Oxidation state governs structural transitions in peroxiredoxin II that correlate with cell cycle arrest and recovery

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Inactivation of eukaryotic 2-Cys peroxiredoxins (Prxs) by hyperoxidation has been proposed to promote accumulation of hydrogen peroxide (H2O2) for redox-dependent signaling events. We examined the oxidation and oligomeric states of Prxl and -II in epithelial cells during mitogenic signaling and in response to fluxes of H2O2. During normal mitogenic signaling, hyperoxidation of Prxl and -II was not detected. In contrast, H2O2-dependent cell cycle arrest was correlated with hyperoxidation of PrxII, which resulted in quantitative recruitment of ~66- and ~140-kD PrxII complexes into large filamentous oligomers. Expression of cyclin D1 and cell proliferation did not resume until PrxII-SO2H was reduced and native PrxII complexes were regenerated. Ectopic expression of Prxl or -II increased Prx-SO2H levels in response to oxidant exposure and failed to protect cells from arrest. We propose a model in which Prxs function as peroxide dosimeters in subcellular processes that involve redox cycling, with hyperoxidation controlling structural transitions that alert cells of perturbations in peroxide homeostasis.
ases that participate in retroreduction of Prx-SO\textsubscript{2}H, regenerating active H\textsubscript{2}O\textsubscript{2} to accumulate to substantial levels, thereby facilitating inactivation through hyperoxidation has been proposed to allow bond formation with the resolving cysteine (Wood et al., 2003a). The fact that the sulfenic acid (Prx-SO\textsubscript{2}H) is not detected above background levels in the neighboring subunit, resulting in an intersubunit disulfide bond. In mammalian cells, the intersubunit disulfide is reduced by thioredoxin (Trx), which is then regenerated by Trx reductase (TrxR) using reducing equivalents from NAD(P)H (Fig. 1). Calcium concentration, pH, and oxidation state influence the assembly of 2-Cys Prx dimers into decamers, and decamers into high molecular mass oligomers, depending on oxidation state, pH, calcium concentrations, and posttranslational modifications such as phosphorylation.

homodimers, and in these enzymes the Cys-SOH of the peroxidatic cysteine in one subunit is attacked by a resolving cysteine in the neighboring subunit, resulting in an intersubunit disulfide bond. In mammalian cells, the intersubunit disulfide is reduced by thioredoxin (Trx), which is then regenerated by Trx reductase (TrxR) using reducing equivalents from NAD(P)H (Fig. 1). Calcium concentration, pH, and oxidation state influence the assembly of 2-Cys Prx dimers into decamers, and decamers into high molecular mass oligomers (for reviews see Wood et al., 2003b; Immenschuh and Baumgart-Voigt, 2005; Rhee et al., 2005). Recent work also provides evidence for a link between structural transitions in the oligomeric state of Prxs and their peroxidase and protein chaperone activities (Wood et al., 2003a; Parsonage et al., 2005; Jang et al., 2006).

In contrast to prokaryotic homologues, eukaryotic 2-Cys Prxs have a particularly interesting biochemical characteristic in that they are readily inactivated by their own substrate, H\textsubscript{2}O\textsubscript{2}. Because of a C-terminal domain that induces a kinetic pause in the catalytic cycle, the peroxidatic cysteine of PrxI and -II is particularly susceptible to hyperoxidation, leading to the formation of sulfinic acid (Cys-SO\textsubscript{2}H), which cannot participate in disulfide bond formation with the resolving cysteine (Wood et al., 2003a). Inactivation through hyperoxidation has been proposed to allow H\textsubscript{2}O\textsubscript{2} to accumulate to substantial levels, thereby facilitating redox-dependent signaling, a concept known as the "floodgate" hypothesis (Wood et al., 2003a). The fact that the sulfenic acid form of 2-Cys Prxs is not a terminal end product but can be reduced in an ATP-dependent manner by sulfynil reductases, such as sulfiredoxins (Biteau et al., 2003; Chang et al., 2004) and p53-inducible sestrins (Budanov et al., 2004), suggests that Prx-SO\textsubscript{2}H may participate in regulatory signaling loops.

We tested the relevance of the floodgate hypothesis during mitogenesis by investigating the connection between the oxidative state of Prx isoforms and cell cycle entry and arrest. Our studies indicate that widespread inactivation of PrxI and -II by hyperoxidation is not a facet of normal mitogenic signaling. Rather, examination of dose-dependent responses to fluxes of H\textsubscript{2}O\textsubscript{2} demonstrate that cell cycle arrest in response to oxidative stress correlates with recruitment of PrxII-SO\textsubscript{2}H into cytoplasmic oligomers and that recovery of cell proliferation occurs after Prx-SO\textsubscript{2}H is reduced. Unexpectedly, transient overexpression of PrxI and -II led to increased levels of hyperoxidized Prxs in response to oxidative stress and failed to protect cells from arrest. We propose that Prx-SO\textsubscript{2}H functions in stress response pathways that warn cells of perturbations in oxidant metabolism and thereby contribute to oxidant-induced cell cycle arrest.

