Low glucose under hypoxic conditions induces unfolded protein response and produces reactive oxygen species in lens epithelial cells

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Aging is enhanced by hypoxia and oxidative stress. As the lens is located in the hypoglycemic environment under hypoxia, aging lens with diabetes might aggravate these stresses. This study was designed to examine whether low glucose under hypoxic conditions induces the unfolded protein response (UPR), and also if the UPR then generates the reactive oxygen species (ROS) in lens epithelial cells (LECs). The UPR was activated within 1 h by culturing the human LECs (HLECs) and rat LECs in <1.5 mM glucose under hypoxic conditions. These conditions also induced the Nrf2-dependent antioxidant-protective UPR, production of ROS, and apoptosis. The rat LECs located in the anterior center region were the least susceptible to the UPR, whereas the proliferating LECs in the germinative zone were the most susceptible. Because the cortical lens fiber cells are differentiated from the LECs after the onset of diabetes, we suggest that these newly formed cortical fibers have lower levels of Nrf2, and are then oxidized resulting in cortical cataracts. Thus, low glucose and oxygen conditions induce the UPR, generation of ROS, and expressed the Nrf2 and Nrf2-dependent antioxidant enzymes at normal levels. But these cells eventually lose reduced glutathione (GSH) and induce apoptosis. The results indicate a new link between hypoglycemia under hypoxia and impairment of HLEC functions.

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Diabetic cataracts are characterized by cortical or posterior sub-capsular (PSC) opacities, and in adults the opacities are known to be due to an acceleration of age-related cataracts (ARCs).1 Cataractous lenses have decreased epithelial cell density and extensive cellular damage in the cortical region of the lens.2 It has been reported that an accumulation of sorbitol under hyperglycemic conditions can generate osmotic stress,3 and the osmotic stress combined with oxidation,4 for example, glycoxidation, increased cytosolic calcium,5,6 cell death and aging,1,7 is considered to be the cause of diabetic cataracts.9

The lens is an avascular organ, and it receives glucose and oxygen from the ciliary body and blood vessels of the iris. The level of glucose in the aqueous fluid is 2–4 mM and that in the vitreous fluid is ~1.6 mM.8 Although intensive insulin therapy is aimed at strictly controlling the glucose level, it can also cause an increase in the incidence of severe hypoglycemia by 4–6-folds.10 If the lens is deprived of glucose for 48 h, a nuclear cataract can develop.11 This cataract bears a striking resemblance to the hypoglycemic cataract seen in children.12

The lens is also located in a hypoxic environment of 0.5–2.3% oxygen.13–15 The oxygen level in the vitreous body of cataract patients is 0.1–0.5%,16 and that in the anterior vitreous immediately adjacent to the lens is reported to be 0.26–0.40% in cats, rabbits, and chickens.15,17 An environment of 0.1–1.0% oxygen is defined as being severely hypoxic. The oxygen level in the ocular lens can easily fall into the severe hypoxic range even in a 13–15% atmospheric oxygen environment,18 for example, in diabetic patients.19 Similar low levels can develop during the progression of arteriosclerosis.20

In addition to the individual levels of glucose and oxygen, the ratio of glucose/oxygen is important and excess levels of either oxygen or glucose can lead to oxidization of the lens. Importantly, normal lenses acquire about 75–80% of their ATP from anaerobic and 20–25% from aerobic respiration.20

