Lack of an Efficient Endoplasmic Reticulum-localized Recycling System Protects Peroxiredoxin IV from Hyperoxidation*

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Background: Peroxiredoxin IV metabolizes endoplasmic reticulum-derived hydrogen peroxide.

Results: In contrast to other peroxiredoxins, peroxiredoxin IV is insensitive to hyperoxidation within its cellular environment.

Conclusion: There is a lack of a robust peroxiredoxin IV recycling system within the endoplasmic reticulum.

Significance: Studying the reduction of peroxiredoxin IV highlights the different mechanisms for maintaining redox status within different cellular compartments.

Typical 2-Cys peroxiredoxins are required to remove hydrogen peroxide from several different cellular compartments. Their activity can be regulated by hyperoxidation and consequent inactivation of the active-site peroxidatic cysteine. Here we developed a simple assay to quantify the hyperoxidation of peroxiredoxins. Hyperoxidation of peroxiredoxins can only occur efficiently in the presence of a recycling system, usually involving thioredoxin and thioredoxin reductase. We demonstrate that there is a marked difference in the sensitivity of the endoplasmic reticulum-localized peroxiredoxin to hyperoxidation compared with either the cytosolic or mitochondrial enzymes. Each enzyme is equally sensitive to hyperoxidation in the presence of a robust recycling system. Our results demonstrate that peroxiredoxin IV recycling in the endoplasmic reticulum is much less efficient than in the cytosol or mitochondria, leading to the protection of peroxiredoxin IV from hyperoxidation.

Peroxiredoxin (Prxs)² are a family of ubiquitous, highly expressed antioxidant enzymes capable of efficiently metabolizing hydrogen peroxide ($H_2O_2$) (1). They are the dominant cellular peroxide-reducing enzymes on the basis of their relative reactivity and protein concentration (2). In human cells, there are six Prxs that differ in subcellular localization and number of catalytic cysteine residues (3). The typical 2-Cys Prxs are localized to the cytosol (PrxI and II), the mitochondrion (PrxIII), and the endoplasmic reticulum (ER) (PrxIV). Under normal conditions, the active-site peroxidatic cysteine rapidly cycles between a sulfenic acid, disulfide, and free thiol, with the reduction of the disulfide being catalyzed by a member of the thioredoxin family of enzymes (3) (Fig. 1). The reactivity of the peroxidatic cysteine toward $H_2O_2$ is dependent upon its presence in the folded active site (4). Upon sulfenylation, there is a local unfolding of the active site to allow the peroxidatic cysteine to form a disulfide with a resolving cysteine located outside of the active site. Crucially, this localized unfolding reduces the reactivity of the peroxidatic cysteine because it is no longer in the optimal environment for reaction with $H_2O_2$. When the disulfide has been reduced, the active site refolds to allow reaction with the next $H_2O_2$ molecule. In the presence of high concentrations of $H_2O_2$, the peroxidatic cysteine can become hyperoxidized to form cysteine sulfenic acid, a modification that renders the protein inactive. Hyperoxidation occurs in competition with disulfide formation and can, therefore, occur more efficiently at high $H_2O_2$ concentrations. Although recycling is not essential for hyperoxidation (5), at lower $H_2O_2$ concentrations, disulfide formation occurs more efficiently, requiring recycling to allow hyperoxidation to occur (6, 7). This recycling is usually catalyzed by a thioredoxin domain-containing protein. It has also been shown that the hyperoxidized, sulfanylated active-site cysteine can be recycled back to the thiol and, thereby, reactivated by sulfiredoxin, an ATP-dependent enzyme localized to the cytosol (8). The inactivation of Prxs by hyperoxidation of the active-site cysteine residue has led to the suggestion that this modification provides a mechanism to allow $H_2O_2$ to act as a signaling molecule (3).

PrxIV reduces $H_2O_2$ produced by the ER oxidase 1 (Ero1) (9). The oxidized PrxIV formed can pass the disulfide bond to a member of the protein disulfide isomerase (PDI) family (10, 11). Therefore, during reduction of one molecule of oxygen to two molecules of water, two disulfide bonds can be introduced to PDI family members by Ero1 and PrxIV. The crystal structure of PrxIV shows that it forms a stable decamer and shares the same active sites as Prxl-III (4). Also like other Prxs, the reactivity of PrxIV toward $H_2O_2$ is high (12), making PrxIV the likely primary target of $H_2O_2$. Under conditions where Ero1 is activated, such as following treatment of cells with DTT, PrxIV can become hyperoxidized (9), although the physiological rele-

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2 The abbreviations used are: Prx, peroxiredoxin; ER, endoplasmic reticulum; PDI, protein disulfide isomerase; IEF, isoelectric focusing; CMVLS, College of Medical, Veterinary and Life Science.
variance of this modification is uncertain. In addition, it is unknown whether the ER contains a sulfiredoxin capable of recycling any sulfinylated PrxIV.

