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Prohibitin as an oxidative stress biomarker in the eye

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Abstract

Identification of biomarker proteins in the retina and the retinal pigment epithelium (RPE) under oxidative stress may imply new insights into signaling mechanisms of retinal degeneration at the molecular level. Proteomic data from an *in vivo* mice model in constant light and an *in vitro* oxidative stress model are compared to controls under normal conditions. Our proteomic study shows that prohibitin is involved in oxidative stress signaling in the retina and RPE. The identity of prohibitin in the retina and the RPE was studied using 2D electrophoresis, immunohistochemistry, western blot, and mass spectrometry analysis. Comparison of expression levels with apoptotic markers as well as translocation between mitochondria and the nucleus imply that the regulation of prohibitin is an early signaling event in the RPE and retina under oxidative stress. Immunohistochemical analysis of murine aged and diabetic eyes further suggests that the regulation of prohibitin in the RPE/retina is related to aging- and diabetes-induced oxidative stress. Our proteomic approach implies that prohibitin in the RPE and the retina could be a new biomarker protein of oxidative stress in aging and diabetes.

Keywords

oxidative stress; proteomics; retina; retinal pigment epithelium; aging

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Introduction

Transduction of visual information from light is an energy-, oxygen-, and vitamin A-dependent process in the retinal pigment epithelium (RPE) and the retina. Light is converted into an electrical signal in photoreceptors using vitamin A as an opsin chromophore through a G-protein-coupled receptor pathway. Relatively high oxygen tension is observed in the retina to allow extensive ATP production in mitochondria. More than 90% of oxygen is reduced to water in mitochondria and 1-5% of consumed oxygen is converted to the reactive oxygen species (ROS) that include superoxide anion (O_2^-), hydroxyl radical, singlet oxygen, and hydrogen peroxide (H_2O_2) in the RPE and the retina. In mammals, the average extracellular concentration of H_2O_2 is around 1 nM and the intracellular level is 0.5 ~ 0.7 μM [1]. However, in the eyes of cataract patients, the H_2O_2 levels in the anterior uvea and aqueous humor is up to 10-660 μM, almost 10-1000 times higher than the average intracellular concentration [2]. Consequently, the RPE has evolved to express anti-oxidative defense molecules such as vitamin E, superoxide dismutase, catalase, glutathione-S-transferases, glutathione and melanin. Furthermore, the RPE contains abundant anti-apoptotic and neuroprotective factors to support retina survival and maintenance. With aging, an imbalance occurs between ROS production and the capacity for detoxification that results in the accumulation of ROS and the diminution of mitochondrial respiratory function. ROS accumulation may contribute to the development of such eye diseases as uveitis [3], glaucoma [4], retinopathy of prematurity[5], age-related macular degeneration (AMD) [6], and diabetic retinopathy [7]. Under sub-lethal conditions of oxidative stress, mitochondrial respiratory function is impaired by electron leakage [8] that leads to decreased ATP production. Moreover, increased production of ROS may initiate mutations in mitochondrial DNA, cross-linking of cellular macromolecules, and accumulation of damaged proteins [9]. As a phenotype of accumulated oxidative stress, aging cells increase the number of mitochondria and induce the expression of genes to reduce oxidants, but this process may induce inflammation [10].

Even though many risk factors and hypotheses were proposed, signaling mechanisms of retinal degeneration at the molecular level have been elusive. Identification of biomarkers and regulators in the retina and the RPE under oxidative stress may imply new insights into the complex pathways. In this study, we have employed proteomic approaches to identify target proteins in the RPE and the retina that are integral to the retinal signaling network under oxidative stress [11,12]. We have identified prohibitin (PHB) as a new mediator of mitochondria shuttling that is operative under conditions of oxidant stress. Prohibitin is highly conserved from bacteria to humans, but its presence and function in the eye has not yet been studied. Prohibitins are ubiquitous proteins that share an evolutionarily conserved stomatin/prohibitin/flotillin/HflK/C (SPFH) domain similar to several prokaryotic and eukaryotic proteins. This family is comprised of membrane associated proteins implicated in roles of protein turnover, senescence and proliferation control. Eukaryotic prohibitin forms a mitochondrial complex of two highly homologous subunits, PHB1 and PHB2. The prohibitin 1 gene is mapped to chromosome 17q21 and encodes a 32kDa protein. PHB1 was so named because it was identified as a potential tumor suppressor with anti-proliferative activity, but this was later attributed to the 3’-UTR of the prohibitin mRNA. PHB2, a 37kDa

