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ORIGINAL RESEARCH COMMUNICATION

# Oxidation of 2-Cys Peroxiredoxins in Human Endothelial Cells by Hydrogen Peroxide, Hypochlorous Acid, and Chloramines

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## Abstract

***Aims:*** Reactive oxygen species released from neutrophils during vascular inflammation could contribute to endothelial dysfunction seen in diseases such as atherosclerosis. Activated neutrophils generate hydrogen peroxide ($H_2O_2$) and hypochlorous acid (HOCl), as well as chloramines that are formed when HOCl reacts with amino compounds. These oxidants preferentially target thiol groups and thiol-containing proteins. The peroxiredoxins (Prxs) are thiol proteins that have high reactivity with $H_2O_2$ and may also be sensitive to HOCl and chloramines. ***Results:*** We have investigated human umbilical vein endothelial cells and shown that their cytoplasmic (Prx1 and Prx2) and mitochondrial (Prx3) Prxs are oxidized when they are exposed to $H_2O_2$, HOCl, or cell-permeable chloramines. $H_2O_2$ converted the Prxs to hyperoxidized, inactive forms, with little accumulation of disulfide-linked dimers. The oxidized Prxs were reduced over hours, presumably due to the action of endothelial sulfiredoxin. In contrast to the hyperoxidation seen with $H_2O_2$, HOCl and the chloramine derivatives of glycine and ammonia converted the Prxs to disulfide-linked dimers and dimerization was reversed within 10–30 min of oxidant removal. HOCl treatment caused thioredoxin reductase (TrxR) inhibition with no reversal of dimerization. The cytotoxicity of ammonia chloramine was increased when cells were pretreated with $H_2O_2$ to hyperoxidize the Prxs, or when the chloramine was added in the presence of the TrxR inhibitor, auranofin. ***Innovation:*** We describe the novel observation that exposure of nucleated cells to inflammatory oxidants results in the accumulation of Prxs in the dimeric form. ***Conclusions:*** Endothelial cell Prxs are sensitive targets for neutrophil-derived oxidants and may protect against their damaging effects. *Antioxid. Redox Signal.* 17, 411–421.

## Introduction

Activated neutrophils generate a range of oxidants, both in the phagosome when they ingest microorganisms and into the extracellular milieu during inflammation. Superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) are generated by the plasma membrane NADPH oxidase complex and these are used by the enzyme myeloperoxidase (MPO) to form hypochlorous acid (HOCl) (19). HOCl is a strong oxidant that reacts with a wide variety of biomolecules, including amines to produce chloramines (12). Chloramines retain the oxidizing equivalents of HOCl and have been proposed to mediate some of its toxic effects (16). How this "cocktail" of potentially harmful neutrophil oxidants affects cell function has important implications for many diseases in which chronic low-grade inflammation is a contributing factor, such as cancer (40), atherosclerosis (6), and hypertension (51).

It is now recognized that inflammation is an important component of atherosclerosis and other vascular diseases, and that endothelial cell dysfunction is a characteristic of these

## Innovation

Peroxiredoxins (Prxs) are well-studied peroxidases that react readily with peroxides and are widely accepted as protective antioxidant enzymes across many cell types and organisms. Here we have reported that Prxs can also be oxidized by hypochlorous acid (HOCl) and chloramines in a dose-dependent manner in intact endothelial cells. In contrast to hydrogen peroxide, chloramines generated only disulfide-linked Prx dimers that were fully reducible by the cells, in the absence of any observed hyperoxidation. As HOCl and chloramines are generated by the neutrophil enzyme, myeloperoxidase, these novel findings are relevant to the function of Prxs at sites of inflammation. Prxs are known to be highly expressed in many disease states where there is significant inflammation, and the data reported here suggest that chloramine-induced Prx oxidation could occur in the endothelium during vascular inflammation and the consequences of this are worthy of further study.

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conditions (6, 51). Neutrophils accumulate within the inflamed vascular wall (3) and released MPO has also been demonstrated in the extracellular space (14), resulting in the generation of HOCl and derived chloramines in the vicinity of endothelial cells (20). Understanding the cellular reactivity of the neutrophil oxidants with endothelial cells should therefore lead to better understanding of the mechanisms of endothelial dysfunction. Previous studies have shown that whereas higher doses of HOCl and chloramines are toxic to endothelial cells, sublethal doses induce a number of biochemical and functional changes. These include depletion of ATP, oxidation of reduced glutathione (GSH) and susceptible thiol enzymes including glyceraldehyde-6-phosphate dehydrogenase and cofilin, inactivation of NF$\kappa$B inhibitor (I$\kappa$B), suppression of cytokine and adhesion molecule expression, activation of mitogen-activated protein (MAP) kinases, and initiation of apoptosis (4, 23, 25, 27, 29, 30, 32, 42, 45, 48, 49). It is unclear whether cells are able to protect themselves against these oxidants.

Peroxiredoxins (Prxs) are an important component of antioxidant defenses against peroxides (17, 22) and it is possible that they could have a wider antioxidant role. HOCl and derived chloramines are known to react preferentially with thiol residues, particularly if they are ionized (36) and the Prxs have a low-pKa Cys residue at their active site. Prxs are highly expressed peroxidases that catalyze the reduction of $H_2O_2$, organic hydroperoxides, and peroxynitrite (18, 34, 47). As well as having an antioxidant role, Prxs are potential sensors in redox signaling and their oxidation status may also be a sensitive indicator of *in vivo* oxidative stress (9, 15, 53). There are six family members in mammals, four of which are classified as typical 2-Cys Prxs: Prxs 1 and 2 in the cytoplasm, Prx3 in mitochondria, and Prx4 in the endoplasmic reticulum.

The 2-Cys Prxs operate by a mechanism in which there is initial oxidation of a highly reactive (peroxidative) Cys that condenses with another (resolving) Cys on an adjacent subunit to give a disulfide-linked dimer. The dimeric form is reduced by the action of thioredoxin reductase (TrxR), with reducing equivalents from Trx and NADPH. The peroxidative Cys can also react with another oxidant molecule prior to dimerization to form the sulfinic acid. This hyperoxidized form is not easily reduced but can be slowly recycled by sulfiredoxin (5, 8). Oxidation of Prx by $H_2O_2$ has been demonstrated for a variety of cell types (11, 24, 38, 39, 44). Recently, we showed that Prx2 in the erythrocyte is oxidized by chloramines (43), but it is unknown whether Prxs are sensitive to chloramines in other cells. In the current study we have investigated the sensitivity of Prxs 1–3 to oxidation in human umbilical vein endothelial cells (HUVECs) exposed to $H_2O_2$, HOCl, and the chloramine derivatives of ammonia ($NH_2Cl$) and glycine (GlyCl), and related this to toxicity. We demonstrate hyperoxidation and slow recycling of the Prxs with $H_2O_2$ compared with readily reversible dimerization with the chloramines.

## Results

### *Prxs in HUVECs*

HUVECs were found to express cytosolic Prxs 1 and 2, and mitochondrial Prx3. Using western blot analysis to compare signal intensity of the Prxs 2 and 3 against known amounts of purified protein, Prx protein levels were determined to be in the range of 4–6 ng per microgram total protein (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/ars).

