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Original Research Communication

Induction of Sulfiredoxin *via* an Nrf2-Dependent Pathway and Hyperoxidation of Peroxiredoxin III in the Lungs of Mice Exposed to Hyperoxia

Soo Han Bae, Hyun Ae Woo, Su Haeng Sung, Hye Eun Lee, Se Kyoung Lee, In Sup Kil, and Sue Goo Rhee

Abstract

The cysteine residue at the active site of peroxiredoxin (Prx) I, Prx II, or Prx III is reversibly hyperoxidized to cysteine sulfinic acid, with concomitant loss of peroxidase activity, during normal catalysis. Sulfiredoxin (Srx) is the enzyme responsible for reversing this hyperoxidation. We now show that the expression of Srx at both the mRNA and protein levels is increased markedly in the lungs of mice exposed to hyperoxia. This hyperoxia-induced expression of Srx was not evident in mice deficient in the transcription factor Nrf2, indicating an essential role for an Nrf2 signaling pathway in this effect. Hyperoxia also elicited the accumulation of the sulfinic form of the mitochondrial enzyme Prx III, but not that of the cytosolic enzymes Prx I or Prx II, in lung tissue. This selective hyperoxidation of Prx III is likely due either to mitochondria being the major site of the hyperoxia-induced production of reactive oxygen species or to the translocation of Srx from the cytosol into mitochondria being rate limiting for the reduction of sulfinic Prx III. Hyperoxia induced the degradation of Prx III in Nrf2-deficient mice but not in wild-type animals, suggesting that, in the absence of a sufficient amount of Srx, sulfinic Prx III is converted to a form that is susceptible to proteolysis. *Antioxid. Redox Signal.* 11, 937–948.

Introduction

Peroxiredoxins (Prxs) protect cells from oxidative stress by removing hydroperoxides produced as a result of normal cellular metabolism. Mammalian cells express six isoforms of Prx (Prx I to Prx VI), which can be classified into three subgroups (2-Cys, atypical 2-Cys, and 1-Cys) on the basis of the number and position of cysteine residues that participate in catalysis. Prx I to Prx IV, which belong to the 2-Cys subgroup, exist as homodimers and possess two conserved cysteine residues (6, 45). Prx I and Prx II are cytosolic proteins, whereas Prx III is localized exclusively to mitochondria, and Prx IV is found in the endoplasmic reticulum and peroxisomes. Prx V and Prx VI, which belong to the atypical 2-Cys and 1-Cys subgroups, respectively, contain only one conserved cysteine residue.

In the catalytic cycle of 2-Cys Prx enzymes, the NH_2-terminal conserved Cys–SH (designated the peroxidatic cysteine, or C_P) is first converted to cysteine sulfenic acid (Cys–SOH) by a peroxide. The unstable sulfenic intermediate then reacts with the COOH-terminal conserved Cys–SH (the resolving cysteine, or C_R) of the other subunit in the homodimer to form a disulfide, which is subsequently reduced by a thiol-containing reductant, such as thioredoxin, to complete the catalytic cycle (6). As a result of the slow rate of its conversion to a disulfide, the sulfenic intermediate is occasionally oxidized further to cysteine sulfinic acid (Cys–SO_2H), leading to inactivation of peroxidase activity (47). Examination of the fate of inactivated sulfinic 2-Cys Prxs led to the unexpected finding that sulfinic acid formation is a reversible process (42). The enzyme responsible for the reduction of the hyperoxidized Prxs was identified in yeast (3) and mammals (42) and was named sulfiredoxin (Srx). Reduction by Srx is specific to 2-Cys Prxs; the sulfinic forms of Prx V and Prx VI are thus not reduced by Srx (9, 23, 43). In addition to its sulfinic reductase function, Srx has been shown to catalyze reversal of the glutathionylation reaction in human lung carcinoma cells (16, 27). An increase in the level of oxidative stress often

Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul, Korea.

induces the expression of antioxidant enzymes. For example, exposure of animals to hyperoxia, which is studied as a model of oxidant-induced lung disease, increases the expression of manganese-dependent superoxide dismutase (Mn-SOD), NAD(P)H:quinine oxidoreductase–1 (NQO-1), heme oxygenase–1 (HO-1), and Prx VI (10, 12, 20, 25, 37). Transcriptional activation of many genes encoding such antioxidant enzymes is mediated by a promoter element termed the antioxidant response element (ARE). The principal ARE-binding protein is nuclear factor erythroid 2–related factor 2 (Nrf2), which is required for the expression of many antioxidant and detoxifying enzymes (7, 11, 22, 24). In unstressed cells, Kelch-like ECH-associating protein 1 (Keap1), a cytoplasmic cysteine-rich protein, sequesters Nrf2 in the cytoplasm. In cells exposed to oxidative stress, such as that induced by hyperoxia, Nrf2 accumulates in the nucleus and activates ARE-containing genes. Hepatic gene-expression profiles in mice fed with the Nrf2-inducer 3H-1,2-dithiole-3-thione indicated that neoplastic progression 3 (the name given to Srx before identification of its sulfinic reductase activity) was highly inducible through the Keap1-Nrf2 pathway (26). The Keap1-Nrf2 pathway was also suggested as the mechanism responsible for the nitric oxide–dependent induction of Srx in macrophages (15). Conversely, a large-scale effort to identify genes that respond to glucose and cAMP in pancreatic β cells revealed the Srx gene to be potentially regulated by AP-1 (19). In addition, upregulation of Srx expression in rat neurons by synaptic activity and in mouse epidermis by tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate was shown to be dependent on AP-1 (32, 41). Conversely, induction of Srx in cortical neurons by chemopreventive inducer 3H-1,2-dithiole-3-thione was shown to be mediated by Nrf2 (38). It is worth noting that the core palindromic sequence [TGA(C/G)TCA] targeted by activator protein–1 (AP-1) is often embedded within most AREs (28, 46).

Exposure of laboratory animals to hyperoxia (>90% O_2) leads to their death within 3 to 7 days, primarily as a result of progressive damage to the lungs (13, 30, 31). This damage is thought to be attributable to an increase in the intracellular production of reactive oxygen species (ROS) (1, 4, 5, 17). We now show that exposure of mice to hyperoxia increased the levels of Srx mRNA and protein in lung tissue and that this upregulation of Srx expression was abolished in mice lacking Nrf2. Hyperoxia also induced hyperoxidation of mitochondrial Prx III but not that of cytosolic Prx I or Prx II. Exposure of Nrf2-deficient mice to hyperoxia resulted in downregulation of the amount of Prx III protein in the lungs without a corresponding change in the amount of Prx III mRNA. These data indicate that the failure of Srx induction in response to hyperoxia and the consequent intracellular accumulation of ROS in $Nrf2^{-/-}$ mice result in the conversion of Prx III to a form that is susceptible to proteolysis.

Materials and Methods

Materials

An antibody specific for Srx was described previously (9). Antibodies specific for Prx isoforms and for the sulfinic forms of 2-Cys Prxs were purchased from Young In Frontier (Seoul, Korea). Antibodies to β-actin and a phosphatase-inhibitor cocktail were obtained from Sigma Aldrich (St. Louis, MO). Aprotinin, leupeptin, and 4-(2-aminoethyl)-benzenesulfonyl fluoride were obtained from ICN Biomedicals (Costa Mesa, CA).

Animal models

Male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained on a 12-h light, 12-h dark cycle. Breeding pairs of Nrf2-knockout ($Nrf2^{-/-}$) mice were obtained from RIKEN BioResource Center (Tsukuba, Japan), and $Nrf2^{+/+}$ littermates were used as wild-type controls. Mice (8–10 weeks of age) were exposed to isobaric hyperoxia for 24, 48, or 72 h in a chamber that was saturated with 95% O_2 and 5% N_2 at a sufficient flow rate. Control animals were exposed to room air. All mice were allowed free access to food and water. Mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body weight), and lung tissue was perfused with phosphate-buffered saline, and excised, and then stored at $-70^{\circ}C$ until analysis. All animal experiments were approved by the Animal Care and Use Committee of Ewha Womans University.

