Role of NRF2 in Protection Against Hyperoxic Lung Injury in Mice

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NRF2 is a transcription factor important in the protection against carcinogenesis and oxidative stress through antioxidant response element (ARE)-mediated transcriptional activation of several phase 2 detoxifying and antioxidant enzymes. This study was designed to determine the role of NRF2 in the pathogenesis of hyperoxic lung injury by comparing pulmonary responses to 95–98% oxygen between mice with site-directed mutation of the gene for NRF2 (*Nrf2*$^{-/-}$) and wild-type mice (*Nrf2*$^{+/+}$). Pulmonary hyperpermeability, macrophage inflammation, and epithelial injury in *Nrf2*$^{-/-}$ mice were 7.6-fold, 47%, and 43% greater, respectively, compared with *Nrf2*$^{+/+}$ mice after 72 h hyperoxia exposure. Hyperoxia markedly elevated the expression of NRF2 mRNA and DNA-binding activity of NRF2 in the lungs of *Nrf2*$^{+/+}$ mice. mRNA expression for ARE-responsive lung antioxidant and phase 2 enzymes was evaluated in both genotypes of mice to identify potential downstream molecular mechanisms of NRF2 in hyperoxic lung responses. Hyperoxia-induced mRNA levels of NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione-S-transferase (GST)-Ya and -Yc subunits, UDP glycosyl transferase (UGT), glutathione peroxidase-2 (GPx2), and heme oxygenase-1 (HO-1) were significantly lower in *Nrf2*$^{-/-}$ mice compared with *Nrf2*$^{+/+}$ mice. Consistent with differential mRNA expression, NQO1 and total GST activities were significantly lower in *Nrf2*$^{-/-}$ mice compared with *Nrf2*$^{+/+}$ mice after hyperoxia. Results demonstrated that NRF2 has a significant protective role against pulmonary hyperoxic injury in mice, possibly through transcriptional activation of lung antioxidant defense enzymes.

Reactive oxygen species (ROS) have been implicated in the pathogenesis of many acute and chronic pulmonary disorders such as adult respiratory distress syndrome and bronchopulmonary dysplasia (1). In laboratory animals, administration of pure oxygen (> 95%, hyperoxia) causes extensive pulmonary damage characterized by inflammation and death of capillary endothelial and alveolar epithelial cells resulting in pulmonary edema and severe impairment of respiratory functions (2, 3). Sufficiently long exposure (≥ 3 d) to hyperoxia is lethal to animals (4). The precise molecular mechanism(s) by which hyperoxia produces lung injury remain(s) unresolved. However, excess production of ROS that could overwhelm endogenous pulmonary antioxidant defense systems has been proposed (5), and a number of studies have focused on enzymatic defense components in the pathogenesis of oxygen-induced lung damage (6–13).

In laboratory rodents, hyperoxia causes increases of "classic" antioxidant enzymes (e.g., superoxide dismutase [SOD], glutathione peroxidase [GPx], glutathione reductase [GR], and catalase) in the lung (6, 7). The protective roles of these enzymes in the development of oxidative lung damage have been proposed in a few *in vivo* studies with genetically engineered mice (i.e., gene knockout mice and transgenic mice). For example, lung inflammation and damage was attenuated in mice that overexpressed SOD3, relative to wild-type (*wt*) mice (8); partial protection against hyperoxic lung injury was also observed in transgenic mice overexpressing SOD2 (9). Tsan and colleagues suggested that SOD2 gene-knockout mice were more susceptible to pulmonary hyperoxic injury than normal mice (10). Enhanced pulmonary antioxidant enzyme activity through exogenous administration of SOD1 and/or catalase also provided protection to rats against hyperoxic insults (11). Heme oxygenase-1 (HO-1), an oxidative stress protein, has been also shown to be protective in hyperoxic pulmonary injury (12, 13). In addition, phase 2 detoxifying enzymes including NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione-S-transferase (GST) have attracted attention due to their protective roles against oxidative processes in malignant cells or tissues (14, 15). As indirect antioxidants, phase 2 enzymes detoxify reactive electrophilic metabolites, such as organic peroxides, lipid peroxides, epoxides, and quinones, and facilitate their excretion through conjugation reaction or two-electron reduction. However, little is known about the contribution of phase 2 detoxifying enzymes to lung defense against oxygen toxicity.