Results

Effects of H\textsubscript{2}O\textsubscript{2} on mitogenic signaling

To examine the oxidation state of Prxs during mitogenic signaling, mouse C10 lung epithelial cells were collected in G0 by serum deprivation, and the formation of Prx-SO\textsubscript{2}H in response to serum stimulation was assessed using an antibody specific for Prx-SO\textsubscript{2}H. Prx-SO\textsubscript{2}H was not detected above background levels in cells stimulated for 15 min with medium containing serum concentrations from 2 to 20%, a range that induces dose-dependent induction of tyrosine phosphorylation (Fig. 2A, lanes 2–5), activation of the ERK1/2 and PI-3 kinase–Akt mitogenic signaling pathways, and expression of cyclin D1 (Ranjan et al., 2006). These results indicate that normal mitogenic signaling does not require inactivation of Prxs by hyperoxidation, in agreement with a recent report on the role of PrxII in PDGF signaling (Choi et al., 2005).

To further explore Prx oxidation in cell cycle control, we adopted an experimental paradigm that utilizes a dose-dependent H\textsubscript{2}O\textsubscript{2} generating system to evoke transient cell cycle arrest (Burch et al., 2004). C10 cells were synchronized in G0 by serum deprivation and induced to reenter the cell cycle by adding medium containing 10% FBS with or without glucose oxidase (GOx). In complete medium with glucose and 10% FBS, GOx caused the dose-dependent production of H\textsubscript{2}O\textsubscript{2} in a linear fashion for at least 8 h (Fig. 2B). For example, in complete medium, 5.0 mU/ml GOx generated ~10 μM H\textsubscript{2}O\textsubscript{2}/h.

During the first 6 h of serum stimulation, 1.0 or 2.5 mU/ml GOx had little effect on the expression of cyclin D1, whereas doses of 5.0 mU/ml or greater blocked expression of cyclin D1 (Fig. 2C, lanes 7–9). In response to continuous exposure to 1.0 mU/ml GOx, the levels of activated ERK1/2 were similar to the serum control, cyclin D1 was expressed, and hyperoxidized 2-Cys Prxs were not observed (Fig. 2C, lane 5), suggesting that C10 cells are able to metabolize considerable amounts of exogenous H\textsubscript{2}O\textsubscript{2} during the G0–G1 transition without accumulating hyperoxidized 2-Cys Prxs. At 2.5 mU/ml, levels of phospho-ERK1/2 were unaffected, Prx-SO\textsubscript{2}H was barely detectable after 6 h of exposure, and cyclin D1 was expressed at nearly normal levels. In contrast, at 5.0 mU/ml, hyperoxidized Prx-SO\textsubscript{2}H accumulated to substantial levels and cyclin D1 was not expressed (Fig. 2C, lane 7). Concentrations of GOx ≥10.0 mU/ml induced accumulation of hyperoxidized Prx-SO\textsubscript{2}H, caused hyperactivation of ERK1/2, and blocked expression of cyclin D1 (Fig. 2C, lanes 8 and 9).

Figure 1. The catalytic cycle of C-Cys eukaryotic Prxs. [A] When exposed to H\textsubscript{2}O\textsubscript{2}, the peroxidatic cysteine [S\textsubscript{H}] of 2-Cys Prxs is oxidized to sulfenic acid [Prx-SOH]. Upon reaction with the resolving cysteine [S\textsubscript{R}], a Prx dimer with an intermolecular disulfide bond is formed, which is then reduced by Trx to regenerate active enzyme. Because of a pause in the catalytic cycle, the S\textsubscript{H} of eukaryotic 2-Cys Prxs is susceptible to hyperoxidation, resulting in the formation of a sulfenic acid form (Prx-SO\textsubscript{2}H) that is catalytically inactive. Sulfiredoxins and sestrins are ATP-dependent sulfynil reductases that participate in retroreduction of Prx-SO\textsubscript{2}H, regenerating active enzyme. 2-Cys Prxs are obligate homodimers that can assemble into decamers and higher molecular mass oligomers, depending on oxidation state, pH, calcium concentrations, and posttranslational modifications such as phosphorylation.
We previously showed that termination of ERK1/2 signaling after 3 h of exposure to the highest dose of GOx (15 mU/ml) restores expression of cyclin D1 but not cell proliferation (Burch et al., 2004). Hence, prolonged activation of ERK1/2 is a useful marker of oxidant-induced arrest at the G0–G1 transition of the cell cycle. Although GOx influenced the levels of phospho-ERK1/2 in a dose-dependent manner as before, it did not induce phosphorylation of JNK in synchronized cells at any dose (Fig. 2 C, lanes 5–9). In asynchronous cells, activation of JNK in C10 cells by H2O2 is associated with cell death (Pantano et al., 2003).

To determine if retroreduction of Prx-SO2H prevented the accumulation of Prx-SO2H, serum-stimulated cells were treated with 1-chloro-2,4-dinitrobenzene (DNCB), with or without GOx. DNCB depletes cells of reduced glutathione (GSH) and blocks reduction of Trx by inhibiting TrxR (Arner et al., 1995), thereby impairing the ability of Trx and GSH to participate in the retroreduction of Prx-SO2H to catalytically active forms. Within 10 min, 5 μM DNCB caused a 90% reduction in GSH levels that persisted for at least 3 h (unpublished data).

