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Mitochondrial-Nuclear Communication by Prohibitin Shuttling Under Oxidative Stress

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Abstract

Mitochondrial-nuclear communication is critical to maintain mitochondrial activity under stress conditions. Adaptation of the mitochondria-nucleus network to changes in the intracellular oxidation and reduction milieu is critical for the survival of retinal and retinal pigment epithelial (RPE) cells, in relation to their high oxygen demand and rapid metabolism. However, the generation and transmittal of mitochondrial signal to the nucleus remains elusive. Previously, our *in vivo* study revealed that prohibitin is up-regulated in the retina but is down-regulated in RPE in the aging and diabetic model. In this study, the functional role of prohibitin in the retina and the RPE was studied using biochemical methods, including lipid binding assay, 2D gel electrophoresis, immunocytochemistry, Western blot, and knockdown approach. Protein depletion by siRNA characterized prohibitin as an anti-apoptotic molecule in mitochondria, while lipid binding assay demonstrated subcellular communications between mitochondria and the nucleus under oxidative stress.

The changes of the expressions and localization of mitochondrial prohibitin triggered by reactive oxygen species are crucial for mitochondrial integrity. We propose that prohibitin shuttles between mitochondria and the nucleus as an anti-apoptotic molecule and a transcriptional regulator under stress environment in the retina and RPE.

Keywords

prohibitin; translocalization; mitochondria; oxidative stress; lipid binding

Mitochondrial-nuclear reciprocal signaling is the central question that needs to be answered to understand the maintenance mechanism of appropriate mitochondrial activities and functions in neurodegeneration. Disruptions of mitochondrial-nuclear network lead to accelerated aging and age-related diseases. Considering the significant consequences of

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SUPPORTING INFORMATION

S1. To confirm the efficiency of subcellular fractionation, positive markers in subcellular organelles were analyzed by Western blot. Proteins were fractionated by Cox and Emili method and separated by SDS-PAGE. RNA polymerase II, transketolase, and cytochrome c were used as the nuclear (N), cytosolic (C), and mitochondrial (M) markers, respectively.

S2. Translocalization of prohibitin under oxidative stress and mitochondrial protection by etoposide treatment. This material is available free of charge via the Internet at <http://pubs.acs.org>.

mitochondrial dysfunction in neurodegeneration and aging, it is crucial to understand the molecular mechanisms of mitochondria-nucleus communication by protein shuttling.^{1,2}

Cellular proteome changes, including expressions, localizations, and modifications, to adapt oxidative environment is critical for the survival of retinal and retinal pigment epithelial (RPE) cells, due to their high oxygen demand and up-regulated metabolism, especially under constant phototransduction. Oxidative stressors, including reactive oxygen species (ROS) and hypoxic environment are a serious risk factor in retinal degeneration. Understanding the molecular mechanisms that mediate oxidative stress-induced proteome changes and protein translocation between mitochondria-nucleus may provide insight into pathogenesis of retinal degeneration. Previously, our proteomic study demonstrated that prohibitin is involved in oxidative stress signaling in the retina and RPE.^{3,4} *In vivo* study revealed that the aging process up-regulated prohibitin in the retina and decreased it in RPE. Prohibitin was first reported as an anti-proliferative protein or a tumor suppressor that binds to p53, E2F, and Rb.^{5,6} It forms a high molecular complex with its isoform, prohibitin 2, in mitochondria.⁷ Localization of prohibitin has been controversial, possibly due to cell type and extracellular stress, as being mitochondrial, nuclear, or cell surface.^{8,9} Prohibitin is ubiquitous in organisms ranging from bacteria, to plants, to humans, but its function is not clearly understood at the molecular level in each subcellular organelle.¹⁰ Prohibitin is proposed to have roles as a cell cycle inhibitor, transcriptional regulator, inflammatory modulator, plasma membrane receptor, and a mitochondrial chaperone.^{6,11–18} Prohibitin is involved in apoptosis and aging by stabilizing newly synthesized respiratory enzymes, however, its presence and function in the eye has never been elucidated.^{19,20} In the current study, we examined the functional role of prohibitin in the retina and the RPE and demonstrated the altered lipid interactions under oxidative stress. Translocation of prohibitin was observed between mitochondria and the nucleus as an apoptotic signaling. The changes of the expression and location of mitochondrial prohibitin triggered by redox imbalance mediated by ROS suggest a critical role of prohibitin translocation to maintain mitochondrial integrity. Here, we demonstrate that prohibitin is a new dynamic regulator of mitochondrial and nuclear functions under stress environment in the retina and RPE. Knockdown of prohibitin upregulated proapoptotic factors, including BAK and caspases, and decreased antiapoptotic molecules such as Bcl-xL. The significance of our study resides in our characterization of prohibitin as a mitochondria-nucleus shuttle to respond ROS as an antiapoptotic protein and also as a transcriptional regulator by binding with p53 in the nucleus.