Non-physiological glucose concentration such as near 0 mM and more than 250 mM of glucose in a 20% atmospheric oxygen environment induce the chronic unfolded protein response (UPR).21 Recent paper reported that aging is associated with hypoxia and oxidative stress.22 However...
there is no information on whether hypoglycemic conditions under in situ hypoxic concentrations (~1% oxygen) can induce the UPR in the lens. The UPR is a protective mechanism that is activated by phosphorylation of PKR-like endoplasmic reticulum kinase (PERK), inositol requiring enzyme-1 (IRE-1), and eukaryotic translation initiation factor 2α (eIF2α). All of these components are the initial cellular response to stress. However, chronic UPR induces apoptosis by activating ATF4 and death factors such as, C/EBP-homologous protein (CHOP)23 and caspase-12.24 ATF4 is identified as an important regulator of mammalian lens development.25 The lens-specific expression of ATF4 in the mutant mice demonstrates its importance in the later stages of lens fiber cell differentiation.26 The UPR also generates excess levels of reactive oxygen species (ROS).27 The ROS are generated by disulfide formation in the ER which is driven by a protein relay27 involving a protein disulfide isomerase (PDI) and ER oxidoreductin 1 (Ero1)-Lt, and -Lt.28 The terminal electron acceptor is molecular oxygen. In the UPR, the PDI level is constant but the level of Ero1-Lt is increased and Ero1-Lt is decreased. In addition, decreased levels of cystolic-free GSH from the mitochondria are an additional source of ROS.7

To counteract the ROS, the UPR activates a PERK-dependent antioxidant defense system and upregulates Nrf2, a master transcription factor for more than 20 antioxidant enzymes such as, glutathione reductase (GR), thioredoxin, thioredoxin-S-transferase, and catalase (Figure 1).29,30

We hypothesized that low glucose under hypoxic condition in LECs induces the UPR, which leads to the production of ROS, although the Nrf2-dependent antioxidant defense protection might fail during low glucose. To test this hypothesis, we studied the UPR induction, ROS production, and evaluated the levels of Nrf2 in HLECs treated with low glucose under low oxygen levels.

Results

Glucose and oxygen levels in cell culture medium. To estimate the consumption of glucose, HLECs in 10-cm Petri dishes at 80% confluence were incubated in 1 or 2 mM glucose-DMEM under 1% atmospheric oxygen. After 2, 4, 6, and 8 h, the glucose concentration in the culture medium was measured. The consumption of glucose by the HLECs under our culture conditions was 1 mM/Petri dish/8 h or 1.0 nM/cell/8 h both in 1 mM or 2 mM glucose-DMEM (Figure 2a). We also measured the oxygen levels in air, culture medium, and culture medium containing 1/106 HLECs (10-cm Petri dish) placed in the vacuum bag containing oxygen absorption chemicals, AnaeroGen (Oxoid Ltd, Haunts, UK). The oxygen level in the air of the vacuum bag was reduced to severe hypoxic levels (~0.1%) within 1 h, in the culture medium with HLECs within 2 h, and in the culture medium without cells within 4.5 h (Figure 2b). Therefore, to achieve severe hypoxic conditions required at least 2 h under our experimental conditions.

Low level of glucose under severe hypoxic condition activates UPR. When HLECs were cultured in GF-DMEM under 0.14% oxygen, the UPR was strongly activated.

Figure 1 A schematic diagram of our working hypothesis. Cataractogenic stress induces the UPR.40 The UPR is a protective mechanism against ER stress, and its activation leads to phosphorylation of PERK (P-PERK). Prolonged or severe ER stress induces the apoptotic UPR, which generates excess levels of ROS and activates M-calpain. P-PERK phosphorylates Nrf2, the P-Nrf2 dissociates from Keap1 and translocates into the nucleus to bind to the antioxidant response element (ARE), which activates the transcription of more than 20 antioxidant enzymes such as, glutathione, GR, thioredoxin, thioredoxin-S-transferase, and catalase. These antioxidant enzymes regenerate GSH, and the resultant GSH eliminates ROS to survive or recover from the stress. We hypothesized that low glucose under hypoxic condition suppresses the levels of Nrf2, which downregulates the antioxidant enzymes and GSH and results in oxidation of the lens. Thus, low glucose under low oxygen stress activates the UPR, production of ROS, and dysfunction of the antioxidant defense protection leading to lens oxidation.

