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.

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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.
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 (p0) 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 p0 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% CO2.

2.4. Flow cytometric analysis of cell viability

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).

2.3. 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).

2.5. Immunoblot detection of the redox state of the Trxs

Treated cells (2 × 10⁶) 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, protease inhibitors, and 100 µg/ml uridine). The eluant was incubated with 5 mM dithiothreitol for 30 min before alkylation with 25 mM N-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 × 10⁷ 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 MgCl2, 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×g for 10 min. The supernatant was removed and centrifuged at 12 500×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×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 ΔA412 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 p0 HL-60 cells. No increase in oxidation was observed following treatment of p0 HL-60 cells with PEITC (Fig. 1A) or AFN (Fig. 1B). Assessment of cell viability 24 h after treatment revealed that the p0 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 oxidation to Trx 2 oxidation in the p0 HL-60 cells. In contrast, only minor Trx 1 oxidation was observed and this was similar in both WT and p0 HL-60 cells.

The ability of PEITC and AFN to inhibit TrxR was assessed in both WT and p0 HL-60 cells. PEITC inhibited TrxR in a dose-dependent manner with an IC50 of 20 µM, while AFN was considerably more potent with an IC50 of 0.25 µM (Fig. 3A). There was no
difference in inhibition between WT and $\rho^0$ cells (Fig. 3A). Close inspection of the AFN dose response data reveals that AFN almost completely inhibited TrxR activity at doses that had little effect on the redox state of Prx3. For example, 0.5 $\mu$M AFN inhibited TrxR by approximately 90%, but significant Prx3 oxidation was not detected until cells were exposed to 2 $\mu$M AFN (Figs. 3A and 1B).

Since PEITC had a limited effect on TrxR activity at concentrations that oxidized Prx3 (Figs. 3A and 1A), we conclude that it is operating independent of its effect on TrxR.

To confirm that both compounds were able to effectively inhibit both TrxR1 (cytosolic) and TrxR2 (mitochondrial), HL-60 cells were fractionated into cytosolic and mitochondrial preparations prior to
the determination of TrxR activity. Consistent with our previous studies [4,5], PEITC and AFN inhibited both isoforms of TrxR, with cytosolic TrxR1 exhibiting a slightly greater extent of inhibition than mitochondrial TrxR2 (Fig. 3B). Consequently, the oxidation of Prx 3 observed with PEITC and AFN does not seem to be associated with differential inhibition of mitochondrial TrxR.
Low doses of AFN were used to determine if TrxR inhibition could sensitize Prx 3 to oxidation. The complex III inhibitor antimycin A increases mitochondrial oxidant production [12], and it triggered Prx 3 oxidation in HL-60 cells (Fig. 4A). Antimycin A did not affect cellular TrxR activity, and it did not cause Prx 3 oxidation in the \( \rho^0 \) HL-60 cells (data not shown). AFN at 0.5 \( \mu \)M promoted the oxidation of Prx 3 in the presence of antimycin A (Fig. 4A), with a synergistic increase in cytotoxicity (Fig. 4C). AFN was also able to sensitize cells to PEITC-triggered Prx 3 oxidation and cytotoxicity (Fig. 4B and D).

4. Discussion

Oxidation of mitochondrial Prx 3 has been observed following treatment of cells with isothiocyanates and AFN [4,5], but the mechanism of oxidation has not been elucidated. Two possibilities exist for promoting the accumulation of oxidized Prx 3: an increase in mitochondrial oxidant production, or direct inhibition of the Trx/TrxR/NADPH system necessary for recycling the oxidized protein. We have determined that the redox state of Prx 3 is resilient to inhibition of TrxR over the short periods examined in this study. While the TrxR inhibitor AFN caused Prx 3 oxidation at high doses, it was unable to trigger rapid accumulation of oxidized Prx 3 at doses where substantial inhibition of TrxR activity occurred. Instead, increased oxidation was dependent on a functional mitochondrial respiratory chain, indicating a central role for hydrogen peroxide generated by dismutation of superoxide derived from respiratory complexes. We therefore propose that PEITC increases the rate of oxidant generation from the respiratory chain. It is also possible that higher concentrations of AFN increase the endogenous flux of respiratory chain oxidants, but that is more difficult to separate from its effects on TrxR activity at lower doses. Regardless, the mitochondrial respiratory chain is vital for driving Prx 3 oxidation and the subsequent cytotoxicity associated with both compounds.

Mitochondria have been reported to be involved in isothiocyanate- and AFN-mediated cytotoxicity [5,13–15], primarily through

![Graphs and figures showing Prx 3 oxidation and cell death percentages](attachment:graphs.png)

**Fig. 4.** TrxR inhibition sensitizes Prx 3 to oxidation by antimycin A and PEITC and enhances cytotoxicity. Prx 3 oxidation was measured in HL-60 cells following exposure to 10 \( \mu \)M antimycin A for 30 min (A) or 5 \( \mu \)M PEITC for 60 min (B) in the presence or absence of 0.5 \( \mu \)M AFN. (C) and (D) Cell viability was measured after 24 h exposure under the same conditions as described above. Values are the mean and S.E. of at least three independent experiments. * Indicates a significant difference (\( p < 0.05 \)) between indicated treatments as determined by a one-way repeated ANOVA with Holm-Sidak multiple comparisons (Systat).
the utilization of conventional mitochondrial-dependent apoptosis pathways. There is, however, evidence for a direct effect of isothiocyanates on mitochondrial function [16–18]. The exact mechanisms are not clear, with one group linking a small inhibitory effect on complex III to apoptosis [18], and the other proposing the effect is independent of the direct targeting of respiratory complexes [16]. Isothiocyanate-affinity probes may prove valuable in elucidating direct targets within mitochondria [19], AFN has been shown to increase hydrogen peroxide production by isolated mitochondria, but not in submitochondrial particles with a functional respiratory chain, therefore it was concluded to primarily act through impairment of thioredoxin-dependent antioxidant defense [20].

Prr3 appears to be a valuable marker of mitochondrial roex homeostasis. It is present endogenously at high concentrations, thereby eliminating the need for chemical targeting or expression, and it is extremely reactive [21,22]. It is difficult to determine how observations obtained with global redox-sensitive probes such as dichlorofluorescein relate to mitochondria [18,23], but the localization of Prr3 ensures specificity. The disadvantage is that the oxidation of Prx 3 by AFN alone does not immediately trigger Prr3 oxidation, means that low dose AFN can be used to improve the sensitivity of Prr3 oxidation as a biomarker. There is also growing interest in the development of anti-cancer agents that act on mitochondria [24]. PETIC has been shown to effectively, and in some cases selectively, sensitize or kill drug-resistant and oncogenically-transformed cells [23,25–30]. AFN and related gold complexes are also being explored as anti-cancer agents that act on mitochondria [24]. PEITC has been shown to increase mitochondrial-dependent apoptosis of TrxR by AFN alone does not immediately trigger Prr3 oxidation, thereby eliminating the need for chemical targeting or expression.

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