We developed a simple assay on the basis of one-dimensional isoelectric focusing (IEF) that allows us to monitor the hyperoxidation of all intracellular Prxs simultaneously. The ability to quantify the oxidation status of the Prxs enabled us to monitor the sensitivity of the Prxs to hyperoxidation within distinct cellular compartments. Here we show that ER-localized PrxIV is insensitive to hyperoxidation even at concentrations of H₂O₂ that cause hyperoxidation of Prxs localized to the cytosol and mitochondria. In addition, we could not find any evidence for the existence of an ER-localized sulfiredoxin.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—An antibody was raised in rabbits to a peptide with the sequence of the first 11 N-terminal amino acids of mature PrxIV (WETEERPRTRC). The commercial rabbit polyclonal antibodies used were anti-Prx II and III (Abcam) and anti-Prx-SO₂⁻³ (AbFrontier). An anti-PrxIV mouse monoclonal antibody (Abcam) was also used. The fluorescently conjugated anti-mouse and anti-rabbit IgG 680 and 800 secondary antibodies were purchased from either Li-Cor IRDye or Thermo-Scientific DyeLight. Human recombinant PrxIV, human PDI, E. coli thioredoxin, and Saccharomyces cerevisiae thioredoxin reductase were purified as described previously (4, 9, 10, 13).

Preparation of Samples for IEF—Cells were maintained in DMEM supplemented with 10% fetal bovine serum before being treated with 20 mM N-ethylmaleimide to prevent disulfide exchange. Cells were lysed in buffer A (50 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, 5 mM EDTA, and 1% (w/v) Triton X-100). Cell lysates were centrifuged (10,000 × g for 10 min at 4 °C) to remove insoluble material, and 5 volumes of ice-cold acetone were added to the supernatant and incubated for at least 2 h at −20 °C. The protein precipitated was isolated by centrifugation (10,000 × g for 10 min at 4 °C) and washed with 80% ice-cold acetone. The precipitate was isolated by centrifugation, and the pellet was air-dried and suspended in IEF sample buffer consisting of 7M urea, 2M thiourea, 2% (w/v) CHAPS, 0.8% (v/v) ampholytes (pH 4 – 6), 50 mM DTT, 4% (v/v) glycerol, and 0.02% (w/v) bromphenol blue. The protein was dissolved in IEF sample buffer for 1 h at room temperature before application to a one-dimensional IEF gel.

One-dimensional IEF—A 10% gel containing 9 M urea, 0.4% (v/v) ampholytes mix (pH4 – 6), and 1% (w/v) CHAPS was cast and run using the mini-VE system (Amersham Biosciences). The cathodic buffer was 10 mM phosphoric acid, and the anodic buffer was 25 mM Tris base. One-dimensional IEF was carried out at 1000 V and 2 mA for 4 h. Focused gels were rinsed in water and soaked in transfer buffer for 20 min at room temperature.

Immunoblotting—Following one-dimensional IEF, proteins were transferred to a nitrocellulose membrane (Li-Cor Biosciences). Nonspecific binding was blocked using 5% (w/v) nonfat dried skimmed milk in Tween/Tris-buffered saline (TTBS) (50 mM Tris buffer (pH 7.5) containing 150 mM NaCl and 0.1% (v/v) Tween 20). Membranes were incubated with primary antibody for 16 h at 4 °C in TTBS. The secondary antibody was cast and run using the mini-VE system (Amersham Biosciences). The cathodic buffer was 10 mM phosphoric acid, and the anodic buffer was 25 mM Tris base. One-dimensional IEF was carried out at 1000 V and 2 mA for 4 h. Focused gels were rinsed in water and soaked in transfer buffer for 20 min at room temperature. Specific proteins were visualized using an Odyssey SA imaging system (Li-Cor Biosciences). For quantification of fluorescent immunoblot analyses, scans were performed at the minimum intensities required to see all relevant proteins. Densitometry was then performed on unmodified output images using ImageJ (National Institutes of Health) (fraction hyperoxidized = SO₂H/(SH + SO₂H)). Statistical analysis was carried out using a one-tailed, paired t test assuming unequal variance between samples.
**Protection of Peroxiredoxin IV from Hyperoxidation**