Reverse transcription–polymerase chain reaction (RT-PCR) and quantitative real-time PCR analysis

Total RNA was prepared from lung tissue with the use of the TRIZOL reagent (Invitrogen, Carlsbad, CA) and was treated with DNA-free DNase I (Ambion, Austin, TX). Portions of the RNA (2 μg) were then subjected to RT with random-hexamer primers and with the use of an ABI cDNA synthesis kit (PE Biosystems, Foster City, CA). The resulting cDNA (1 μg) was subjected to PCR with *Taq* DNA polymerase and primer pairs (forward and reverse, respectively) specific for mouse Nrf2 (5′-TCTCCTCGCTGGAAAAAGAA-3′ and 5′-AATGTGCTGGCTGTGCTTTA-3′) and for mouse β-actin (5′-AGAGCATAGCCCTCGTAGAT-3′ and 5′-CCCAGAGCA AGAGAGGTATC-3′). Quantitative real-time PCR (qPCR) analysis was performed with the use of SYBR Green (Perkin Elmer, Boston, MA) and an ABI PRISM 7700 system (PE Biosystems). Data were normalized by the amount of 18S rRNA. The primer sequences for qPCR analysis of Srx and Prx I to Prx VI were described previously (15). Those (forward and reverse, respectively) for tumor necrosis factor–α (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein–1 (MCP-1) were 5′-GCCACCACGCTCTTCTG-3′ and 5′-GGTG TGGGTGAGGAGCA-3′, 5′-ACAACCACGGCCTTCCCTAC TT-3′, 5′-CACGATTTCCCAGAGAACATGTG-3′, and 5′-CC ACTCACCTGCTGCTACTCAT-3′ and 5′-TGGTGATCCTCT TGTAGCTCTCC-3′, respectively (18).

Immunoblot analysis

Lung tissue was homogenized in lysis buffer [50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride, aprotinin (10 μg/ml), leupeptin (10 μg/ml)] containing 1% Nonidet P-40, and the homogenates were centrifuged to remove cell debris. The resulting supernatants were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE), and the separated proteins were transferred electrophoretically to a polyvinylidene difluoride membrane. The membrane was incubated with primary antibodies, and immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence reagents (Young In Frontier).

Protein carbonylation assay

Carbonylation of total lung proteins (10 μg) was examined by analysis of tissue homogenates prepared as described earlier with an Oxyblot Protein Oxidation Detection Kit or OxyELISA Oxidized Protein Quantitation Kit (Chemicon, Temecula, CA).

Statistical analysis

Data are expressed as mean $\pm$ SEM. In the case of comparison between two different samples, a two-tailed Student's t test was used. The significant difference was defined by $p < 0.05$, except where indicated.

Results

Hyperoxia increases the levels of Srx protein and mRNA in lung tissue

Exposure of animals to isobaric hyperoxia has been studied as a noninvasive model of oxidative stress and of oxidant injury to lung tissue. To examine the effects of hyperoxia on the expression of Srx and Prxs in lung tissue, we exposed mice to hyperoxia (1 atm of 95% O_2 and 5% N_2) or room air for 24, 48, or 72 h. The amounts of Srx and Prx I relative to Prx VI in lung tissue were then measured with immunoblot analysis with antibodies specific for each protein. The amount of Srx increased gradually with time of exposure to hyperoxia, whereas the amounts of Prx I to Prx VI did not change significantly (Fig. 1A). The levels of Srx and Prx mRNAs were also measured by qPCR analysis (Fig. 1B). The abundance of Srx mRNA increased gradually with time of exposure to hyperoxia, with that apparent after 3 days of hyperoxia being $\sim$6 times that apparent in room air. The amounts of Prx I, Prx III, and Prx VI mRNAs also showed $\sim$1.5-fold to twofold increases after 3 days of hyperoxia, whereas those of Prx II, Prx IV, and Prx V mRNAs were unaffected by hyperoxia. Exposure of rats to hyperoxia was previously shown to induce 1.5- and 1.7-fold increases in the amounts of Prx VI protein and mRNA, respectively, in lung tissue (25). Oxidative damage in the lungs of mice exposed to hyperoxia was evident from an increased level of protein carbonylation compared with that apparent in the lungs of animals maintained in room air (Fig. 1C). Hyperoxic damage also is characterized by inflammation, and the lungs of mice exposed to hyperoxia have been found to manifest increased amounts of mRNAs for chemokines and other cytokines (2, 14). We also found that the levels of TNF-α, IL-6, and MCP-1 mRNAs in lung tissue were all markedly increased after exposure of mice to hyperoxia for 3 days (Fig. 1D).

Induction of Srx expression by hyperoxia is reversible

We next determined the levels of Srx protein and mRNA in lung tissue after exposure of mice to hyperoxia for 3 days and subsequent exposure to room air for 2 or 4 days. The amount of Srx protein was increased after exposure to hyperoxia for 3 days and then returned gradually to basal levels during subsequent exposure to room air for 4 days (Fig. 2A). The hyperoxia-induced increase in the abundance of Srx mRNA was almost fully reversed after exposure to room air for only 2 days (Fig. 2B). These data thus indicated that the induction of Srx expression by hyperoxia is reversible.

We also investigated whether Prx enzymes accumulate in the inactivated sulfinic form in the lungs of mice exposed to hyperoxia (Fig. 2A). Sulfinic 2-Cys Prxs can be detected with immunoblot analysis with rabbit polyclonal antibodies that recognize a specific sequence surrounding the cysteine sulfinic acid (C_P–SO_2H) at the active site (44). Although the antibodies were actually generated in response to a peptide containing cysteine sulfonic acid (Cys–SO_3H), they recognize both sulfinic and sulfonic forms of 2-Cys Prxs (44). Given that the amino acid sequence (DFTFVCPTEI) at the active site is the same for 2-Cys Prxs (Prx I to Prx IV) and because the sizes of Prx I and Prx II are almost identical, the sulfinic form of Prx I cannot be differentiated from that of Prx II with immunoblot analysis. Sulfinic Prx III, however, can be distinguished because its apparent size is larger than the sizes of Prx I and Prx II. Exposure of mice to hyperoxia for 3 days caused accumulation of sulfinic Prx III but not Prx I/II (Fig. 2A). The band intensity of sulfinic Prx III return to basal level after 2 days of normoxia (Fig. 2A), suggesting that the generation of sulfinic Prx III is reversible. Protein carbonylation was measured with immunoblot analysis as well as with a sensitive ELISA method (Fig. 2C and E). The hyperoxia caused an $\sim$2.5-fold increase in the extent of protein carbonylation, and the increase was fully reversed after exposure of animals to room air for 4 days (Fig. 2E). The increased abundance of mRNAs for TNF-α, IL-6, and MCP-1 also was reversible (Fig. 2D).

The hyperoxia-induced upregulation of Srx expression is mediated by Nrf2

Given the microarray data suggesting that Srx expression can be induced *via* the Nrf2-ARE pathway in mouse liver (26), we examined whether the same pathway contributes to the hyperoxia-induced expression of Srx with the use of mice lacking Nrf2. The genotype of $Nrf2^{-/-}$ mice was confirmed with RT-PCR analysis, which also showed that exposure of $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice to hyperoxia had no effect on the abundance of Nrf2 mRNA (Fig. 3B). Exposure to hyperoxia failed to induce Srx expression at both the protein and mRNA levels in the lungs of $Nrf2^{-/-}$ mice (Fig. 3A and C). The level of lung protein carbonylation in $Nrf2^{-/-}$ mice exposed either to room air or to hyperoxia was higher than that in their similarly treated $Nrf2^{+/+}$ littermates (Fig. 3D and E). Similarly, the amount of TNF-α mRNA, but not the amounts of IL-6 or MCP-1 mRNAs, was greater in the lungs of $Nrf2^{-/-}$ mice than in wild-type animals maintained in room air, whereas the abundance of all three mRNAs was greater in $Nrf2^{-/-}$ mice than in $Nrf2^{+/+}$ mice after exposure to hyperoxia (Fig. 3F).