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Critical concentrations of glucose and oxygen to induce the UPR. Next, we determined the lowest concentration of glucose that would induce the UPR in HLECs. The cells were cultured in GF-DMEM (Figure 3a), 1 mM glucose-DMEM, and 2 mM glucose-DMEM under 1% atmospheric oxygen for 0, 2, 4, 6, and 8 h (Figures 3b–d). During these culture periods, glucose is consumed by the cells, and the levels decreased from 1 to 0 mM (Figure 3c) or from 2 to 1 mM (Figure 3d). Then, we examined the phosphorylation of PERK and eIF2α by sampling the HLECs after 2, 4, 6, and 8 h of incubation. Protein blot analysis showed that increased levels of P-PERK and P-eIF2α after 4 h in GF-DMEM and 1 mM glucose-DMEM. These results indicated that both GF-DMEM and 1 mM glucose-DMEM induced the phosphorylation of PERK and eIF2α after 4 h. Thus, 1 mM of glucose can induce the UPR after 4 h of culture, which does not activate the UPR, but thereafter when the glucose concentration has decreased to 1.5 mM or less it would induce the UPR. These results further indicated that glucose concentration less than 1.5 mM induces the UPR in cultured HLECs under 1% atmospheric oxygen.

Activity of UPR in rat LECs by ischemia. Under ischemic conditions, the oxygen level in the eye is reduced very rapidly but the glucose level remains normal for relatively longer times. Thus, ischemia might be a condition to study the effects of hypoxic condition on lenses. To accomplish this, four groups of three rats each were euthanized by CO₂ inhalation, and the right eyes were immediately removed and the left eyes were kept in the AnaeroGen vacuum bag for less than 1.5 h and then returned to the normal culture conditions (5 mM glucose-DMEM in 4% atmospheric oxygen) for 20 h did not produce ROS and apoptosis (Figure 5a). However, when the LECs were treated for more than 1.5 h and then returned to normal culture conditions for 20 h, 5–10% of these HLECs produced ROS and apoptosis in the LECs (Figure 5a). These results indicated that the protective UPR was activated by 0.5–1.5 h of GF-DMEM, but the apoptotic UPR was activated in a relatively small number of LECs.
when the HLECs were exposed to longer durations (> 1.5 h) of the hypoxic conditions.

To confirm this UPR activation, HLECs were cultured for 3 or 5 h in GF-DMEM in the AnaeroGen vacuum bag, return to 5 mM glucose-DMEM, and then cultured for 1, 6, 14, 17, and 20 h under 1% atmospheric oxygen. We assayed for the levels of the Nrf2, glutathione reductase (GR), and catalase in the HLECs cultured for 0, 1, 6, 14, 17, and 24 h by protein blot analysis. One striking finding was that the level of Nrf2, GR, and catalase were significantly increased in HLECs treated for 3 h (Figure 6a). These results indicated that HLECs treated for 3 h activated the survival UPR to protect cells from stress by increasing Nrf2, GR, and catalase. In contrast, HLECs treated for 5 h (clock time) induced the apoptotic UPR and significant levels of the production of ROS and resulted in apoptosis (Figure 6b).

Proliferating HLECs are more susceptible to low glucose and low oxygen conditions. Next, we asked what type of LECs becomes apoptotic among all the LEC types cultured in GF-DMEM under hypoxic conditions. We hypothesized that proliferating cells might be more susceptible to the UPR. To test this, HLECs were exposed to BrdU for 30 min, and the cells were washed and cultured in GF-DMEM for an additional 15 h in 4% atmospheric oxygen. At the end of the culture period, the cells were stained with EthD and a fluor-labeled antibody to BrdU.