When untreated control cells were lysed in the presence of the alkylating agent *N*-ethylmaleimide (NEM) to preserve the intracellular oxidation state of the Prxs, and the proteins were separated by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the Prxs migrated predominantly at ~21–23 kDa, the molecular weight of the reduced monomers. Some disulfide-linked dimer was detected, particularly for Prx3 (Fig. 1A, left panel, first lane). The hyperoxidized form of the Prxs, which would be evident as a monomer band in the absence of NEM, was not observed in control cells (Fig. 1A, first lanes in right panel).

### *Effect of $H_2O_2$ on Prx redox state*

Treatment of HUVECs with the lowest concentrations of $H_2O_2$ resulted in only modest dimer accumulation, which was most evident with Prx1 (Fig. 1A, left panel). With higher $H_2O_2$ concentrations, the Prxs remained primarily as monomers. This would be expected if they became hyperoxidized (and therefore unable to dimerize). Hyperoxidation was confirmed by examining cells prepared in the absence of NEM. Accumulation of monomeric Prxs 1, 2, and 3, which corresponds to the hyperoxidized protein under these conditions, was observed with increasing doses of $H_2O_2$ (Fig. 1A right panel, and 1B). Prx2 was the most hyperoxidized and Prx3 the least at each $H_2O_2$ concentration, with maximal effect at 120 $\mu M$. Analysis of the $H_2O_2$ remaining in the supernatant showed that for each concentration added to the cells, ~30% was consumed over the 10 min treatment (Supplementary Fig. S2). Thus, only this fraction of the applied $H_2O_2$ doses was responsible for mediating the Prx redox changes.

It has been reported that hyperoxidation of purified Prx1 requires the presence of all catalytic components that enable turnover (Trx, TrxR, and NADPH) (55). To test this in HUVECs, auranofin (AFN) was added to inhibit TrxR (31), and thereby restrict catalytic cycling. Treatment with AFN for 20 min caused dose-dependent inhibition of HUVEC TrxR activity with 4 $\mu M$ giving 92% inhibition (mean from four experiments; Supplementary Fig. S3A). Concomitantly, there was dose-dependent dimerization of the Prxs (Supplementary Fig. S3B). However, 18% of Prx1, 48% of Prx2, and 37% of Prx3 remained in the monomeric form after 4 $\mu M$ AFN pretreatment. When AFN-pretreated cells were exposed to $H_2O_2$ then lysed in the absence of NEM, western blotting showed only the dimer band (Fig. 2). Thus, when the Prx catalytic cycle was blocked, HUVECs were insensitive to $H_2O_2$-induced Prx hyperoxidation.

To establish whether the Prx hyperoxidation induced by $H_2O_2$ in endothelial cells was reversible, cells were treated for 10 min with sufficient $H_2O_2$ to give maximum hyperoxidation (shown as monomer in Fig. 3), then allowed to recover in fresh medium. Because the hyperoxidized and reduced forms are both monomeric when analyzed by SDS-PAGE, regeneration was monitored by lysing in the absence of alkylating NEM; this allowed the detection of hyperoxidized species (which remained monomeric) *versus* reduced species (which became oxidized upon lysis). For all three Prxs, hyperoxidation was slowly reversed over a period of 4–6 h.

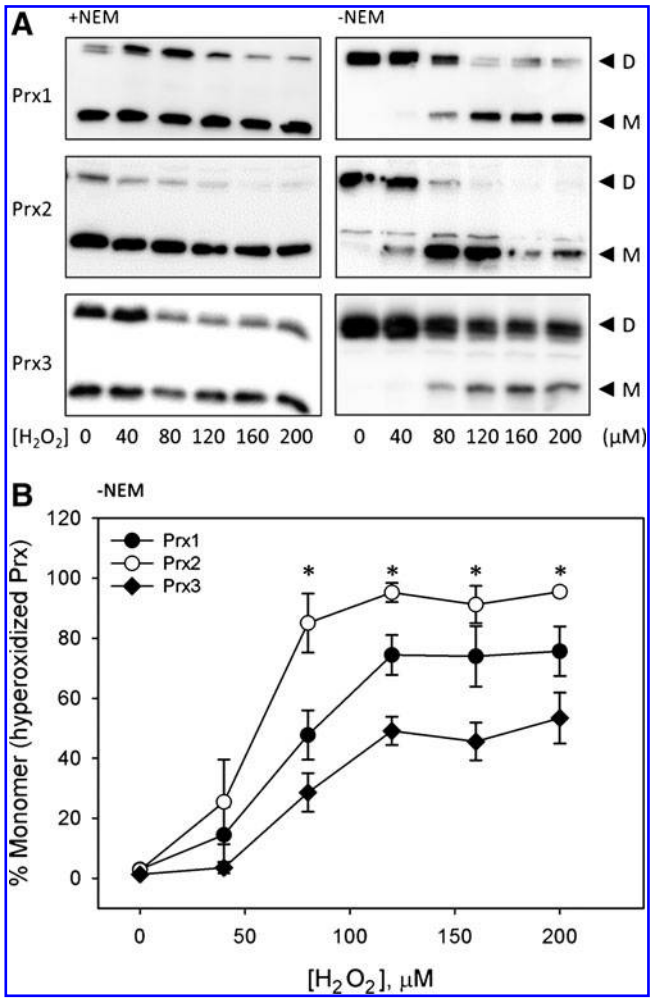


**FIG. 1. Peroxiredoxin (Prx) redox state in hydrogen peroxide ($H_2O_2$)–treated human umbilical vein endothelial cells (HUVECs). (A)** Nonreducing western blot with antibodies against Prx1, Prx2, and Prx3, demonstrating the redox status of Prxs in HUVECs subjected to 10 min treatments with $H_2O_2$ of varying doses. Cells treated in Medium 199 (M199) were harvested in extract buffer with (*left panel*) or without (*right panel*) *N*-ethylmaleimide (NEM) present during lysis. In the *left panel*, monomer (M) is the sum of any reduced and/or hyperoxidized monomeric enzyme, and dimer is disulfide-linked oxidized Prx. In the *right panel* (−NEM), monomer (M) represents hyperoxidized species only, while dimer (D) in this assay represents the sum of reduced and dimeric protein at the time of lysis. **(B)** Prx hyperoxidation (% monomer in the absence of NEM) quantified by densitometry of blots as in **A** (*right panel*). Data (means ± standard error of the mean [SEM] for at least three separate experiments) were analyzed by one-way analysis of variance (ANOVA) with Holm-Sidak multiple comparison ($p \leq 0.05$). The symbol * indicates $H_2O_2$ concentrations at which all three Prxs are significantly more hyperoxidized than untreated cells. There was also a significant difference in the extent of hyperoxidation between each of the Prxs at all concentrations $\geq 120\ \mu M$.