In the absence of GOx, DNCB blocked the ability of serum to induce expression of cyclin D1 but did not prevent phosphorylation of ERK1/2 (Fig. 2 C, lanes 4) or cause the accumulation of hyperoxidized Prxs. In contrast, DNCB markedly sensitized 2-Cys Prxs to hyperoxidation by GOx (Fig. 2 C, compare lanes 6–9 with lanes 10–13), suggesting that Prx retroreduction pathways are active during cell cycle reentry. Although phospho-ERK1/2 levels were increased in cells treated with GOx and enhanced in cells treated with DNCB and GOx, only with DNCB were high concentrations of GOx able to induce phosphorylation of JNK (Fig. 2 C, lanes 10–13).

Cell proliferation was then examined in serum-stimulated cells treated with DNCB and/or GOx (Fig. 2 D). GOx and/or DNCB were added to serum-stimulated cells, and proliferation was examined over a 3-d period without changing the culture media. C10 cells exposed to 1.0 or 2.5 mU/ml GOx proliferated as well as untreated controls, whereas those exposed to doses of GOx ≥ 5.0 mU/ml failed to proliferate by 3 d (Fig. 2 C). Greater than 70% of cells arrested in response to all but the highest dose of GOx (15.0 mU/ml) remained viable for at least 3 d (Fig. 2 D and not depicted). Caspase 3 was not activated in serum-stimulated cells at any dose of GOx, although it was readily activated after exposure to GOx by staurosporin (unpublished data), indicating that proapoptotic pathways were functional in arrested C10 cells. Cells treated with DNCB alone recovered slowly (Fig. 2 D), whereas cells treated with DNCB and any dose of GOx did not proliferate (not depicted).

Although DNCB sensitized Prxs to hyperoxidation by GOx, it did not sensitize Prxs to hyperoxidation in response to serum at any time point. Together, these studies indicate that formation of Prx-SO2H may not be required for mitogenic signaling during the GO–G1 transition of the cell cycle. In contrast, dose-response experiments with GOx revealed a sharp transition from unimpeded cell proliferation to cell cycle arrest that occurred between concentrations of 2.5 and 5.0 mU/ml, and that arrest was reflected in failure to express cyclin D1.

**Oxidation of Prxl and -II and cell cycle progression**

Transitions between dimers, decamers, and high molecular mass oligomers of Prxs are governed by oxidation state (Wood et al., 2002; Moon et al., 2005), phosphorylation during G2/M
fi ed by densitometry varied less than

tal PrxI and -II levels detected by immunoblotting and quanti-

probed fi rst for Prx-SO2H and then for either PrxI or -II after

after serum stimulation, with or without GOx (Fig. 3). When

medium (Fig. 3). At 2.5 mU GOx/ml,

centration after 3 h of exposure and after 3 h of recovery in fresh

nal for reduced PrxI (Fig. 3, lane 15; and Fig. 4 A), confi rming

mU/ml GOx (Fig. 3, lane 6), only

peared to be less sensitive to hyperoxidation than PrxI; at 2.5

the activity of retroreduction pathways in C10 cells. PrxII ap-

reduction pathways in C10 cells. PrxII ap-

the limitation that the Prx-SO2H antibody recognizes hyperoxi-

date the fraction of catalytically active PrxI and -II despite

signals that refl ected the fraction of PrxI or -II that was not cata-

were stripped and reprobed for Prx or -II (see Materials and methods).

When extracts were resolved by standard SDS-PAGE, to-
tal PrxI and -II levels detected by immunoblotting and quanti-
fied by densitometry varied less than ±8% during the fi rst 6 h
fter serum stimulation, with or without GOx (Fig. 3). When
robbed fi rst for Prx-SO2H and then for either PrxI or -II after
stripping the membrane, immunoblotting produced reciprocal
signals that refl ected the fraction of PrxI or -II that was not cata-
litically inactivated versus the fraction that was inactivated by
yperoxidation. Using densitometry, the levels of reduced/oxi-
dized PrxI (Fig. 4 A), reduced/oxidized PrxII (Fig. 4 B), and
Prx-SO2H (Fig. 4 C) were estimated as a function of GOx con-
centration after 3 h of exposure and after 3 h of recovery in fresh
medium (Fig. 3). At 2.5 mU GOx/ml, >85% of PrxI was hyperoxi-
adized after a 3-h exposure (Fig. 3, lane 6). After recovery,
<50% of PrxI was hyperoxidized, and the reduction in Prx-
SO2H levels (Fig. 4 C) was accompanied by recovery of the sig-
nal for reduced PrxI (Fig. 3, lane 15; and Fig. 4 A), confi rming
the activity of retroreduction pathways in C10 cells. PrxII ap-
peared to be less sensitive to hyperoxidation than PrxI; at 2.5
mU/ml GOx (Fig. 3, lane 6), only ~25% of PrxII had been in-
activated by 3 h (Fig. 4 B). At 10 or 15 mU/ml, both PrxI and -II
were quantitatively hyperoxidized (Fig. 3 A, compare lanes 8
and 9 with lanes 17 and 18), and little signal for reduced PrxI
and -II was regained after a 3-h recovery period (Fig. 4 A and B).
In cells treated with GOx, expression of cyclin D1 was inversely
correlated with the levels of Prx-SO2H (Fig. 3).

