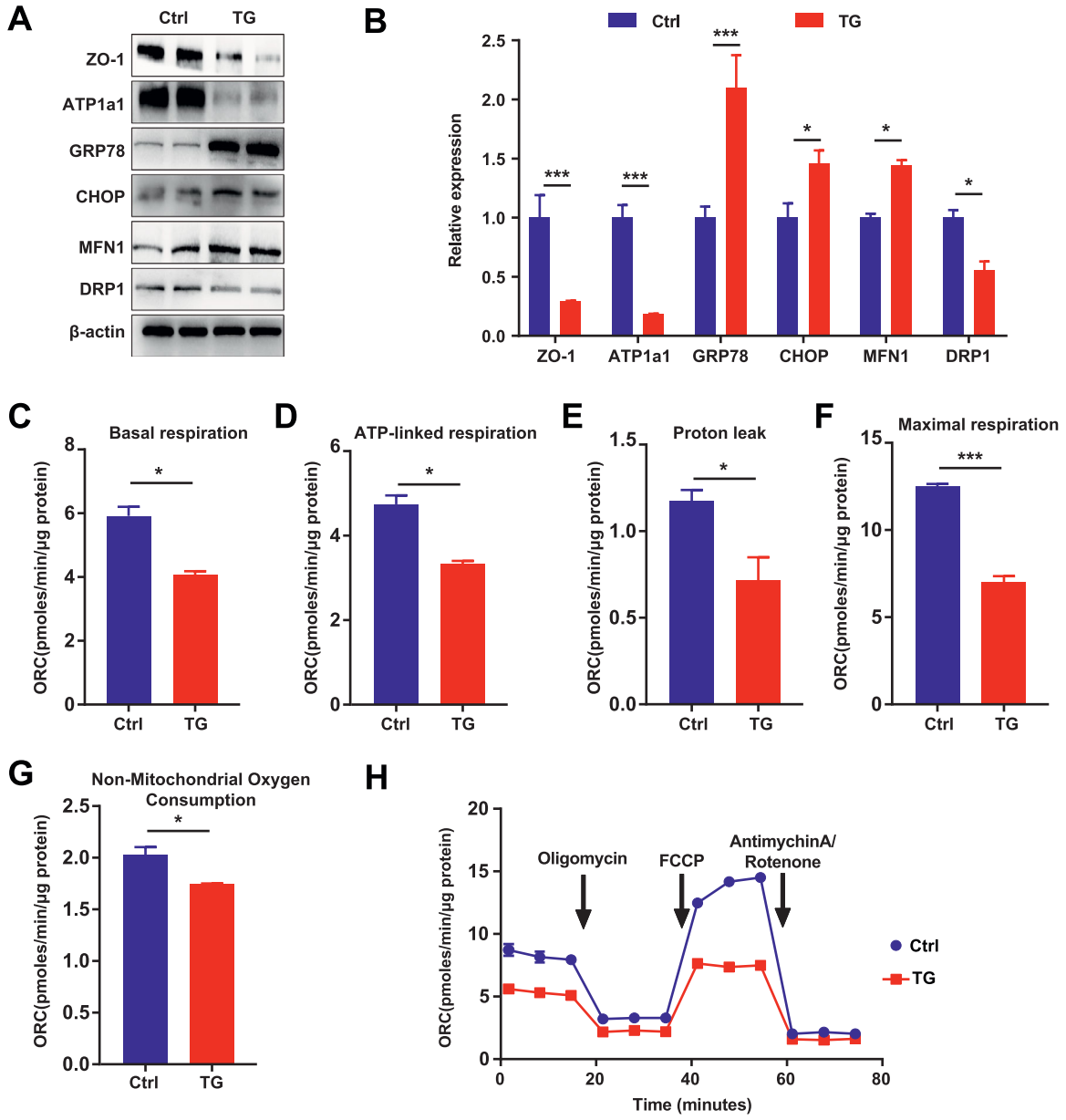

Figure 4. Activation of ER stress induces mitochondrial dysfunction in cultured cells. (**A**) Western blot bands of ZO-1, ATP1a1, GRP78, CHOP, MFN1, dynamin-related protein 1 (DRP1), and cytochrome *c* (CytoC) expression with or without TG treatment ($n = 3$). (**B**) Quantified intensities of western blot bands compared with those of β-actin. The mitochondrial function of HCECs with or without TG treatment was evaluated with a Seahorse XFp Analyzer. After measurement of (**C**) basal OCR, (**D**) ATP-linked respiration, and (**E**) proton leak was determined following the injection of oligomycin (1.5 μM final), (**F**) maximal respiration was determined after FCCP injection (0.5 μM), and all the parameters were calculated by subtracting (**G**) the non-mitochondrial respiration. (**H**) Mitochondrial respiration measured by a Seahorse XFp Analyzer ($n = 3$). *$P < 0.05$, ***$P < 0.001$.

compelling evidence that targeting ER stress is a potential method for treating these complications.

