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Mitochondrial respiratory chain involvement in peroxiredoxin 3 oxidation by phenethyl isothiocyanate and auranofin

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ABSTRACT

Mitochondrial peroxiredoxin 3 (Prx 3) is rapidly oxidized in cells exposed to phenethyl isothiocyanate (PEITC) and auranofin (AFN), but the mechanism of oxidation is unclear. Using HL-60 cells deplete of mitochondrial DNA we show that peroxiredoxin 3 oxidation and cytotoxicity requires a functional respiratory chain. Thioredoxin reductase (TrxR) could be inhibited by up to 90% by auranofin without direct oxidation of peroxiredoxin 3. However, inhibition of thioredoxin reductase promoted peroxiredoxin 3 oxidation and cytotoxicity in combination with phenethyl isothiocyanate or antimycin A. We conclude that rapid peroxiredoxin 3 oxidation occurs as a consequence of increased oxidant production from the mitochondrial respiratory chain.

1. Introduction

Peroxiredoxin 3 (Prx 3) is an abundant mitochondrial protein that reacts rapidly with hydrogen peroxide, and is likely to be a key regulator of mitochondrial hydrogen peroxide levels [1]. Prx 3 is oxidized by hydrogen peroxide to a disulfide-linked dimer, and is dependent on the thioredoxin system for recycling to the reduced form. Accumulation of oxidized Prx 3 has been detected during ischemia [2] and apoptosis [3–5] and in all of these models the redox status of the cytoplasmic Prxs are unaltered, indicating a specific mitochondrial event.

Prx 3 has been proposed to play a role in the regulation of apoptosis, with alterations in expression modulating cellular sensitivity to pro-apoptotic stimuli [6,7]. While various redox events are proposed to be involved in apoptosis signaling, it is difficult to determine whether they are a cause or consequence of apoptosis induction. Two inducers, phenethyl isothiocyanate (PEITC) and auranofin (AFN), trigger substantial oxidation of Prx 3 within 30 min and prior to other major apoptotic events [4,5], indicating an early disruption of mitochondrial redox homeostasis. However, the mechanism underlying the oxidation of Prx 3 and its significance is unclear.

Prx 3 oxidation could result from increased mitochondrial hydrogen peroxide production or impairment of the thioredoxin (Trx)/thioredoxin reductase (TrxR)/NADPH system responsible for reducing Prxs. The major source of mitochondrial hydrogen peroxide is dismutation of superoxide generated by respiratory complexes [8]. In this study we compared the response of wild-type HL-60 leukemia cells with those lacking mitochondrial DNA to determine the importance of a functional respiratory chain for Prx 3 oxidation. AFN, an inhibitor of TrxR [9], was also used to determine the relative importance of thioredoxin system in maintaining Prx 3 in its reduced form.

2. Materials and methods

2.1. Materials

Cell culture materials were from Invitrogen New Zealand Ltd. (Auckland, New Zealand). PEITC was from Sigma Chemical Co. (St. Louis, MO, USA). Auranofin was from ICN Biomedicals Inc. (Costa Mesa, CA, USA). Complete™ protease inhibitors and CHAPS were from Roche Diagnostics (Manheim, Germany). Rabbit polyclonal antibodies to Prx 3 and Trx 1 were from Abfrontier Co.

Abbreviations: AFN, auranofin; NEM, N-ethylmaleimide; PEITC, phenethyl isothiocyanate; Prx, peroxiredoxin; ρ^0, mitochondrial DNA deplete; TrxR, thioredoxin reductase

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Ltd. (Seoul, Korea) and goat polyclonal antibody to Trx 2 was from R&D Systems (Minneapolis, MN, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG were from Sigma Chemical Co. Hybond-PVDF membrane and enhanced chemiluminescence (ECL™) western blotting system were from GE Healthcare (Buckinghamshire, England). All other chemicals and reagents were from Sigma Chemical Co. and BDH Laboratory Supplies (Poole, England).

2.2. Cell culture

The mitochondrial DNA deplete (ρ^0) and WT HL-60 cells used in this study were generously provided by Prof. Mike Berridge of the Malaghan Institute of Medical Research (Wellington, New Zealand). The ρ^0 cells had been generated by culturing HL-60 cells in the presence of ethidium bromide for 6–8 weeks and the lack of mtDNA confirmed by PCR [10]. Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamate, 1 mM pyruvate and 50 μg/ml uridine, and maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cells were harvested in the exponential phase of growth (between 0.4 and 0.8×10^6 cells/ml). WT and ρ^0 HL-60 cells were cultured and treated in full DMEM media containing pyruvate and uridine. Removal of pyruvate and uridine halted growth of the ρ^0 cells but had no effect on WT cell growth (not shown).

2.3. Immunoblot detection of the Prxs

Treated cells were pelleted and resuspended in N-ethylmaleimide (NEM) buffer (40 mM HEPES, pH 7.4, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, protease inhibitors, and 100 mM NEM). After 15 min incubation cells were lysed by addition of 1% CHAPS and clarified by centrifugation. Lysates containing 10 μg protein were combined in a 2:1 ratio with non-reducing sample loading buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue) and resolved by SDS–PAGE. Proteins were transferred to PVDF membrane, probed with primary and secondary antibody and visualized by using a peroxidase system with enhanced chemiluminescence (ECL™). Images were obtained using a ChemiDoc™ XRS system (Bio-Rad, Hercules, CA) and densitometry of scanned images was undertaken using Quantity One® software (Bio-Rad, Hercules, CA).