To assess the relationship between Prx hyperoxidation and
and cellular redox status, GSH levels were measured as a func-
tion of GOx concentration after exposure and recovery (Fig. 4 D).
A considerable drop in GSH levels was not observed at 3 h until
concentrations of GOx exceeded 5.0 mU/ml, and at all concen-
trations of GOx, GSH levels increased after recovery in fresh
medium (Fig. 4 D). These results agree well with a report that
shows PrxII is hyperoxidized in response to levels of H2O2 that
do not inhibit the TrxR–Trx system or deplete cells of GSH
(Baty et al., 2005). Hence, cells treated with 5.0 mU/ml GOx
for 3 h that retained near normal levels of GSH underwent tran-
sient cell cycle arrest, whereas those treated with either 10 or
15 mU/ml GOx that accumulated hyperoxidized PrxI and -II that
could not be reduced after 3 h of recovery (Fig. 3), perhaps be-
cause of low GSH levels (Fig. 4 D), were not able to proliferate.

**Serum stimulation engages Prxl and -II in peroxide metabolism**

When assessed under standard conditions, the total levels of
Prxl and -II did not change during the fi rst 6 h of serum stimulation
(Fig. 3). When samples were denatured in the presence of
SDS, but without reducing agents to preserve disulfi de bonds,
gel electrophoresis showed that both Prxl (Fig. 5, lane 1) and
PrxII (lane 7) from serum-starved cells were partitioned be-
tween 23-kD Prx-SH/Prx-SOH monomers and 38-kD Prx-S-S-
Prx homodimers. Upon addition of serum, the levels of Prxl
(Fig. 5, lanes 2–6) and Prx II (lanes 8–12) monomers decreased,
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and PrxI and -II homodimers with intersubunit disulfide bonds increased (Fig. 5, lanes 2–6 and 8–12, respectively). After exposure to 15 mU/ml GOx, all dimers with intersubunit disulfide bonds were lost by 30 min, and only hyperoxidized PrxI and -II monomers were detected for the duration of the experiment (Fig. 5, lanes 13–17; and not depicted). Because homodimers with intersubunit disulfide bonds are produced only during peroxide catalysis (Fig. 1), these results indicate that PrxI and -II metabolize H₂O₂ produced in response to serum stimulation. Upon hyperoxidation, a condition in which intersubunit disulfide bonds cannot form, only Prx-SO₂H monomers were observed, as expected.

**Recruitment of hyperoxidized PrxII into high molecular mass oligomers**

At 2.5 mU/ml GOx, 85% of PrxI was hyperoxidized, and yet cells expressed cyclin D1 and proliferated normally. In contrast, cells treated with 5.0 mU/ml GOx did not express cyclin D1 or proliferate. To better understand this difference, native gel electrophoresis was used to examine the effect of GOx on the oligomerization state of PrxI and -II. When cell extracts were resolved by electrophoresis in the absence of reducing agents and SDS, immunoblotting indicated that PrxI was organized exclusively in complexes >660 kD (unpublished data). In contrast, PrxII was detected in two sets of bands that we refer to as A–A’ and B–B’ (Fig. 6). Compared with the mobility of native molecular mass markers, A–A’ migrated with an apparent molecular mass of ~66 kD and B–B’ with a mass of ~140 kD. Although similar PrxII complexes have been observed in other cell types (Moon et al., 2005), the precise constituents of these complexes are not known.

In extracts of serum-starved cells, bands A and B were the predominant form of PrxII (Fig. 6 A, lane 1). Addition of DNCB or FBS alone for 3 h did not change the mobility of PrxII on native gels (Fig. 6 A, lanes 2 and 3), but together DNCB and FBS increased the signal of band B’ (lane 4). Because FBS and DNCB do not induce Prx hyperoxidation (Fig. 3), changes in band B may reflect structural transitions during formation of PrxII-S-S-PrxII dimers during peroxide metabolism (Fig. 5).

**Figure 5.** Serum stimulation increases the levels of Prx-S-S-Prx dimers. At the indicated times, extracts were prepared from serum-stimulated C10 cells in the absence of reducing agents and resolved by gel electrophoresis in the presence of SDS. After transfer, blots were probed for PrxI or -II as indicated. Extracts from serum-stimulated cultures treated with 15 mU/ml GOx were prepared in the same fashion and probed for Prx-SO₂H. Serum-starved cells (time 0) were used as controls.

**Figure 6.** Hyperoxidation of PrxII induces structural transitions that correlate with cell cycle arrest. (A and B) Serum-starved C10 cells (time 0) were stimulated with medium containing 10% FBS and the indicated concentration of GOx with or without 5 μM DNCB. Cell extracts prepared in the absence of reducing agents were resolved by native gel electrophoresis to preserve protein complexes. After transfer, immunoblots first were probed for PrxII [A], and after stripping, for Prx-SO₂H [B]. Complexes A–A’ and B–B’ migrated with apparent molecular masses of ~66 and ~140 kD, respectively. HMCs detected by the anti–Prx-SO₂H antibody in B migrated with apparent molecular masses >500 kD. To examine the dynamics of these complexes in response to oxidative stress, extracts were prepared at the indicated times from cells stimulated with FBS alone (C) or from cells stimulated with FBS containing 2.5 mU/ml (D) or 5.0 mU/ml (E) GOx for the first 3 h of serum stimulation. Samples were resolved under reducing conditions for assessing total levels of Prx-SO₂H and cyclin D1 and native conditions for visualizing HMCs and PrxII complexes A–A’ and B–B’.
agreement with studies that show PrxII metabolizes H$_2$O$_2$
produced in response to growth factors (Choi et al., 2005) and terminates H$_2$O$_2$-activated signaling by phospholipase D1 (Xiao et al., 2005).

