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Parkin elimination of mitochondria is important for maintenance of lens epithelial cell ROS levels and survival upon oxidative stress exposure

Lisa Brennan, Josef Khoury, Marc Kantorow *

Charles E. Schmidt College of Medicine, Florida Atlantic University, Boca Raton, FL 33431, USA

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ABSTRACT

Age-related cataract is associated with oxidative stress and death of lens epithelial cells (LECs) whose survival is dependent on functional mitochondrial populations. Oxidative stress-induced depolarization/damage of LEC mitochondria results in increased reactive oxygen species (ROS) levels and cell death suggesting the need for a LEC mechanism to remove mitochondria depolarized/damaged upon oxidative stress exposure to prevent ROS release and LEC death. To date, a mechanism(s) for removal of depolarized/damaged LEC mitochondria has yet to be identified and the importance of eliminating oxidative stress-damaged mitochondria to prevent LEC ROS release and death has not been established. Here, we demonstrate that Parkin levels increase in LECs exposed to H_2O_2-oxidative stress. We establish that Parkin translocates to LEC mitochondria depolarized upon oxidative stress exposure and that Parkin recruits p62/SQSTM1 to depolarized LEC mitochondria. We demonstrate that translocation of Parkin results in the elimination of depolarized/damaged mitochondria and that Parkin clearance of LEC mitochondria is dependent on its ubiquitin ligase activity. Importantly, we demonstrate that Parkin elimination of damaged LEC mitochondria results in reduced ROS levels and increased survival upon oxidative stress exposure. These results establish that Parkin functions to eliminate LEC mitochondria depolarized/damaged upon oxidative stress exposure and that elimination of damaged mitochondria by Parkin is important for LEC homeostasis and survival. The data also suggest that mitochondrial quality control by Parkin could play a role in lens transparency.

1. Introduction

The eye lens is composed of a single layer of organelle-containing lens epithelial cells (LECs) that overlies a core of transparent organelle-free lens fiber cells [1]. The homeostasis and survival of LECs is essential for the transparency of the entire lens [1–4] since damage to LECs [5,6] and their sub-cellular components [7–10] has been suggested to result in cataract formation. Despite advances in surgical techniques, cataract remains a significant cause of world blindness and it has been estimated that any therapy that could delay the onset of cataract by just ten years could half the number of cataract surgeries required annually [11], improving the quality and reducing the cost of visual healthcare.

A key contributor to LEC death and cataract formation is exposure of the lens to oxidative stress [12–16]. Oxidative stress exposure results in damage to a wide-array of LEC components including LEC mitochondria that are particularly sensitive to oxidative stress exposure [7,12,13,17,18]. Oxidative stress damage to LEC mitochondria is characterized by mitochondrial depolarization [12], increased production of mitochondrial reactive oxygen species (ROS) [12,17,18] and LEC death [12,17].

To prevent oxidative stress-induced damage to LEC mitochondria and thereby prevent LEC death, multiple anti-oxidant [3,12,17,19,20] and chaperone systems [3,21–24] function to defend LEC mitochondria against depolarization/damage and thereby prevent increased ROS levels. However, despite the presence of these protective systems, high-level or chronic exposures of LECs to oxidative stress results in increased ROS levels and LEC death [12,17] and long-term exposure of the lens to hyperbaric oxygen-oxidative stress results in cataract formation in animal models [7,25–27]. These data suggest the need for an LEC mechanism(s) to remove damaged mitochondria to prevent increased ROS levels and LEC death. However, to date, no LEC mechanism for removal of damaged mitochondria has been identified and the effect of removing damaged LEC mitochondria on ROS levels or survival has not been established.

Evidence for the existence of a mechanism that could function to remove damaged LEC mitochondria was recently provided through the identification of mitochondria contained within autophagolysosomes of embryonic chick and adult human LECs [28]. This observation suggests that the selective autophagy process called mitophagy that

Abbreviations: LEC, Lens epithelial cells; ROS, Reactive oxygen species.