Effects of hyperoxia on Prxs in the lungs of $Nrf2^{-/-}$ mice

We exposed $Nrf2^{+/+}$ and $Nrf2^{-/-}$ littermates to room air or hyperoxia for 3 days and then subjected lung proteins to immunoblot analysis with antibodies to the sulfinic forms of 2-Cys Prxs (Fig. 4A). Lysates of NIH 3T3 cells that had been exposed to 100 μM H_2O_2 for 10 min were included as a standard for sulfinic Prx I/II and Prx III. An immunoreactive band that migrated at a position corresponding to that of sulfinic Prx III was detected in lung homogenates of wild-type mice exposed to hyperoxia but not of those maintained in room air (Fig. 4A). No band at a position corresponding to that of

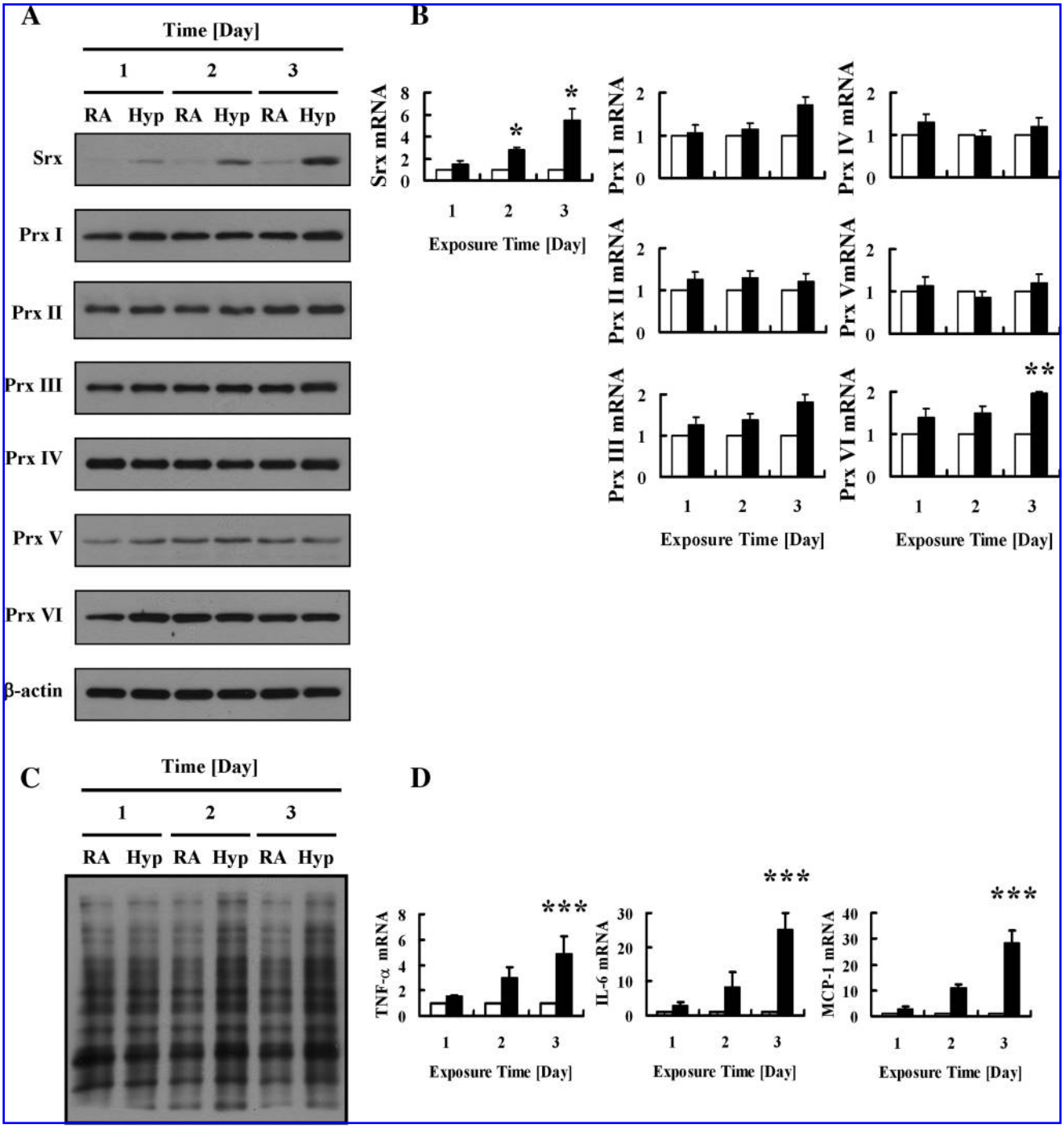


FIG. 1. Induction of Srx expression in the lungs of mice exposed to hyperoxia. (**A**) Homogenates (20 μg of protein) of lung tissue from mice exposed to room air (RA) or hyperoxia (Hyp) for 24, 48, or 72 h were subjected to immunoblot analysis with antibodies specific for Srx, for Prx I to Prx VI, or for β-actin (loading control). Data are representative of three independent experiments. (**B**) Total RNA prepared from lung tissue of mice treated as in (**A**) was subjected to qPCR analysis for determination of the amounts of Srx and Prx mRNAs. *White and black bars* correspond to the animals exposed to room air and hyperoxia, respectively. Data are expressed as mean ± SEM for three mice of each group and are expressed relative to the corresponding value for the RA group at each exposure time. $^{*}p < 0.003$ *vs.* RA for indicated time. $^{**}p < 0.05$ *vs.* RA for indicated time. (**C**) Homogenates (10 μg of protein) of lung tissue from mice treated as in (**A**) were subjected to immunoblot analysis of protein carbonylation. Data are representative of three independent experiments. (**D**) Total RNA prepared from lung tissue of mice treated as in (**A**) was subjected to qPCR analysis for determination of the amounts of TNF-α, IL-6, and MCP-1 mRNAs. *White and black bars* correspond to the animals exposed to room air and hyperoxia, respectively. Data are expressed as mean ± SEM for three mice of each group and are expressed relative to the corresponding value for the RA group at 1 day of exposure. $^{***}p < 0.05$ *vs.* RA for indicated time.

sulfinic Prx I/II was detected in $Nrf2^{+/+}$ mice exposed to room air or hyperoxia (Fig. 4A). To confirm the identification of sulfinic Prx III, we subjected lung proteins from wild-type mice exposed to room air or hyperoxia to two-dimensional (2D) PAGE followed by immunoblot analysis (Fig. 4B). Sulfinylation of proteins induces an acidic shift (shift to the left) in their position on 2D gels. Immunoblot analysis of the 2D gels with antibodies to Prx III revealed four spots including a faint one in the left-most position for mice exposed to room air or hyperoxia. Only the middle two spots for the tissue of mice exposed to hyperoxia were recognized by the antibodies to sulfinic 2-Cys Prxs. None of the four spots for the tissue of mice maintained in room air were recognized by these antibodies. These results suggested that exposure to hyperoxia resulted in the formation of sulfinic Prx III in $Nrf2^{+/+}$ mice. They also suggested that sulfinylation (hyperoxidation) was not the only cause of the acidic shift of Prx III in 2D gels. Prx I and Prx II have been shown to undergo acetylation and phosphorylation (8, 34). In Fig. 4A, the position of a band slightly above that for sulfinylated Prx III in the samples from wild-type mice exposed to hyperoxia is similar to that expected for Prx IV. However, the sulfinic form of Prx IV has not been previously detected, and our effort to identify the protein in this band as sulfinic Prx IV was not successful.