protein, was identified, along with PHB1, in its binding to the IgM antigen receptor, after which both proteins were named B-cell-receptor complex-associated proteins (BAP32 and BAP37). Both PHB1 and PHB2 are attributed to cell cycle progression, transcription regulation and cell surface signaling. PHB1 and PHB2 associate to form a macromolecular structure of approximately 1 MDa at the mitochondrial inner membrane, with no homodimers detected. Instead, the prohibitins bind to each other to form heteromeric building blocks; 12-16 PHB heterodimers associate to form a ring-like chaperone that stabilizes unassembled membrane proteins. This complex plays a potential part in stabilizing the mitochondrial genome and is indicated in mitochondrial morphogenesis as a scaffold that recruits proteins to a specific lipid environment. This mitochondrial chaperone function has more recently received attention as a modulator that responds to oxidative stress. Our proteomic study shows that prohibitin is involved in oxidative stress signaling in the retina and the RPE. *In vivo* study reveals that prohibitin is up-regulated in the retina in aging and diabetic models. Finding a new oxidant-target molecule in the retina under oxidative stress provides a foundation to examine pathophysiological network signaling of retinal degeneration.

Methods

Primary retinal cell culture

Newborn Sprague-Dawley rats were handled in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. Primary cell cultures including neurons, astrocytes and photoreceptor cells were generated from the retinas of newborn (postnatal day 1 or 2) Sprague-Dawley rats as previously described [13].

Human, bovine RPE cells of early passages, and human D407 RPE cells of long passages

According to a previously described method [14], eyes were opened posterior to the ora serrata and the vitreous and the retinal tissues were removed. D407 cells were maintained in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine at 37°C and 5% CO_2. When cells grown in a 6-well tissue culture plate reached 75-80% confluence, they were treated with 200 μM H_2O_2 or PBS for 1 hour after which H_2O_2 was removed. Cells were then maintained in a conditioning media for 6 hrs.

Assessment of cell survival

Survival of retinal cells was quantitatively assessed using a cell proliferation assay kit (CyQUANT NF Cell Proliferation Assay Kit, Molecular Probes, Eugene, OR) according to the manufacturer's protocol as described previously [15].

H_2O_2 treatment of bovine RPE cells

Fresh bovine eyes were obtained from a local slaughterhouse immediately after excision from the animal (Brown Packing Company, Gaffney, SC). With the anterior part upward and the posterior part immersed in Hank's balanced salt solution (HBSS) the retina was adapted to darkness for 30 minutes at room temperature prior to the experiment. One ml of H_2O_2 (50

or 100 μM) was injected into the vitreous followed by incubation in the dark in a humidified chamber at room temperature for 1 hr. After incubation, the cornea, iris, lens, vitreous body, and retina were removed and proteins in the RPE were obtained in a lysis buffer consisting of 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF).

In vivo Animal Study

Female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) at 4-5 weeks of age were housed as previously reported [15]. Mice were treated with 12 h light/12 h dark or in continuous light for 7 days. At 15 weeks of age, the mice were euthanized and the eyes were harvested. Retinas were obtained by microsurgery and proteins were isolated. In a diabetic animal model, male Sprague-Dawley rats weighing 250 to 300 g were used. As an aging model, 12-month-old male Sprague-Dawley rats were used. Diabetes was induced by intravenous injection of streptozotocin (STZ, 50 mg/kg) dissolved in 0.1 M sodium citrate buffer at pH 4.5. As a control, age-matched rats received only the vehicle. Rats were considered diabetic if their blood glucose was greater than 350 mg/dL. After 4 weeks, the animals were euthanized, and their blood was collected for analysis of blood glucose, cholesterol, and triglyceride levels. Retinas from different subgroups of animals were prepared for biochemical and morphologic analysis.

Subcellular fractionation

Cox's method was used for subcellular protein fractionation [16]. In brief, isolated bovine RPE proteins were extracted in a pre-chilled Dounce homogenizer in a buffer consisting of 250 mM sucrose, 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT, 25 mg Spermine per ml, 25 mg Spermidine per ml and 1 mM PMSF. Cells were centrifuged at 661 g for 15 minutes at 4 °C. The resulting pellet was kept for further isolation of nuclear proteins and the supernatant fraction was re-centrifuged at 4228 g for 15 minutes at 4 °C to obtain a cytosolic fraction. For total mitochondrial protein extraction, the pellet was suspended in an extraction buffer consisting of 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mg leupeptin per ml and 1 mM PMSF. The pellet was suspended in homogenization buffer and centrifuged at 661 g for 15 minutes at 4 °C. The resulting pellet was resuspended in nuclear extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.5M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton X-100, 1 mM DTT, and 1 mM PMSF). Subsequently, the suspension was sonicated, centrifuged, and the supernatant fraction was used for nuclear prohibitin analysis.

2D SDS-PAGE, western blot, and mass spectrometry analysis

2D electrophoresis, western blot, and mass spectrometry analysis were described previously in detail [11,12,17]. Prohibitin primary antibodies from Genemed (peptide specific polyclonal, San Antonio, TX), Thermo Scientific (mouse monoclonal) and Abcam (mouse monoclonal) showed a specific band in 1D-SDS-PAGE.