2.4. Flow cytometric analysis of cell viability

Treated cells (2×10^5) were resuspended in PBS containing 5 μg/ml PI. Cells were incubated in the dark for 10 min before analysis of the percentage PI-positive cells using a Cytomics FC500 MPL flow cytometer (Beckman Coulter, Fullerton, CA).

2.5. Immunoblot detection of the redox state of the Trxs

Treated cells (2×10^6) were pelleted and resuspended in 80 μl NEM buffer containing 1% CHAPS. After 30 min incubation lysates were clarified by centrifugation and desalted using a micro spin column (Bio-Rad, Hercules, CA, USA) pre-equilibrated with extract buffer (40 mM HEPES, pH 7.4, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA). The eluant was incubated with 5 mM dithiothreitol for 30 min before alkylation with 25 mM α-methoxy-ω-ethylmaleimide poly(ethylene glycol) 2 kDa (MalPEG-2kDa, Iris Biotech GmbH, Marktredwitz, Germany) for 30 min. Alkylated protein was extracted by acetone precipitation, resuspended in reducing sample buffer (sample loading buffer containing 700 mM β-mercaptoethanol), resolved by SDS–PAGE and detected by immunoblotting as described above.

2.6. Preparation of mitochondrial and cytosolic fractions

Following treatment, 3×10^7 HL-60 cells were collected and resuspended in 1 ml mitochondrial isolation buffer (20 mM HEPES–KOH, pH 7.5, containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.25 M sucrose and protease inhibitors). Selective permeabilization of the plasma membrane was achieved by addition of 100 μg digitonin. Samples were briefly vortexed before centrifugation at $650 \times g$ for 10 min. The supernatant was removed and centrifuged at $12\,500 \times g$ for 10 min. The resulting supernatant (cytosolic fraction) was removed while the pellet (mitochondrial fraction) was resuspended in 50 μl of mitochondrial isolation buffer containing 2% CHAPS. Mitochondrial fractions were clarified by centrifugation at $15\,000 \times g$ for 4 min. The purity of the cytosolic and mitochondrial preparations was confirmed by immunoblotting against Prx 2 (cytosolic) and Prx 3 (mitochondrial) under reducing conditions.

2.7. TrxR assay

The activity of TrxR was measured using a modified DTNB reduction assay [11]. Treated cells were pelleted and resuspended in extract buffer (40 mM HEPES, pH 7.4, containing 50 mM NaCl, 1 mM EDTA, 1 mM EGTA) containing 1% CHAPS and protease inhibitors. Cell lysates (or subcellular fractions) were transferred to a microplate and mixed with 200 μl of 5 mM DTNB in PE buffer (100 mM potassium phosphate, pH 7.0, 10 mM EDTA) and the reaction was initiated by addition of 200 μM NADPH. The relative activity of TrxR was determined as the difference between $\Delta A_{412\ nm}$ before and after the addition of NADPH.

2.8. Statistics

Statistical analyses were performed with the software package SigmaStat (Systat, San Rafael, CA, USA).

3. Results

Conversion of Prx 3 reduced monomer to oxidized dimer was observed following treatment of wild-type (WT) HL-60 cells with PEITC and the TrxR inhibitor AFN (Fig. 1A and B). To determine the role of mitochondrial respiratory complexes in the oxidation of Prx 3, we also assessed the response of ρ^0 HL-60 cells. No increase in oxidation was observed following treatment of ρ^0 HL-60 cells with PEITC (Fig. 1A) or AFN (Fig. 1B). Assessment of cell viability 24 h after treatment revealed that the ρ^0 cells were also considerably more resistant than WT HL-60 cells to both compounds (Fig. 1C and D). This indicates that a fully functional respiratory chain is critical for both Prx 3 oxidation and subsequent cytotoxicity.

Given that the redox state of Prx 3 is regulated by Trx 2, we examined the redox status of Trx 1 (cytosolic) and Trx 2 (mitochondrial) following treatment with PEITC or AFN. The redox state of Trx was investigated by labeling reversibly oxidized thiols with MalPEG-2kDa and visualizing migration shifts by immunoblotting. Consistent with the Prx 3 results, Trx 2 was almost completely converted to an oxidized form in WT HL-60 cells following treatment with PEITC (Fig. 2A) or AFN (Fig. 2B), yet there was only a subtle increase in Trx 2 oxidation in the ρ^0 HL-60 cells. In contrast, only minor Trx 1 oxidation was observed and this was similar in both WT and ρ^0 HL-60 cells.

The ability of PEITC and AFN to inhibit TrxR was assessed in both WT and ρ^0 HL-60 cells. PEITC inhibited TrxR in a dose-dependent manner with an IC_{50} of 20 μM, while AFN was considerably more potent with an IC_{50} of 0.25 μM (Fig. 3A). There was no