MATERIALS AND METHODS

ARPE-19 Cell Culture

ARPE-19 cells were transferred to tissue culture flasks (Nunc, Roskilde, Denmark) containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin, placed in a 5% CO₂ incubator at 37°C. After proliferation, cells were trypsinized with a 0.1% trypsin-EDTA solution (Sigma, St. Louis, MO) for 5 minutes at 37°C. After centrifugation (125 g, 7 min), cells were plated in 24 or 6-well plates (Nunc) at 2×10^4 or 8×10^4 cells/well and allowed to

grow to confluence for 2 to 4 days. Eight to twelve-passage cells were used for experiments.^{21,22}

Oxidative stress treatment

ARPE-19 cells were incubated with fresh media for 12 hrs before treatment. Hydrogen peroxide (Mallinckrodt Inc.) was diluted (50, 100, 200, 250 μ M) in FBS-free media. Cells were washed with PBS 3 times before treatment. Cells were treated with H₂O₂ for 1, 2, 4, 6, 8, 12, and 24 hrs in 37 °C 5% CO₂ incubator. Media was removed immediately after treatment and cells were washed with PBS and harvested. Cells were lysed by RIPA buffer (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA, Millipore) containing protease inhibitor cocktail (Thermo Scientific) for SDS-PAGE/ Western blot or immunocytochemistry.

Immunocytochemistry

ARPE-19 cells were incubated with the final concentration of 100 nM MitoTracker Orange CMTMRos (Molecular Probes, Carlsbad, CA) in FBS-free culture media for 30 min at 37°C. After washing in PBS, cells were fixed with 10% formaldehyde in PBS for 25 min at room temperature and the membrane was permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 20 min. Cells were blocked with complete media containing 10% FBS, 0.05% Tween-20 (Sigma) for 1 hr. Cells were labeled with Anti-prohibitin antibody (1:500; Genemed Synthesis Inc, San Antonio, TX) overnight at 4 °C. After washing with PBS 3 times, cells were stained with Alexa Fluor-488 conjugated anti-rabbit IgG (1:700; Molecular Probes, Carlsbad, CA) for 1 hr at 25 °C. Finally, cells were mounted with VECTASHIELD Mounting Medium with DAPI (4, 6-diamidino-2-phenylindole) to counterstain the nucleus. Images were acquired using Zeiss AxioVert 200 M Apo Tome fluorescent microscope with 63X magnification.

Co-immunoprecipitation

ARPE-19 cells were homogenized in lysis buffer (25mM Tris, 120 mM NaCl, 0.3% Triton X 100, 15% glycerol, 2 mM PMSF, 10 mM EDTA, 0.2% sodium orthovanadate and protease inhibitor cocktail). Cells were lysed by repeated freeze thaw cycles and sonication (5 min $\times$ 3), followed by centrifugation at 13,000 g for 30 min. Proteins in supernatant were purified by using control agarose resin, crosslinked by 4% bead agarose, to minimize non-specific binding. Purified anti-prohibitin antibody was immobilized with amino-linked protein A beads in the column with coupling buffer (0.5 mM sodium phosphate, 7.5mM NaCl, pH 7.2) and incubated with 3 μ l of 5 M sodium cyanoborohydride on a shaker at 25 °C for 2 hrs. Columns were washed with a washing buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, pH 7.4). Protein samples were incubated in the protein A-antibody column with gentle mixing for overnight at 4 °C. Columns were washed three times to remove non-specific binding proteins. Samples were equilibrated with 1 $\times$ sample buffer containing 100 mM DTT. Eluted proteins were separated by SDS-PAGE and visualized by Western blot using primary antibodies against prohibitin and p53. Protein bands on the gel were visualized by silver staining (Bio-Rad, CA).

Two dimensional SDS-PAGE

Protein samples were purified by ReadyPrep 2-D Cleanup Kit (Bio-Rad, CA) and quantified using BCA protein assay kit (Pierce, Rockford, IL). One hundred fifty μg of proteins were incubated in 200 μl of rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS and 50 mM DTT) supplemented with 0.5% destreak IPG buffer, pH 3–10 (GE healthcare, PA). Isoelectric focusing was carried out using 11 cm immobiline dry strips (Bio-Rad, CA) with a linear pH 3–10 and 4–7 gradients. Strips were rehydrated for 12 hrs at 20 °C. Proteins were separated by the Ettan IPGphor-3 (GE healthcare) using programmed voltage gradients at 20 °C for a total of 12 kVh (1 h at 500 V, 1 h at 1000 V, 2 hrs at 6000 V and 40 min at 6000 V). The IPG strips were reduced in equilibration buffer I (0.375 M Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, and 50 mM DTT) for 20 min at 25 °C and alkylated for 20 min in equilibration buffer II containing 150 mM iodoacetamide. The equilibrated strips were placed on top of polyacrylimide gels (8–16%, Bio-Rad, CA) and sealed with 1% agarose buffer. Proteins were visualized by Coomassie staining using Imperial Protein Stain (Pierce, Rockford, IL) and Western blot.