In response to exposure to 1.0 or 2.5 mU/ml GOx, band B' increased in abundance relative to band B, perhaps reflecting increased engagement of the PrxII 140-kD complex in peroxide metabolism (Fig. 6 A, lanes 5 and 6). At concentrations of GOx of 5.0 mU/ml or higher, bands B and B' disappeared, band A decreased, and band A' appeared (Fig. 6 A, lanes 7–9). As observed in Fig. 3, DNCB shifted the dose response for the A–A' and B–B' complexes to lower concentrations of GOx (Fig. 6 A, lanes 10–14).

When reprobed for Prx-SO$_2$H, little hyperoxidized PrxII was observed for cells treated with 1.0 mU/ml GOx (Fig. 6 B, lane 5), whereas hyperoxidized Prx-SO$_2$H was observed to comigrate with band B' in extracts from cells treated with 2.5 mU/ml GOx (Fig. 6 B, lane 6). At concentrations of GOx ≥5.0 mU/ml, Prx-SO$_2$H was incorporated into several discrete high molecular mass complexes (HMCs) with apparent molecular masses >500 kD and considerable levels of A' accumulated (Fig. 6 B, lanes 7–9). As signal from the PrxII B–B' complex (Fig. 6 A, lanes 7–9).

**PrxII complexes accumulate during cell proliferation**

In time course experiments, the A–A' and B–B' complexes responded to serum stimulation and cell proliferation and, during recovery from exposure, to 5.0 mU/ml GOx. The levels of the 140-kD B–B' complex fluctuated during the first 12 h of serum stimulation (Fig. 6 C, lanes 1–6) and increased markedly in abundance as cells reached confluence 48–96 h later (lanes 8–10). As cells reached confluence, increases in the A–A' also were observed (Fig. 6 C, lanes 8–10). Serum stimulation and cell proliferation for >3 d caused no change in the signal for total Prx-SO$_2$H detected under reducing and denaturing conditions or Prx-SO$_2$H in HMCs detected by native gel electrophoresis (Fig. 6 C, lanes 2–10). The PrxII complexes were largely unaffected by exposing cells to 2.5 mU/ml GOx for the first 3 h of serum stimulation (Fig. 6 D, lanes 1–9), even though substantial levels of Prx-SO$_2$H were observed under these conditions (Fig. 6 D, lanes 1–4) and the cultures took slightly longer to reach confluence. Note that 2.5 mU/ml GOx did not increase HMCs containing Prx-SO$_2$H.

At 5.0 mU/ml GOx, the B–B' complex was not observed during the 3-h exposure, HMCs containing Prx-SO$_2$H increased in abundance, and cyclin D1 was not expressed (Fig. 6 E, lanes 1 and 2). After GOx was removed at 3 h, total Prx-SO$_2$H levels were reduced over time, and Prx-SO$_2$H in HMCs returned to background levels (Fig. 6 E, lanes 3–9). As signal for Prx-SO$_2$H diminished in HMCs, A' was lost, the B–B' complex reappeared, and cyclin D1 was expressed (Fig. 6 E, lanes 5–9). By 96 h, the HMCs and PrxII A–A' and B–B' complexes observed by native gel electrophoresis were identical in extracts from cells exposed to all three conditions, even though proliferation to confluence for >3 d caused no change in the signal for total Prx-SO$_2$H detected under reducing and denaturing conditions or Prx-SO$_2$H in HMCs detected by native gel electrophoresis (Fig. 6 C, lanes 2–10). The PrxII complexes were largely unaffected by exposing cells to 2.5 mU/ml GOx for the first 3 h of serum stimulation (Fig. 6 D, lanes 1–9), even though substantial levels of Prx-SO$_2$H were observed under these conditions (Fig. 6 D, lanes 1–4) and the cultures took slightly longer to reach confluence. Note that 2.5 mU/ml GOx did not increase HMCs containing Prx-SO$_2$H.

At 5.0 mU/ml GOx, the B–B' complex was not observed during the 3-h exposure, HMCs containing Prx-SO$_2$H increased in abundance, and cyclin D1 was not expressed (Fig. 6 E, lanes 1 and 2). After GOx was removed at 3 h, total Prx-SO$_2$H levels were reduced over time, and Prx-SO$_2$H in HMCs returned to background levels (Fig. 6 E, lanes 3–9). As signal for Prx-SO$_2$H diminished in HMCs, A' was lost, the B–B' complex reappeared, and cyclin D1 was expressed (Fig. 6 E, lanes 5–9). By 96 h, the HMCs and PrxII A–A' and B–B' complexes observed by native gel electrophoresis were identical in extracts from cells exposed to all three conditions, even though proliferation to confluence was delayed in cells treated with 5.0 mU/ml GOx (e.g., total cellular protein at 72 h was ~50% of the 10% FBS control).