NF-E2–related factor 2 (NRF2) is a recently identified cap'n'collar basic leucine zipper transcription factor. It was originally detected in erythroid cells, but abundant NRF2 mRNA expression has subsequently been described in murine liver, intestine, lung, and kidney, where detoxification reactions occur routinely (16, 17). High similarity exists between the NRF2 binding sequence (NF-E2 consensus sequence) and antioxidant response element (ARE, also referred to as electophilic response element). Conse-

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Abbreviations: antioxidant response element, ARE; bronchoalveolar lavage fluid, BALF; butylated hydroxyanisole, BHA; butylated hydroxytoluene, BHT; 1-chloro 2,4-dinitrobenzene, CDNB; α-dithiothreitol, DTT; ethylenediaminetetraacetic acid, EDTA; electrophoretic mobility shift assay, EMSA; flavine adenine dinucleotide, FAD; γ-glutamate cystein ligase regulatory subunit, GCLS; glutathione peroxidase, GPx; glutathione reductase, GR; glutathione-S-transferase, GST; N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid, HEPES; tris buffered EDTA, TBE; heme oxygenase-1, HO-1; 3-(N-morpholino)propanesulfonic, MOPS; nicotinamide adenine dinucleotide, reduced form, NADH; nuclear factor, erythroid 2, NF-E2; NAD(P)H:quinone oxidoreductase 1, NQO1; NF-E2–related factor 2, NRF2; ICR/Sv129 mice, *Nrf2*$^{+/+}$; ICR/Sv129 mice with site-directed mutation in *Nrf2*, *Nrf2*$^{-/-}$; polymorphonuclear leukocyte, PMN; phenylmethanesulfonyl fluoride, PMSF; polydeoxyinosinic-deoxycytidylic acid, polydI-dC; reactive oxygen species, ROS; reverse transcriptase polymerase chain reaction, RT-PCR; standard error of the mean, SEM; superoxide dismutase, SOD; UDP glycosyl transferase, UGT.

quently, NRF2 has induced mRNA expression for ARE-bearing phase 2 detoxifying enzymes such as NQO1, GST–Ya subunit, and γ-glutamate cysteine ligase regulatory subunit (GCLS), classic antioxidant enzymes (e.g., catalase, SOD1), and HO-1, and protected cells against carcinogenesis and oxidative stress in various *in vivo* (17–19) and *in vitro* (20–23) models. The role of NRF2 in the pathogenesis of oxygen toxicity, however, has not been studied in the lungs of laboratory animals.

The present study was designed to test the hypothesis that NRF2 contributes to pulmonary protection against hyperoxic injury in mice. Mice with site-directed mutation (knockout) of *Nrf2* ($Nrf2^{-/-}$) and *wt* mice ($Nrf2^{+/+}$) were exposed to hyperoxia, and pulmonary permeability, inflammatory, and epithelial injury responses in bronchoalveolar lavage fluid (BALF) were compared in both genotypes of mice. The effects of hyperoxia exposure on lung NRF2 mRNA expression and DNA-binding activity in *wt* mice were determined by Northern blot analysis and electrophoretic mobility shift analysis (EMSA), respectively. Lung mRNA expression for selected ARE-responsive defense enzymes were examined in $Nrf2^{-/-}$ and *wt* mice to identify molecular mechanisms through which NRF2 may contribute to the protection against oxidative lung injury.

Materials and Methods

Animals

Breeding pairs of ICR/Sv129-$Nrf2^{+/-}$ mice were obtained from a colony at Tsukuba University (17) and maintained in the Johns Hopkins facility. Mice were fed a purified AIN-76A diet. Water was provided *ad libitum*. Mice were bred, and progeny were genotyped for $Nrf2^{+/+}$ and $Nrf2^{-/-}$ (17). Cages were placed in laminar flow hoods with high-efficiency particulate-filtered air. Sentinel animals were examined periodically (titers and necropsy) for infection. All experimental protocols conducted in the mice were performed in accordance with the standards established by the US Animal Welfare Acts, set forth in NIH guidelines and the Policy and Procedures Manual (Johns Hopkins University School of Hygiene and Public Health Animal Care and Use Committee).

Oxygen Exposure

Mice were placed on a fine mesh wire flooring in a sealed 45-liter glass exposure chamber. The chamber bottom was lined with CO_2 absorbent (Soda-sorb; WR Grace, Lexington, MA). Food and water were provided *ad libitum*. Sufficient humidified pure oxygen was delivered to the chamber to provide 10 changes/h (7 liters/min flow rate). The concentration of oxygen in the exhaust from the chamber was monitored (OM-11; Beckman, Irvine, CA) throughout the experiments. The oxygen concentration for all experiments ranged from 95–99%. The chambers were opened once a day for 10 min to replace CO_2 absorbent, food, and water. Age- and gender-matched (6- to 8-wk) mice of each genotype ($Nrf2^{+/+}$ and $Nrf2^{-/-}$) were exposed to either room air or hyperoxia for 48 and 72 h ($n = 4$ per group).