Morphological changes are the most remarkable characteristic of endothelial dysfunction; however, these changes in diabetes mellitus remain controversial.[29–31] A previous study revealed that patients who have had diabetes for over 10 years exhibited significantly lower corneal endothelial cell density and enlarged cells.[32] Here, we used a type 1 diabetic mouse model and examined the changes in corneal endothelial cell morphology, corneal thickness, endothelial barrier, and pump function 4 to 6 months after the establishment of diabetes. We observed apparent corneal edema and increased corneal thickness in the diabetic mice over the 6 months, which was consistent with the manifestation of corneal thickening and endothelial cell loss of diabetic patients in the clinic.[8,32–34] Further, studies have shown that microvascular complications are more common among children with type 2 diabetes at the time of presentation than among those with type 1 diabetes.[35] Mice with type 2 diabetes not only had hyperglycemia but also had increased insulin and glucagon, which may be the reason for greater vulnerability.[36] Recent studies have generally agreed that the corneal endothelium of diabetic patients is more vulnerable to trauma associated with ocular surgeries and

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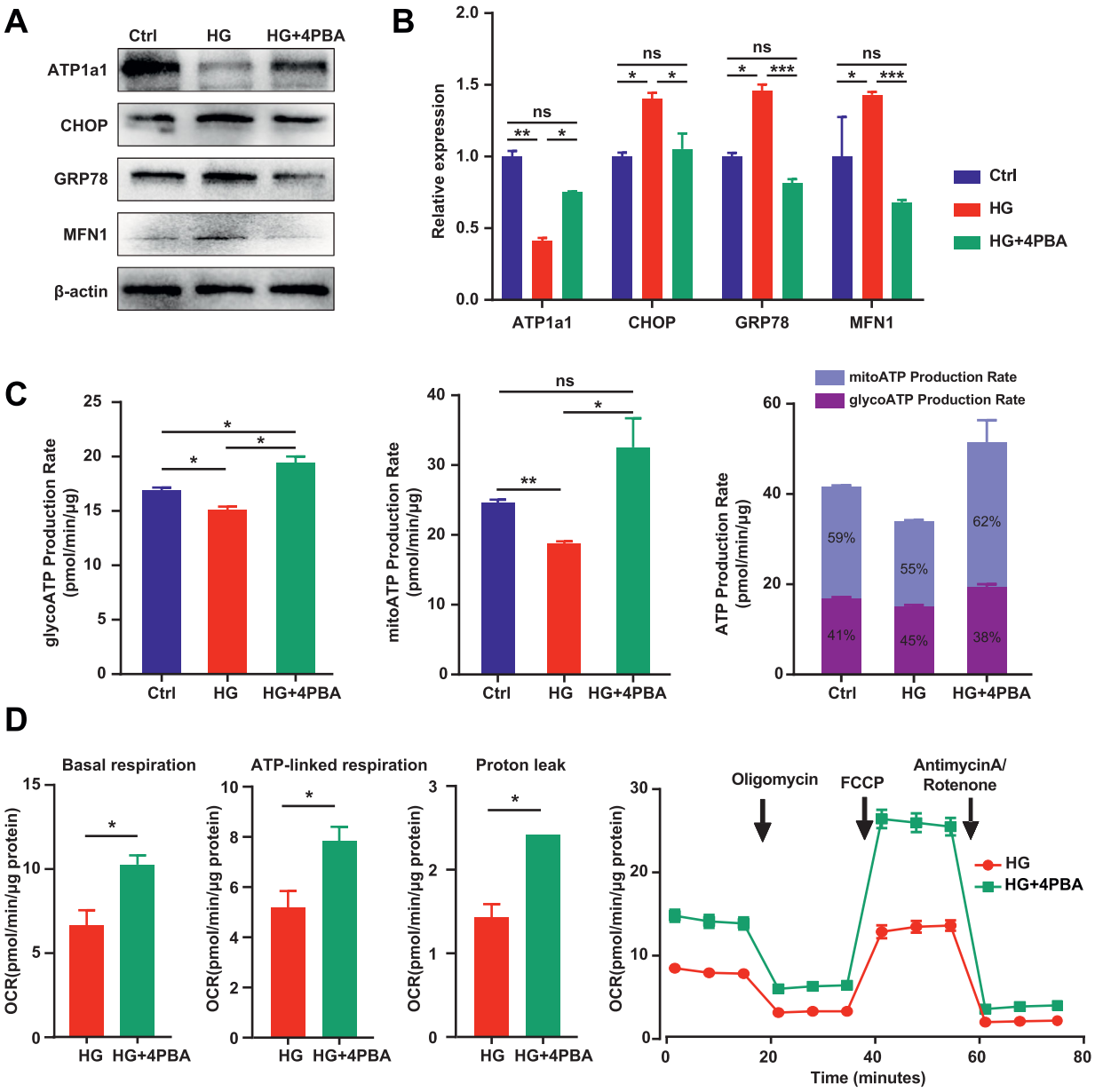