Failure of hyperoxia to induce Srx expression in the lungs of $Nrf2^{-/-}$ mice would be expected to accelerate the accumulation of sulfinic Prx III. However, the amount of sulfinic Prx III in $Nrf2^{-/-}$ mice exposed to hyperoxia was about half of that in similarly treated $Nrf2^{+/+}$ littermates (Fig. 4A and C). Immunoblot analysis also revealed that, whereas hyperoxia induced a small increase in the total amount of Prx III in lung tissue of wild-type mice, it resulted in a marked decrease in that in $Nrf2^{-/-}$ mice. The total abundance of Prx III in $Nrf2^{-/-}$ mice exposed to hyperoxia was thus about one-fourth that in $Nrf2^{+/+}$ controls (Fig. 4A and C). In contrast, the amount of Prx III mRNA was increased by hyperoxia in both $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice, and it did not differ between mice of the two genotypes under either condition (Fig. 4D). Neither hyperoxia nor the absence of Nrf2 had an effect on the amount of Prx II protein or mRNA (Fig. 4A, C, and D). These results suggested that sulfinic Prx III molecules might be degraded when they cannot be reactivated, as a result of an insufficient amount of Srx. However, when exposed to hyperoxia for 2 days, the amounts of sulfinic Prx III in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ littermates were similar (Fig. 5A). These results suggest that degradation of hyperoxidized Prx III is a slow process.

Effects of oxidative stress on Srx expression and Prx sulfinylation in A549 cells

To investigate the mechanisms responsible for the hyperoxia-induced upregulation of Srx expression and hyperoxidation of Prx III, we first examined the effect of hyperoxia on the amount of Srx in A549 human lung adenocarcinoma cells, NIH 3T3 mouse embryonic fibroblasts, HEK 293 human embryonic kidney cells, and HeLa human cervical carcinoma cells. The abundance of Srx varied markedly among the four cell lines and was not affected by exposure to hyperoxia for 3 h (Fig. 6A, data not shown). The induction of proteins regulated by the Nrf2-ARE pathway is usually complete within 3 h (33). Srx mRNA in A549 cells was also unaffected by hyperoxia (Fig. 6B). Exposure of A549 cells to hypoxia (1 atm of 3% O_2 and 97% N_2) also did not affect the expression of Srx (Fig. 6A). Whereas hyperoxia-induced oxidative stress triggered hyperoxidation of Prx III, but not that of Prx I or Prx II, in mouse lung, the sulfinic forms of 2-Cys Prxs were not detected in A549 cells exposed to hyperoxia for 12 h (Fig. 5B).

To determine whether Prx III is more responsive to hyperoxidation than are Prx I and Prx II, we monitored the accumulation of sulfinic Prxs in A549 cells after their exposure to various concentrations (0, 50, 100, and 200 μM) of H_2O_2 for 10 min. A weak band corresponding to sulfinic Prx I/II was detected in the cells exposed to 50 μM H_2O_2; a strong band for sulfinic Prx I/II and a faint band for sulfinic Prx III were apparent in the cells exposed to 100 μM H_2O_2; and strong bands for both sulfinic Prx I/II and sulfinic Prx III were observed in the cells exposed to 200 μM H_2O_2 (Fig. 6C). Analysis with 2D-PAGE of the lysate of A549 cells that had been exposed to H_2O_2 at 200 μM for 10 min indicated that Prx I, Prx II, and Prx III were fully converted to their sulfinic forms (data not shown). These results suggested that, unlike in lungs exposed to hyperoxia, Prx III was not selectively hyperoxidized in A549 cells exposed to extracellular H_2O_2, which is freely diffusible through cell membranes. Rather, hyperoxidation of Prx I and Prx II was faster than that of Prx III. Analysis of A549, HeLa, and NIH 3T3 cells that had been exposed to various levels of extracellular H_2O_2 indicated that, among Prx I, Prx II, and Prx III, Prx II is the most sensitive to hyperoxidation (data not shown).

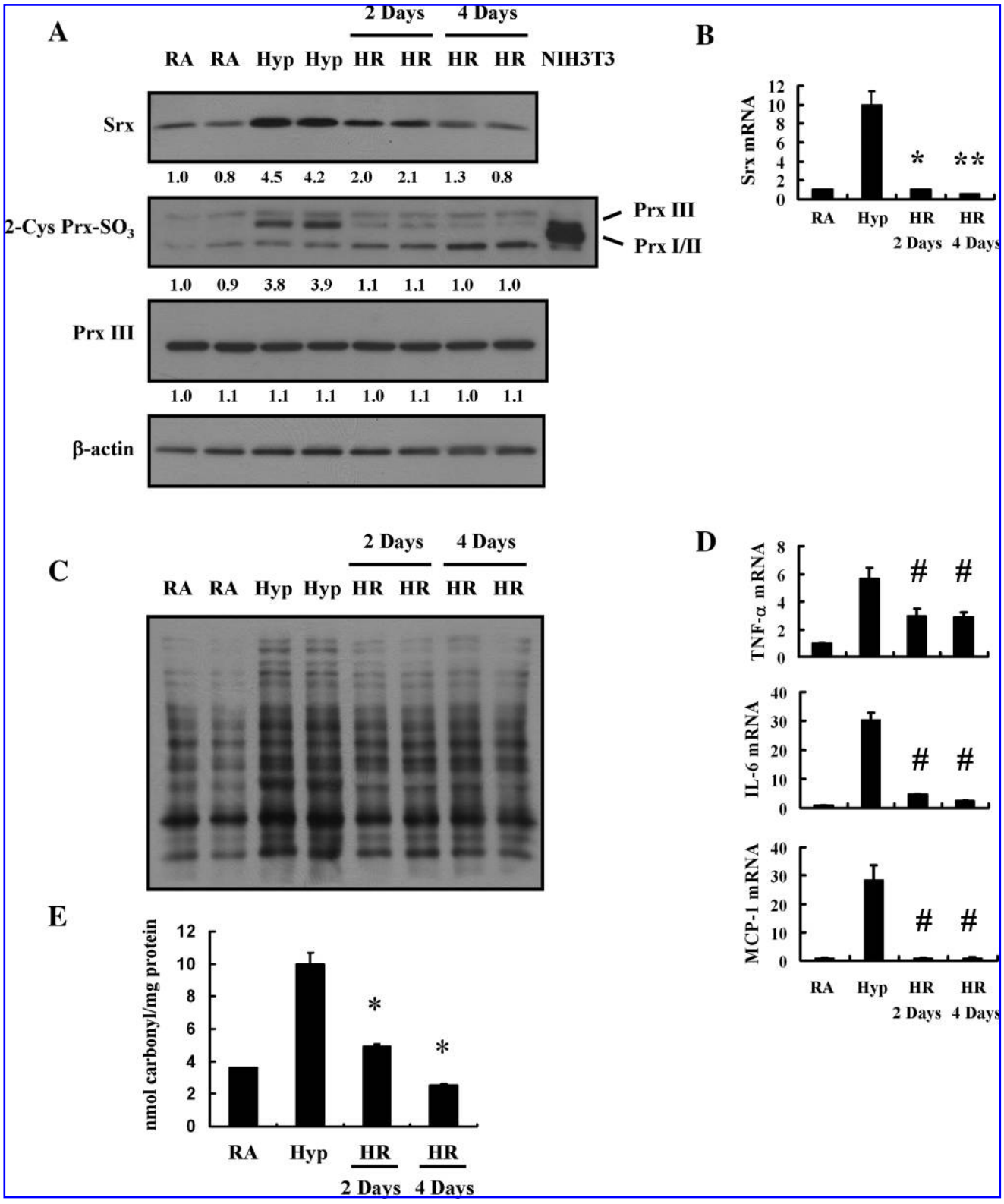
The sulfinylation of Prx III molecules in the lungs of $Nrf2^{-/-}$ mice resulted in their degradation, likely because of the lack of a sufficient amount of Srx to catalyze their reduction. We examined whether depletion of Srx in A549 cells might have a similar effect. The amount of Srx was reduced by >90% in A549 cells transfected with a small interfering RNA (siRNA) specific for Srx mRNA (Fig. 6D). As shown earlier, exposure of A549 cells to 200 μM H_2O_2 for 10 min resulted in hyperoxidation of Prx I/II and Prx III (Fig. 6E), with complete hyperoxidation of Prx III being apparent with 2D-PAGE (Fig. 6F). When the H_2O_2-treated cells were allowed to recover for 8 h in the absence of H_2O_2, the amounts of the sulfinic forms of Prx I/II and Prx III decreased markedly in control cells but not in those depleted of Srx (Fig. 6E and F). Nevertheless, no decrease in the total abundance of Prx I or Prx III was apparent in the control or Srx-depleted cells (Fig. 6E and F). Depletion of Srx increased the sensitivity of A549 cells to extracellular H_2O_2, such that their exposure to a constant supply of 100 μM H_2O_2 over a 30-min period resulted in detachment of the cells (data not shown). Furthermore, exposure of the Srx-depleted A549 cells to hyperoxia for 12 h did not cause accumulation of sulfinic Prx III (Fig. 5B).