In-gel digestion and mass spectrometry

Selected protein spots from 2D electrophoresis were excised from gels. The gel pieces were destained twice with 200 μl of 50% acetonitrile (MeCN)/25 mM NH_4HCO_3 buffer (pH 8.0) at room temperature for 20 min, washed once with 200 μl of 100% MeCN and vacuum dried by a Speed Vac concentrator (Savant, Holbrook, NY). The gel pieces were rehydrated with 13 ng/ μl sequencing grade modified trypsin (Promega; Madison, WI) in 25 mM NH_4HCO_3 and incubated at 37 °C overnight. Peptides were subsequently extracted twice with 50 μl of 50% MeCN/5% formic acid for 15 min at 37°C. All extracts were combined and dried. The peptide extracts were purified using the C18 ZipTip (Millipore, USA). The peptides were eluted with 5 μl of 75% MeCN/0.1% TFA. The samples were analyzed by MALDI-TOF or tandem ESI MS/MS as previously described.^{3,4,24–26}

Lipid-protein interaction assay

Lipid strips were purchased from Echelon Biosciences or prepared using nitrocellulose membrane in the lab. For commercially available lipid strips, membranes with a solvent blank control and 15 different lipids were initially blocked with SuperBlock (Pierce, IL) blocking buffer for 1 hr at room temperature. To prepare own lipid strips, different amounts of lipids (100 pmole–10 nmole), including cardiolipin, cholesterol, and phosphatidylserine, were spotted on the nitrocellulose membrane. The membrane was air dried for 1 hr at room temperature. Crude proteins, fractionated samples, or partially purified prohibitin by Co-IP (1 $\mu\text{g}/\text{ml}$) from control ARPE-19 cells or cells under oxidative stress (200 μM H_2O_2) were incubated with nitrocellulose membrane overnight. The membrane was washed three times with a TTBS buffer (tris-buffered saline with 0.1% v/v Tween-20), followed by incubation with prohibitin antibody (1:1000, Genemed synthesis Inc, San Antonio TX) overnight at 4°C with gentle agitation. Blots were washed with TTBS buffer, incubated with horse radish peroxidase-conjugated anti-rabbit secondary antibody (1:10,000, Agrisera Vännäs, Sweden) for 1 hr at 25 °C. Strips were developed with Super Signal West Pico Chemiluminescence

substrate (Pierce, IL) and proteins were visualized using LAS 4000 mini luminescent image analyzer (Fuji).

siRNA transfection

ARPE-19 cells were seeded in 24-well plates at 6×10^4 cells/well with 0.1 ml of DMEM containing 10% FBS and 1% penicillin-streptomycin. Cells were incubated at 37 °C with 5% CO₂. Cells were treated with 175 ng (final concentration of 23 nM) of siRNA (AAAGCCAGCTTCCTCGCATCT) against prohibitin (FlexiTube siRNA) and random sequence control siRNA in 100 µl culture medium without serum. Transfection was carried out by HiPerFect transfection reagent (QIAGEN, Valencia, CA) and cells were harvested 48 hrs posttransfection. Proteins were assayed by Western blot to detect AIF (EMD Millipore), Bcl-xL (sc-634, Santa Cruz Biotechnology), Caspase-9 (AB16969, EMD Millipore), BAK (ab32371, Abcam) and actin (sc-47778, Santa Cruz Biotechnology). Protein localizations and expressions were also analyzed by immunocytochemistry.

Subcellular fractionation

Bovine retinal tissues were separated and proteins were isolated by Cox method, which uses different centrifugation in density gradients to generate nucleus, mitochondria and cytosolic protein components.²⁷ Cold PBS pre-rinsed tissue was homogenized in homogenization buffer and centrifuged at 800 g for 15 minutes. The pellet was resuspended for further isolation of nuclear proteins. The supernatant was collected and centrifuged at 6,000 g for 15min. The pellet was saved for mitochondria and supernatant for cytosol/microsome separation. Crude nuclear fraction was suspended in nuclear fraction buffer and separated by ultracentrifuge at 80,000 g for 35 min. In order to obtain mitochondrial membrane and matrix protein the pellet was resuspended in membrane extraction hypotonic lysis buffer, and centrifuged in lysate at 9,000 g for 30 minutes. Cytosolic proteins were obtained by spinning the supernatant for 1 hr at 100,000 g.