Immunohistochemistry

Ten μm cryostat sections of whole rat and mouse eyes were fixed in a buffered 4% paraformaldehyde solution and subsequently incubated with mouse prohibitin antibody (Lab Vision Corporation). Biotin-coupled secondary antibody (Jackson ImmunoResearch Laboratories) was used with horseradish peroxidase-streptavidin. Images were acquired at 20X with an Olympus BX51 microscope.

Statistical Analysis

Values are presented as the mean ± the standard error of the mean (SEM) with three independent experiments. Statistical significance of differences among the analyzed groups was determined by performing one way ANOVA and post hoc comparison. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of prohibitin in the eye

Comparative proteomics was used to identify proteins in the bovine RPE cells under H_2O_2-induced oxidative stress. One of down-regulated proteins (Fig 1A, open arrow head) was analyzed by tandem time-of-flight (TOF-TOF) mass spectrometry to reveal fourteen peptides that matched with prohibitin with 63% sequence coverage. Other oxidative stress markers are reported separately elsewhere. The identity of prohibitin was further confirmed by 2D western blotting (Fig. 1B). Several prohibitin spots at 32 kD, pI 5.5 and a more acidic prohibitin spot at pI 5 were observed. Down-regulation of prohibitin was compared to control as well as the inflammatory factor NF-κB *in vitro* using RPE D407 cells in H_2O_2-induced oxidative stress, followed by 6 hrs incubation (Fig. 1C). Prohibitin under oxidative stress was decreased compared to controls and NF-κB.

The expression pattern of prohibitin was partially controlled by light

Localization of prohibitin was examined in the eye by immunohistochemical analysis under light and dark conditions *in vivo* (Fig. 2A). Prohibitin was localized in ganglion cell, outer nuclear, inner nuclear, and RPE cell layers (Fig. 2A, left). Two hrs of light exposure after a 12 hrs dark cycle increased prohibitin in the outer nuclear, inner nuclear, and RPE layers (Fig. 2A, right). To confirm the light effect on prohibitin regulation *in vivo*, mice were kept in continuous light for 7 days and prohibitin expression was compared to that of mice entrained with a 12hrs light/dark cycle. Retinal proteins were separated by 2D SDS-PAGE and prohibitin was visualized by 2D western blot (Fig. 2B). Prohibitin expression in the retina was up-regulated almost threefold as compared to actin in constant light exposure.

The expression pattern of prohibitin is changed in aging and diabetic rodent eyes

To investigate whether prohibitin is involved in pathologic conditions or aging, we examined the expression of prohibitin in aged and diabetic rats. The immunohistochemistry of control animals *in vivo* revealed that the basal level of prohibitin is high in the eye, particularly in the ganglion cell and RPE cell layers (Fig. 3). Prohibitin in the RPE is down-regulated or even absent in old and diabetic rat eyes, which is consistent with known decreased levels of

prohibitin in cellular senescence [24]. In the old rat model (12 months), prohibitin is highly expressed, especially in the ganglion cell layer. In diabetic eyes, the level of prohibitin increased significantly in the ganglion cell and inner nuclear layers.

The RPE and the retina show different susceptibility to hydrogen peroxide

The RPE and the retina have higher O_2 tension and ROS concentration with aging, and this oxidative environment may contribute to the pathogenesis and progression of eye disease. To verify whether H_2O_2, a major ROS in the eye, provokes cytotoxicity in the RPE and the retinal cells, human RPE cell cultures and primary rat retinal cell cultures were treated with different concentrations of H_2O_2 for 24 hrs. Cell viability was estimated by relative intensity (%) of DNA fluorescence, which is proportional to the number of live cells. Cell viability of the retina dramatically decreased from 70 to 30% in 50~100 μM and inhibitory concentration (IC_{50}) was shown also in this range. However, the viability of RPE cells linearly decreased at much higher concentrations of H_2O_2 and when the IC_{50} was 1 mM. RPE cell susceptibility to H_2O_2 was further tested over a period of time. Human RPE cells were exposed to 1 mM H_2O_2 for 0.5, 1, 3, and 6 hrs. More than 80% of cells were alive in 1 hr, but less than 50% after 6 hrs (Fig. 4).