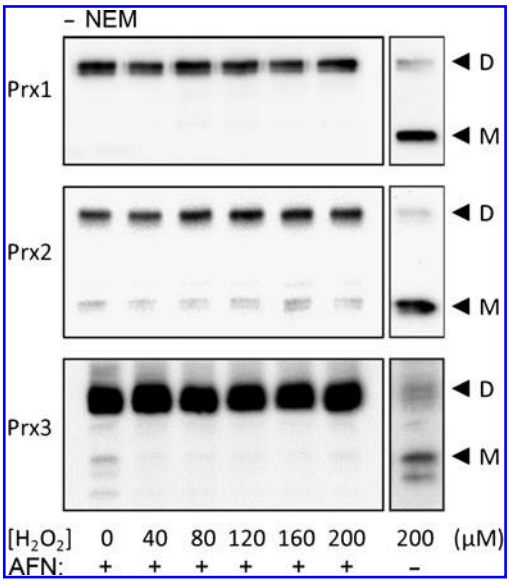


**FIG. 2. Lack of Prx hyperoxidation in auranofin (AFN)–treated HUVECs.** Nonreducing western blots of HUVECs treated with AFN (4 $\mu M$) then exposed to $H_2O_2$ in M199 and lysed in the absence of NEM. Control lanes are shown at *right*, from samples prepared and analyzed alongside the +AFN samples, but from separate gels. Absence of monomer (M) indicates a lack of hyperoxidized species. Blots are representative of three separate experiments.

### *HOCl- or chloramine-induced oxidation of Prxs*

Treatment of HUVECs with GlyCl, $NH_2Cl$, or HOCl gave a dose-dependent decrease in monomer for each Prx and concomitant dimer accumulation (Fig. 4 and Supplementary Fig. S4). No monomer was detected in the absence of NEM, indicating that no hyperoxidized Prx species were formed. This was also verified using an antibody against hyperoxidized Prx, which recognized Prxs from $H_2O_2$-treated but not HOCl- or chloramine-treated cells (Supplementary Fig. S5). Prx1 and Prx2 showed similar sensitivity to GlyCl, with almost complete dimerization after exposure to 500 $\mu M$ of the oxidant (Fig. 4A). No significant Prx3 dimerization was seen at any concentration. In contrast, all three Prxs were progressively oxidized with 5–25 $\mu M$ $NH_2Cl$ (Fig. 4B). Prx2 appeared to be slightly more sensitive, with significant oxidation at 10 $\mu M$ (Fig. 4B). Treatment of the cells with 20–100 $\mu M$ HOCl also caused progressive oxidation, with the three Prxs exhibiting similar susceptibility (Fig. 4C).

The extent of oxidant reaction with the HUVECs will depend on the cellular permeability of the oxidant and the length of exposure (35, 46). We found that although relatively high concentrations of GlyCl were used, <10% of the oxidant was consumed in 30 min (Table 1). In contrast, ~70% of the more permeable $NH_2Cl$ was consumed. As consumption was proportional to chloramine concentration, we can assume exponential decay. On this basis 2%–3% of GlyCl and about a third of $NH_2Cl$ would be consumed in 10 min, implying that complete oxidation of Prx2 required ~10 $\mu M$ GlyCl and 4 $\mu M$ $NH_2Cl$. By comparison, >50% of the HOCl was consumed within 10 min, with near-complete consumption by 30 min (Table 1).

### *Reduction of Prx dimers generated by HOCl and chloramines*

To determine whether chloramine-induced Prx oxidation was reversible, cells were treated with sufficient chloramine to

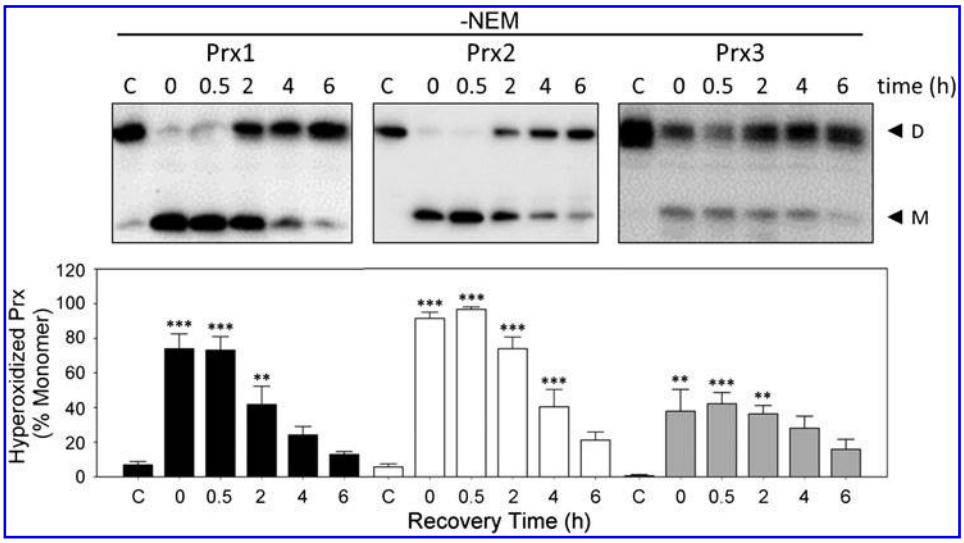


**FIG. 3. Regeneration of reduced Prxs following $H_2O_2$-induced hyperoxidation.** HUVECs (grown in six-well plates) were treated with 400 $\mu M$ $H_2O_2$ for 10 min in 2.5 ml M199 (this concentration gave similar levels of oxidation to 120–200 $\mu M$ in the smaller well format; Fig. 1); C indicates control (untreated) cells. Following treatment, fresh culture medium was applied and cells were incubated for the given times until harvesting in the absence of NEM. Treatment and recovery were performed in a humidified incubator at 37°C and 5% $CO_2$. Prx hyperoxidation was monitored as in Figure 1A (*right panel*). Bar graphs represent means ± SEM from three or four experiments. Significant differences compared with control cells are shown, as determined by one-way ANOVA with Holm-Sidak multiple comparison (**$p < 0.005$; ***$p < 0.001$).