The results showed that about 5% of cells were BrdU- and EthD-positive and more than 78% of these cells were co-stained (Figure 5b). These results clearly indicated that the proliferating cells were the cells most susceptible to the UPR-dependent cell death. These findings are consistent with those of Endo et al.36

**Figure 3** Activation of UPR by phosphorylation of PERK and eIF2α in human LECs. (a) Representative western blots of P-PERK and P-eIF2α of human LECs cultured in GF-DMEM for 0.5, 1.5, 2.5, and 4.5 h at < 0.14% atmospheric oxygen. (b–d) Representative western blots of P-PERK and P-eIF2α of human LECs cultured in GF-DMEM (b), 1 mM glucose-DMEM (c), and 2 mM glucose-DMEM (d) for 0, 2, 4, 6, and 8 h at 1% atmospheric oxygen. Protein expressions were normalized to intensity of GAPDH expression. The results are the means ± S.D.s of three experiments for each protein.
Similarly, rat lenses subjected to ischemia for 1 or 5 h were reperfused with 5 mM glucose-DMEM in under 1% atmospheric oxygen for 22 h. The production of ROS and cell death in the treated and untreated control lenses was determined. The lenses exposed to ischemic conditions for 1 h had very few ROS-producing cells in the germinative zone of LECs and no apoptotic cells (Figure 7a). Neither ROS nor apoptotic cells were found in the untreated control LECs. On the other hand, lenses treated with ischemia for 5 h had ROS that were found predominantly in the peripheral and cortical LECs of all lenses (Figure 7b). In contrast, we found very few apoptotic cells in these lenses. These results indicated that the proliferating cells in the germinative zone were most sensitive to hypoglycemic insults under hypoxia, and these conditions activated the UPR. Although brain and cardiac cell die after 4–9 min of anoxia, LECs have an unusually strong tolerance to ischemic insults.

Loss of GSH in HLECs cultured in GF-DMEM under 0.14% oxygen. There is no information on how much GSH is decreased in HLECs treated with GF-DMEM under severe hypoxic conditions. This is relevant because GSH is oxidized rapidly in the presence of ROS. Initially, we examined the

![Figure 4](image)

**Figure 4** Expression of UPR-specific proteins in rat LECs. (a) The glucose and oxygen levels present in the anterior chamber were measured at 0, 1 and 3 h after killing the rats and the respective values were presented. (b) Representative western blot of P-PERK, P-IRE1α, and P-eIF2α of rat lens exposed to ischemic conditions for 1 and 3 h. Intensity of each band was normalized with GAPDH

![Figure 5](image)

**Figure 5** Responses of HLECs against low glucose and hypoxic conditions. (a) Results of representative experiments of ROS production and cell death in HLECs cultured under <0.14% atmospheric O₂ for 0.5, 1.5, 2.5, and 4.0 h and then reperfused with 5 mM glucose-DMEM under 4% atmospheric oxygen for 20 h. (b) Results of representative experiments of human LECs proliferation after a short-term BrdU pulse. Left panel show the light photomicrographs of human LECs, middle and right panel represent the BrdU-incorporated cells (proliferating cells) and the EthD-positive cells (apoptotic cells), respectively. Total BrdU- and EthD-positive cells from four separate experiments were counted and are presented as the means ± S.D. (c) Level of GSH in human LECs cultured in 5 mM glucose-DMEM under 0.14, 1, 4, and 20% atmospheric oxygen and GF-DMEM under <0.14% atmospheric oxygen for 3 h and then reperfused with 5 mM glucose-DMEM under 1% (AN-1), 4% (AN-4), and 20% (AN-20) of atmospheric oxygen for 22 h. The results are the means ± SDs. The experiments were repeated 4 times and were compared between the anoxia treated and untreated human LECs (Student’s t-tests).
levels of GSH in HLECs cultured in 5 mM glucose-DMEM at 0, 1, 4, and 20% atmospheric oxygen for 22 h. The GSH levels in the HLECs cultured in 0.14% were lowest, followed by 1 and 4%. The 20% atmospheric oxygen condition had the highest levels of GSH (Figure 5c). These results are consistent with the idea that aerobic respiration generates more ATP and more NADPH in the mitochondria which then produces higher levels of free GSH.37