Prohibitin subcellular localization was changed by oxidative stress in mitochondria and in the nucleus

Prohibitin was mainly expressed in RPE mitochondria. Prohibitin was also detected in the nucleus and was exported from the nucleus in the presence of 100 μM H_2O_2 (Fig. 5A). Proapoptotic cytoplasmic cytochrome C leakage increased in RPE cells and anti-apoptotic Bcl $X_{L/S}$ was up-regulated as well under higher concentrations of H_2O_2 (Fig. 5B and C). Leaking of cytochrome C from the mitochondria to cytoplasm indicates induced mitochondrial membrane depolarization by oxidative stress. Nuclear translocalization of NF-κB increased and the total amount was up-regulated (Fig. 5D). Active NF-κB has been shown to be localized in the nucleus and bound to promoter regions of genes involved in cell survival, differentiation, inflammation, and growth [18]. Taken together, acute oxidative stress by H_2O_2 treatment may trigger anti-oxidative defenses and pro-apoptotic responses in the RPE simultaneously.

DISCUSSION

Adaptation to changes in an oxidative environment is critical for the survival of RPE and retina cells, considering their high oxygen demand and rapid rate of metabolism. RPE and retina cells control their gene expression as a basic regulatory mechanism in response to external environmental factors, including light, nutrient supply, or oxidative stress, as well as internal cellular signals such as ROS, calcium concentration, or DNA damage. To understand the molecular mechanisms of early signaling under oxygen imbalance conditions, we investigated proteomic changes in oxidative stress using *in vitro* and *in vivo* models. The decreased prohibitin in H_2O_2- treated RPE cells may indicate an anti-oxidative role, which is consistent with the previous studies of decreased prohibitin levels by H_2O_2 treatment [19] or in *ex situ* lung tissue exposed to hyperoxia [16]. Prohibitin is known to act as a molecular chaperone to stabilize newly synthesized respiratory enzymes in

mitochondria [20]. In the RPE, phagocytosis generates excess ROS and prohibitin maintains mitochondrial integrity to prevent oxidation of mitochondrial proteins. Perhaps, under elevated oxidative stress that may exceed mechanisms that inhibit apoptosis, prohibitin might be exported from the nucleus to mitochondria. Translocation of prohibitin may imply correlations with its post-transcriptional regulation and mitochondrial membrane depolarization. NF-κB could be translocated into the nucleus during oxidative stress and NF-κB may act as a survival factor under this condition [18]. It is important to note that nuclear prohibitin and NF-κB translocated in opposite directions. This coordinated movement may determine cell viability and apoptosis in the RPE and the retina.

Outside of the eyes, prohibitin has been proposed to have a role as a cell cycle inhibitor, transcription regulator, inflammatory modulator, plasma membrane receptor, or a mitochondrial chaperone [21-23]. We identified multiple prohibitin spots that might represent post-translational modifications. Phosphorylated prohibitin in human fibroblasts was more acidic in 2D gels and lower pI prohibitin was up-regulated in active younger cells [22]. We also showed that prohibitin expression is perturbed in aged and diabetic rodent eyes. Prohibitin levels decreased in aging RPE, and prohibitin was undetectable in the RPE of diabetic rats. Down-regulation of prohibitin in the RPE in aged and diabetic rats may suggest a cumulative impairment in mitochondria by ROS, which causes cellular senescence [21,23]. Prohibitin deficiency in these eyes may increase the ROS production to initiate apoptotic signals and cause more cellular damage [24]. Increased prohibitin in the retina of these animals, especially in ganglion cell and inner nuclear layers of diabetic eyes, implies possible connections between prohibitin and diabetic pathways, when accompanied by oxidative stress and inflammation. As the susceptibility of the RPE and the retina to the oxidative stress should be different, as shown in Fig. 4, decreased and increased expression of prohibitin in the RPE and the retina of these animals is anticipated. Better knowledge of prohibitin changes in the RPE and the retina may allow us to understand pathophysiological pathway, especially for the earlier stages of retinal degeneration.