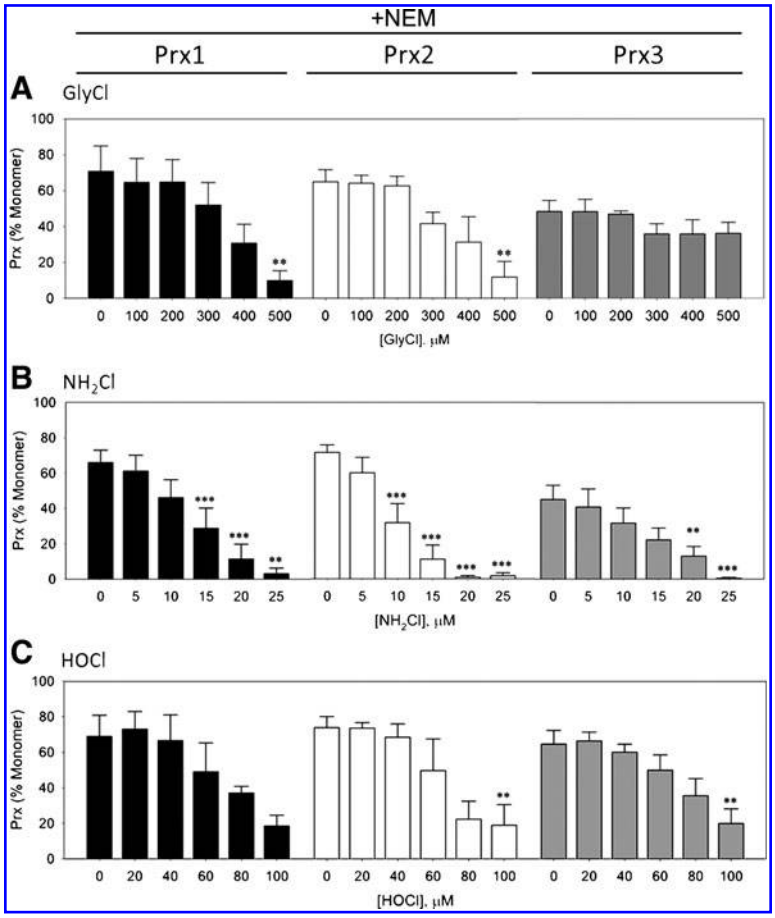


**FIG. 4. Chloramine-induced oxidation of Prxs.** HUVECs cultured in 24-well plates were treated with the given concentrations of **(A)** glycine chloramine (GlyCl), **(B)** monochloramine, or **(C)** hypochlorous acid (HOCl) in 1 ml Hanks' balanced salt solution (HBSS) for 10 min at 37°C. Cells were lysed in the presence of NEM; the relative concentration of monomer was quantified by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by western blotting with antibodies against Prx1 (*left panels*), Prx2 (*middle panels*), and Prx3 (*right panels*). The percentage of Prx present in the monomer position, indicating the reduced form of the enzyme, was determined by densitometry. Data are means ± SEM for at least three separate experiments. Statistically significant differences were determined by one-way ANOVA with Holm-Sidak multiple comparison (**$p < 0.005$; ***$p < 0.001$). Refer to Supplementary Figure S4 for representative blots.

dimerize most of the Prxs, then incubated in fresh medium (Fig. 5). This resulted in rapid reduction of all three Prxs within 10 min and complete recovery at 30 min. In comparison, cells treated with sufficient HOCl (100 $\mu M$) to induce high levels of Prx dimer did not recover monomer when incubated in medium; indeed, loss of protein was observed upon longer incubation, even following removal of HOCl and the application of fresh medium. This is evident in Supplementary Figure S6. Cell morphology appeared to deteriorate over time following high HOCl treatment, with loss of protein (presumably due to cell permeabilization/lysis) and evidence of toxicity (see Fig. 7).

As inhibition of TrxR activity can be sufficient to induce accumulation of Prx dimer in HUVECs, we investigated whether Prx dimer accumulation observed in cells treated with chloramines or HOCl could be an indirect effect of the inhibition of the reductase. There was no detectable change in TrxR activity with up to 500 $\mu M$ GlyCl or 25 $\mu M$ $NH_2Cl$ (data not shown). However, with HOCl, a dose-dependent linear decline in TrxR activity was observed with ~90% inhibition at 100 $\mu M$ (Supplementary Fig. S7). Therefore, loss of TrxR activity could contribute to HOCl-dependent Prx oxidation and lack of recycling.

### *Comparison of Prx oxidation with loss of reduced GSH*

GSH loss was monitored to determine whether the oxidation of HUVEC Prxs was selective or a reflection of broad-spectrum thiol oxidation. There was no significant decrease in the level of reduced GSH with up to 200 $\mu M$ $H_2O_2$ (Fig. 6A) and only 20% loss with 500 $\mu M$ or higher GlyCl (Fig. 6B). These are concentrations that gave almost complete oxidation of Prx1 and Prx2 (Fig. 4). At 25 $\mu M$ $NH_2Cl$, only 35% of the GSH was lost (Fig. 6C) whereas Prxs 1–3 were fully oxidized. In contrast, HOCl caused a decrease in reduced GSH over the same concentration range that caused Prx oxidation (Fig. 6D).

### *$H_2O_2$-, HOCl-, and chloramine-induced loss of viability*

To assess whether the oxidants caused loss of viability, uptake of propidium iodide (PI) was measured as an index of membrane integrity either immediately after exposure, or after 24 h incubation in fresh Medium 199 (M199). At the concentrations that caused Prx hyperoxidation, $H_2O_2$ caused no significant increase in PI uptake at either time (shown for 24 h in Fig. 7A). Neither GlyCl nor $NH_2Cl$ caused any immediate loss of viability (not shown), indicating that the observed Prx changes occurred while the cells were intact. However, at 24 h, there was a concentration-dependent increase in PI-positive cells (Fig. 7B, C). $NH_2Cl$ was the most cytotoxic, with loss of viability in up to 80% of cells. In

Table 1. Chloramine and Hypochlorous Acid Consumption by Human Umbilical Vein Endothelial Cells

| *Treatment* | | *30 min consumption (chloramine consumed, nmoles)* |
|---|---|---|
| *Concentration* | *nmoles* | |
| 200 $\mu M$ GlyCl | 200 | 15 ± 5 |
| 500 $\mu M$ GlyCl | 500 | 29 ± 16 |
| 10 $\mu M$ $NH_2Cl$ | 10 | 7 ± 2 |
| 20 $\mu M$ $NH_2Cl$ | 20 | 13 ± 1 |
| 40 $\mu M$ HOCl | 40 | 27 ± 1 |
| 80 $\mu M$ HOCl | 80 | 58 ± 2 |

Values were measured by reaction with TMB and represent loss of oxidant from 1 ml treatment of confluent 24-well plates of HUVECs ($n = 3$).

GlyCl, glycine chloramine; HOCl, hypochlorous acid; HUVECs, human umbilical vein endothelial cells; TMB, 3,3′,5,5′-tetramethylbenzidine.