Figure 5. Restoration of endothelial cell function through the ER stress–mitochondrial pathway in HG-treated cells. (**A**) Western blot analysis of ATP1a1, GRP78, and MFN1 expression in the control, HG-treated, and HG with 4-PBA groups ($n = 3$). (**B**) The western blot bands were quantified by densitometric analysis and normalized to β-actin as the internal control. (**C**) Seahorse XFp Analyzer and quantification of ATP levels contributed by mitochondria (ATPmito) and glycolysis (ATPglyco). Cells were cultured under control, HG, and HG conditions with 4-PBA ($n = 3$). (**D**) Mitochondrial function in HG-stimulated HCECs with or without 4-PBA treatment was evaluated with a Seahorse XFp Analyzer ($n = 3$). After measurement of basal OCR, ATP-linked respiration and proton leakage were determined following oligomycin injection (1.5 μM final), and all of the parameters were calculated by subtracting the nonmitochondrial respiration. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

glaucoma.[37–40] Therefore, additional methods should be developed to mitigate the risk of corneal decompensation before performing intraocular surgeries in diabetic patients.

ER stress has been observed and considered a potential target for treating multiple diabetic complications, including retinopathy, glaucoma, and corneal epitheliopathy,[26,41] whereas its mechanism in endothelial dysfunction is vague. Here, we performed western blot and corneal whole-mount staining analyses of diabetic mice and vehicle-treated mice. Interestingly, ER stress was observed in the corneal endothelium of diabetic mice at 4 months with no significant morphological changes. Consistently, we confirmed the upregulated expression of the ER stress-related GRP78 and CHOP proteins in diabetic human corneal endothelium by immunohistochemical staining. To validate the strategy of targeting ER stress to treat diabetic corneal endothelial dysfunction, we further used a mode of acute injuries in glaucoma. The results confirmed that inhibition of ER stress with 4-PBA, either by subconjunctival injection before surgery or via perfusion supplementation during surgery, resulted in a significant improvement in corneal edema and thickening, as well as a reduction in corneal endothelial cell