SDS PAGE-Western Blot analysis

Protein quantification was determined by BCA protein assay kit (Pierce, Rockford, IL). Samples were dissolved in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8 25% glycerol, 2% SDS, 0.01% Bromophenol Blue). Proteins were resolved using 8–16% polyacrylamide precast gels (Bio-Rad, Hercules, CA), followed by electrotransfer onto the methanol activated PVDF membrane and blocked in 5% of nonfat milk prepared in TTBS 0.1% v/v Tween-20) for 1 hr at 25 °C. The membranes were incubated with primary antibodies at 4 °C overnight and subsequently incubated with corresponding horseradish peroxidase-conjugated secondary antibodies for 1 hr at 25 °C. Proteins were visualized by using West Pico Chemiluminescent Substrate (Pierce, IL, USA). Immunoreactive proteins bands were acquired using LAS 4000 mini luminescent image analyzer and quantified using Quantity One software (Bio-Rad, Hercules, CA). Proteins in gels were stained with Imperial Protein Stain and scanned using GS800 imaging densitometer (Bio-Rad, Hercules, CA).

RESULT

Mitochondrial-Nuclear Trafficking of Prohibitin

Oxidative stress induced by hydrogen peroxide has been implicated in many ocular diseases. RPE cells maintain a reducing environment within cells to balance the production of reactive oxygen species and detoxification. Recently, we studied early molecular signaling events during oxidative stress in the retina and RPE using a comparative proteomic approach to identify altered protein levels.^{3,4,23–26} In the RPE, one of the down-regulated proteins under oxidative stress was prohibitin. Prohibitin level changes in both diabetic and aging eyes suggest that it may have an apoptosis related function in the RPE, especially under chronic stress and pathophysiological conditions.³

First we examined the cellular localization of prohibitin which is a controversial question. Translocation of prohibitin in response to oxidative stress was examined using immunocytochemistry. Subcellular organelles and prohibitin were visualized by DAPI (nuclear DNA, blue, 369/460 nm absorbance/emission), MitoTracker Orange (mitochondria, red, 578/599 nm absorbance/emission), and Alexa Fluor 488 (prohibitin, green, 495/519 nm absorbance/emission), by taking three color fluorescent images every hour. Kinetic assay confirmed that the signaling pathway was involved in prohibitin transit to the nucleus from mitochondria. Confluent ARPE-19 cells were treated with 200 μ M H₂O₂ for 24 hrs. H₂O₂ treated cells showed nuclear translocation of prohibitin, whereas untreated cells showed localization in mitochondria (Figure 1). After 8 hrs, prohibitin showed an accumulation in the nucleus as compared to the control group. After 24 hrs, more nuclear prohibitin was observed and much less colocalized with mitochondria.

To examine prohibitin response in apoptosis signaling in detail, we then examined protein expression level changes *in vitro* using ARPE-19 cells. We examined prohibitin level changes under H₂O₂ in time and dose dependent manner. Protein analysis by SDS-PAGE and Western blot demonstrated that prohibitin is down-regulated under oxidative stress (200 μ M H₂O₂, 1–24 hrs) (Figure 2). Cells treated by 200 μ M H₂O₂ showed a 40% decrease of prohibitin, but the levels between treated groups in 24 hrs revealed no significant difference by t-test, indicating that decreased prohibitin caused by oxidative stress is an acute process that occurs within 2 hrs. Down-regulation of prohibitin under stress condition suggests that the nuclear prohibitin may not be a newly synthesized protein as there was no prohibitin increase for 24 hrs; the nuclear prohibitin might derive from mitochondria since prohibitin was not colocalized with mitochondria after extended H₂O₂ treatment.

To address the issue of membrane binding property of prohibitin, we investigated the stress effect on protein solubility. First we examined the correlation between oxidative stress and soluble prohibitin and found that the ratio of soluble prohibitin to membrane binding prohibitin decreased under oxidative stress. Prohibitin exists in both detergent-resistant insoluble and soluble fractions and was separated by SDS-PAGE using 8–16% gradient gels and maximum running time. Soluble prohibitin in the cytosolic fraction (32 kDa) moved faster when compared to membrane-bound mitochondrial and microsomal prohibitin (34 kDa). Membrane-binding prohibitin showed slightly higher molecular weight when compared to soluble prohibitin. As shown in Figure 3A and 3B, soluble prohibitin from

ARPE-19 cells decreased and detergent-resistant, membrane-binding insoluble prohibitin increased under stress condition in 4 hrs. To compare insoluble and soluble forms from subcellular organelles, prohibitins from mitochondrial membrane (MM), cytosol (CYT), and whole cell lysate (WCL) were separated by SDS-PAGE and visualized by Western blot (Figure 3C). The insoluble, detergent-resistant, membrane binding prohibitin showed slightly higher band compared to cytosolic, soluble prohibitin. Prohibitins in whole cell lysate from both retinal and RPE cells showed two bands as insoluble and soluble forms. It is possible that the membrane-binding and soluble prohibitins may exist in different subcellular organelles, so we next examined the subcellular localization of prohibitin.