**Localization of hyperoxidized 2-Cys Prxs**

Immunofluorescence confocal microscopy was used to localize Prx-SO$_2$H within C10 cells treated with various doses of GOx. In all cells, the Prx-SO$_2$H antibody reacted with the cell nucleus, but this signal did not correlate with the level of Prx hyperoxidation detected by immunoblotting. In cells treated with 1.0 mU/ml GOx for 3 h, immunostaining was occasionally observed in small patches at the cell periphery (Fig. 7 D), and this pattern was more obvious in cells treated with 2.5 mU/ml GOx (Fig. 7 E). At 5.0 mU/ml GOx staining was observed in a filamentous pattern in the cell cytoplasm (Fig. 7 F). Prx-SO$_2$H in cytoplasmic filaments was particularly evident in cells treated with 10.0 mU/ml GOx, and at 15 mU/ml GOx, staining was prominent around the cell periphery (Fig. 7 G and H). At higher doses of GOx, the peripheral Prx-SO$_2$H staining pattern correlated with changes in morphology that included a considerable increase in cell diameter. A filamentous cytoplasmic staining pattern for Prx-SO$_2$H was not observed in asynchronous cells at any dose of GOx (Fig. 7 I and not depicted).

**Ectopic expression of HA-PrxI and -PrxII and oxidant-induced arrest**

Up-regulation of PrxI is thought to counteract the effects of enhanced oxidant production in tumor cells and thereby promote cell survival and proliferation (Chang et al., 2005; Park et al., 2006).
To test the effects of Prx expression on responses to GOx, we generated expression vectors for HA-tagged PrxI, PrxII, and PrxII-ΔC, a robust mutant of PrxII that is 100-fold less sensitive to inactivation by H$_2$O$_2$ (Koo et al., 2002; Wood et al., 2003a). HA-PrxI interacts with endogenous PrxI in coimmunoprecipitation experiments, and HA-PrxI and -PrxII are hyperoxidized in response to GOx and reduced during recovery (unpublished data), indicating that HA-tagged Prxs function in peroxide metabolism in a manner similar to their endogenous counterparts. C10 cells were first transfected with expression constructs, and 24 h later the cultures were trypsinized and cells were plated at identical cell densities and synchronized by serum deprivation for 72 h as before. The transfected and serum-starved cell cultures were then treated with 5.0 mU/ml GOx as before.

In synchronized cells, immunoblotting showed HA-PrxI (Fig. 8 A, lanes 10–12) and HA-PrxII (lanes 13–15) were expressed at levels about fourfold that of their endogenous counterparts. Because of addition of the HA epitope tag and deletion of the PrxII C-terminal domain, HA-PrxII-ΔC comigrated with endogenous PrxII. As compared with untransfected cells (Fig. 8 A, lane 3) or vector controls (lane 6), expression of catalase (lane 9) and the robust PrxII-ΔC mutant (lane 18) reduced but did not eliminate Prx-SO$_2$H levels generated in response to GOx during a 3-h exposure, with 3 h of recovery period as before. HA-PrxI (Fig. 8 A, lane 12) and HA-PrxII (Fig. 8 A, lane 15) were hyperoxidized under these conditions and thereby increased the total cellular levels of Prx-SO$_2$H as measured by densitometry (Fig. 8 B). After recovery, expression of HA-PrxI or -PrxII did not reduce the levels of phospho-ERK1/2 or promote expression of cyclin D1 (Fig. 8 A). Although cells expressing catalase (Fig. 8 A, lanes 7–9) or PrxII-ΔC (Fig. 8 A, lanes 16–18) showed lower levels of total Prx-SO$_2$H and pERK1/2 after recovery, cells had not expressed cyclin D1 or resumed proliferation by this time. Expression of HA-PrxI or -PrxII did not affect expression of cyclin D1 in response to serum alone (Fig. 8 A, lanes 11 and 14). When cells treated with 5.0 mU/ml GOx were examined after 72 h of recovery, cells expressing HA-PrxI and -PrxII proliferated in a manner similar to vector controls, whereas cells expressing PrxII-ΔC resumed proliferation earlier during recovery (Fig. 8 C). Thus, as in serum-stimulated cells, the accumulation of Prx-SO$_2$H in cells overexpressing PrxI or -II was correlated with delays in cell cycle progression during recovery.

To confirm that HA-PrxII-ΔC was cytoprotective, stable cell lines were generated and treated with 5.0 mU GOx/ml continuously for 16 h. Flow cytometry showed that after 16 h ~30% of control cells exhibited a sub-G1 DNA content, whereas in comparison, ~10% of the cell population expressing HA-PrxII was detected in the sub-G1 fraction. In contrast, <0.5% of cells expressing PrxII-ΔC were detected in the dead cell fraction (unpublished data).