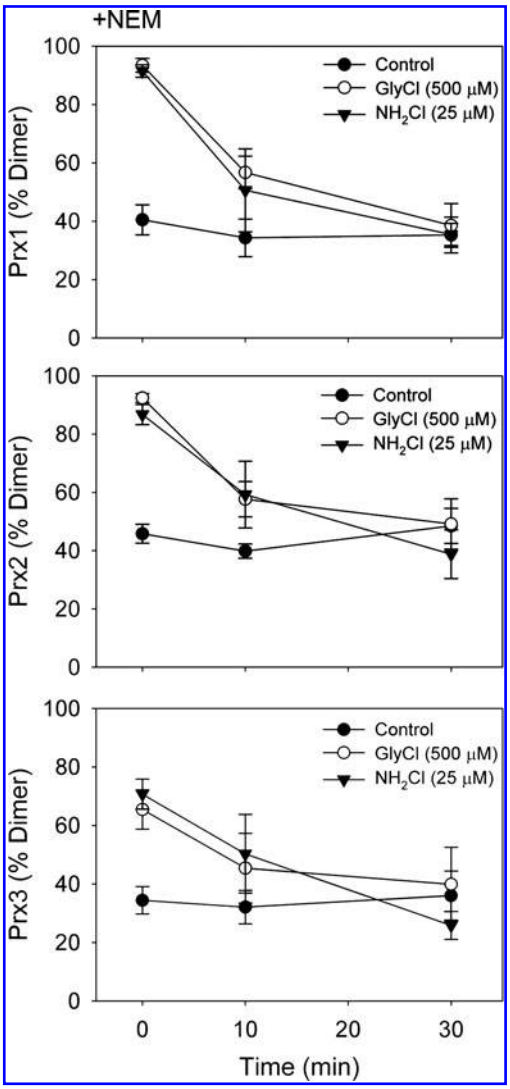


**FIG. 5. Regeneration of reduced Prxs following chloramine-induced oxidation.** HUVECs (grown in six-well plates) were treated for 10 min with oxidant in HBSS; after treatment, cells were washed with phosphate-buffered saline. Following 10 or 30 min recovery in M199, cells were lysed in the presence of NEM. Prx oxidation was quantified as in Figure 1A. Values are means ± SEM for four to eight separate experiments. Statistically significant differences were determined by one-way ANOVA with Holm-Sidak multiple comparison. Prx1 was significantly regenerated after only 10 min recovery regardless of treatment (GlyCl or $NH_2Cl$). Following GlyCl treatment, Prx2 was significantly regenerated after 10 min recovery. Prxs 2 and 3 underwent significant monomer regeneration by 30 min recovery when oxidation was induced by $NH_2Cl$.

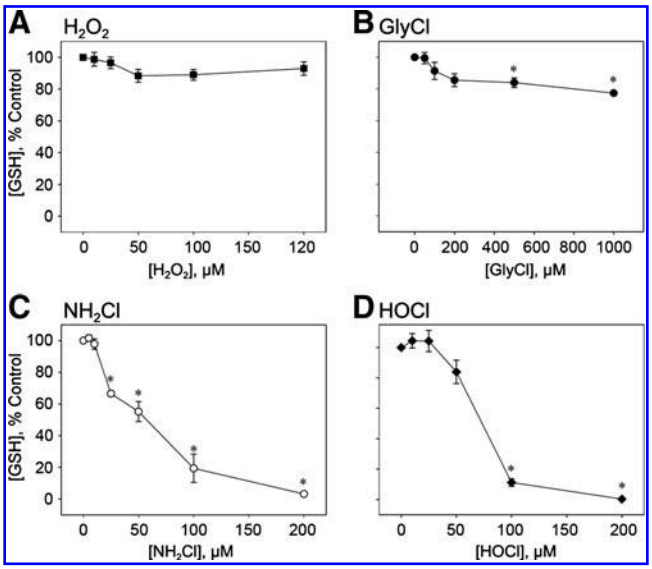


**FIG. 6. Concentration of reduced glutathione (GSH) in oxidant-treated cells.** GSH was measured by high-performance liquid chromatography following 10 min exposure of HUVECs (grown in 24-well plates) to the given concentrations of **(A)** $H_2O_2$; **(B)** GlyCl; **(C)** $NH_2Cl$; and **(D)** HOCl. Amounts were calculated as % control in each experiment. Values shown are means ± SEM for at least three separate experiments. Control cells contained 2–3 nmol reduced GSH per well ($\sim 10^5$ cells). Treatments that induced significant loss of GSH compared with untreated cells are indicated (*$p < 0.05$, by one-sample *t*-test [expected mean = 100%]).

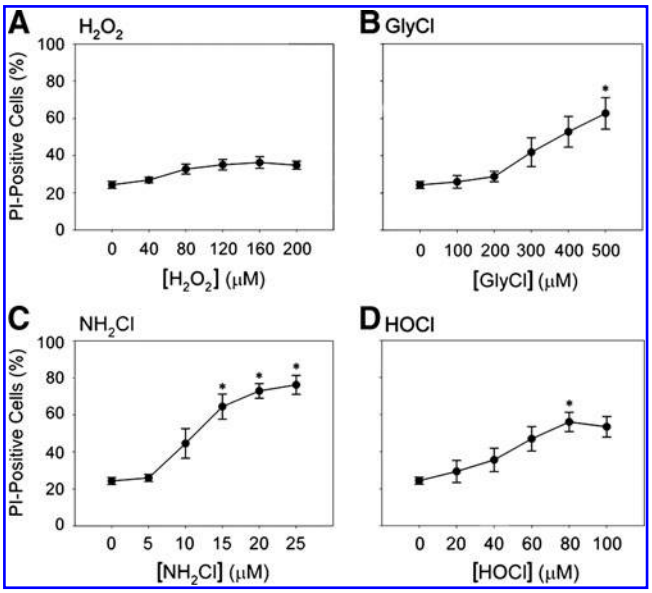


**FIG. 7. Dose-dependent loss of viability in oxidant-treated HUVECs.** Cells were interrogated by flow cytometry for fluorescence from propidium iodide (PI) 24 h after treatment with **(A)** $H_2O_2$; **(B)** GlyCl; **(C)** $NH_2Cl$; and **(D)**, HOCl. Data are means ± SEM for at least three separate experiments; treatments that induced significantly higher cell death compared with untreated cells are indicated (*$p < 0.05$, by one-way ANOVA with Holm-Sidak multiple comparison).

contrast, HOCl caused an immediate loss of viability that did not increase further over the next 24 h (Fig. 7D), suggesting a rapid loss of cell membrane integrity.

Linear regression analysis revealed a strong relationship between Prx oxidation and cell viability measured as PI uptake when all of the data for chloramines as well as HOCl were considered. This is shown in Figure 8 for Prx1; similar relationships were observed for Prx2 ($R^2 = 0.78$, $p < 0.001$) and Prx3 ($R^2 = 0.48$, $p = 0.001$; Supplementary Fig. S8).

We investigated whether the correlation between Prx oxidation and cell death might be explained by the Prxs protecting the cells against chloramine toxicity. This was investigated in two ways: HUVECs were pretreated with $H_2O_2$ prior to $NH_2Cl$ to hyperoxidize the Prxs and prevent their reactions with the chloramine, or they were treated with $NH_2Cl$ in the presence of AFN to inhibit TrxR and prevent Prx recycling. In these experiments, analysis of cell viability was measured by the MTT assay. HUVECs treated with either 15 $\mu M$ $NH_2Cl$ or $H_2O_2$ alone underwent no loss of viability (Fig. 9). However, pretreatment with $H_2O_2$ immediately before exposure to $NH_2Cl$ resulted in a dramatic 84% decrease in cell viability. When $NH_2Cl$ was added before $H_2O_2$, the decrease in viability was only 35%, indicating that it was not simply a synergistic effect and it could be related to Prx inactivation. Treatment with AFN and then $NH_2Cl$ also decreased HUVEC viability (Fig. 9) but there was only a modest difference between adding the AFN before (75% loss on average) or after the $NH_2Cl$ (65% decrease compared with control cells).

