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Peroxiredoxin 3 levels regulate a mitochondrial redox setpoint in malignant mesothelioma cells

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ABSTRACT

Peroxiredoxin 3 (PRX3), a typical 2-Cys peroxiredoxin located exclusively in the mitochondrial matrix, is the principal peroxidase responsible for metabolizing mitochondrial hydrogen peroxide, a byproduct of cellular respiration originating from the mitochondrial electron transport chain. Mitochondrial oxidants are produced in excess in cancer cells due to oncogenic transformation and metabolic reorganization, and signals through FOXM1 and other redox-responsive factors to support a hyper-proliferative state. Over-expression of PRX3 in cancer cells has been shown to counteract oncogene-induced senescence and support tumor cell growth and survival making PRX3 a credible therapeutic target. Using malignant mesothelioma (MM) cells stably expressing shRNAs to PRX3 we show that decreased expression of PRX3 alters mitochondrial structure, function and cell cycle kinetics. As compared to control cells, knockdown of PRX3 expression increased mitochondrial membrane potential, basal ATP production, oxygen consumption and extracellular acidification rates. shPRX3 MM cells failed to progress through the cell cycle compared to wild type controls, with increased numbers of cells in G2/M phase. Diminished PRX3 expression also induced mitochondrial hyperfusion similar to the DRP1 inhibitor mdivi-1. Cell cycle progression and changes in mitochondrial networking were rescued by transient expression of either catalase or mitochondrial-targeted catalase, indicating high levels of hydrogen peroxide contribute to perturbations in mitochondrial structure and function in shPRX3 MM cells. Our results indicate that PRX3 levels establish a redox set point that permits MM cells to thrive in response to increased levels of mROS, and that perturbing the redox status governed by PRX3 impairs proliferation by altering cell cycle-dependent dynamics between mitochondrial networking and energy metabolism.

Introduction

Oxidative stress, defined as the imbalance between the production and the elimination of cellular oxidants by antioxidants, contributes to cancer initiation, progression and survival [1]. Due to their ability to damage cellular macromolecules, reactive oxygen species (ROS) must be dynamically regulated for normal and cancer cells to maintain steady state levels below the cytotoxic threshold [1]. In normal cells oncogenic stimuli, such as activated Ras, increases the production of cellular oxidants, leading to oxidative stress and ultimately inducing senescence [2]. Tumor cells must adapt in order to evade this fate and therefore commonly over-express antioxidant enzymes, such as superoxide dismutase 2 (MnSOD, SOD2) and peroxiredoxin 3 (PRX3), which permits escape from oncogene-induced senescence [3].

Mitochondria are dynamic cellular organelles responsible for producing the majority of adenosine triphosphate (ATP), the primary energy source of the cell. Mitochondria are the primary producers of cellular ROS, both as a byproduct of aerobic respiration [4] and from other important mitochondrial sources [5]. The inner mitochondrial membrane contains the electron transport chain (ETC), which provides the driving force for ATP synthesis via electron flow, proton pumping, and the formation of an electrochemical gradient fueling ATP synthase (complex V). Electron leakage, primarily at complexes I and III, leads to the incomplete reduction of molecular oxygen which forms superoxide radical [6]. Superoxide is an unstable intermediate that is spontaneously or enzymatically dismutated to hydrogen peroxide (H_2O_2), the primary oxidant implicated in redox signaling [7]. Under basal conditions resident cytosolic and mitochondrial antioxidant enzymes maintain proper redox status while changes in the rate of oxidant

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production and metabolism activate redox-dependent signaling pathways. Numerous signaling networks responsive to cellular oxidants have been identified, and these influence survival, proliferation and stress signaling pathways in normal and pathological settings [8].

Peroxiredoxin 3 (PRX3) is a member of the typical 2-Cys peroxiredoxin family (PRX 1–4) and functions as the primary oxidoreductase in the mitochondria responsible for metabolizing H_2O_2 [9] . PRX3 exists as a head to tail homodimer that utilizes a peroxidatic cysteine that reacts with a molecule of H_2O_2, thereby forming a sulfenic acid (–SOH) intermediate. After local unfolding of the active site, the resolving cysteine located on the adjacent monomer then forms a disulfide bond with the oxidized peroxidatic cysteine [10]. Thioredoxin 2 (TRX2) reduces this disulfide bond and thereby reactivates PRX3 [11]. A structural C-terminal extension found in typical 2-cys peroxiredoxins slows disulfide bond formation, allowing another molecule of H_2O_2 to further oxidize the peroxidatic cysteine to sulfinic (–SO2H) acid [12]. Typically these additional oxidation events are irreversible and lead to an inactive protein, but a system comprised of sulfiredoxin and ATP specifically regenerates active PRX3 [13,14]. This is a slow, energy-dependent reaction that has been hypothesized to allow transient and local increases in ROS levels to modulate redox-dependent signaling pathways [12].