Subcellular localization of prohibitin in the retina and RPE

Our previous experiments demonstrated that prohibitin may respond differently in the retina and RPE under various oxidative stress conditions, including diabetes and aging. To specify the location of prohibitin in the retina, we fractionated bovine retina using serial centrifugation and specific detergents, including polyamines.²⁷ In this method, proteins were separated by nuclear and mitochondrial extraction buffer. Denatured and native gel electrophoresis and Western blot of subcellular organelle showed that prohibitin is widely distributed in retina cells but is centralized in mitochondria (Figure 4). We used cytochrome c, RNA polymerase II and transketolase as positive controls to show mitochondrial, nuclear, and cytosolic fractions, respectively (Supplement Figure 1). Quantitative analysis showed that 60–80% of prohibitin was localized in mitochondrial membrane pellet (Figure 4, Mitochondria II) in bovine retina cells, but the cytosol and nucleus (soluble I and membrane II) also contains a substantial amount of prohibitin. Then we explored the prohibitin levels in different subcellular locations in the RPE. Our fractionation experiments using ARPE-19 cells showed that prohibitin has a membrane-binding and soluble form. In ARPE-19, prohibitin was mainly expressed in mitochondria (60–80%) as determined by Cox fractionation method.²⁷

As prohibitin is widely spread in each subcellular fraction, we asked whether different subcellular prohibitins have the same isoelectric point (pI). We used 2D electrophoresis to isolate prohibitin from fractionated subcellular proteome. Mitochondrial, nuclear, cytosolic and microsomal prohibitin was separated and visualized by Coomassie staining and Western blot (Figure 5). In mitochondrial fraction, prohibitin showed 32 kDa molecular weight and pI=5.6 as expected. Prohibitin was more acidic in the nucleus showing pI=5.3, due to post-translational modifications such as phosphorylation. Cytosolic prohibitin showed very basic spots of pI=6–7 and higher molecular weight as shown in the box, representing potential ubiquitination.

As each subcellular prohibitin showed different pI values in 2D electrophoresis, we examined potential post-translational modifications in bovine retinal cells, including phosphorylation, using a different fractionation method.²⁷ Mitochondrial and nuclear fractions were further separated by matrix or supernatant (Mito and Nucl I) vs. membrane proteins (Mito and Nucl II). Prohibitin in mitochondrial matrix (Figure 6A, Lane 1) and cytosol (Figure 6A, Lane 5) may exist as putative heterodimer at 75 kDa, whereas it showed multiple bands around 32 kDa in the nucleus and cytosol, possibly due to phosphorylation or

solubility change. Proteins in each fraction were separated by electrophoresis and prohibitin was visualized by Western blot using prohibitin and phosphatidyl-serine (p-Ser) antibodies. Only the nuclear fraction showed phosphorylated prohibitin demonstrated by p-Ser Western blot (Figure 6A, Lane 9 and 10). Mitochondrial, cytosolic, and microsomal prohibitins did not show phosphorylation. To confirm this data, we performed native gel electrophoresis and received the same result (Figure 6B, Lane 9 and 10). Mass spectrometry (MALDI-TOF-TOF) analysis revealed that S101 and S151 sites are phosphorylated (data not shown).

Prohibitin Binds Cardiolipin under Oxidative Stress

We next asked whether prohibitin localization could be controlled by membrane lipid binding. We speculated that the localization and trafficking of prohibitin might be determined by mitochondrial-specific lipid such as cardiolipin, when considering mitochondrial specific lipid composition. Prohibitin in the bovine retina binds with cardiolipin, a major mitochondrial phospholipid (Figure 7A). Lipid interaction assay demonstrated that mitochondrial prohibitin from bovine retina has a strong affinity at relatively low cardiolipin concentration (10–15 μ g, 6–10 nmole) whereas nuclear prohibitin showed a weaker affinity to cardiolipin (Figure 7B).

However, prohibitins in the RPE showed different lipid interactions under oxidative stress when we examined prohibitin using ARPE-19 cells. Under normal condition, prohibitin from ARPE-19 cells demonstrated a strong interaction with phosphatidylinositol 3,4,5 triphosphate (PIP3) but not with cardiolipin (Figure 8). Lipid binding affinity changed dramatically under oxidative stress induced by H_2O_2 (200 μ M). Prohibitin under stress showed a strong affinity toward cardiolipin as shown in Figure 8. This observation led us to conclude that prohibitin has an affinity towards negatively charged phospholipids, especially cardiolipin. Lipid analysis by mass spectrometry demonstrated that cardiolipin concentration decreased 20–70% under 200 μ M H_2O_2 (data not shown).