Discussion

Hyperoxia increases the intracellular production of both H_2O_2 and the superoxide anion (O_2^-), with this effect having been suggested to be a major factor in the etiology of lung damage caused by hyperoxia (11, 21, 40). To protect against oxidative damage, lung cells increase their expression of antioxidant enzymes such as manganese-dependent superoxide dismutase (Mn-SOD), NAD(P)H:quinine oxidoreductase–1 (NQO-1), heme oxygenase–1 (HO-1), and glutathione peroxidase–2 *via* the Nrf2-ARE pathway (10, 12, 21, 33, 37).

The availability of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mouse littermates has aided efforts to elucidate the functional association between Nrf2 and such antioxidant enzymes.

We have now shown that exposure of mice to hyperoxia resulted in a marked increase in Srx expression in the lungs. This effect was apparent at both the mRNA and protein levels, suggesting that it is mediated by transcriptional activation of the Srx gene. With the use of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mouse littermates, we found that Nrf2 is essential for the hyperoxia-induced upregulation of Srx expression in lung tissue.



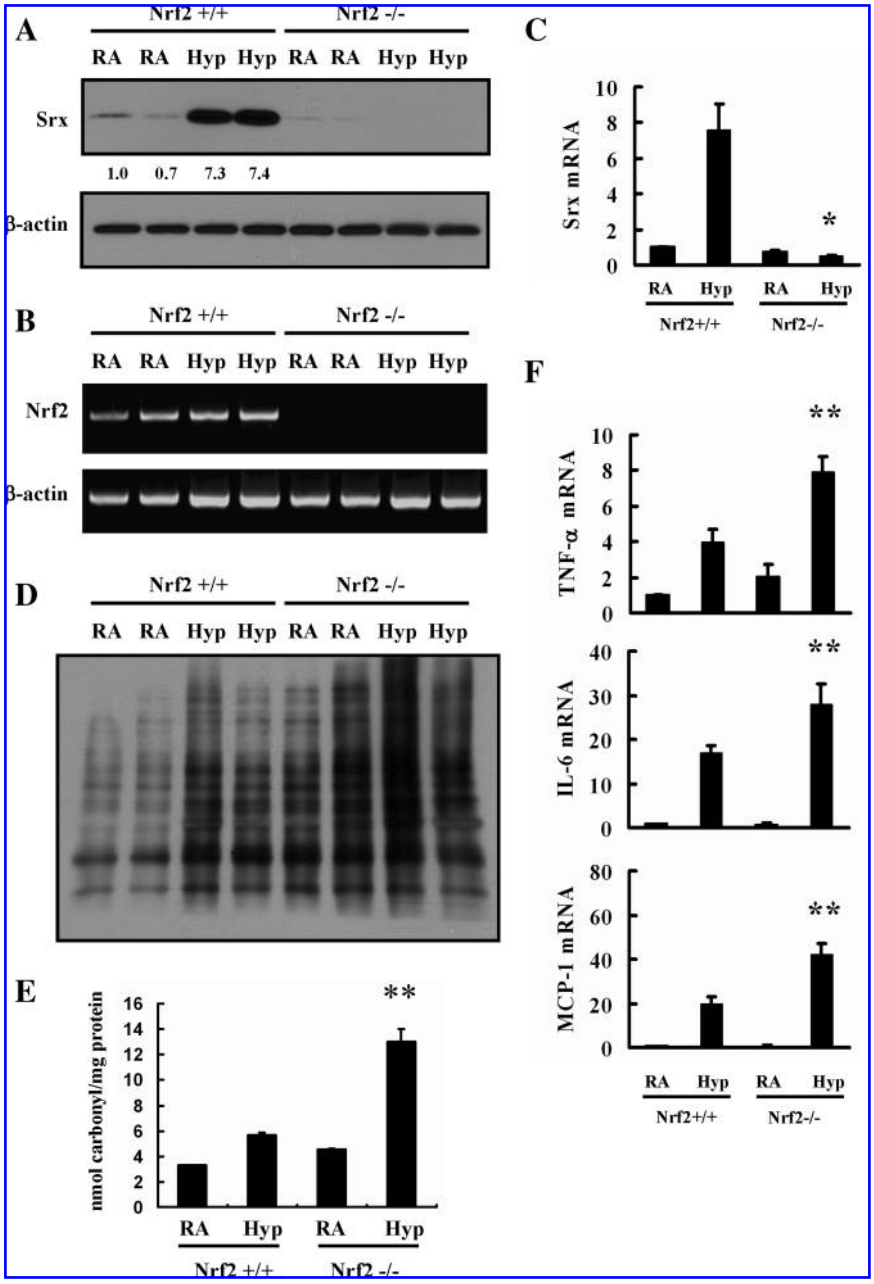


FIG. 3. Failure of hyperoxia to induce Srx expression in the lungs of $Nrf2^{-/-}$ mice. (**A**) Homogenates (20 μg of protein) of lung tissue from $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice exposed to room air (RA) or hyperoxia (Hyp) for 3 days were subjected to immunoblot analysis with antibodies specific for Srx or β-actin. Data are shown for two mice of each group and are representative of three independent experiments. (**B**) Total RNA extracted from the lungs of $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice treated as in (**A**) was subjected to RT-PCR analysis with primers specific for Nrf2 and β-actin (internal control) mRNAs. Each lane corresponds to a different animal. (**C**) Total RNA prepared from lung tissue of mice treated as in (**A**) was subjected to qPCR analysis for determination of the amount of Srx mRNA. Data are expressed as mean $\pm$ SEM for three mice of each group and are expressed relative to the corresponding value for $Nrf2^{+/+}$ mice exposed to room air. $^{*}p < 0.02$ *vs.* Hyp in $Nrf2^{+/+}$ mice. (**D**) Homogenates (10 μg of protein) of lung tissue from mice treated as in (**A**) were subjected to immunoblot analysis of protein carbonylation. Data are shown for two mice of each group and are representative of three independent experiments. (**E**) Homogenates (10 μg of protein) of lung tissue from $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice exposed to room air (RA) or hyperoxia (Hyp) for 3 days were subjected to carbonylation assay with OxyELISA Oxidized Protein Quantitation Kit. $^{**}p < 0.02$ *vs.* Hyp for 72 h. (**F**) Total RNA prepared from lung tissue of mice treated as in (**A**) was subjected to qPCR analysis for determination of the amounts of TNF-α, IL-6, and MCP-1 mRNAs. Data are expressed as mean $\pm$ SEM for three mice of each group and are expressed relative to the corresponding value for $Nrf2^{+/+}$ mice exposed to room air. $^{**}p < 0.05$ *vs.* Hyp in $Nrf2^{+/+}$ mice.