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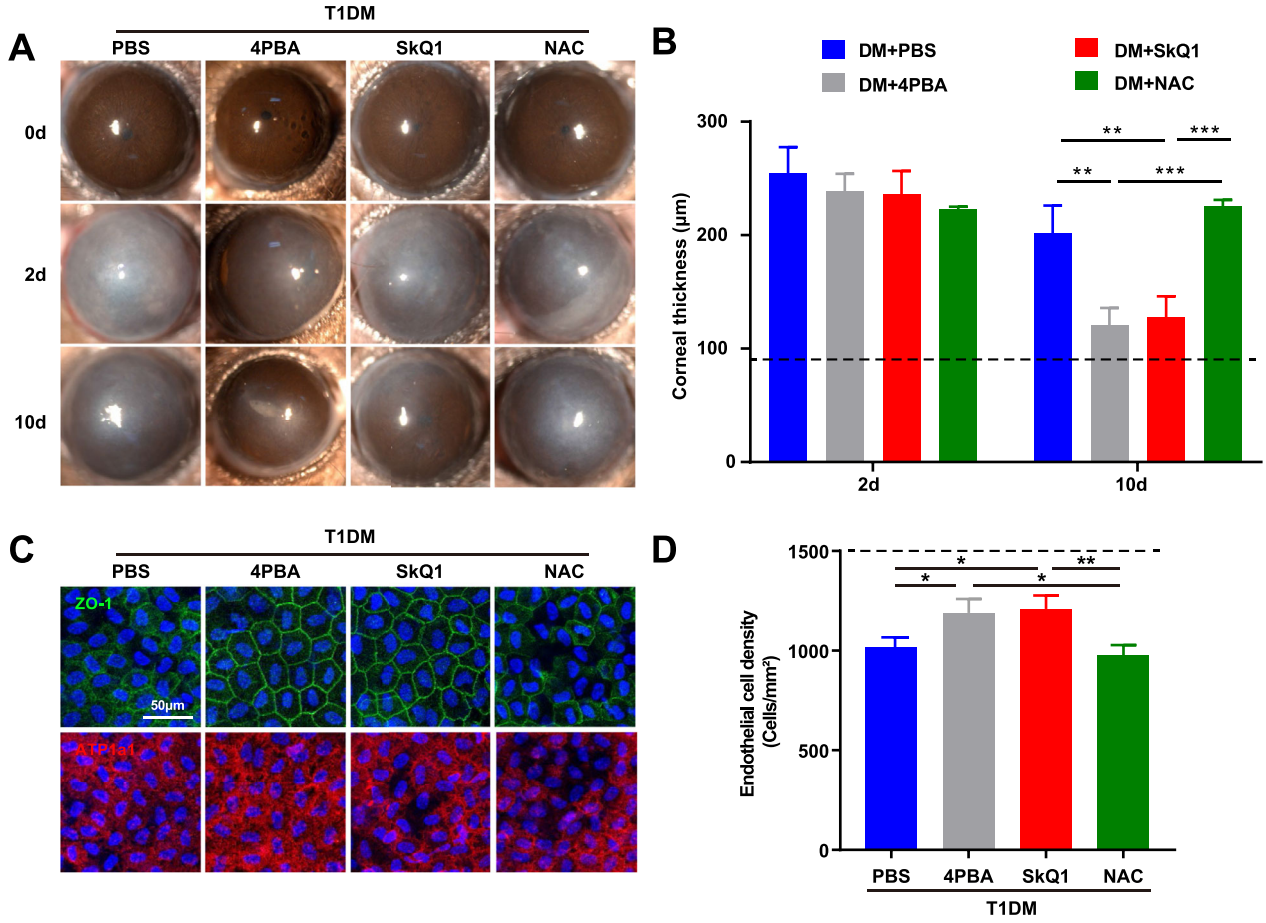