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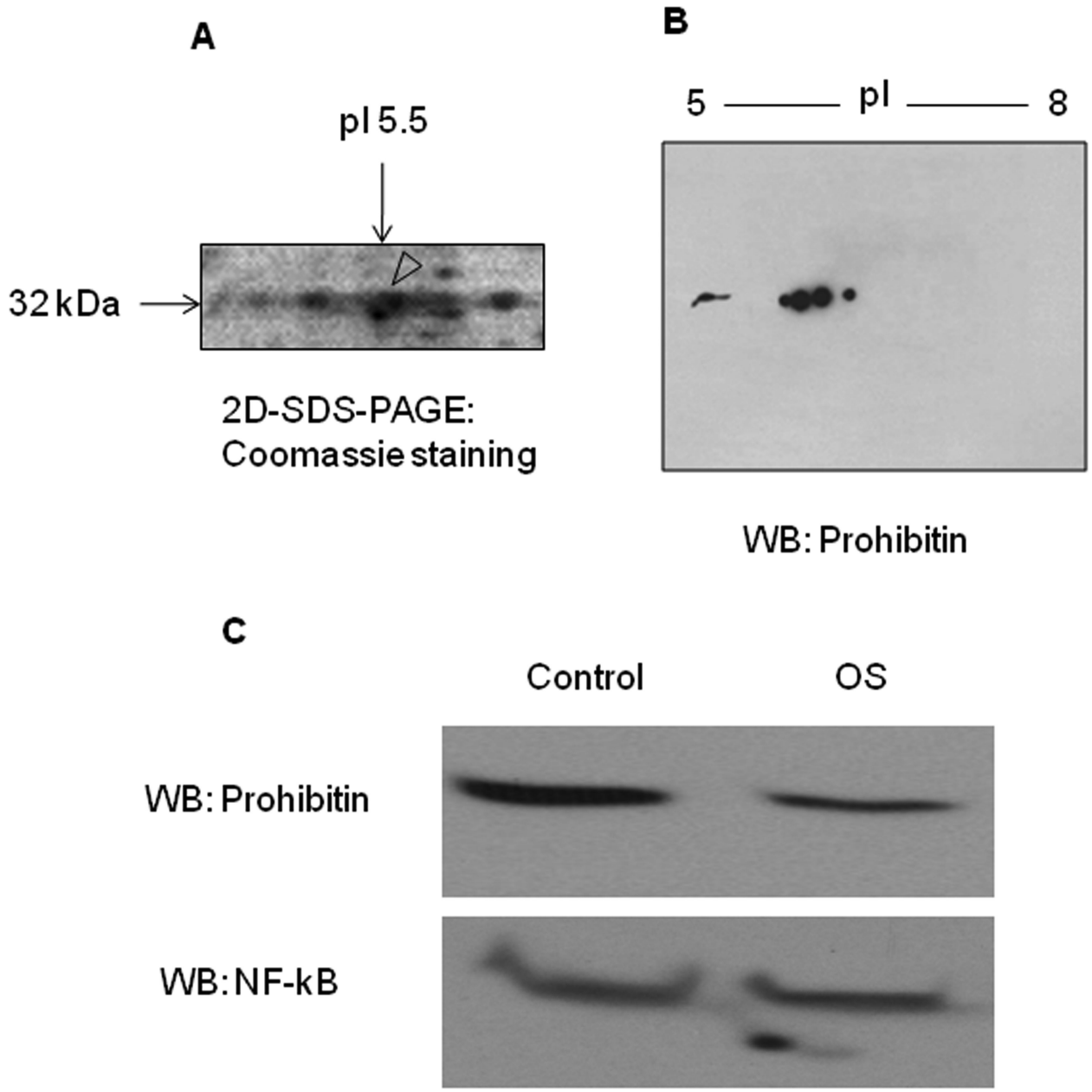

Figure 1.

Proteomics analysis of prohibitin by 2D SDS-PAGE and mass spectrometry. Proteins from bovine RPE cells under oxidative stress induced by H_2O_2 were analyzed by 2D electrophoresis and mass spectrometry. (A) The open arrow head represents one of down-regulated proteins. Up- or down-regulated gel spots were excised, trypsin-digested, and analyzed by MALDI-TOF-TOF mass spectrometry. In H_2O_2 treated RPE, prohibitin was down-regulated in 50 μM H_2O_2 treated group. (B) 2D western blot of prohibitin in RPE proteome. Proteins were separated by 2D SDS-PAGE, and visualized by western blot using anti-prohibitin antibody. (C) Down-regulation of RPE prohibitin under oxidative stress *in vitro*. RPE D407 cells were treated with 200 μM H_2O_2 (OS) or PBS (Control) for 1 hr. H_2O_2 was removed and then cells were maintained in conditioning media for another 6 hrs before

harvest. Proteins were separated by SDS-PAGE (10 μg/lane), then prohibitin and NF-κB were analyzed by western blot. RPE cells were maintained in DMEM supplemented with 10% FBS and 2 mM glutamine at 37 °C and 5 % CO_2.