Metabolic Labeling and Pulse-Chase Analysis—Cells were starved for 30 min in cysteine/methionine-free DMEM and then radiolabeled in the same medium containing 22 μCi/ml EXPRESS<sup>35</sup>S protein labeling mix (PerkinElmer Life Sciences). After 30 min of incubation at 37 °C, the radiolabel was removed, and cells were washed with PBS and incubated in complete DMEM for various lengths of time. Cells were washed with PBS before being lysed in buffer A. Cell debris was removed by centrifugation (10,000 × g for 10 min at 4 °C). The lysates were precleared by adding protein A-Sepharose (Generon) and incubated for 30 min at 4 °C. For immunoisolation, anti-PrxIV antibody was added to protein A-Sepharose. Samples were incubated for 16 h at 4 °C with end-over-end mixing. The Sepharose beads were pelleted by centrifugation (600 × g for 1 min) and washed three times with buffer A. Proteins were eluted with IEF sample buffer prior to loading onto a one-dimensional IEF gel. Gels were fixed, washed, and exposed to a phosphorimaging plate. Radioactivity was detected using a FujiFilm FLA-7000 PhosphorImager. Quantification of band intensities was carried out using ImageJ software.

In Vitro Assays—Recombinant human PrxIV (2.8 μM) was expressed and purified as described previously (9) and mixed with GSH (1 mM), GSH/GSSG (3:1 mM), reduced PDI (2.8 μM), DTT (1 mM), or a thioredoxin recycling system (2.8 μM thioredoxin, 2.8 μM thioredoxin reductase, and 1.5 mM NADPH) in 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl and 1 mM EDTA. Various concentrations of H<sub>2</sub>O<sub>2</sub> were added, and samples were incubated for 10 min at 30 °C. IEF sample buffer (5 volumes) was added to each sample and mixed for 1 h before being loaded onto a one-dimensional IEF gel. An immunoblot analysis using the polyclonal PrxIV antibody was carried out, and band intensities were quantified using ImageJ software.

**RESULTS**

Quantitative Assay for the Hyperoxidation of Prxs—The hyperoxidation of the members of the typical 2-Cys family of Prxs has been detected previously, either by using an antibody (α-SO<sub>2</sub>) that recognizes the sulfenylation active-site peptide of all 2-Cys Prxs (14) or by separation of the various oxidation states of the proteins by two-dimensional gel analysis (15). Although the antibody approach is very sensitive and provides a qualitative analysis of hyperoxidation, it suffers from a lack of specificity toward individual members of the family (9) and is non-quantitative in that it cannot assess the fraction of the total peroxiredoxin hyperoxidized. Such quantification can be provided by a two-dimensional gel analysis, but this approach is not suitable for screening large numbers of samples (16). To overcome this problem, we used a one-dimensional IEF approach that separates the unmodified or sulfenylated forms from the sulfenylated proteins on the basis of charge. To validate this approach, we treated HT1080 cells with increasing concentrations of DTT, separated the resulting cell lysates by one-dimensional IEF, and visualized specific proteins with different species, we could use different fluorescently labeled secondary antibodies on the same blot to determine the comigration of proteins. As the concentration of DTT increases, so does the appearance of a more acidic form of PrxIV (α-PrxIV) or the α-SO<sub>2</sub> antibody (Fig. 2A). We have shown previously that DTT generates H<sub>2</sub>O<sub>2</sub> in the ER by an Ero1-dependent mechanism and leads to hyperoxidation of PrxIV (9). Because the antibodies are raised in different species, we could use different fluorescently labeled secondary antibodies on the same blot to determine the comigration of proteins. As the concentration of DTT increases, so does the appearance of a more acidic form of PrxIV (Fig. 2A, top panel) that comigrates with protein recognized by the α-SO<sub>2</sub> antibody (bottom panel). The additional bands seen with the α-SO<sub>2</sub> antibody (center panel) reflect cross-reactivity of this antibody with other 2-Cys Prxs that show some increased reactivity at high concentrations of DTT (see below). Strikingly, at higher concentrations of DTT, most of the PrxIV was modified. To demonstrate that PrxIV was being modified following DTT treatment by the Ero1-dependent production of H<sub>2</sub>O<sub>2</sub>, we repeated the experiment with a cell line that contains low levels of Ero1α because of stable shRNA knockdown (17). No increase in hyperoxidized PrxIV was seen, even at high concentrations.
of DTT (Fig. 2B). Hence, the one-dimensional IEF system can separate unmodified from sulfinylated PrxIV and can be used to specifically quantify the levels of hyperoxidized PrxIV as well as other members of the 2-Cys Prx family.