Figure 6. Mitochondria-targeted antioxidants protect the corneal endothelium in diabetic mice. The ER stress inhibitor 4-PBA, mitochondrial-targeted antioxidant SkQ1 (0.155 μg/mL in PBS), and ROS inhibitor NAC (15 mM in PBS) were applied to diabetic mice with high IOP. (**A**) Slit-lamp microscopy images of the changes in corneas ($n = 3$). (**B**) Evaluation of corneal thickness by OCT ($n = 3$). (**C**) Corneal whole-mount staining shows the expression of ZO-1 and ATP1a1 in diabetic mice treated with 4-PBA, SkQ1, or NAC compared with their expression in PBS-treated mice ($n = 3$). DAPI was used for nuclei staining (*blue*). *Scale bar:* 50 μm. (**D**) ZO-1 immunostaining-based analysis of cell density ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

loss. Collectively, our study results confirmed for the first time, to the best of our knowledge, the effect of ER stress on the pathogenesis of diabetic corneal endothelial dysfunction.

Mitochondria provide the large levels of ATP that are necessary for the endothelial Na^+/K^+-ATPase active transport pump to regulate corneal hydration.[42] Mitochondrial dysfunction has been confirmed in Fuchs endothelial corneal dystrophy.[43] Here, we demonstrated mitochondrial dysfunction using a flux analyzer and found that basal respiration, maximal respiration, ATP production, and spare respiratory capacity were decreased in HG-stimulated corneal endothelial cells. These findings are consistent with the conclusion that hyperglycemia leads to reduced mitochondrial bioenergetics in renal tubules and mesangial cells in diabetic nephropathy[44] and in epithelial cells in diabetic keratopathy.[21] We further demonstrated that the topical administration of mitochondria-targeted antioxidants significantly attenuated corneal edema and endothelial cell loss in diabetic mice. Previous studies have shown that mitochondrial function can be mediated by prolonged activation of ER stress.[45] A study on diabetic nephropathy showed that mitochondrial dysfunction is associated with ER stress in proximal tubule epithelial cells of the kidney.[46] Here, we found that mitochondrial function was disturbed by an ER stress inducer. Moreover, 4-PBA administration to hyperglycemia–HCEC cells rapidly improved mitochondrial function. Similarly, inhibition of ER stress was reported to be associated with the improvement of mitochondrial function in individuals with cardiovascular disease.[47] The ER stress–mitochondrial pathway may provide a new therapeutic strategy for diabetic corneal endothelial dysfunction.

In conclusion, the present study first demonstrates that endothelial cells from diabetic mice and humans exhibit high levels of ER stress. ER stress appears before morphological changes of the endothelium are observed. Activation of ER stress can promote corneal endothelial dysfunction by triggering mitochondrial dysfunction. Acute injuries, such as glaucoma, can lead to corneal edema and endothelial cell loss, whereas inhibition of ER stress can alleviate these injuries. ER stress inhibition attenuates diabetic corneal endothelial dysfunction by blocking hyperglycemia-induced ER stress through the mitochondrial signaling pathway. Mitochondrial-targeted antioxidants can imitate the effects of ER stress inhibition on diabetic corneal endothelial dysfunction. Here, our findings suggest that targeting the ER stress–mitochondrial pathway is a potential strategy for ameliorating corneal endothelial cell function in

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diabetic patients. Further studies are still required to validate the effectiveness and sustainability of these treatments for patients with diabetic corneal endothelial dysfunction in the clinic.

Acknowledgments

The authors thank Qun Wang and Haoyun Duan (Shandong Eye Institute) for their help with animal tissue collection, statistical analysis, and instrument use. QZ and LX are the guarantors of this work and, as such, had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Supported by grants from the Academic Promotion Program and Innovation Project of Shandong First Medical University (2019ZL001 and 2019RC008) and the Taishan Scholar Program (20161059).

Disclosure: **C. Chen**, None; **B. Zhang**, None; **J. Xue**, None; **Z. Li**, None; **S. Dou**, None; **H. Chen**, None; **Q. Wang**, None; **M. Qu**, None; **H. Wang**, None; **Y. Zhang**, None; **L. Wan**, None; **Q. Zhou**, None; **L. Xie**, None

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