## Discussion

Consistent with previous reports (44) we have detected the 2-Cys Prxs—Prx1, Prx2, and Prx3—in endothelial cells. As isolated, each Prx was mostly reduced, with a fraction present as the disulfide-linked dimer. Inhibition of TrxR led to further dimer accumulation in unstressed cells. This implies that the Prxs are in a dynamic state of redox cycling involving the Trx system, and that they are continuously scavenging endogenously generated reactive oxidants. At inflammatory sites where neutrophils accumulate, endothelial cells will also be exposed to exogenous oxidants, particularly $O_2^-$, $H_2O_2$, and the products of MPO. $O_2^-$ has low membrane permeability

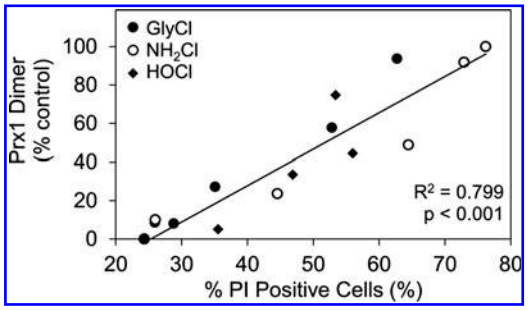


**FIG. 8. Regression analysis of Prx1 oxidation and cell death measured by uptake of PI.** The proportion of cells that were positive for PI 24 h after treating HUVECs for 10 min with chloramines or HOCl (data from Fig. 7) were plotted against the amount of Prx1 oxidation in cells given the same treatment (data from Fig. 4). Untreated control values were included in the analysis. $R^2$ values and significance were obtained by linear regression with ANOVA.

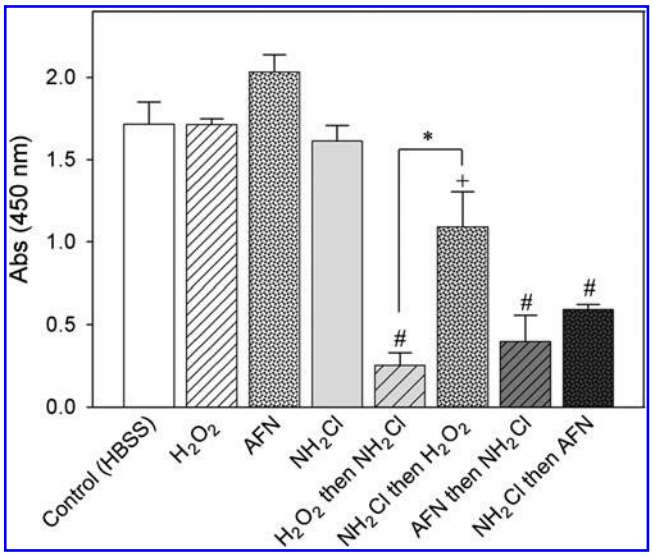


**FIG. 9. Thiazolyl blue tetrazolium bromide (MTT) measurement of HUVEC viability 24 h after treatment.** HUVECs were treated with $H_2O_2$ (400 $\mu M$, 20 min), AFN (4 $\mu M$, 20 min), or $NH_2Cl$ (15 $\mu M$, 10 min) either alone or in combination; where in combination, order of treatments is shown beneath the graph ($n = 3$; data are means ± SEM). Cells were returned to culture medium for 24 h, whereupon viability was measured using MTT assay as described under the "Methods" section. Assays were performed in 6-well plates where higher $NH_2Cl$ concentrations than with 24-well plates were required to see equivalent cytotoxicity. Significance was determined by one-way ANOVA with Holm-Sidak multiple comparison: #significantly different from control, $NH_2Cl$ alone, and $H_2O_2$ or AFN alone, $p < 0.001$; +significantly different from control and from $H_2O_2$, but not $NH_2Cl$ alone, $p < 0.005$; *paired treatments are significantly different from each other, $p < 0.001$.

and is unlikely to affect intracellular constituents (52). However, $H_2O_2$, HOCl, and some chloramines are able to enter cells (1, 7, 26, 45). The findings of the present study show that endothelial cell Prxs are sensitive targets for these neutrophil-derived oxidants. Neutrophils are present in the blood at $2–4 \times 10^6$ cells/ml, and accumulate to much higher numbers at inflammatory sites (50); in *in vitro* experiments, physiological cell concentrations of stimulated neutrophils are capable of generating HOCl that amount to 100 $\mu M$ producing in under an hour (33). Because of its reactivity, HOCl is not expected to accumulate but instead will react with biological targets; reaction of HOCl with amines, for example, generates chloramines. Because of the nature of their generation and reactivity, HOCl and chloramines would likely be in flux rather than at a steady-state concentration. It is therefore reasonable that the doses applied in this study would fall within the range encountered by cells *in vivo* under conditions of inflammation. In this study we used bolus addition rather than a flux. With either method of delivery, oxidation of the Prxs is likely to be similar. However, with a low flux, recycling may result in no accumulation of the oxidized forms. Therefore, while a flux may be more physiological, bolus addition is more useful for demonstrating what oxidation products are formed. It also allows short-term experiments to be carried out without requiring the cells to be in culture medium, thus avoiding complex interactions between the specific oxidants and medium constituents.

We found that each class of oxidant caused oxidation of endothelial cell Prxs 1–3. $H_2O_2$ treatment caused hyperoxidation to the inactive sulfinic (or sulfonic) acid, with a small amount of dimer evident only for Prx1 at the lowest $H_2O_2$ concentration. This result is consistent with the hyperoxidation that others have observed with HUVECs and other cell types (38, 39, 55), with the exception of erythrocytes in which Prx2 forms dimers (24). Although the dose response was similar for the three Prxs, the maximum level of hyperoxidation was less for Prx3. This may in part be due to less $H_2O_2$ reaching its mitochondrial location, but the greater resistance of purified Prx3 compared with Prx2 (11) suggests that there could also be a structural basis for this phenomenon.

Others have observed that Prx hyperoxidation occurs during redox cycling, with a small amount of sulfinic acid accumulating at each turn of the cycle (55). Hyperoxidation of the Prxs was observed in HUVECs exposed to $H_2O_2$, as inhibition of recycling by AFN prevented hyperoxidation and caused the Prx dimers to accumulate. The catalytically inactive hyperoxidized Prxs can be reduced to the monomeric form by sulfiredoxin, an enzyme with a slow catalytic rate (5, 8). Our results suggest that sulfiredoxin is present in HUVECs and is able to regenerate the reduced forms of the three Prxs over several hours. Although sulfiredoxin is normally cytosolic, it has been shown to translocate to mitochondria under situations of oxidative stress (28). This could explain why Prx3 reduction appeared slower than for Prxs 1 and 2.

Endothelial cell Prxs were readily oxidized by HOCl, $NH_2Cl$ and GlyCls. However, there was a striking difference between these oxidants and $H_2O_2$. They caused no hyperoxidation and instead converted each Prx to the reversibly oxidized dimer. With the chloramines, this was not due to impaired recycling by the Trx system, because there was no loss of TrxR activity in the treated cells. Furthermore, once the chloramines were removed from the cells, the Prx dimers were rapidly reduced. With HOCl, inhibition of TrxR limited reversal of Prx dimerization and could have contributed to the lack of hyperoxidation. However, the most likely explanation for why $H_2O_2$ but not chloramines caused hyperoxidation lies in the kinetics of Prx oxidation. With $H_2O_2$, the initial product is a sulfenic acid on the peroxidatic Cys, which condenses with the resolving Cys to form a dimer. This occurs in competition with oxidation by a second $H_2O_2$ to form the sulfinic acid (18). Chloramines would initially form a sulfenyl chloride (53) that could either condense directly with the resolving Cys or hydrolyze to the sulfenic acid. Our results would be explained if condensation was much faster for the sulfenyl chloride, and/or the second oxidation step by the chloramine was less favorable than for $H_2O_2$. Further study of the purified Prxs is needed to resolve the mechanism.