* Corresponding author at: Charles E. Schmidt College of Medicine, Florida Atlantic University, 777 Glades Rd., Boca Raton, FL 33431, USA.
E-mail address: mkantoro@health.fau.edu (M. Kantorow).

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eliminates mitochondria [29–32], could function to eliminate LEC mitochondria damaged upon oxidative stress exposure.

One protein that functions to remove damaged mitochondria in multiple cell-types is Parkin, Parkin operates in conjunction with the actions of phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (Pink1), mitochondrial-processing protease (MPP), and presenillin-associated rhomboid-like protein (PARL) [29–32]. Under normal conditions, Pink1 is cycled through the inner mitochondrial membrane where it is cleaved by several proteases including MPP and inner membrane PARL [29]. In depolarized/damaged mitochondria, unprocessed Pink1 is retained on the outer mitochondrial membrane where it phosphorylates Parkin [33,34]. Phosphorylation of Parkin by PINK1 results in its conversion into an active phospho-ubiquitin-dependent E3 ligase that ubiquitinates itself [35] and multiple mitochondrial outer membrane (OMM) proteins [35–39]. These ubiquitinated mitochondrial proteins are then recognized by ubiquitin-binding adaptor proteins such as p62/sequestosome 1 (p62/SQSTM1) [40–43]. p62/SQSTM1 contains an LC3B-interacting region (LIR) that recruits LC3B-labeled autophagosomes to engulf and degrade the damaged mitochondria [41–43]. Consistent with a potential role for Parkin in the removal of LEC mitochondria damaged upon oxidative stress exposure, DNA microarray [44] and RNA sequencing analysis [45] revealed that Parkin, Pink1, MPP, PARL and p62/SQSTM1 are expressed by LECs.

Here, we demonstrate that Parkin levels increase in LECs exposed to H_2O_2-oxidative stress. We establish that Parkin translocates to LEC mitochondria depolarized upon oxidative stress exposure and that Parkin recruits p62/SQSTM1 to depolarized LEC mitochondria. We demonstrate that translocation of Parkin results in the elimination of depolarized/damaged LEC mitochondria resulting from oxidative stress exposure and that Parkin elimination of damaged LEC mitochondria is dependent on its ubiquitin ligase activity. Importantly, we demonstrate that Parkin elimination of damaged LEC mitochondria results in reduced LEC ROS levels and increased LEC survival upon oxidative stress exposure. These results establish that Parkin functions to eliminate LEC mitochondria damaged upon oxidative stress exposure and that elimination of damaged mitochondria by Parkin is important for LEC homeostasis and survival. The data also provide evidence that LEC mitochondrial quality control by Parkin could play an important role in the maintenance of lens transparency.

2. Materials and methods

2.1. Cell culture of human lens epithelial cells

Human lens epithelial cells (SRA 01/04) [46] were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 15% FBS (Invitrogen), gentamicin (50 units/ml; Invitrogen), penicillin-streptomycin antibiotic mix (50 units/ml; Invitrogen) and fungizone (5 μl/ml; Invitrogen) at 37 °C in the presence of 5% CO_2.

2.2. Preparation of chicken primary lens epithelial cells

Primary chicken LEC cultures were prepared from the lenses of Embryonic day 10 (E10) White Leghorn embryonated chicken eggs (Charles River Laboratories, Storrs, CT) using the method of Menko et al. [47]. Briefly, primary lens cells were isolated from chicken lenses by trypsinization and agitation. Cells were plated onto glass bottom dishes coated with mouse laminin (Invitrogen) and cultured in Medium 199 (Invitrogen) supplemented with 10% FBS (Invitrogen) and penicillin-streptomycin antibiotic mix (50 units/ml; Invitrogen).