Recycling of Hyperoxidized PrxIV—The modification of the 2-Cys Prxs to the sulfinylated form blocks reactivity of the active-site peroxidatic cysteine (7). However, a cytosolic enzyme called sulfiredoxin has been identified that catalyzes recycling of the sulfinylated peroxidatic cysteine back to an active thiol (8). To investigate whether hyperoxidized PrxIV could also be recycled, we first treated cells with a pulse of DTT to hyperoxidize about 80% of the protein (Fig. 3A), removed the DTT, and determined the recovery over 24 h. The hyperoxidized fraction dropped to <40%, indicating that either the pre-existing PrxIV was being recycled or that newly synthesized protein was replacing the hyperoxidized PrxIV. To determine whether pre-existing hyperoxidized PrxIV was being recycled, we first radiolabeled newly synthesized protein for 30 min, treated it with DTT to hyperoxidize the PrxIV, and chased it for up to 16 h in fresh medium. For this experiment, we used a cell line overexpressing PrxIV (18) because the endogenous protein was not radiolabeled efficiently. We immunoprecipitated the radiolabeled PrxIV at various time points and separated the oxidized forms of PrxIV by one-dimensional IEF (Fig. 3B). None of the hyperoxidized PrxIV radiolabeled during the pulse period was recycled, suggesting that, for PrxIV, the only mechanism to regenerate the active enzyme following hyperoxidation is by new protein synthesis. An immunoblot analysis of the recovery of PrxIV from hyperoxidation in this overexpressing cell line gave a similar result as that from HT1080s (Fig. 3C). That this recovery was due to new protein synthesis was confirmed when we analyzed the recovery of hyperoxidized PrxIV following a DTT pulse in the presence of the protein synthesis inhibitor cycloheximide (Fig. 3D). Some recovery was observed after 3 h in the absence of cycloheximide, but no recovery was seen in the presence of the protein synthesis inhibitor. Taken together, these results strongly suggest that there is no sulfiredoxin activity to recycle hyperoxidized PrxIV in the ER.

Differential Hyperoxidation of Prx Located in Different Intracellular Compartments—The ability to quantify the extent of hyperoxidation of 2-Cys Prxs enabled us to evaluate the consequence of the addition of H$_2$O$_2$ to HT1080 cells on the hyperoxidation of Prxs localized to the cytosol (PrxII), mitochondria (PrxIII), or the ER (PrxIV). A marked transition from mostly unmodified (basic) to mostly hyperoxidized (acidic) PrxII was seen after the addition of 100 μM H$_2$O$_2$ (Fig. 4A). As demonstrated previously, PrxIII was slightly less sensitive to hyperoxidation, requiring higher concentrations to become hyperoxidized (5, 19). PrxIV was largely resistant to hyperoxidation, with only low levels of hyperoxidation, even at 10 mM H$_2$O$_2$. The difference in sensitivity to hyperoxidation between PrxII and PrxIV was statistically significant ($p < 0.01$ at 0.1 and 1 mM H$_2$O$_2$). Similar differential susceptibility of the Prxs to hyperoxidation was also observed when the experiment was repeated with HeLa cells (results not shown). The addition of H$_2$O$_2$ exogenously to cells is unlikely to reflect a physiological increase in concentration, so to generate a more gradual increase, we added various concentrations of glucose oxidase to the cell medium to generate H$_2$O$_2$, in situ and assayed the extent of hyperoxidation after 3 h (Fig. 4B). Similar differential hyperoxidation of the Prxs in different cellular compartments was observed, with PrxIV being less sensitive to hyperoxidation than PrxII or PrxIII ($p < 0.01$ at 33 units/liter and $< 0.05$ at 100 units/liter, comparing PrxII to PrxIV). Taken together, these results demonstrate that there are distinct sensitivities to hyperoxidation of the various 2-Cys Prxs in different cellular compartments, with PrxIV being particularly resistant to exogenously added H$_2$O$_2$. 

Protection of Peroxiredoxin IV from Hyperoxidation
We have shown above that PrxIV can become hyperoxidized by an Ero1-dependent process in the presence of DTT. We evaluated whether the Ero1-generated H₂O₂ could also hyperoxidize PrxII or PrxIII in HT1080 cells (Fig. 4C). When DTT was added to cells, PrxIV was mostly hyperoxidized. However, only modest, but statistically significant (p < 0.05), levels of hyperoxidation of PrxII and PrxIII were observed at higher concentrations of DTT. These results indicate that ER-generated H₂O₂ can enter the cytosol at a sufficient concentration to hyperoxidize PrxII. In addition, it is clear that, in the presence of Ero1-generated H₂O₂ and a reducing agent, PrxIV does become sensitive to hyperoxidation.