Increases in mitochondrial oxidant levels may lead to the activation of stress signaling pathways and can cause cellular damage when oxidant levels reach a cytotoxic threshold. In order to escape oxidative stress, mitochondria have been shown to undergo structural rearrangements during which damaged and healthy mitochondria fuse, effectively reducing the number of damaged mitochondrion and ameliorating oxidative stress [15]. In addition to decreasing ROS, mitochondrial fusion has been shown to support increased ATP output from mitochondria [16].

A unique form of mitochondria at the G1/S phase of the cell cycle has been identified and described as an extensive hyperfused network with higher ATP producing capacity [16]. During cell cycle progression, mitochondrial fragmentation is required for proper segregation of mitochondria to daughter cells during mitosis [17,18]. Loss of dynamin-related protein 1 (DRP1), the primary enzyme responsible for mitochondrial fission, leads to cell cycle arrest at the G2/M phase. Furthermore, loss of DRP1 leads to hyperfusion of mitochondrial networks and increased ATP levels [19]. The redox status of the cell has also been shown to play a fundamental role in cell cycle progression where transient increases in ROS activate cell cycle signaling pathways [20]. These findings provide evidence for a functional relationship between mitochondrial structure and function and cell cycle progression.

In this report we identify PRX3 as an important regulator of mitochondrial metabolism and structure in human malignant mesothelioma cells. Changes in the basal redox status of mitochondria through stable knockdown of PRX3 led to alterations in energy production and changes to the mitochondrial network through inactivation of DRP1 by dephosphorylation at Serine 616. These alterations in mitochondrial structure induced a cell cycle pause at the G2/M phase. Mitochondrial structural perturbations in shPRX3 cells, as well as cell cycle abnormalities, were rescued by expression of catalase or mito-catalase, both of which corrected mitochondrial redox status back to control levels. Together these results indicate that PRX3 establishes a mitochondrial redox set point that supports proper integration of metabolic status, mitochondrial structure and cell cycle dynamics.

Results

Knockdown of PRX3 increases mitochondrial oxidant levels in HM cells

Using human malignant mesothelioma cells (HM) as a model for a ROS-driven tumor cell line [21] we sought to investigate the phenotype associated with lowering expression of the mitochondrial oxidoreductase peroxiredoxin 3 (PRX3), the antioxidant enzyme responsible for metabolizing the majority of mitochondrial peroxide [22]. HM cell lines stably expressing short hairpin RNA against PRX3 were generated (HMshPRX3) and successful knockdown was confirmed by immunoblotting (Fig. 1A) and immunofluorescence microscopy (Fig. 1B). The relative mean fluorescence intensity (MFI) for anti-PRX3 signal was quantified from immunofluorescence images and found to be approximately 6-fold lower than HM cells (Fig. 1C). PRX3 is estimated to metabolize approximately 90% of mitochondrial H_2O_2 [22] and therefore it would be expected that reduced expression of PRX3 would alter the redox status of the mitochondria. HM and HMshPRX3 cells were loaded with MitoSOX Red, a fluorescent indicator of general oxidant levels in the mitochondrial matrix, and analyzed by flow cytometry. HMshPRX3 cells showed a 30% increase in MitoSOX RED fluorescence, thereby indicating an increase in the levels of mitochondrial oxidants in these cells (Fig. 1D).

Knockdown of PRX3 alters the metabolic profile of HM cells

Among the many processes of mitochondria, including calcium buffering [23], propagation of apoptotic signals [24] and production of ROS [4], their primary role is to provide energy in the form of adenosine triphosphate (ATP). The transport of electrons through the electron transport chain (ETC) with oxygen as the final electron acceptor supports the pumping of hydrogen atoms across the inner mitochondrial membrane, thereby producing the electrochemical gradient that drives ATP production at complex V (ATP synthase). We measured ATP levels in HMshPRX3 cells and found them to be significantly elevated above those of HM cells, and both cell lines were responsive to the ATP synthase inhibitor oligomycin (Fig. 2A). Mitochondrial membrane potential supports the flow of electrons through the ETC, and ATP levels fluctuate based on changes to mitochondrial membrane potential. Supporting the evidence that HMshPRX3 cells have increased relative ATP levels as compared to HM cells, they also have an increased mitochondrial membrane potential compared to HM cells as measured with the cell permeant positively charged ethyl ester TMRE (Fig. 2B).

Using Seahorse extracellular flux analysis we compared metabolic features of HM and HMshPRX3 cells. Basal oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were increased in HMshPRX3 cells compared to HM cells (Fig. 2C and D). The observed increase in ATP levels, membrane potential and oxygen consumption suggests increased mitochondrial metabolism in HMshPRX3 cells. We could not directly measure this change as HMshPRX3 cells failed to respond to ETC inhibitors to reveal reproducible mitochondrial metabolism profiles using the Seahorse Bioanalyzer (data not shown, see discussion). To control for differences in mitochondrial mass, which would confound the observed metabolic changes, mitochondrial mass was quantified from fluorescence images and found to be equal for both the shPRX3 and HM control cell lines (Supplemental Fig. 1).