BALF and Phenotyping

Immediately following exposure, mice were removed from the chamber, anesthetized with sodium pentobarbital (104 mg/kg), and weighed. Hyperoxia-induced changes in lungs were assessed by total protein concentration and total and differential cell counts in BALF following procedures described previously (24). Briefly, the right lung of each mouse was lavaged *in situ* four times with Hanks' balanced salt solution (HBSS, 17.5 ml/kg, pH 7.2–7.4), and the recovered BALF was immediately cooled to 4°C. For each mouse, the four BALF returns were centrifuged (500 × *g* at 4°C), and the supernatant from the first BALF return was decanted for determination of total protein (an indicator of lung permeability). Protein concentration was measured following the method of Bradford as indicated in the manufacturer's procedure (Bio-Rad, Hercules, CA). The cell pellets from all lavage returns were combined and resuspended in 1 ml of HBSS. The numbers of cells (per ml total BALF return) were counted with a hemocytometer as indicators of lung injury and inflammation. An aliquot (200 μl) of BALF cell suspension was cytocentrifuged (Shandon Southern Products, Pittsburgh, PA) and stained with Wright-Giemsa stain (Diff-Quik; Baxter Scientific Products, McGaw Park, IL) for differential cell analysis. Differential counts for epithelial cells, macrophages, and PMNs were done by identifying 300 cells according to standard cytologic techniques (25). Epithelial cells in particular were identified by the presence of cilia.

Total Lung RNA Isolation and Northern Blot Analysis for NRF2 mRNA Expression

Total RNA was isolated from nonlavaged lung homogenate of each mouse according to the method of Chomczynski and Sacchi (26) as indicated in the Trizol (Life Technologies, Gaithersburg, MD) reagent specifications. Pooled total RNA from each group (15 μg) was separated on a 1.2% formaldehyde-agarose gel in 1× MOPS acid buffer and transferred overnight to a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH). The membrane was UV-crosslinked, and the blot was hybridized with a double-stranded [γ^{32}P]dCTP-labeled NRF2 cDNA probe (1.8 kb) in the Perfect Hybridization Buffer (Sigma, St. Louis, MO) and evaluated autoradiographically. As a control for loading of total RNA, 18S RNA on the gel was examined by staining with ethidium bromide. The intensity of each NRF2 band was quantitated using a Gel Doc 2000 System (Bio-Rad) and normalized by the intensity of the corresponding 18S RNA band.

Lung Nuclear Protein Extraction and EMSA for NRF2 Activity

Nuclear protein extracts were prepared from pooled lung tissues of four mice in each group as previously described (27). An aliquot of 2 μg nuclear proteins was incubated on ice with a binding buffer (10 mM HEPES [pH 7.9], 60 mM KCl, 0.5 mM EDTA, 4% Ficoll, 1 mM DTT, 0.2 μg PolydI-dC, 1 mM PMSF) in a total volume of 19 μl. After 15 min incubation, 1 μl (2×10^4 cpm) of [γ^{32}P]dATP end-labeled double-stranded oligonucleotide containing a NF-E2-consensus sequence (5′-TGG GGA ACC TGT GCT GAG TCA CTG GAG-3′) or ARE-consensus sequence (5′-AGT CAC AGT GAC TCA GCA GAA TCT-3′) was added to the reaction and followed by an additional 30-min incubation at room temperature. The mixture was subjected to electrophoresis on a 4% polyacrylamide gel with 0.25× TBE buffer for 2 h at 180 V. The gel was autoradiographed using an intensifying screen at −70°C. The intensity of each shifted band was quantitated using a Gel Doc 2000 System (Bio-Rad).

RT-PCR for Lung Antioxidant Enzyme mRNA Expression

Total RNA (500 ng) was reverse transcribed into cDNA in a volume of 50 μl, containing 1× PCR buffer (50 mM KCl and 10 mM Tris [pH 8.3]), 5 mM $MgCl_2$, 1 mM each dNTPs, 125 ng oligo $(dT)_{15}$, and 50 U of Moloney Murine Leukemia Virus reverse transcriptase (Life Technologies), at 45°C for 15 min and 95°C for 5 min using gene amp PCR System 9700 (Perkin Elmer Applied Biosystems, Foster City, CA). Separate but simultaneous PCR amplifications were performed with aliquots of cDNA (10 μl) at a final concentration of 1× PCR buffer, 4 mM $MgCl_2$, 400 μM