The differential sensitivity of the 2-Cys Prxs to hyperoxidation by exogenously added H₂O₂ may be explained if the individual Prxs differed in their intrinsic sensitivity to hyperoxidation. It has been demonstrated that the efficiency of reduction of the disulfide formed between the peroxidatic and the resolving cysteine of the 2-Cys Prxs has a dramatic effect on the sensitivity to hyperoxidation (6, 7), so variations in the reductive pathways in each of the cellular compartments could also account for the variations in sensitivity to hyperoxidation. Alternatively, the lack of hyperoxidation of PrxII or PrxIII by ER-generated H₂O₂ and the resistance of PrxIV to exogenously generated oxidant could be due to the ER membrane being impermeable to H₂O₂.

To assess whether PrxIV is intrinsically insensitive to hyperoxidation, we used purified components in an attempt to reconstitute a pathway of hyperoxidation. Note that purified recombinant PrxIV is primarily oxidized (9), so the disulfide would need to be reduced to allow oxidation. In the absence of any

FIGURE 4. Differential sensitivity of Prxs located in different intracellular compartments to hyperoxidation. HT1080 cells were exposed to increasing concentrations of H₂O₂ (0–10 mM) for 10 min (A), units of glucose oxidase (0–100 units/L) for 3 h (B), or concentrations of DTT (C). Cell lysates were prepared as described in Fig. 2 and separated by one-dimensional IEF. Immunoblot analyses were developed with polyclonal antibodies to PrxII (α-prxII), PrxIII (α-prxIII), or PrxIV (α-prxIV), as indicated. The fraction of hyperoxidized Prx for the displayed image was calculated and plotted graphically for each treatment. Each experiment was carried out in triplicate with similar results obtained. Error bars indicate the mean ± S.D.
other components, PrxIV does not become hyperoxidized, even at 10 mM H₂O₂ (Fig. 5). However, in the presence of thioredoxin, thioredoxin reductase, and NADPH, PrxIV is readily hyperoxidized at concentrations of H₂O₂ as low as 100 μM. These results demonstrate that PrxIV will become hyperoxidized at similar concentrations of H₂O₂ that modified PrxII in cells if a robust recycling system is present. Within the ER, the initial reductase for PrxIV is likely to be a member of the PDI family (9). Indeed, when a stoichiometric amount of reduced PDI was incubated with PrxIV, hyperoxidation occurred. The sensitivity of PrxIV to hyperoxidation was maximized when GSH (1 mM) was included with reduced PDI, conditions that, as we have shown previously, fully reduce PrxIV (10). The ER lumen is unlikely to contain such reducing conditions, so the level of hyperoxidation of PrxIV in cells will most likely reflect the prevailing ratio of GSH:GSSG. Indeed, in the presence of a 3:1 ratio of GSH:GSSG and reduced PDI, PrxIV was less sensitive to hyperoxidation than with GSH alone (p < 0.03). An absolute requirement for PDI in this system was evidenced by the lack of PrxIV hyperoxidation when incubated in the presence of increasing concentrations of H₂O₂ and GSH at 1 mM. Analysis of the extent of hyperoxidation of PrxIV confirmed that, at 10 mM H₂O₂, statistically significant differences were seen between the presence and absence of all reductant apart from GSH alone (p < 0.05 (PDI) to p < 0.005 (thioredoxin, thioredoxin reductase, and NADPH). Hence, the level of hyperoxidation of PrxIV reflects the efficiency of the recycling system and requires a member of the PDI family to catalyze the initial reduction of the active site disulfide.

Cellular PrxIV Becomes Hyperoxidized in the Presence of a Reducing Pathway—The results with purified protein components provide compelling evidence that PrxIV can become hyperoxidized at quite low concentrations of H₂O₂. To determine whether an equivalent sensitivity of the three Prxs to hyperoxidation could occur when they were exposed to the same recycling pathway, we first lysed cells with detergent and then assessed the sensitivity of the three Prxs to increasing concentrations of H₂O₂ (Fig. 6A). Under these conditions, the cytosolic and mitochondrial reduction pathways would be diluted, so to provide a source of reducing equivalents, we included DTT (1 mM). Each of the Prxs was now sensitive to hyperoxidation, with PrxIV as sensitive as PrxII.

To determine whether PrxIV would be as sensitive as the other Prxs when a source of reductant was present in the ER, we took advantage of the fact that cells depleted of Ero1α do not produce H₂O₂ when DTT is added (Fig. 2B). Hence, we can add DTT to these cells, where it will act as a reductant, directly or indirectly, for PrxIV without generating H₂O₂. When the Ero1α-depleted cells were incubated with H₂O₂ in the presence of DTT, PrxIV became as sensitive to hyperoxidation as PrxII (>80% hyperoxidation at 100 μM H₂O₂) (Fig. 6B). This result demonstrates that ER-localized PrxIV is sensitive to hyperoxidation when a reducing agent is included and explains why PrxIV is sensitive to hyperoxidation in non-Ero1α-depleted cells when DTT is present. In addition, it shows that H₂O₂ must be able to permeate the ER membrane, proving that the lack of hyperoxidation of PrxIV was not due to a lack of H₂O₂ in the ER.