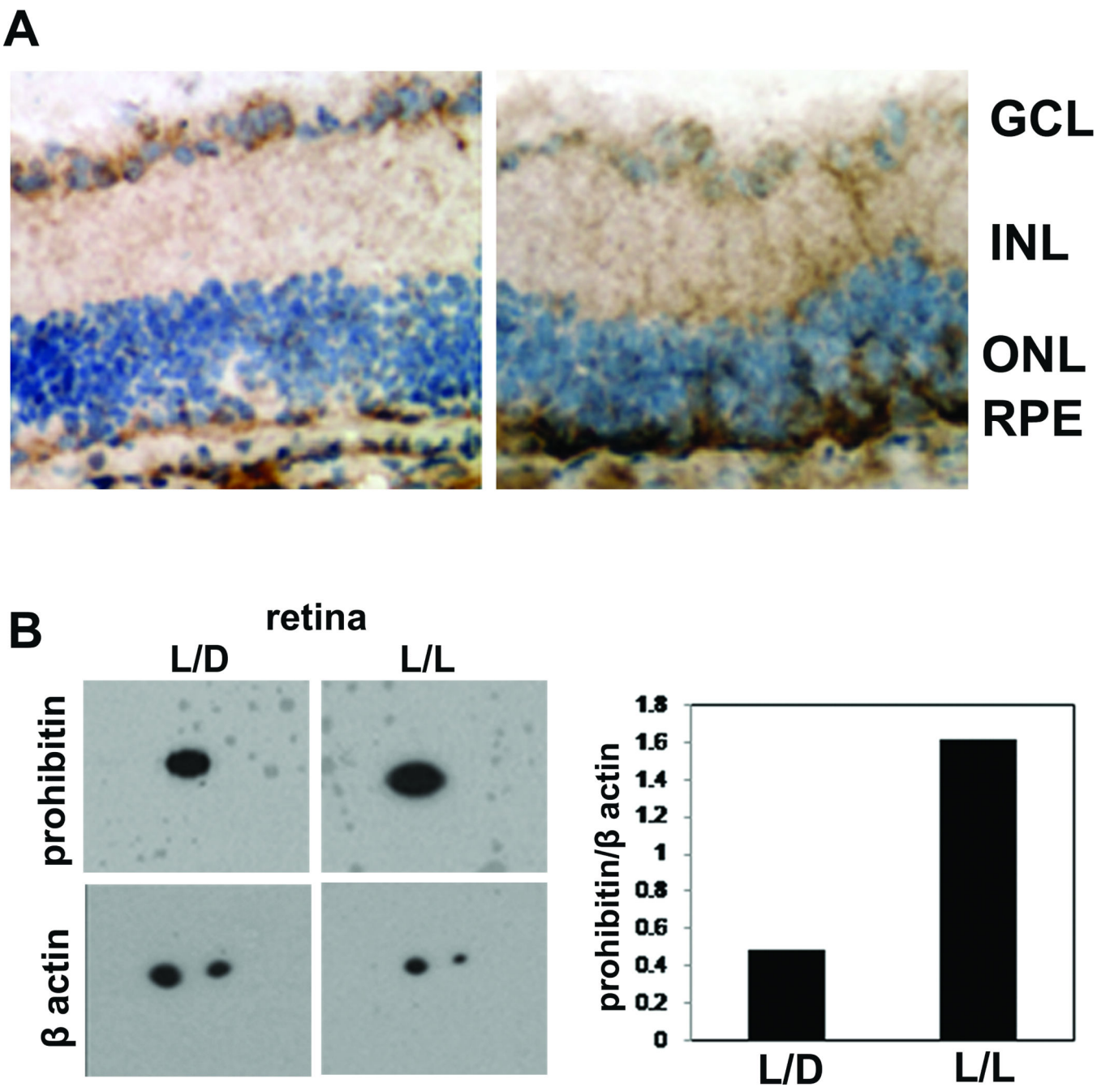

Figure 2.

Prohibitin in the retina was regulated by light *in vivo*. (A) Immunohistochemical analysis of prohibitin in mouse model. Mice were kept in the 12 h light/dark cycle. Prohibitin was shown in brown and hematoxylin was shown in blue as nuclear counter stain. Two hrs of light exposure increased immunostaining of prohibitin in outer nuclear, inner nuclear, and RPE layers. (B) Mice were housed in 12 h light/dark cycle or constant light for 7 days. Proteins in the retina were separated by 2D SDS-PAGE and prohibitin was visualized by western blot.

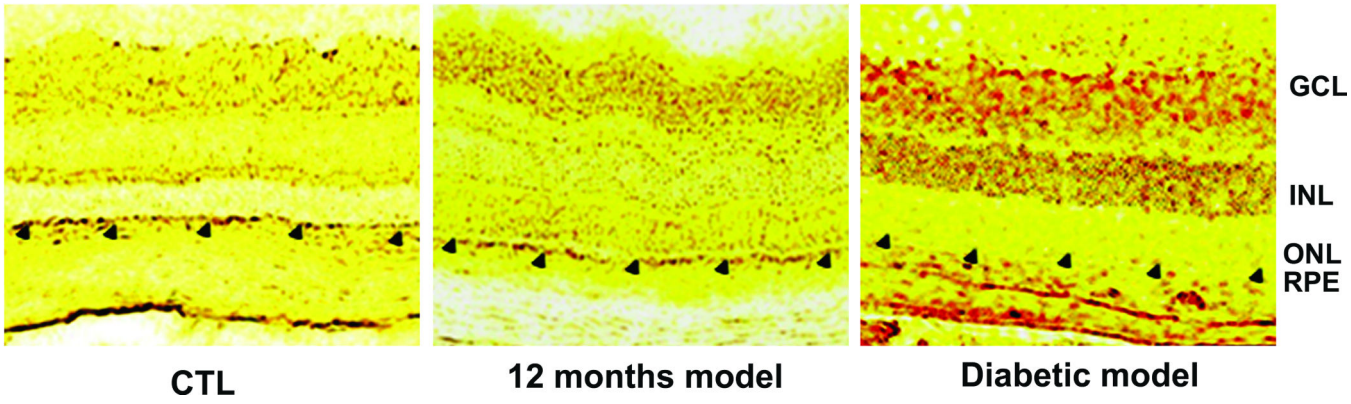

Figure 3.