Apart from the lack of Prx3 oxidation with GlyCl, there was little difference in sensitivity between the three Prxs for each of the oxidants. Although a much lower concentration of $NH_2Cl$ than GlyCl was required to give equivalent Prx oxidation, GlyCl has a lower cell permeability (35, 46) and much less GlyCl than $NH_2Cl$ was consumed. Therefore, when related to chloramine loss, GlyCl was almost as effective. More HOCl than chloramine was required to see equivalent oxidation. This can be explained by HOCl being a stronger, less selective oxidant (37, 52). GlyCl and $NH_2Cl$ were selected as

model chloramines for this study. Both are likely to be formed physiologically, with one source of ammonia being activated neutrophils themselves (16). Other chloramines would be expected to have similar effects, provided they are able to enter cells. Taurine chloramine has been much studied, largely because neutrophils contain large amounts of taurine. We saw no effect of taurine chloramine on HUVEC Prxs (unpublished observations), which is in keeping with its very low membrane permeability (35, 46). However, in a physiological context, the nature of the initial chloramine may not be critical. HOCl or any chloramine added to physiological media undergoes chlorine exchange reactions with the range of amino compounds present (35). It is likely that cell-permeable chloramines so generated are responsible for many of the intracellular effects of taurine chloramine (16, 26).

Our findings suggest that Prxs could scavenge HOCl-derived oxidants in endothelial cells. How well they perform this function will depend on selectivity. They are unlikely to be effective against HOCl, which showed no preference for Prxs over GSH or TrxR. This is in keeping with the low selectivity of HOCl and exemplifies the problem of any antioxidant acting as an effective HOCl scavenger. However, the chloramines caused Prx oxidation under conditions where there was little loss of reduced GSH or TrxR activity. Other oxidant-sensitive proteins, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (35), I$\kappa$B (26) (at a methionine site), and cofilin (23), have also been shown to be more readily oxidized than GSH in chloramine-treated cells. It is difficult to compare between studies, but our preliminary proteomic studies (unpublished) suggest that GAPDH and the Prxs show similar chloramine sensitivity. The sensitivity of the Prxs is surprising because in isolation they are not highly reactive with chloramines (43). One explanation could be that they have relatively higher reactivity in a cellular environment. Alternatively, intracellular GSH could be oxidized but recycled more rapidly than the Prxs. Either way, it appears that Prxs are intracellular targets for chloramines and could potentially protect against their damaging effects. As many of the effects of HOCl are likely to be mediated by secondary chloramines (16), Prxs may also provide physiological protection against HOCl.

Prx oxidation was seen at sublethal $H_2O_2$ concentrations, whereas oxidation by HOCl was accompanied by some initial loss of viability. The chloramines were not immediately toxic, but they did cause concentration-dependent loss of viability after 24 h. We observed striking correlations between the extent of oxidation of each Prx by the chlorinated oxidants immediately after treatment and cell death at 24 h. No such relationship was seen with loss of reduced GSH. The relationship cannot simply be explained by loss of Prx activity leading to cell death, as oxidation by the chloramines was rapidly reversed and there was no cell death associated with the less reversible hyperoxidation by $H_2O_2$. A link between Prx redox status and viability is also suggested from the results of pretreating HUVECs with $H_2O_2$ prior to $NH_2Cl$, thereby hyperoxidizing the Prxs and preventing their reaction with chloramines. Although the combination of $H_2O_2$ and $NH_2Cl$ was more harmful than either alone, long-term loss of viability was much greater when $H_2O_2$ preceded $NH_2Cl$ treatment. AFN also enhanced the cytotoxicity of $NH_2Cl$ although this outcome did not depend on the order in which the treatments were applied. One possible explanation is that with hyperoxidation (or inhibition of recycling) preventing removal of the chloramines by the Prxs, the cells become more vulnerable to damage from other chloramine-mediated reactions. However, other mechanisms can be proposed and understanding the role of Prxs in cell death pathways will require further study involving modification of expression levels of these enzymes.

The endothelium is a vulnerable tissue in terms of oxidative injury due to inflammation. There is cross-talk between the endothelium and immune cells [*e.g.*, *via* cytokines and their receptors; see (41) for a review] and endothelial dysfunction is a known consequence of many inflammatory conditions. It can even contribute to the disease pathologies, as in patients with cardiovascular disease (6, 51). The results presented here support the notion that Prxs could act as sensors of oxidative stress in endothelia. It is easy to imagine that accumulation of Prxs in their dimer forms as a result of the cell's exposure to chloramines could act as part of a signaling cascade; this could be involved in controlling any number of processes from actin contraction/loss of adhesion or increased endothelial permeability, to transcription factor activation and heightened expression of antioxidant proteins.

Our findings suggest that in endothelial cells, Prxs could be targeted and possibly inactivated by $H_2O_2$ produced by neutrophils during inflammation. However, as much of this $H_2O_2$ is converted by MPO to HOCl and chloramines (12), the effects of the latter on the endothelium could be more relevant. Sublethal doses have been shown to deplete ATP (42); activate MAP kinase signaling pathways (27); prevent nuclear factor kappa B activation (26, 29); suppress cytokine, adhesion molecule, and inducible nitric oxide synthase expression (4, 25, 32); increase cell permeability (45); and initiate apoptosis (23, 30, 49). We have now shown that they can also reversibly oxidize Prxs and activate the Prx/Trx catalytic cycle. This suggests that Prxs could also act as antioxidants for removal of chloramines and thus modulate these other effects. Further study is required to establish whether this is the case.

## Materials and Methods

### *Materials*

Cell culture media and supplies were from Invitrogen. Complete™ protease inhibitors, tris(hydroxymethyl)aminomethane (Tris) and CHAPS, were from Roche. AFN was from ICN Biomedicals, Inc. Monobromobimane (MBB) was purchased from Calbiochem. Rabbit polyclonal antibodies were sourced as follows: anti-Prx1 antibodies were from AbCam, anti-Prx2 antibodies were from Sigma-Aldrich, and anti-Prx3 antibodies were from AbFrontier. Rabbit anti-Prx-$SO_{2/3}$ was from AbFrontier, and goat anti-rabbit IgG conjugated to horseradish peroxidase was from Sigma. Polyvinylidene difluoride (PVDF) membrane and enhanced chemiluminescence reagents were purchased from GE Healthcare. HyClone New Zealand Cosmic Calf Serum was from Global Science & Technology Ltd. All other reagents were from Sigma-Aldrich, BDH Laboratory Supplies, and Biolab.