Anti-apoptotic prohibitin function in mitochondria

We performed a loss of function study with prohibitin-specific small interfering RNA (siRNA) resulting in an increase of apoptotic signaling under decreased prohibitin levels (Figure 9). We tested two siRNA constructs that inhibit prohibitin expression against a control siRNA that has a random sequence. A solvent vehicle was used as a negative control. We chose conditions where prohibitin expressions were reduced by 80–90% when compared to random sequence control. We examined whether anti-apoptotic molecules such as BCL-xL and pro-apoptotic molecules that include apoptosis inducing factor (AIF), caspases, and BCL-2 homologous antagonist/killer (BAK), are also activated as essential markers in apoptosis (Figure 9A). The activation of AIF and caspase-9 cleavage, the upstream regulators in apoptosis, showed early apoptotic signaling under prohibitin knockdown condition. However, anti-apoptotic BCL-xL decreased when prohibitin was down-regulated. Further, we investigated whether prohibitin knockdown cleaves poly (ADP ribose) polymerase (PARP) as a pro-apoptotic marker. PARP cleavage is an early marker of caspase activation. Transfection of siRNA of prohibitin into ARPE-19 cells resulted in increased cleavage of PARP which represents that an apoptotic signal is induced when prohibitin is down-regulated (data not shown). Our results demonstrate that knockdown of prohibitin

increases apoptotic signaling. These observations suggest that prohibitin may act as an anti-apoptotic protein in mitochondria by forming a functional complex with apoptotic proteins. Prohibitin concentration is inversely correlated with the initiation of apoptosis. Then we examined whether decreased prohibitin disrupts the filamentous reticular network of mitochondria and results in fragmented mitochondria. Immunocytochemical analysis showed that prohibitin knockdown led to accumulation of fragmented mitochondria as shown in Figure 9B. Fragmented mitochondria imply that the fusion of mitochondrial membranes is impaired when prohibitin concentration is decreased.

Prohibitin function in the nucleus

To address a putative functional role of prohibitin in the nucleus, protein-protein interaction was examined. Immunoprecipitation of prohibitin demonstrated that prohibitin is involved in direct regulation of transcription through p53 binding. Prohibitin-protein binding assay using anti-prohibitin primary antibody, followed by SDS-PAGE, and mass spectrometry demonstrated that prohibitin has interactions with transcription factors that include tumor suppressor p53. Western blot of immunoprecipitated fractions confirmed prohibitin-p53 interaction as shown in Figure 10. To test oxidized cardiolipin dependent translocalization, a known cardiolipin antioxidant etoposide was examined. Etoposide is an antitumor drug that acts as a topoisomerase II inhibitor and is used to block cardiolipin oxidation and prohibitin translocalization. One hour treatment induced prohibitin translocalization from the nucleus to cytosol (Supplement Figure 2).

DISCUSSION

In the current study, we demonstrate that prohibitin is translocated from mitochondria to the nucleus as a mitochondrial response to oxidative stress *in vitro*. Previously, we investigated oxidative stress-induced proteome changes in the RPE using the differential gel electrophoresis (DIGE) technique to identify early biomarkers of eye diseases^{3,4}. We were seeking a novel protein that shuttles between mitochondria and the nucleus as the initiation step in retinal pathogenesis. We found that prohibitin changes its subcellular location under stress conditions. In the current study, dynamic translocation of prohibitin was examined by subcellular fractionation, immunocytochemistry, lipid binding assay, and knockdown approach. This study challenges a question of mitochondrial-nuclear (MT-NU) communication by protein translocation through the lipid interaction change mechanism. Knockdown and immunoprecipitation studies suggest that trafficking prohibitin may have a dual function as an anti-apoptotic molecule that maintains mitochondrial structure, and acts as a transcriptional activator that interacts with p53 in the nucleus. We tested the hypothesis that a lipid binding property of prohibitin may determine mitochondrial localization. Since prohibitins are expected to be dynamically translocated within retinal and RPE cells, there is an essential need for time-dependent assay of cellular prohibitin movement. Under oxidative stress, cardiolipin decreased and cardiolipin-prohibitin interactions increased. Under relatively low cardiolipin concentration (below 6–10 nmole), mitochondrial prohibitin competes with nuclear prohibitin for cardiolipin binding. We examined whether levels of prohibitin in mitochondria and the nucleus are altered by oxidative stress. We found that the level of prohibitin initially decreased by mild oxidative stress with H₂O₂ treatment

especially under IC₅₀ concentration.^{3,23} Translocalization of prohibitin under stress condition led us to investigate post-transcriptional regulation. A proteomic approach using SDS-PAGE and phospho-Western blot demonstrated that soluble nuclear prohibitin is phosphorylated. Up-regulation of pro-apoptotic molecules and down-regulation of anti-apoptotic proteins under prohibitin depletion by siRNA suggest an anti-apoptotic response of prohibitin depending on the prohibitin levels.