Next, we studied the GSH level in HLECs exposed to GF-DMEM and placed under severe hypoxic conditions for 3 h and then return to 5 mM glucose-DMEM in 1, 4, or 20% oxygen for 22 h. The results were compared with those obtained from HLECs cultured at the different atmospheric oxygen levels without the prior treatment with GF-DMEM (Figure 5c). The cells exposed to the GF-DMEM only once had 5% lower GSH levels than the HLECs cultured in 5 mM glucose-DMEM at comparable oxygen levels. We suggest that multiple exposures to low glucose conditions might lead to significant oxidation of the lenses.

Immunohistochemistry of UPR proteins. The levels of UPR-specific proteins in two groups of four rat lenses treated with ischemia for 3 h and reperfused for 21 h were determined. The intensity of immunostaining with antibodies specific to ATF4, Bip, CHOP, PDI, and Ero1-Lβ were significantly increased in the germinative zone, in the LECs that covered the cortical region of the lens, and lens fiber cells at the very early stage of differentiation but not in the central LECs and in matured lens fiber cells. The intensity of immunostaining was also lower in the untreated control lenses (Figure 8a). Interestingly, Ero1-Lβ is an oxidative enzyme that is increased by prolonged UPR.

Protein blot analyses of the proteins obtained from an ARC lens showed that most of the UPR proteins are degraded but Ero1-Lβ was still detectable, and its level remained for a long time in the lens fiber cells (data not shown). A diabetic cataractous lens from a 57-year-old individual had a comparatively high intensity of gold particles at a depth of a few millimeters in the cortical lens fiber cells (Figure 8b). We estimated that this region developed in the past 3.5–4 years, which suggests that the UPR-specific oxidative protein, Ero1-Lβ might be accumulated in the LECs produced over the past several years. The control lens did not have the higher intensity of the gold particles. Thus, we were able to see the effects of UPR activation in the human diabetic cataractous lens.

Discussion
Severe hypoglycemia in patients has been statistically shown to be associated with adverse consequences, although a direct causal relationship has not been demonstrated.
between hypoglycemia and cataracts. Our results showed that a short duration exposure to low glucose conditions activated the protective UPR in HLECs, and prolonged hypoglycemic condition under severe hypoxic condition induced the apoptotic UPR and production of ROS. These changes resulted in lens oxidation, and the death of LECs. Diabetic patients with tight glycemic control by insulin therapy can develop hypoglycemia, in which the glucose level can be \(< 3\) mM in the blood and \(< 1.5\) mM in the vitreous fluid. This is a critical concentration in the lens because the UPR is activated below this concentration.\(^9\) These observations are consistent with earlier results that diabetic cataracts are strongly associated with age-dependent circulatory disorders, duration of diabetes, elevated glycosylated hemoglobin levels, lower intraocular pressure, and diastolic blood pressure.\(^1,7\) Furthermore, extreme fasting and metabolic diseases induce hypoglycemia, and our results showed that only 30 min exposure to low glucose is sufficient to induce the UPR in the LECs in the germinative zone of rat lenses. Generally, hyperglycemia and hyperoxia have been reported to induce lens oxidation and cataracts.\(^38,39\) We propose that lower than physiological concentrations of glucose and oxygen also induce oxidation of the lens by activating the UPR. Thus, it is highly possible that hypoglycemia is also one of the factors contributing to oxidation in lenses.