Prohibitin showed different expressions in an aged and diabetic rat models. The basal expression level of prohibitin was higher in the control, particularly in ganglion cells, and RPE cells. However, in aged model, the RPE prohibitin gradually decreased and retinal prohibitin increased especially in ganglion cells. In a diabetic model, the RPE prohibitin was disappeared and retinal prohibitin increased in ganaglion cells and inner nuclear layers. The closed arrow head represents RPE layer.

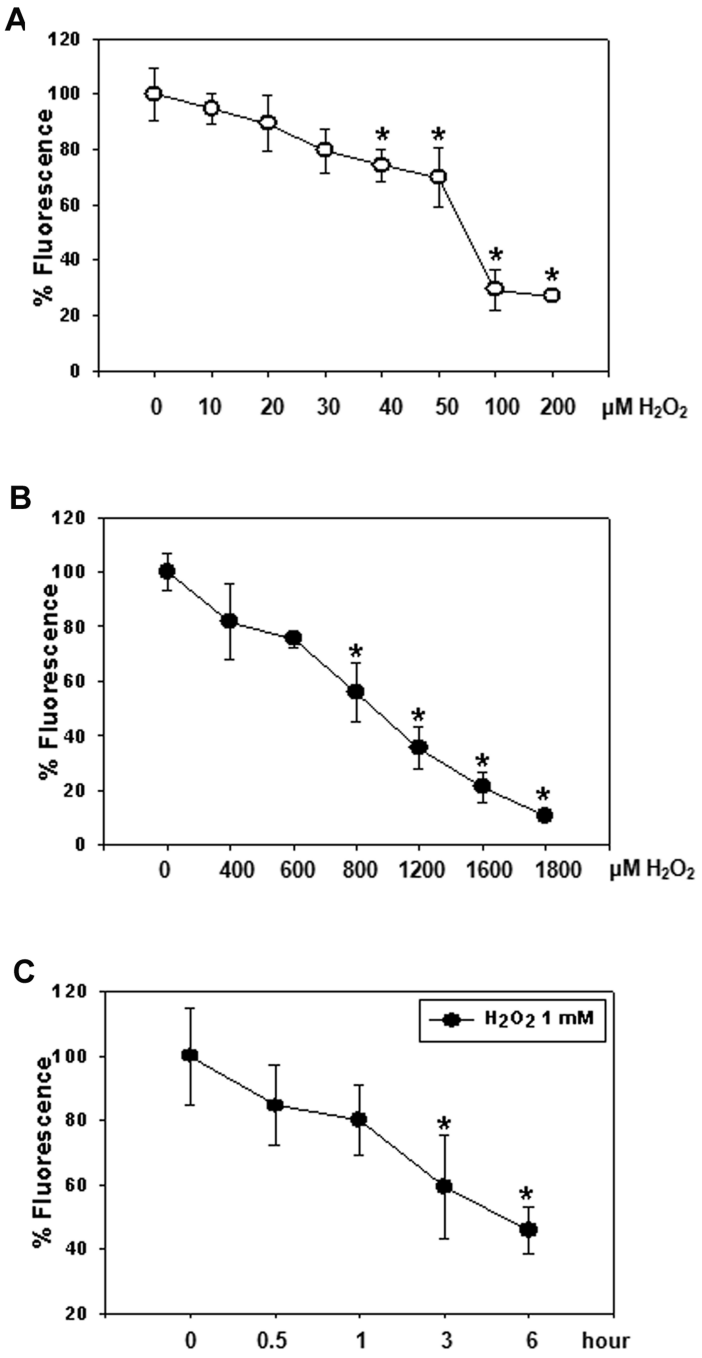

Figure 4.

Viability of the retina and the RPE cell cultures against H_2O_2. Cell viability was estimated by relative intensity (%) of DNA fluorescence, which is proportional to the number of live cells. (A) Primary rat retinal cells and (B) Human RPE cells of early passages were exposed to the indicated concentrations of H_2O_2 for 24 hours. Cell viability of the retina was dramatically decreased from 70 to 30 % in 50~100 μM, whereas RPE cells shows resistance to oxidative stress at the same condition. (C) Human RPE cells were exposed to 1 mM H_2O_2

for 30 min, 1, 3 and 6 hr. All values are presented as means ± S.E. (n = 6) and statistical significance relative to the appropriate vehicle control (*, p = 0.05).