FIG. 2. Reversibility of the hyperoxia-induced increase in Srx expression. (**A**) Homogenates (20 μg of protein) of lung tissue from mice exposed to room air for 3 days (RA), to hyperoxia for 3 days (Hyp), or to hyperoxia for 3 days followed by room air (HR) for 2 or 4 days were subjected to immunoblot analysis with antibodies specific for Srx, 2-Cys Prx–SO_2, Prx III, and for β-actin. Data are shown for two mice of each group and are representative of three independent experiments. (**B**) Total RNA prepared from lung tissue of mice treated as in (**A**) was subjected to qPCR analysis for determination of the amount of Srx mRNA. Data are expressed as mean $\pm$ SEM for three mice of each group and are expressed relative to the corresponding value for the RA group. $^{*}p < 0.02$ *vs.* Hyp for 72 h. $^{**}p < 0.01$ *vs.* Hyp for 72 h. (**C**) Homogenates (10 μg of protein) of lung tissue from mice treated as in (**A**) were subjected to immunoblot analysis of protein carbonylation. Data are shown for two mice of each group and are representative of three independent experiments. (**D**) Total RNA prepared from lung tissue of mice treated as in (**A**) was subjected to qPCR analysis for determination of the amounts of TNF-α, IL-6, and MCP-1 mRNAs. Data are expressed as mean $\pm$ SEM for three mice of each group and are expressed relative to the corresponding value for the RA group. $\#p < 0.03$ *vs.* Hyp for 72 h. (**E**) Homogenates (10 μg of protein) of lung tissue from mice exposed to room air for 3 days (RA), to hyperoxia for 3 days (Hyp), or to hyperoxia for 3 days followed by room air (HR) for 2 or 4 days were subjected to carbonylation assay with OxyELISA Oxidized Protein Quantitation Kit. $^{*}p < 0.01$ *vs.* Hyp for 72 h.

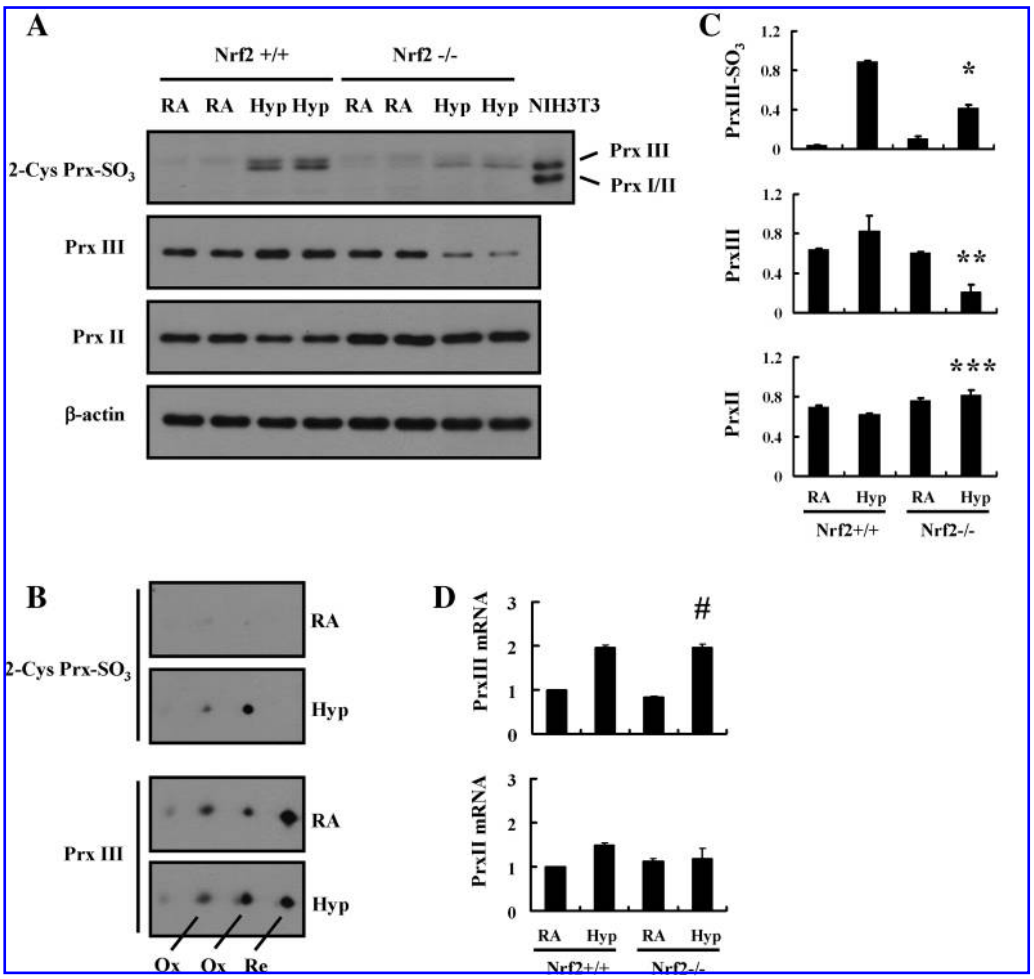


FIG. 4. Hyperoxidation and degradation of Prx III. (**A**) Homogenates (20 μg of protein) of lung tissue from Nrf2$^{+/+}$ or Nrf2$^{-/-}$ mice exposed to room air (RA) or hyperoxia (Hyp) for 3 days were subjected to immunoblot analysis with antibodies specific for 2-Cys Prx–SO_2, for Prx III, for Prx II, or for β-actin. A total lysate of NIH 3T3 cells that had been treated with 100 μM H_2O_2 for 10 min was used as a positive control for sulfinic forms of Prx I/II and Prx III. Data are shown for two mice of each group and are representative of three independent experiments. (**B**) Homogenates (250 μg of protein) of lung tissue from Nrf2$^{+/+}$ mice treated as in (**A**) were subjected to 2D-PAGE followed by immunoblot analysis with antibodies to the sulfinic forms of 2-Cys Prxs and to Prx III. Ox and Re indicate spots corresponding to oxidized and reduced forms, respectively, of Prx III. (**C**) Immunoblot intensities for the sulfinic form of Prx III, total Prx III, and total Prx II in experiments similar to those shown in (**A**) were determined by densitometry and normalized by the corresponding value for β-actin. Data are expressed as mean ± SEM of values from three independent experiments. *$p < 0.002$, *vs.* Hyp in Nrf2$^{+/+}$ mice. ** and ***$p < 0.03$ *vs.* Hyp in Nrf2$^{+/+}$ mice, respectively. (**D**) Total RNA prepared from lung tissue of mice treated as in (**A**) was subjected to qPCR analysis for determination of the amounts of Prx III and Prx II mRNAs. Data are expressed as mean ± SEM for three mice of each group and are expressed relative to the corresponding value for Nrf2$^{+/+}$ mice exposed to room air. #$p < 0.05$ *vs.* Hyp in Nrf2$^{+/+}$ mice.

The induction of Srx expression was shown to be reversible: On reintroduction of hyperoxia-exposed mice to room air, the amounts of Srx mRNA and protein returned to basal levels within 2 days (the earliest time of observation) and 4 days, respectively. The amounts of mRNAs for the inflammatory cytokines TNF-α and IL-6, as well as for the chemokine MCP-1, were also increased in the lungs of mice exposed to hyperoxia. Furthermore, the extent of carbonylation of lung proteins was increased in mice exposed to hyperoxia compared with that in those maintained in room air. Hyperoxia induced 1.5-fold to twofold increases in the abundance of mRNAs for Prx I, Prx III, and Prx VI, but it had no substantial effect on the amounts of these Prxs at the protein level.