2.3. Transfection of human and primary chick lens epithelial cells

For transient transfection, SRA 01/04 were plated onto 35 mm^2 glass bottom tissue culture dishes (CellVis, Mountain View, CA) in 2 ml of media or 12 well glass bottom dishes (Matek, Ashland MA) in 1 ml of antibiotic free media and transfected with YFP-wt-Parkin or mutant YFP-C431N-Parkin vectors that were gifts from Dr. Richard Youle, [48] (Addgene, Cambridge, MA; Plasmid #23955 and #46924 respectively) using Lipofectamine 2000® transfection reagent (Invitrogen) according to the manufacturer's instructions. For transient transfection the efficiency was approximately 50% in SRA 01/04 cells transfected with either vector. Transient transfection was confirmed by visualizing YFP in transfected cells using a Zeiss LSM 700 Confocal microscope.

Primary chicken LECs were plated onto 12 well glass bottom dishes in 1 ml of media, allowed to adhere and reverse transfected with YFP-wt-Parkin or mutant YFP-C431N-Parkin vectors using Lipofectamine 2000® (Invitrogen) transfection reagent according to the manufacturer's instructions. Transfection efficiency was approximately 60% in primary chicken LECs transfected with either vector. Transient transfection was confirmed by visualizing YFP in transfected cells using a Zeiss LSM 700 Confocal microscope.

For both SRA 01/04 and primary chick LECs, cells were treated 48 h post transfection.

2.4. Creation of stable overexpressing SRA 01/04 cell lines

SRA 01/04 cells were transfected as described above with either YFP-wt-Parkin or mutant YFP-C431N-Parkin and incubated for 48 h. After 48 h cells were passaged into media containing 1.4 mg/ml Geneticin for selection of transfected cells. Clonal populations of cells remaining after a number of passages were transferred to 96 well plates and grown in the presence of Geneticin. Three cell lines were created for each vector and stored in media + 10% DMSO in liquid nitrogen. YFP-wt-Parkin or mutant YFP-C431N-Parkin transcripts levels were evaluated relative to a stable GFP overexpressing SRA 01/04 cell line by semi-quantitative RT-PCR using the SuperScript® III one-step RT-PCR system with Platinum Taq polymerase (Invitrogen) according to the manufacturer's instructions. Total RNA was purified from cells using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. 200 ng of total RNA was assayed following isolation from cell lines. Parkin transcripts were amplified for 30 PCR cycles with a 52 °C annealing temperature and the primer sequences: Forward primer – CTGT GCAGAATTGTGACCT and reverse primer – GCAAAGCTACTGATGTTTCC. GAPDH was amplified as the internal control transcript for 20 PCR cycles with a 60 °C annealing temperature using forward primer – CCACCCATG GCAAATTCCATGGCA and reverse primer - TCTAGACGGCAGGTCAGGTCC ACC. Parkin protein levels were also evaluated by western blot analysis using a Parkin-specific antibody (Santa Cruz Biotech, Dallas, TX).

2.5. Oxidative stress treatment of lens epithelial cells

To examine the effect of oxidative stress on Parkin mediated clearance of mitochondria, SRA 01/04 LECs that were transiently or stably transfected with YFP-wt-Parkin or mutant YFP-C431N-Parkin vectors were plated onto 35 mm^2 tissue culture dishes in 2 ml of media or 12 well glass bottom dishes in 1 ml of media. Cells were transferred to serum free media and treated with indicated amounts of H_2O_2 for indicated times. The volume of H_2O_2 added was proportional to the volume of media i.e. the stock concentration was consistent. Following treatment, cells were fixed and stained as detailed below or cells were lysed in NP40 lysis (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.25% sodium deoxycholate) buffer supplemented with a protease inhibitor cocktail (Sigma, St Louis, MO). Following lysis, samples were briefly sonicated and centrifuged for 5 min at 10,000 rpm. The supernatant was stored at −20 °C.

2.6. Analysis of Parkin protein levels in human lens cells following exposure to oxidative stress

SRA 01/04 LECs were seeded onto 6 well plates at a density of 2×10^5 cells per well. Cells were incubated in serum free medium for 2 h and