The DTT added to cells could reduce PrxIV directly or indirectly via PDI. To determine whether DTT can directly accelerate the recycling of PrxIV and lead to hyperoxidation, we incubated purified PrxIV with DTT and added increasing concentrations of H₂O₂ (Fig. 6C). The presence of DTT sensitized PrxIV to hyperoxidation. However, this effect was more pronounced when PDI was also included in the incubation. Taken together with the more efficient hyperoxidation of PrxIV in the presence of PDI and GSH (Fig. 5), we conclude that PDI or a PDI family member is the primary reductant of PrxIV and that maximal sensitivity can be achieved when either DTT or GSH is present to reduce PDI. In the lumen of the ER, PrxIV is sensitive to hyperoxidation only when PDI is efficiently reduced with DTT. The resistance of PrxIV to hyperoxidation indicates that an efficient mechanism to reduce PDI is absent from the ER.
DISCUSSION

We developed a simple quantitative assay to address the extent of hyperoxidation of members of the 2-Cys family of Prxs. This assay, on the basis of separation by one-dimensional IEF of the hyperoxidized from the unmodified form, can be used to evaluate the efficiency of the thiol reduction pathways in different intracellular organelles. The assay could also be used to quantify hyperoxidation of Prxs during the cell cycle (20) or to determine whether the oxidation status of Prxs changes during circadian oscillations (21). Here we used the assay to assess the sensitivity of Prxs in different cellular compartments to hyperoxidation following exposure to H$_2$O$_2$. Although the pathway for reduction of PrxII and PrxIII is fairly well characterized, the pathway for reduction of PrxIV was unclear. It has been shown previously that the disulfide formed in PrxI, II, and III following sulfenylation of the peroxidatic cysteine is reduced by a thioredoxin, which itself is reduced by a thioredoxin reductase present in the cytosol (PrxI and II) or mitochondria (PrxIII), with the ultimate electron donor being NADPH (6, 7). The fact that both Prx II and III were sensitive to hyperoxidation in the presence of H$_2$O$_2$ confirms that the thioredoxin reduction pathway present in the cytosol or the mitochondria was not compromised in the presence of this oxidant. The absence of hyperoxidation of PrxIV suggests that no equivalent pathway exists in the ER lumen for the reduction of thioredoxin domain-containing proteins. The presence within the...
ER of several thioredoxin domain-containing proteins that make up the PDI family allows the initial reduction of PrxIV (10). In the absence of a thioredoxin reductase to reduce the PDI family member, the disulfide within PrxIV will persist, thereby protecting the peroxidatic cysteine from hyperoxidation.

The protection of Prx from hyperoxidation means that, under normal physiological conditions, it is unlikely that PrxIV becomes extensively inactivated by this mechanism. This is in contrast to the other Prxs, which are regulated by hyperoxidation to allow H₂O₂ to act as a signaling molecule and modify proteins such as members of the protein tyrosine kinase family (22). In contrast to the cytosolic enzymes that are recycled from their hyperoxidized form by sulfiredoxin, hyperoxidized PrxIV could not be recycled, suggesting that there is no ER-localized sulfiredoxin.

Many studies regarding disulfide formation in the ER have used DTT as an experimental tool to prevent disulfide formation during a short pulse and, upon its removal, have followed the posttranslational formation of disulfides (23, 24). The assumption has been that the effect of DTT over a short pulse was to prevent disulfide formation because the reagent has no effect on the secretion of non-disulfide-bonded proteins. The demonstration that DTT also generates H₂O₂ in an Ero1-dependent fashion and that this leads to the hyperoxidation and inactivation of PrxIV should be taken into account when interpreting these previous studies. It should be noted that other peroxidases, such as Gpx7 or 8, could contribute to the removal of H₂O₂ under these conditions (25). However, like PrxIV, both these enzymes require a disulfide exchange protein to maintain optimal activity. Although it is clear that the ER redox status rapidly recovers from the added DTT by an Ero1-dependent process (17, 26), the inactivation of PrxIV will have a consequence for the metabolism of H₂O₂ for several hours following the addition of DTT. Hence, the interpretation of the effect of DTT addition to cells on their ability to recover ER redox status, to form disulfides, and to mount a stress response needs to take into account the fact that H₂O₂ metabolism will be impaired because of the inactivation of PrxIV.