Discussion

The susceptibility of 2-Cys Prxs to inactivation by hyperoxidation is highly conserved in eukaryotes, inspiring the hypothesis that the Prx inactivation loop evolved to support peroxide-dependent signaling (Wood et al., 2003a). Here, we have examined the relationship between the oxidation state of PrxI and -II and transition from G0 into G1, a portion of the cell cycle known to respond to peroxide-dependent signaling (Finkel, 2003). Based on the presence of homodimers containing intersubunit

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**Figure 8. Elevated expression of Prxl and -II does not prevent cell cycle arrest in response to oxidative stress.** (A) C10 cells were transfected with the indicated expression vectors or vector control, synchronized by serum deprivation, and treated with 5.0 mU/ml GOx for 3 h as in Fig. 3. After 3 h of recovery in fresh medium, cell lysates were assayed for Prx-SO$_2$H, HA, cyclin D1, pERK1/2, and ERK1/2 levels using standard immunoblotting or for active Prxl and -II using the strip-reprobe blotting method. Nontransfected (NT) cells were included as controls for transfection. (B) Densitometry was used to quantify the total Prx-SO$_2$H signal for each sample presented in A. (C) After 72 h, cell numbers were determined to assess cell proliferation in cultures transfected with the indicated expression vectors. Error bars indicate mean ± SD.
disulfide bonds that are generated only during the Prx catalytic cycle, both PrxI and -II appear to metabolize H2O2 produced in response to serum stimulation (Fig. 5), although the source of H2O2, rate of catalysis, and sites of metabolism are unknown. In cells treated with DNBC (Fig. 3), which depletes cells of GSH and disrupts both Trx and GSH-dependent steps in the retroreduction cycle (Jeong et al., 2006), Prxs were sensitized to hyperoxidation by GOx. Nonetheless, in the presence of DNBC, hyperoxidation of Prxs was not observed in serum-stimulated cells at any time point, indicating that hyperoxidation of PrxI or -II may not be required during mitogenic signaling, a result that is in agreement with studies on the role of PrxII in PDGF signaling (Choi et al., 2005). Rather, our studies suggest that oligomers of hyperoxidized PrxII play a role in cell cycle arrest.

Hyperoxidized PrxI accumulated more rapidly in response to exogenous fluxes of H2O2 than did hyperoxidized PrxII (Fig. 3), but levels of PrxI-SO2H did not correlate with arrest. In contrast to PrxI, cell cycle progression, arrest, and recovery were correlated with changes in the oligomeric state of PrxII. As assessed by native gel electrophoresis, PrxII existed in two complexes of ∼66 kD (A–A’) and ∼140 kD (B–B’). As cells proliferated to confluence, both A–A’ and B–B’ increased in abundance and B–B’ increased in complexity (Fig. 6 C). In confluent cells, the B–B’ complex encompassed three distinct bands, suggesting recruitment of additional factors as cells exited the cell cycle, a matter presently under investigation.

In GOx dose-response experiments, C10 cells were able to accumulate substantial levels of hyperoxidized PrxI or -II during mitogenic signaling without marked effects on cell cycle progression. For example, exposure to 2.5 mU/ml GOx resulted in nearly complete hyperoxidation of PrxII and considerable levels of hyperoxidized PrxII, yet C10 cells were able to express cyclin D1 and proliferate. At these levels of exposure, GSH levels were unaffected, and hyperoxidized PrxI and -II were readily reduced once GOx was removed (Fig. 3). At levels of GOx that induced transient cell cycle arrest upstream of cyclin D1, but did not alter GSH levels, the B–B’ PrxII complexes disappeared and hyperoxidized PrxII appeared to be incorporated into HMCs. When oxidative stress was terminated, Prx-SO2H/H11601 by native gel electrophoresis, PrxII existed in two complexes of 66 kD (A–A’) and 140 kD (B–B’). As cells proliferated to confluence, both A–A’ and B–B’ increased in abundance and B–B’ increased in complexity (Fig. 6 C). In confluent cells, the B–B’ complex encompassed three distinct bands, suggesting recruitment of additional factors as cells exited the cell cycle, a matter presently under investigation.

In synchronized cells, a fourfold increase in expression of HA-PrxI and -PrxII relative to endogenous PrxI and -II did not reduce the level of hyperoxidized endogenous PrxI or -II in response to GOx but, rather, resulted in increased levels of total cellular Prx-SO2H. Expression of HA-PrxI and -PrxII also did not promote cell proliferation during recovery (Fig. 8 C). Together with the GOx dose-response studies, these results indicate that oligomers of hyperoxidized Prx-SO2H may be sensed as an anti-mitogenic signal.

Although the propensity of eukaryotic 2-Cys Prxs to be inactivated by H2O2 may provide a “flooding” for permitting H2O2 to accumulate for redox-dependent signaling, our data provide evidence for an additional hypothesis for the conservation of the inactivation shunt in mammals. Rather than simply buffering intracellular peroxide, Prx enzymes may continuously interpret and report peroxide levels, using their redox and oligomeric states as posttranslational modifications to interface with and modulate redox-sensitive cellular events (Fig. 9). Thus, Prxs may serve as highly sensitive peroxide dosimeters that link oxidant metabolism to a variety of redox-dependent processes required for cell cycle reentry. Upon serum stimulation, these enzymes become engaged in metabolizing H2O2 produced in response to activation of growth factor receptors, actin stress fiber formation, cell migration, and other processes. If oxidant metabolism goes awry or the cell is exposed to threshold levels of exogenous ROS, structural transitions regulated by hyperoxidation would terminate the Prx catalytic cycle, thereby interfering with redox cycling of other factors. Alternatively, PrxII-SO2H oligomers may be sensed directly as an anti-mitogenic signal. Linking Prx hyperoxidation to cell cycle progression would allow cells to respond to perturbations in peroxide homeostasis well before depletion of GSH or disruption of the TrxR–Trx system.