The major enzymes responsible for the elimination of H_2O_2 in mammalian cells include catalase, glutathione peroxidase, and Prxs. Prxs are thought to be responsible for eliminating low concentrations of peroxides, whereas catalase scavenges H_2O_2 efficiently at high concentrations of the oxidant (35). The primary physiologic substrates of glutathione peroxidase have been proposed to be organic peroxides rather than H_2O_2 (39). The sulfenic intermediate (C_P–SOH) of Prxs generated during catalysis occasionally undergoes further oxidation to sulfinic acid (C_P–SO_2H), leading to inactivation of peroxidase function (47). This hyperoxidation occurs only when Prx is engaged in the catalytic cycle. Analysis of Prx I inactivation in the presence of a low steady-state level (<1 μM) of H_2O_2 indicated that

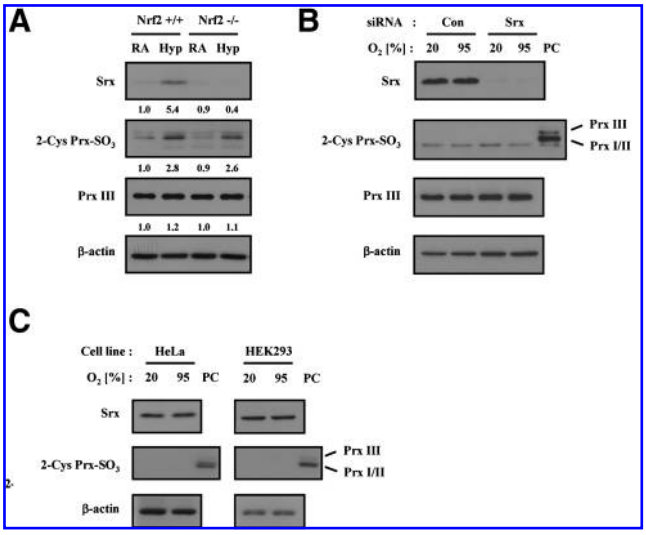


FIG. 5. (A) Homogenates (20 μg of protein) of lung tissue from Nrf2$^{+/+}$ or Nrf2$^{-/-}$ mice exposed to room air (RA) or hyperoxia (Hyp) for 2 days was subjected to immunoblot analysis with antibodies specific for Srx, 2-Cys Prx-SO$_2$, Prx III, and for β-actin. (B) A549 cells were transfected with either a control (Con) siRNA or an siRNA specific for human Srx mRNA, cells were exposed to normoxia (20%), or hyperoxia (95%) for 12 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies specific for Srx, 2-Cys Prx-SO$_2$, Prx III, and for β-actin. A total lysate of NIH 3T3 cells that had been treated with 100 μM H_2O_2 for 10 min was used as a positive control (PC) for sulfinic forms of Prx I/II and Prx III. **(C)** HeLa and HEK293 cells were exposed to normoxia (20%) or hyperoxia (95%) for 12 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies specific for Srx, 2-Cys Prx-SO$_2$, and for β-actin. A total lysate of NIH 3T3 cells that had been treated with 100 μM H_2O_2 for 10 min was used as a positive control (PC) for sulfinic forms of Prx I/II and Prx III.

Prx I was hyperoxidized at a rate of 0.072% per turnover (47). The hyperoxidized 2-Cys Prxs (Prx I to Prx IV) are reactivated by the action of Srx. Thus, when the rate of hyperoxidation exceeds the capacity of Srx, sulfinic Prxs accumulate.

Sulfinic Prxs were not detected in the lungs of normoxic mice, whereas the sulfinic form of Prx III was evident in the lungs of hyperoxic mice. These results suggest that the low amount of Srx present in normoxic lung tissue is sufficient to counteract the hyperoxidation of 2-Cys Prxs that occurs during elimination of the H_2O_2 produced during normal lung function. However, despite the marked increase in Srx expression induced in lung tissue by hyperoxia, the activity of Srx was insufficient to maintain the mitochondrial enzyme Prx III in its active form. We found that among Prx I, Prx II, and Prx III, Prx II was most sensitive to hyperoxidation by H_2O_2 imported into A549 cells from the external environment. Given that Prx II is abundant in mouse lung (data not shown) and that the sulfinic form of Prx III, but not that of Prx II, accumulated in hyperoxic lung tissue, hyperoxia-induced H_2O_2 production may occur predominantly in mitochondria. The precise source of H_2O_2 production in hyperoxic lung tissue is not known, with both the mitochondrial electron-transport chain (5, 17) and NADPH oxidase at the plasma membrane (33) having been implicated. Alternatively, the preferential accumulation of the sulfinic form of Prx III might be explained if the import of Srx from the cytosol into mitochondria is rate limiting for Prx III reactivation. Srx molecules, which are localized predominantly in the cytosol (9), translocate to mitochondria in response to oxidative stress (T. S. Chang, personal communication).

The amount of sulfinic Prx III in hyperoxic lung tissue of Nrf2$^{-/-}$ mice was found to be about half of that in wild-type littermates. This finding was unexpected, given that the amount of Srx in the lungs of hyperoxic Nrf2$^{-/-}$ mice is much less than that in the wild-type animals. Exposure to hyperoxia for 3 days also reduced the level of Prx III in Nrf2$^{-/-}$ mice to one-fourth that in Nrf2$^{+/+}$ mice. The sulfinic/reduced ratio for Prx III in Nrf2$^{-/-}$ mice was thus twice that in Nrf2$^{+/+}$ mice. No difference was found in the amount of Prx III between normoxic Nrf2$^{+/+}$ and Nrf2$^{-/-}$ mice, and hyperoxia induced a slight increase in the amount of Prx III in Nrf2$^{+/+}$ mice. Together, these observations suggest that Prx III was degraded in the lungs of Nrf2$^{-/-}$ mice exposed to hyperoxia. Nrf2 deficiency prevents the induction of various antioxidant enzymes in response to oxidative stress, resulting in the accumulation of ROS, and Nrf2$^{-/-}$ mice are more susceptible to oxidative lung injury than are wild-type animals (11). In addition, failure to induce Srx in hyperoxic lung tissue of Nrf2$^{-/-}$ mice would be expected to result in the rapid accumulation of inactive sulfinic Prx III in mitochondria. These effects of Nrf2 deficiency would thus be expected to result in higher concentrations of ROS in lung cells, especially in mitochondria. Under these conditions, the cysteine sulfinic residue of Prx III is likely further oxidized to cysteic acid (cysteine sulfonic acid). The sulfonic form of Prxs has been detected in human Jurkat T cells and yeast cells during incubation with a strong oxidant such as *tert*-butyl hydroperoxide or with a high concentration of H_2O_2 (29, 36). Such sulfonylation might change the conformation of Prx III to a state that is susceptible to proteolytic degradation.

We carried out the experiments with A549, HeLa, and HEK293 cells to determine whether an established cell line could serve as a model system for studying the mechanisms responsible for the hyperoxia-induced upregulation of Srx expression and degradation of Prx III. Unfortunately, our attempts were stymied by the fact that exposure of these cells to oxidative stress did not induce either Srx expression or degradation of Prx III (Fig. 5C). First, baseline Srx expression in these well-established lines was found to be much higher than that in lung tissue. The higher intrinsic expression of Srx in these cell lines appears to be sufficient to maintain Prx III in its reduced form, even when exposed to hyperoxia. Second, the hyperoxia-induced Prx III degradation was not observed even in the Srx-depleted A549 cells. This is likely because Nrf2$^{-/-}$ cells are deficient not only in the induction of Srx expression but also in that of additional antioxidant enzymes such as MnSOD, Gpx-2, HO-1, and NQO-1. Thus, on exposure to oxidative stress, Nrf2$^{-/-}$ cells likely experience much higher levels of ROS than do Srx-depleted cells. Consequently, the oxidation of sulfinic acid to sulfonic acid might be observed in Nrf2$^{-/-}$ cells but not in Srx-depleted cells.