Studies into the variation in the sensitivity of the 2-Cys peroxiredoxin family members to hyperoxidation has focused on differences between the intrinsic ability of each enzyme to be inactivated by H₂O₂ (5). Using purified protein, it has been shown that PrxIII is more resistant to hyperoxidation than PrxII because of subtle sequence differences within the C-terminal region. In contrast to PrxIII, we show here that PrxIV is resistant to hyperoxidation because of a lack of an efficient recycling system in the ER. To demonstrate that the lack of hyperoxidation was not due to an intrinsic resistance of this enzyme, we showed that purified Prx IV was susceptible to hyperoxidation in the presence of either a thioredoxin recycling system or PDI with a reducing agent such as DTT or GSH. In addition, ER-localized PrxIV also became sensitive to hyperoxidation in the presence of DTT. These results confirm the requirement for a PDI family member to directly reduce PrxIV in the ER (10, 27) and show that the oxidized PDI can be efficiently reduced in the presence of a reducing GSH buffer or DTT. The requirement for a GSH buffer to maintain a reductive pathway in the ER has been suggested previously because lowering the levels of intracellular GSH does not prevent disulfide formation but does lead to the persistence of non-native disulfides in the secreted protein tissue-plasminogen activator (28). The resistance of PrxIV to hyperoxidation in the ER indicates that an efficient pathway for the reduction of GSSG is also absent from the ER. Hence, a mechanism for GSSG/GSH exchange between the ER and the cytosol most likely exists to maintain a sufficiently reducing GSH:GSSG ratio to facilitate the reduction of members of the PDI family and allow non-native disulfide reduction.