### *Cell culture*

HUVECs were harvested from umbilical cords obtained with informed consent and the study was approved by Upper South A Regional Ethics Committee (ethics reference CTY/

02/12/2009). Cells were isolated by collagenase digestion (21) and grown in M199 supplemented with 15% calf serum, 100 $\mu$g/ml heparin, 30 $\mu$g/ml endothelial cell growth factor, 25 U/ml penicillin, and 25 $\mu$g/ml streptomycin. HUVECs were grown to confluence ($\sim 6\times10^4$ cells/cm$^2$) at 37°C with 5% $CO_2$, in flasks and plates pre-coated with 0.1% (w/v) gelatine. Cells were grown in 24-well or 6-well plates and used by the 5th passage.

### *Exposure of HUVECs to $H_2O_2$, HOCl, and chloramines*

Reagent HOCl was diluted in phosphate-buffered saline (PBS; 140 m*M* NaCl, 13 m*M* KCl in 10 m*M* sodium phosphate buffer, pH 7.4); this solution was standardized spectrophotometrically by dilution in NaOH, final pH > 10 ($\varepsilon_{292} = 350\ M^{-1}\cdot cm^{-1}$). GlyCl and $NH_2Cl$ were prepared in Hanks' balanced salt solution (HBSS; PBS containing 0.5 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, and 5.5 m*M* glucose) by mixing a 10:1 molar excess of glycine or ammonia, respectively, with HOCl (added dropwise with vortexing). This ratio ensured that no dichloramine was produced (46). Chloramine concentration was determined by reaction with 5-thio-2-nitrobenzoic acid, monitored at 412 nm (TNB; $\varepsilon = 14{,}100\ M^{-1}\cdot cm^{-1}$) (46). The concentration of $H_2O_2$ solutions was determined by measuring $A_{240}$ ($\varepsilon = 43.6\ M^{-1}\cdot cm^{-1}$).

Prior to addition of oxidants, HUVEC cultures were washed extensively with PBS to remove any medium containing potential scavengers. Solutions of $H_2O_2$, HOCl, or chloramines were added to each well in 1 ml HBSS and incubated for 10 min at 37°C. Any remaining oxidant was quenched by adding methionine (final concentration of 5 m*M*) and the cells were washed with PBS and either harvested or fresh M199 was added for regeneration studies. Control cells were similarly handled except that they were treated with HBSS alone.

### *Measuring chloramine and $H_2O_2$ consumption*

Consumption of chlorinated oxidants by HUVECs was assessed using a spectrophotometric assay that measures the iodide-catalyzed oxidation of 3,3′,5,5′-tetramethylbenzidine (13). Loss of $H_2O_2$ from the medium was measured using ferrous sulfate/xylenol orange (54).

### *SDS-PAGE and redox western blotting for Prxs*

To monitor the transition from monomeric to dimeric Prx, cell extracts were analyzed by western blotting under nonreducing conditions. The cells were lysed in the presence of 10 $\mu$g/ml catalase to scavenge any small amounts of peroxide in the extract buffer (and prevent any subsequent dimer formation), and 100 m*M* NEM to alkylate reduced thiols (and prevent disulfide bond formation during extraction). Extraction buffer consisted of 40 m*M* HEPES buffer (pH 7.4) with 50 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid, and 1 m*M* EGTA, supplemented by 1% Triton X-100 and Complete™ protease inhibitors.

To monitor Prx hyperoxidation (Prx-$SO_{2/3}$H), cells were lysed in extract buffer lacking both NEM and catalase; under these conditions the Prxs become oxidized to dimers by trace amounts of peroxide present in the buffer, enabling the amount of hyperoxidized protein to be quantified as the sole species running in the monomer position on nonreducing SDS-PAGE (11).

The protein content of cell extracts was assessed using a detergent-compatible protein assay (Bio-Rad). For electrophoresis, samples were denatured by boiling for 5 min in SDS-PAGE sample buffer (final concentrations 2% SDS, 10% glycerol, and 62.5 m*M* Tris, pH 6.8), then equal amounts of protein (generally between 15 and 25 $\mu$g) were loaded onto each lane of a nonreducing, 12% (unless otherwise indicated) acrylamide gel. In some cases protein was precipitated before resolubilization in sample buffer. Protein bands were transferred to PVDF membrane, probed with antibodies against Prx1, Prx2, Prx3, or Prx-$SO_{2/3}$H, and detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence. Bands were visualized using the ChemiDoc XRS gel documentation system and quantified using Quantity One analysis software (Bio-Rad).

### *TrxR activity assay*

HUVECs were grown to confluence in 24-well plates, treated, and then lysed in extraction buffer. TrxR activity was measured as NADPH-dependent conversion of 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB) to TNB by monitoring the absorbance change at 412 nm ($\varepsilon = 14{,}150\ M^{-1}\cdot cm^{-1}$) in a 96-well plate (2). Activity was measured in extracts containing 20 $\mu$g total protein.

### *Glutathione assay*

Intracellular GSH in HUVECs grown in 24-well plates was derivatized by addition of 1 m*M* MBB. Proteins were precipitated with trichloroacetic acid and GSH was quantified in the supernatant by high-performance liquid chromatography with fluorescence detection (10).

### *Cytotoxicity assays*

Plasma membrane integrity was monitored using PI. After 10 min exposure to oxidant, methionine was added to quench any remaining oxidant, and then the cells were washed and incubated in M199 for 24 h. Adherent cells were disrupted by trypsinization, pooled with any nonadhering cells, and resuspended in M199 containing 2 $\mu$g/ml PI. Cell fluorescence was measured using an FC500 MPL Flow Cytometry system (Beckman Coulter, Inc.).

Cell viability was also monitored through the ability of HUVEC mitochondrial reductases to convert thiazolyl blue tetrazolium bromide (MTT) to purple formazan. Cells were treated with oxidants in six-well culture dishes and incubated for 24 h in fresh M199. Then MTT solution (5 mg/ml in PBS; 150 $\mu$l) was added to each well. After 3 h incubation, 1 ml of 0.04 M HCl in isopropanol was added, cells were incubated in the dark with gentle agitation for 10 min, insoluble cell material was pelleted by centrifugation (10,000 *g*), and the absorbance was measured at 570 nm with background subtraction at 650 nm.

### *Statistics*

Statistical analyses were performed with the software package SigmaStat 11.0 (Systat).

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### Author Disclosure Statement

No competing financial interests exist.

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**Abbreviations Used**

AFN = auranofin
ANOVA = analysis of variance
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
GlyCl = glycine chloramine
GSH = reduced glutathione
HBSS = Hanks' balanced salt solution
HUVEC = human umbilical vein endothelial cell
IκB = NFκB inhibitor
M199 = Medium 199
MAP = mitogen-activated protein
MBB = monobromobimane
MPO = myeloperoxidase
MTT = thiazolyl blue tetrazolium bromide
NEM = *N*-ethylmaleimide
PAGE = polyacrylamide gel electrophoresis
PBS = phosphate-buffered saline
PI = propidium iodide
Prxs = peroxiredoxins
PVDF = polyvinylidene difluoride
SDS = sodium dodecyl sulfate
SEM = standard error of the mean
TMB = 3,3′,5,5′-tetramethylbenzidine
TNB = 5′-thio-2-nitrobenzoic acid
Trx = thioredoxin
TrxR = thioredoxin reductase