In summary, we showed that hyperoxia upregulates Srx expression in mouse lungs through Nrf2-dependent transcription (Fig. 7). Hyperoxia promotes peroxide production, which in turn results in the activation of Nrf2-mediated gene transcription (10, 12, 21, 33, 37). Hyperoxia also induced the

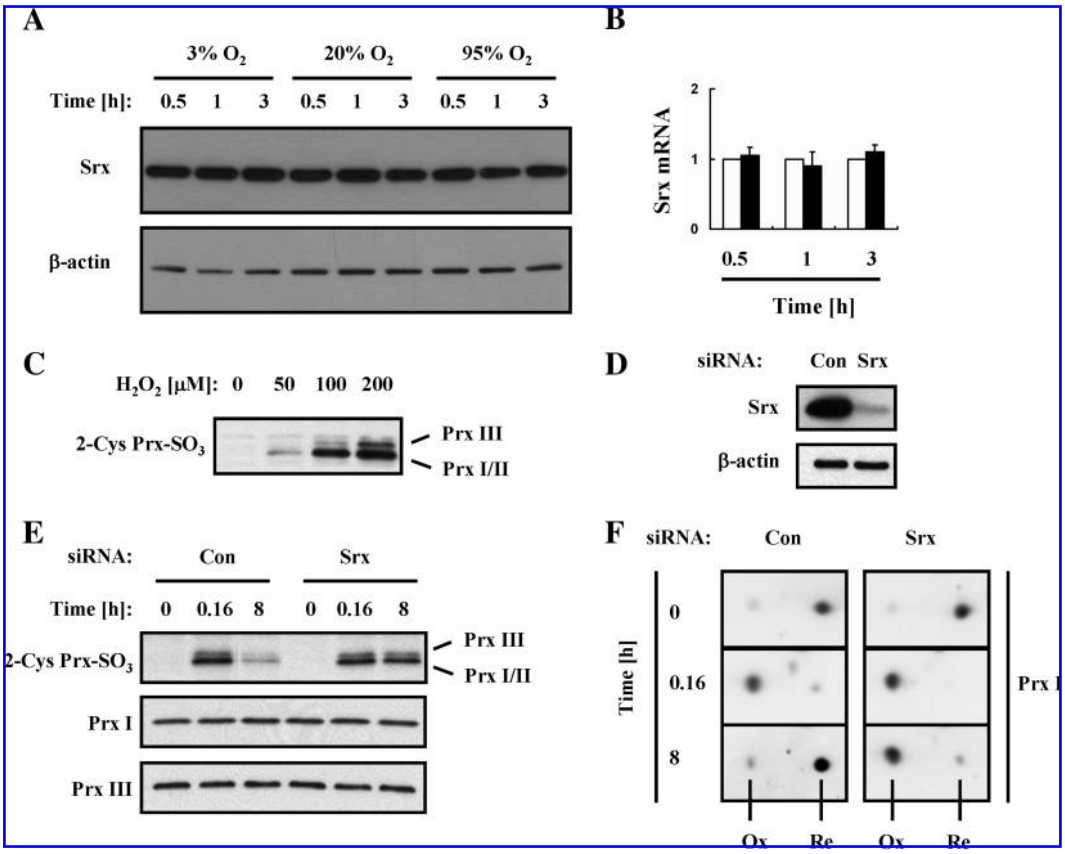


FIG. 6. Effects of oxidative stress on Srx expression and Prx sulfinylation in A549 cells. (**A**) Cells were exposed to hypoxia (3%), normoxia (20%), or hyperoxia (95%) for the indicated times, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to Srx and to *β*-actin. (**B**) A549 cells were exposed to normoxia (20%) or hyperoxia (95%) for the indicated times, after which total RNAs were prepared and subjected to qPCR analysis for determination of the amounts of Srx mRNA. *White and black bars* correspond to the cells exposed to normoxia (20%) and hyperoxia (95%), respectively. Data are expressed relative to the corresponding value for the normoxia group at each incubation time. (**C**) Cells were incubated for 10 min with the indicated concentrations of H_2O_2, lysed, and subjected to immunoblot analysis with antibodies to the sulfinic forms of 2-Cys Prxs. (**D**) Cells were transfected with either a control (Con) siRNA or an siRNA specific for human Srx mRNA, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to Srx or to *β*-actin. (**E**) Cells transfected as in (**D**) were either left untreated (0 h) or exposed to 200 μM H_2O_2 for 10 min (0.16 h) with or without subsequent incubation for 8 h in the absence of H_2O_2 and in the presence of cycloheximide (10 mg/ml). Cell lysates were analyzed with SDS-PAGE (**E**) or 2D-PAGE (**F**), followed by immunoblot analysis with the indicated antibodies. All data are representative of three independent experiments.

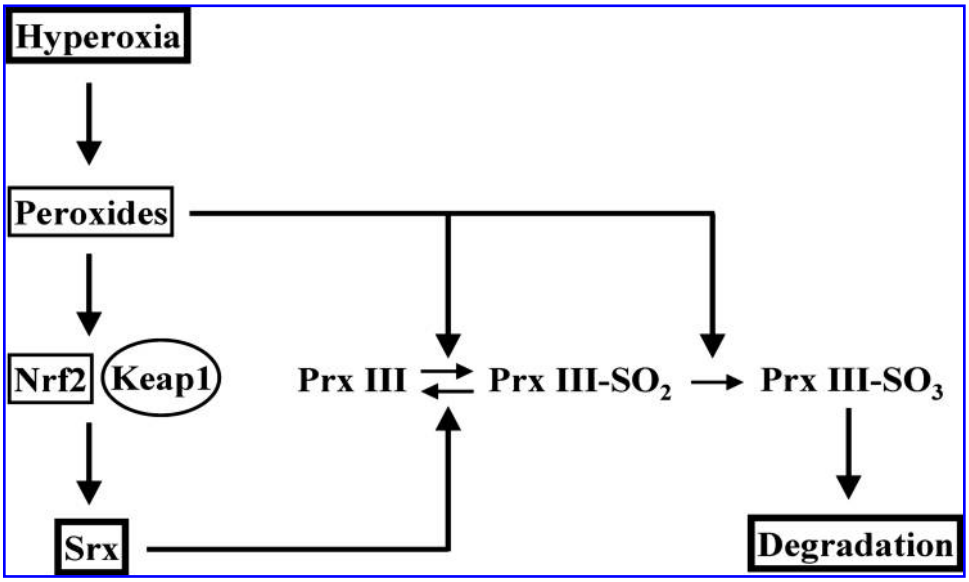


FIG. 7. Model for hyperoxia-induced Srx expression and Prx III degradation in mouse lung. Hyperoxia increases the intracellular level of peroxides and thereby triggers Nrf2-dependent induction of Srx expression. The reduction of peroxides by Prx III results in its hyperoxidation to the sulfinic form (Prx III–SO_2), the reversal of which is catalyzed by Srx. If a sufficient amount of Srx is not available, inactive sulfinic Prx III accumulates in mitochondria, leading to severe oxidative stress. This strong oxidative environment appears to result in the degradation of Prx III after its further oxidation to the sulfonic form (Prx III–SO_3). Keap1, Kelch-like ECH-associating protein 1.

accumulation of the sulfinic form of the mitochondrial enzyme Prx III but not that of the corresponding forms of the cytosolic enzymes Prx I and Prx II. The selective hyperoxidation of Prx III is likely due either to mitochondria being the major site of peroxide production or to translocation of Srx from the cytosol into mitochondria being rate limiting for the reduction of sulfinic Prx III. Exposure of $Nrf2^{-/-}$ mice to hyperoxia resulted in the degradation of Prx III, likely because sulfinic Prx III was further oxidized by high levels of ROS to the sulfonic form, which might be more susceptible to proteolysis.

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Abbreviations

2D, two-dimensional; AP-1, activator protein-1; ARE, antioxidant response element; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein–1; Nrf2, nuclear factor erythroid 2–related factor 2; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Prx, peroxiredoxin; qPCR, quantitative real-time PCR; ROS, reactive oxygen species; RT, reverse transcription; siRNA, small interfering RNA; Srx, sulfiredoxin; TNF-α, tumor necrosis factor–α.

Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Sue Goo Rhee
Division of Life and Pharmaceutical Sciences
Ewha Womans University
11-1 Daehyun-dong
Seodaemun-gu, Seoul 120-750, Korea

E-mail: rheesg@ewha.ac.kr

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