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REFERENCES

1. Wood, Z. A., Poole, L. B., Hantgan, R. R., and Karplus, P. A. (2002) Dimers to doughnuts. Redox-sensitive oligomerization of 2-cysteine peroxiredoxins. Biochemistry 41, 5493–5504
2. Winterbourn, C. C. (2008) Reconciling the chemistry and biology of reactive oxygen species. Nat. Chem. Biol. 4, 278–286
3. Hall, A., Karplus, P. A., and Poole, L. B. (2009) Typical 2-Cys peroxiredoxins. Structures, mechanisms and functions. FEBS J. 276, 2469–2477
4. Cao, Z., Tavender, T. J., Roszak, A. W., Cogrell, R. J., and Bulleid, N. J. (2011) Crystal structure of reduced and of oxidized peroxiredoxin IV enzyme reveals a stable oxidized decamer and a non-disulfide-bonded intermediate in the catalytic cycle. J. Biol. Chem. 286, 42257–42266
5. Haynes, A. C., Qian, J., Reisz, J. A., Furdui, C. M., and Lowther, W. T. (2013) Molecular basis for the resistance of human mitochondrial 2-cys peroxiredoxin 3 to hyperoxidation. J. Biol. Chem. 288, 29714–29723
6. Stacey, M. M., Vissers, M. C., and Winterbourn, C. C. (2012) Oxidation of 2-cys peroxiredoxins in human endothelial cells by hydrogen peroxide, hypochlorous acid, and chloramines. Antioxid. Redox Signal. 17, 411–421
7. Yang, K. S., Kang, S. W., Woo, H. A., Hwang, S. C., Chae, H. Z., Kim, K., and Rhee, S. G. (2002) Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfenic acid. J. Biol. Chem. 277, 38029–38036
8. Biteur, B., Labarre, J., and Toledano, M. B. (2003) ATP-dependent reduction of cysteine-sulphinic acid by S. cerevisiae sulfiredoxin. Nature 425, 980–984
9. Tavender, T. J., and Bulleid, N. J. (2010) Peroxiredoxin IV protects cells from oxidative stress by removing H₂O₂ produced during disulphide formation. J. Cell Sci. 123, 2672–2679
10. Tavender, T. J., Springate, J. J., and Bulleid, N. J. (2010) Recycling of peroxiredoxin IV provides a novel pathway for disulphide formation in the endoplasmic reticulum. EMBO J. 29, 4185–4197
11. Zito, E., Melo, E. P., Yang, Y., Wahlander, Å., Neubert, T. A., and Ron, D. (2010) Oxidative protein folding by an endoplasmic reticulum-localized peroxiredoxin. Mol. Cell. 40, 787–797
12. Wang, X., Wang, L., Wang, X., Sun, F., and Wang, C. C. (2012) Structural insights into the peroxidase activity and inactivation of human peroxiredoxin 4. Biochem. J. 441, 113–118
13. Baker, K. M., Chakravarthi, S., Langton, K. P., Sheppard, A. M., Lu, H., and Bulleid, N. J. (2008) Low reduction potential of Ero1α regulatory disulfides ensures tight control of substrate oxidation. EMBO J. 27, 2988–2997
14. Woo, H. A., Kang, S. W., Kim, H. K., Yang, K. S., Chae, H. Z., and Rhee, S. G. (2003) Reversible oxidation of the active site cysteine of peroxiredoxin IV to cysteine sulfenic acid. Immunoblot detection with antibodies specific for the hyperoxidized cysteine-containing sequence. J. Biol. Chem. 278, 47361–47364
15. Wagner, E., Luche, S., Penna, L., Chevallet, M., Van Dorselaer, A., Leize-Wagner, E., and Rabilloud, T. (2002) A method for detection of overoxidation of cysteines. Peroxiredoxins are oxidized in vivo at the active-site cysteine during oxidative stress. Biochem. J. 366, 777–785
16. Chevallet, M., Wagner, E., Luche, S., Van Dorselaer, A., Leize-Wagner, E., and Rabilloud, T. (2003) Regeneration of peroxiredoxins during recovery
Protection of Peroxiredoxin IV from Hyperoxidation after oxidative stress. Only some overoxidized peroxiredoxins can be reduced during recovery after oxidative stress. J. Biol. Chem. 278, 37146–37153
17. van Lith, M., Tiwari, S., Pediani, J., Milligan, G., and Bulleid, N. J. (2011) Real-time monitoring of redox changes in the mammalian endoplasmic reticulum. J. Cell Sci. 124, 2349–2356
18. Tavender, T. J., Sheppard, A. M., and Bulleid, N. J. (2008) Peroxiredoxin IV is an endoplasmic reticulum-localized enzyme forming oligomeric complexes in human cells. Biochem. J. 411, 191–199
19. Cox, A. G., Pearson, A. G., Pullar, J. M., Jönnsson, T. J., Lowther, W. T., Winterbourn, C. C., and Hampton, M. B. (2009) Mitochondrial peroxiredoxin 3 is more resilient to hyperoxidation than cytoplasmic peroxiredoxins. Biochem. J. 421, 51–58
20. Phalen, T. J., Weirather, K., Deming, P. B., Anathy, V., Howe, A. K., van der Vliet, A., Jönnsson, T. J., Poole, L. B., and Heintz, N. H. (2006) Oxidation state governs structural transitions in peroxiredoxin II that correlate with cell cycle arrest and recovery. J. Cell Biol. 175, 779–789
21. Edgar, R. S., Green, E. W., Zhao, Y., van Ooijen, G., Olmedo, M., Qin, X., Xu, Y., Pan, M., Valekunja, U. K., Feeney, K. A., Maywood, E. S., Hastings, M. H., Baliga, N. S., Merrow, M., Millar, A. J., Johnson, C. H., Kyriacou, C. P., O’Neill, J. S., and Reddy, A. B. (2012) Peroxiredoxins are conserved markers of circadian rhythms. Nature 485, 459–464
22. Veal, E. A., Day, A. M., and Morgan, B. A. (2007) Hydrogen peroxide sensing and signaling. Mol. Cell 26, 1–14
23. Lodish, H. F., and Kong, N. (1993) The secretory pathway is normal in dithiothreitol-treated cells, but disulfide-bonded proteins are reduced and reversibly retained in the endoplasmic reticulum. J. Biol. Chem. 268, 20598–20605
24. Braakman, I., Helenius, J., and Helenius, A. (1992) Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. EMBO J. 11, 1717–1722
25. Nguyen, V. D., Saaranen, M. J., Karala, A. R., Lappi, A. K., Wang, L., Raykhe, I. B., Alane, H. I., Salo, K. E., Wang, C. C., and Ruddock, L. W. (2011) Two endoplasmic reticulum PDI peroxidases increase the efficiency of the use of peroxide during disulfide bond formation. J. Mol. Biol. 406, 503–515
26. Appenzeller-Herzog, C., Riemer, J., Zito, E., Chin, K. T., Ron, D., Spiess, M., and Ellgaard, L. (2010) Disulphide production by Ero1-PDI relay is rapid and effectively regulated. EMBO J. 29, 3318–3329
27. Sato, Y., Kojima, R., Okumura, M., Hagiwara, M., Masui, S., Maegawa, K., Saiki, M., Horibe, T., Suzuki, M., and Inaba, K. (2013) Synergistic cooperation of PDI family members in peroxiredoxin 4-driven oxidative protein folding. Sci. Rep. 3, 2456
28. Chakravarthi, S., and Bulleid, N. J. (2004) Glutathione is required to regulate the formation of native disulfide bonds within proteins entering the secretory pathway. J. Biol. Chem. 279, 39872–39879