To maintain mitochondrial integrity, nuclear and mitochondrial genes should communicate with each other, especially under stress conditions that include hypoxia, abnormal temperature, nutrient deficiency, and aging. Our results suggest that prohibitin may fulfill dual roles as a phospholipid scaffold switch and an MT-NU shuttle under stress condition in the retina and RPE. Coordinated prohibitin-lipid interactions, including cardiolipin and PIP3, support the phospholipid scaffold switch mechanism hypothesis.^{28,29} Cardiolipin, a negatively charged diphosphatidyl glycerol derived phospholipid, is mainly localized in mitochondria and plays a significant role in maintaining mitochondrial membrane function and structure. Cardiolipin decreases with aging and is involved in the up-regulation of mitochondria-derived reactive oxygen species. Considering cardiolipin's role in mitochondria, it is reasonable to speculate that cardiolipin composition may affect targeted trafficking of mitochondrial proteins, especially under oxidative stress. The age-related decrease of cardiolipin is correlated with an increased cholesterol/phospholipid ratio.³⁰ Cholesterol depletion using β -cyclodextrin and simvastatin up-regulated prohibitin expression and cholesterol treatment decreased prohibitin in ARPE-19 cells (data not shown). Post-translational modifications that include hydrophobic palmitoylation and negatively charged phosphorylation may affect membrane binding and translocalization in subcellular organelles.^{25,31–33}

Consistent with our results, recent studies using pro-apoptotic peptide targeting prohibitin demonstrated that prohibitin might be connected to upregulation of lipid turnover and an increased metabolic rate.¹⁴ Changes in cellular metabolism are a central hallmark in the aging process in the retina and RPE. When apoptosis was induced by prohibitin-peptide binding, it was shown that lipid metabolism, energy expenditure, oxygen consumption, and heat generation increased. Using *C. elegans* as a model system, it was reported that prohibitin depletion affected ATP levels, lipid content, and mitochondrial proliferation in an age-specific manner.³⁴ Our results support the hypothesis that prohibitin may have a similar role in modulating mitochondrial structure by binding cardiolipin to maintain mitochondrial integrity. The current study also demonstrated that prohibitin expression levels may determine mitochondrial morphology and distribution shown by knockdown approach.

Recent studies examined the versatile functions of prohibitin as a drug-resistance mediator, plasma membrane receptor, obesity regulator, and cancer target.^{14,35–40} A metabolic switch mechanism of prohibitin was shown by pyruvate carboxylase inhibition and PIP3 binding.⁴¹ Prohibitin may modulate glucose and fatty acid oxidation by balancing between oxidative phosphorylation and anaerobic glycolysis under rapid proliferation, which are associated with malignant transformation.⁴² Down-regulation of prohibitin may activate a retrograde communication that induces mitochondrial proliferation and increased reactive oxygen species (ROS).^{30,34,43–48}

Nuclear function of prohibitin was suggested to be a transcriptional regulator by observing its interactions with p53, E2F, and Rb.⁴⁷ In cancer cells, prohibitin is localized in the nuclear matrix.⁴⁹ Cholesterol-induced down-regulation of prohibitin was shown as a regulator of cell cycle transit in the nucleus.^{45,50} Our previous study suggested that NF- κ B was translocated into the nucleus under oxidative stress, and protein translocation may act as a cell survival mechanism under stress conditions.³ Nuclear tumor suppressor p53 also translocated into mitochondria under apoptotic condition. The coordinated translocation of prohibitin may determine cell viability and the apoptotic cell population in the retina and RPE. Prohibitin under oxidative stress and altered lipid environments may determine the cell survival or death pathway by changing protein-lipid interactions. In the beginning of retinal degeneration, prohibitin may act as an anti-apoptotic molecule as the default function whereas it may become a transcriptional regulator under prolonged or repeated stress condition. This dual function of prohibitin might be the essential mechanism to control apoptotic signaling as a cell fate determinant from uncontrolled proliferation as suggested by the conserved gene evolution under oxidative environment.⁵¹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

DAPI	4',6-diamidino-2-phenylindole
DIGE	differential gel electrophoresis
DMEM	Dulbecco's Modified Eagle Medium
IP	immunoprecipitation
PARP	poly (ADP ribose) polymerase
ROS	reactive oxygen species
RPE	retinal pigment epithelium
siRNA	small interfering RNA

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Figure 1.

Translocation of prohibitin under oxidative stress. ARPE-19 cells treated with H_2O_2 (200 μM , 8, 24 hrs) were analyzed by immunocytochemistry. Subcellular organelles and prohibitin were visualized by DAPI (blue, nucleus), prohibitin-specific primary antibody, Alexa Fluor 488-conjugated secondary antibody (green, prohibitin), and Mito Tracker Orange (red, mitochondria). Prohibitin was translocated from mitochondria to the nucleus under oxidative stress in time dependent manner.

Figure 2.