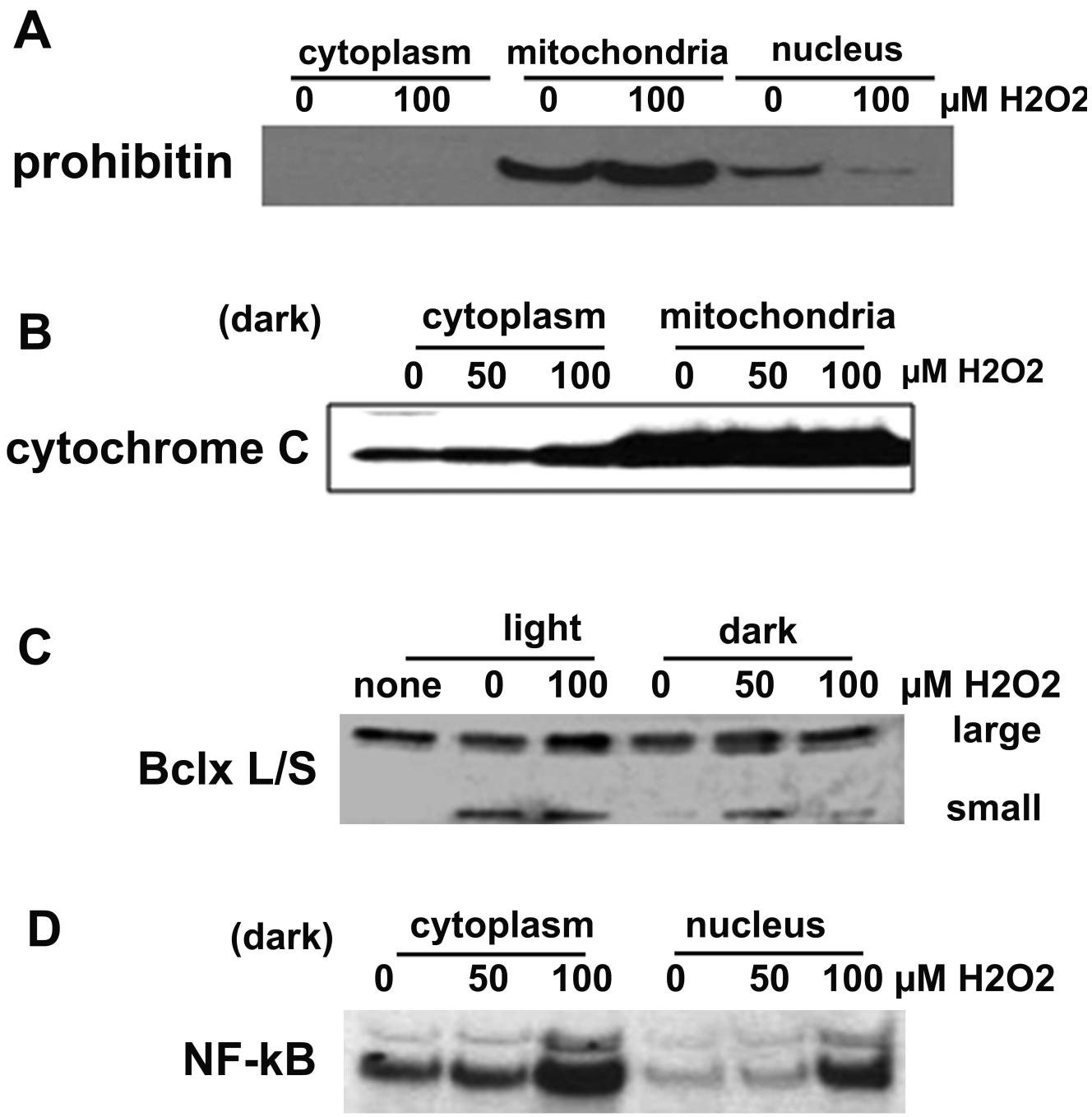

Figure 5.

Translocalization of prohibitin and apoptotic responses of RPE cells. H_2O_2 was injected to bovine eyes (final concentration 50 or 100 μM) in HBSS buffer. PBS injected eyes were used as a control. Proteins in the RPE were separated by SDS-PAGE and visualized by western blot. (A) Prohibitin was mainly localized in mitochondria but also detected in the nucleus. Mitochondrial prohibitin was increased and nuclear prohibitin was translocated to mitochondria in 100 μM H_2O_2. (B) Cytochrome C leakage into the cytosol was observed in 100 μM H_2O_2 concentration. (C) Bcl $X^{L/S}$ as an anti-apoptotic factor was up-regulated under

oxidative stress. (D) Nuclear translocalization of NF-κB in the RPE increased under oxidative stress.