Hyperglycemic cataracts have been extensively studied, and the results showed that increased glucose concentrations in the lenses generate hyperosmotic stress.\(^3\) The hyperosmolarity combined with glyoxidation, increased cytosolic Ca\(^{++}\), cell death, and aging\(^1,7\) is considered to be the etiological factor that causes diabetic cataracts.\(^8\) We suggest that low glucose under severe hypoxia induces the apoptotic UPR, which leads to the production of ROS, releases of Ca\(^{++}\) from the ER, increases of cytosolic Ca\(^{++}\), and

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**Figure 8** (a) Immunohistochemistry of UPR-specific proteins (ATF4, Bip, CHOP, PDI, and Ero1-L\(\beta\)) in rat lenses exposed to \(< 0.14\%\) atmospheric oxygen for 3 h and reperfused in 5 mM glucose-DMEM at 1\% atmospheric oxygen for 21 h. (b) Immunohistochemistry of a human cortical ARC and a clear lens stained with Ero1-L\(\beta\) antibody. (c) Schematic diagram hypothesizes the cortical and posterior sub-capsular cataract formation in diabetic patients.
induced apoptosis. However, hypoglycemia activates a very strong UPR within a short time but hyperglycemia requires a prolonged time to induce only a very mild UPR in the lens. The hypoglycemia would be combined with the hyperglycemic conditions rather than act alone, and it is a transient condition that develops in diabetics. Thus, serious damage can be caused by transient hypoglycemia in the lens.

We hypothesized that diabetic individuals develop cortical or posterior sub-capsular cataracts (PSC) because of the induction of the UPR and production of ROS. The lens grows throughout life by generating new fiber cells on the old lens fiber cells. Our studies, ROS increased in the LECs in the germinative zone which differentiate into the cortical fiber cells (Figure 7a). We suggest that these lens fiber cells have less Nrf2 dependent antioxidant protection, and the changes result in oxidation and crystallin aggregation in the cortical and posterior regions (Figure 8c). This suggestion is consistent with the earlier finding that diabetic cataracts are strongly associated with the duration of diabetes, that is, longer the diabetes, the thicker is the layer of cortical lens fiber cells.

Materials and Methods
Human lens epithelial cell (HLEC) Cultures. To examine cell growth, human lens epithelial cells (HLECs; SRA 01/04) were cultured overnight in 25 mM glucose-Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen Corp., Carlsbad, CA, USA) with 10% fetal calf serum (FCS) at 37°C under 20% atmospheric oxygen. Before each experiment, the HLECs were pre-cultured overnight in 5 mM glucose-DMEM with 1% atmospheric oxygen. Some of the HLECs were cultured in glucose free (GF)-DMEM with 10 or 0.2% FCS at 37°C in 1, 4 or 20% atmospheric oxygen. HLECs were also cultured in <0.14% atmospheric oxygen by placing the cells in a vacuum bag with AnaeroGen. The 1 and 4% atmospheric oxygen environments were generated in an O2/CO2 incubator (Sanyo, Osaka, Japan) with an attached 50 gallon liquid nitrogen gas tank. The 20% atmospheric oxygen environment was generated in a normal tissue culture incubator.

Rat lens organ culture. Sprague Dawley rats (Charles River Laboratories Inc., Wilmington, MA, USA) were euthanized by CO2, and the eyes were enucleated. The lenses were extracted and cultured in 2 ml of modified medium-199 (Lonza eShop, Melnik, Czech Republic) with penicillin (100 units/ml) and streptomycin (100 units/ml) (Invitrogen Corp., Island, NY, USA) for 20–24 h. The cells were treated with 1% trypsin for 3 min and collected by centrifugation at 900 g/ml of 2–5-Bromo-2-deoxyuridine (BrdU) labeling. HLECs were cultured with 25 mM glucose-DMEM and 10 μg/ml of BrdU (Sigma Chemicals, St. Louis, MO, USA) on a glass cover slides for 30 min. The cells were further cultured in GF-DMEM for an additional 16 h and then stained with EthD for 30 min. The cells were then fixed with 4% paraformaldehyde in PBS for 1 h, treated with cold methanol/acetone (1:1) for 10 min, and then treated with 2 N HCl at 37°C for 30 min. The cells were washed three times with PBS and incubated in 1:200 diluted Fluor-labeled anti-BrdU antibody for 1 h to 72 h. Photographs were taken with a camera on a Nikon fluorescence microscope using a red or a green filter.