Down-regulation of prohibitin under oxidative stress. ARPE-19 cells were treated with H_2O_2 . At specific time points, cells were harvested and proteins were separated by SDS-PAGE. Prohibitin was visualized by Western blot. Prohibitin decreased in all treated groups. ANOVA was used to demonstrate the differences among groups. Statistical analysis showed that there is a significant difference among groups ($P < 0.01$). Tukey's Honestly Significant Difference (HSD) test was applied to find the source of the difference. Results showed that control group was significantly different from the treated groups (4 hrs and 12 hrs $P < 0.01$; 2 hrs, 8 hrs, and 24 hrs $P < 0.05$). No significant difference was found among treated groups.

Figure 3.

Detergent-resistant membrane prohibitin under oxidative stress. (A) Detergent-resistant (insoluble) and soluble prohibitins were separated by SDS-PAGE and visualized by Western blot. Under oxidative stress (OS, 250 µM H₂O₂, 4 hrs), detergent-resistant prohibitin was up-regulated compared to control. (B) Quantitative analysis of insoluble/soluble prohibitin under oxidative stress. (C) Prohibitins from bovine retinal cells and ARPE-19 cells were compared to analyze insoluble and soluble forms. Mitochondrial membrane (MM) is slightly moved slower compared to cytosolic form (CYT). Whole cell lysate (WCL) from both bovine retinal cells and ARPE-19 cells shows detergent-resistant insoluble and soluble forms of prohibitin.

Figure 4. Subcellular localization of prohibitin. Subcellular fractions were separated by Cox and Emili method. Proteins in the retina were fractionated based on serial centrifugations with extraction buffers and separated by SDS-PAGE. Main fraction of prohibitin in bovine retina was localized in mitochondria as insoluble form (I, mitochondrial membrane) rather than soluble (S, mitochondrial matrix). Prohibitin was also detected in the nucleus. Prohibitin concentration was quantitatively analyzed. Relative amount of prohibitin is presented as soluble (S), insoluble (I) in mitochondria, nucleus (Soluble and Insoluble), cytosol, and microsome.

Figure 5. 2D SDS-PAGE analysis of prohibitin. Each fractions from bovine retinal cells were separated by isoelectric focusing and gel electrophoresis. Prohibitin was visualized by Western blot. Nuclear fraction showed phosphorylated prohibitin as one acidic spot at pI=5.3 shown by the arrow, whereas many modified prohibitin spots in the box were detected in cytosolic fraction.

Figure 6.

Phosphorylation of prohibitin in the nucleus. (A) Each fraction was further separated by SDS-PAGE and phospho-Western blot. Prohibitin in the nuclear fraction was phosphorylated shown by using p-Ser antibody. Mitochondrial I (matrix), II (membrane), nuclear I (supernatant), II (membrane), cytosolic, and microsomal prohibitins are shown. (B) Proteins were separated by native gel electrophoresis and prohibitin was visualized by Western blot using anti-prohibitin primary antibody and p-Ser antibody.

Figure 7. Prohibitin-cardiolipin interactions. (A) Prohibitin in the nucleus and mitochondria from bovine retina showed different cardiolipin affinity depending on cardiolipin concentration. (B) Quantitative analysis of mitochondrial prohibitin (filled square) vs. nuclear prohibitin (filled circle) with cardiolipin binding.

A. Control

B. Oxidative Stress

Figure 8.

Fractionated or IP purified proteins were incubated with the nitrocellulose membrane coated with various lipids. The membrane was incubated with immunoprecipitated prohibitin and lipid-prohibitin interaction was visualized by Western blot. Prohibitin-lipid binding was changed under oxidative stress (200 μ M H₂O₂). Under normal conditions, prohibitin interacts with phosphatidylinositol phosphates (PIP₃) whereas under oxidative stress prohibitin binds with cardiolipin.

Figure 9.

Anti-apoptotic prohibitin shown by knockdown analysis. (A) ARPE-19 cells were transfected by siRNA and random sequence. Anti-apoptotic BCL-xL increased when prohibitin was down-regulated. Pro-apoptotic proteins, including AIF, caspase-9, and BAK, were up-regulated under prohibitin knockdown condition. (B) ARPE-19 cells under siRNA or random sequence were analyzed by immunocytochemistry. Prohibitin, mitochondria, and the nucleus were visualized by Alexa-488 (green), Mito Tracker Orange (red), and DAPI (blue), respectively. When prohibitin was down-regulated by siRNA, mitochondrial structure was disrupted, and mitochondrial prohibitin was moved to the nuclear membrane.

Figure 10.

Prohibitin binding with p53. Immunoprecipitation using prohibitin antibody demonstrated the interaction between prohibitin and p53. Purified anti-prohibitin antibody was immobilized with amino-linked protein A and washed. Protein samples were incubated in the protein A-antibody column with gentle mixing for overnight at 4 °C. Columns were washed three times to remove non-specific binding proteins. Eluted fractions were separated by SDS-PAGE and visualized by Western blot using primary antibodies against prohibitin and p53.