It is intriguing that p53 is activated by oxidative stress and that downstream targets of p53 include factors that influence cellular redox state, including stress proteins that regulate Prx activity (Budanov et al., 2004). Retrosynthesis of Prxs by sulfhydryl reductases is a reasonable facet of stress responses only if restoration of Prx activity contributes to recovery of activity in cell
Protein-protein interactions
Actin stress fiber formation
Normal oxidant metabolism
Cell cycle arrest and stress responses
Srx and sestrins
Recovery

Figure 9. A model for Prx hyperoxidation in cell signaling. Prxs may serve as dosimeters for redox-dependent signaling events, with structural transitions induced by hyperoxidation disrupting interactions with regulatory factors or modulating other redox-dependent processes, such as actin stress fiber formation. In addition, Prx-SO\textsubscript{2}H oligomers may be directly recognized as a signal that warns cells of perturbations in oxidant metabolism and thereby contribute to stress responses that mediate oxidant-induced cell cycle arrest.

Materials and methods

Cell culture, cell cycle synchronization, and oxidant exposure
C10 mouse lung epithelial cells (Malkinson et al., 1997) were cultured, synchronized, and stimulated with serum as described previously (Burch et al., 2004). For oxidant exposures, recombinant GOx (Roche) in 10 mM phosphate buffer, pH 7.4, was diluted in medium immediately before use. Levels of H\textsubscript{2}O\textsubscript{2} generated by GOx in complete medium were determined by centrifugation in a microfuge for 5 min, and the protein concentration of the soluble fraction was determined using a protein assay (Bio-Rad Laboratories).

For reducing SDS-PAGE, lysates were diluted 1:5 with 5 mM DTT, 300 mM Tris, pH 8.0, 1 mM PMSF, 1 mM EGTA, 2 mM Na\textsubscript{3}VO\textsubscript{4}, and 50% glycerol, heated at 95°C for 5 min before resolution on 12% SDS-PAGE gels. Nonreducing SDS-PAGE was performed in the same buffer (Promega) as cell cycle arrest.

Electrophoresis and immunoblotting
A model for Prx hyperoxidation in cell signaling.

- Levels of H\textsubscript{2}O\textsubscript{2} generated by GOx in complete medium were determined by centrifugation in a microfuge for 5 min, and the protein concentration of the soluble fraction was determined using a protein assay (Bio-Rad Laboratories).
- For reducing SDS-PAGE, lysates were diluted 1:5 with 5 mM DTT, 300 mM Tris, pH 8.0, 1 mM PMSF, 1 mM EGTA, 2 mM Na\textsubscript{3}VO\textsubscript{4}, and 50% glycerol, heated at 95°C for 5 min before resolution on 12% SDS-PAGE gels. Nonreducing SDS-PAGE was performed in the same buffer (Promega) as cell cycle arrest.

GSH measurements
C10 cells were lysed in 1% Triton, 50 mM Hepes, 250 mM NaCl, 10% glycerol, 1.5 mM MgCl\textsubscript{2}, 1 mM PMSF, 1 mM EGTA, 2 mM Na\textsubscript{3}VO\textsubscript{4}, 10 μg/ml aspirin, and 10 μg/ml leupeptin, pH 7.4. GSH was measured as previously described with some modifications (van der Vliet et al., 1998). In brief, samples were mixed 1:1 with 2 mM monobromobimane (Thioleyte; Calbiochem) in 50 mM phosphate buffer, pH 7.4, and incubated at RT for 5 min in the dark. Trichloroacetic acid was added to the reaction mixture to a final concentration of 5%. Samples were centrifuged at 3,000 g for 5 min, and supernatants were injected onto a Waters Symmetry C18 column (150 × 4.6 mm). The GSH-monobromobimane adduct was eluted with 10% CH\textsubscript{3}CN/0.25% glacial acetic acid and detected by fluorescence emission of 480 nm after excitation at 395 nm.

Confocal microscopy
C10 cells were plated on glass coverslips in 100-mm tissue culture dishes, synchronized or allowed to grow asynchronously to 70% confluence, and treated as indicated. Coverslips were rinsed with PBS, fixed with 3% paraformaldehyde for 15 min at RT, and washed several times with PBS, and cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min at RT. After gentle washing, coverslips were blocked for 1 h at RT with 10% normal goat serum in PBS and incubated with 1 μg/ml Prx-SO\textsubscript{2}H antibody in PBS with 1% BSA overnight at 4°C. Alexa Fluor 594 (Invitrogen) conjugated goat anti-rabbit secondary antibody at 1 μg/ml in PBS was added for 25 min at RT in the dark. Coverslips were mounted on slides, and
images were generated at RT using a confocal scanning laser microscope (MRC 1024 ES; Bio-Rad Laboratories) on a stand (BX50; Olympus), using a 40× Plan-Apo lens (Olympus) with a 0.95 NA and a correction collar. Digital images were collected with Laser Sharp Capture Software (Bio-Rad Laboratories) and processed as black-and-white images. Contrast was adjusted using Photoshop (Adobe).

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