Glutathione assay. The level of GSH was determined by a GSH quantification Kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, HLECs (1 x 104 cells) were cultured in 10-cm Petri dishes under different levels of atmospheric oxygen for 20–24 h. The cells were treated with 1% trypsin for 3 min and collected by centrifugation at 900 x g for 10 min at 4°C. The cells were washed with 300 μl of PBS, then 80 μl of 10 mM HCl was added, and the cells were lysed by freezing and thawing. Then, 20 ml of 5% 5-sulfosalicylic acid was added to the lysate, and the mixture was centrifuged at 8000 x g for 10 min. The GSH level was determined by measuring absorbance at 405 nm in an ELISA reader.

Histology and immunohistochemistry. Rat lenses with ARC and a clear lens were obtained from the National Eye Institute Vision and Ophthalmology resource on the use and treatment of animals in ophthalmic and vision research were followed.

Protein blot analysis. Cultured LECs were lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA). A total of 10–20 µg of soluble proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes, and the membranes were blocked for 1 h in a buffer containing 5% nonfat milk. Then the membranes were incubated with primary antibody overnight at 4°C, and with secondary antibody at room temperature for 1 h. The membranes were placed in luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then exposed to X-ray film to view the protein bands. Antibodies to Bip (BD Biosciences, Franklin Lakes, NJ, USA), P-ERK, P-IRE1α, P-eIF2α, ATF4, Erp1-Ut, GR, catalase (Santa Cruz Biotechnology), and GAPDH (Novus Biological, Littleton, CO, USA) were purchased. The intensity of each band was normalized to that of GAPDH, and the data are presented as a relative intensity using the ImageJ analysis software.

Cell viability/death and ROS staining. Rat lenses or cultured HLECs were stained with a mixture of two probes; calcein AM and ethidium homodimer-1 (EthD) (Viability/Cytotoxicity Assay Kits; Biotium Inc., Hayward, CA, USA) for 30 min as recommended by the manufacturers. The cytosolic ROS level was determined by adding 1 µg/ml of 2’,7’-dichlorodihydrofluorescein diacetate (H2-DCFH) (Invitrogen Corp., Island, NY) into PBS for 40 min at 20°C, then washed twice with PBS, followed by fluorescent microscopic imaging (Nikon, Eclipse TE2000-U, Tokyo, Japan). The oxidation of H2-DCFH required cytchrome c, which is present in LECs.

5-Bromo-2-Deoxyuridine (BrdU) labeling. HLECs were cultured with 25 mM glucose-DMEM and 10 µg/ml of BrdU (Sigma Chemicals, St. Louis, MO, USA) on a glass cover slides for 30 min. The cells were further cultured in GF-DMEM for an additional 16 h and then stained with EthD for 30 min. The cells were then fixed with 4% paraformaldehyde in PBS for 1 h, treated with cold methanol/acetone (1:1) for 10 min, and then treated with 2 N HCl at 37°C for 30 min. The cells were washed three times with PBS and incubated in 1:200 diluted Fluor-labeled anti-BrdU antibody for 1 h to 72 h. Photographs were taken with a camera on a Nikon fluorescence microscope using a red or a green filter.

Statistical analysis. The results are expressed as the means ± S.D., and the significance of differences was evaluated by Student’s t-test using SPSS (version 15.0) software (SPSS Inc., Chicago, IL, USA). Values were considered statistically significant when P < 0.05.

Conflict of Interest
The authors declare no conflict of interest.

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Author Contributions
RE and TS contributed intellectually to the study design and interpretation, performed the studies, analyzed the data, and wrote the article. PP, CJM, MLM, DWL, AMT, and JU performed laboratory analyses.
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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)