dNTPs, and 1.25 U *Taq* Polymerase (Life Technologies) in a total volume of 12.5 μl using 240 nM each of forward and reverse primers (Table 1) specific for mouse GST-Ya, -Yc, and -Yp_1 and rat GST-Yb_1; mouse NQO1; UDP glycosyl transferase (UGT); GCLS; HO-1; GPx1 and 2; GR; SODs 1, 2, and 3; and catalase. β-actin was used as an internal control. PCR was started with 5 min incubation at 94°C followed by a three-step temperature cycle: denaturation at 94°C for 30 s, annealing at 55–60°C for 30 s, and extension at 72°C for 1 to ~ 2 min for 25 to ~ 30 cycles (*see* Table 1). A final extension step at 72°C for 10 min was included after the final cycle to complete polymerization. The number of cycles was chosen to ensure that amplification product did not reach a plateau level. Reactions were electrophoresed in 2% agarose gel containing ethidium bromide. The volume of each cDNA band was quantitated using a Gel Doc 2000 System (Bio-Rad), and the ratio of each gene cDNA to β-actin cDNA was determined.

Lung Cytosol Preparation

For preparation of crude cytosol, right lung tissues from four mice of each group were pooled and homogenized in ice-cold 10 mM Tris-HCl (pH 7.8). The homogenates were centrifuged at 10,000 × *g* for 20 min at 4°C. Protein concentration of the resulting supernatant was determined using the Bradford assay (Bio-Rad). Aliquots of the supernatant were stored at −70°C.

Lung NQO1 Assay

Dicoumarol-sensitive NQO1 activity was measured in cytosolic fractions at 25°C by a method reported by Shaw and colleagues (28). The reaction mixture contained 30 μg cytosolic protein, 25 mM Tris-HCl (pH 7.4), 0.23 mg/ml crystalline bovine serum albumin, 0.01% (vol/vol) polyethylene sorbitan monolaurate (Tween 20, Bio-Rad), 5 μM FAD, 0.2 mM NADH, and 0 or 10 μM dicoumarol in a final volume of 200 μl. To initiate the reaction, 40 μM of 2,6-dichloroindophenol (electron acceptor) was added, and the initial velocity of the reduction of dichloroindophenol was measured spectrophotometrically at 600 nm ($a_M = 2.1 \times 10^4$ M/cm). The nonenzymatic rate measured in the presence of dicoumarol was subtracted from the uninhibited rate (i.e., rate in the absence of dicoumarol). Activity was measured three times, and group mean activity was expressed as nmol/min/mg protein.

Lung Total GST Assay

Total GST activity of the cytosolic preparation was measured spectrophotometrically at 25°C according to procedures published previously (29). Cytosolic protein (45 μg) was added to a 200-μl reaction mixture containing 100 mM KH_2PO_4 (pH 6.5) and 1 mM glutathione. Formation of the thioether between glutathione and CDNB was monitored at 340 nm ($a_M = 9.6 \times$ mM/cm) by adding 1 mM CDNB to the reaction. Measurements were performed three times, and activity was expressed as nmol/min/mg protein.

Statistics

Data were expressed as the group mean ± standard error of the mean (SEM). Three-way analysis of variance was used to evaluate the effects of hyperoxia exposure on BALF protein and cells as well as lung antioxidant enzyme mRNA expression and activity between *Nrf2* knockout ($Nrf2^{-/-}$) and *wt* ($Nrf2^{+/+}$) mice ($n = 4$ per group). The factors in the analysis were exposure (hyperoxia or air), genotype ($Nrf2^{-/-}$ or $Nrf2^{+/+}$), and exposure time (48 or 72 h). Data sets were tested for homoscedasticity as required for parametric analyses, and data that did not meet this requirement (that is, heteroscedastic) were natural log transformed. The Student–Newman–Keuls test was used for *a posteriori* comparisons of means. All analyses were performed using a commercial statistical analysis package (SigmaStat; Jandel Scientific Software, San Rafael, CA). Statistical significance was accepted at $P < 0.05$.

Results

Effects of Targeted Disruption of *Nrf2* on Hyperoxia-Induced Lung Injury

The role of NRF2 in hyperoxic lung injury was evaluated by comparing pulmonary responses to hyperoxia in $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice. Statistically significant ($P < 0.05$) effects of genotype and time were detected on total protein and numbers of macrophages and epithelial cells recovered by BALF. No statistically significant effects of genotype, exposure, or time were found for BALF lymphocytes or PMNs. Compared with genotype-matched air controls, hyperoxia induced statistically significant increases in mean total protein concentration and numbers of BALF macrophages and epithelial cells in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice at 72 h (Figure 1). However, the mean numbers of BALF macrophages and epithelial cells were 47 and 43% greater, respectively, in $Nrf2^{-/-}$ mice compared with those in $Nrf2^{+/+}$ mice after 72 h of hyperoxia (Figure 1). Furthermore, total protein concentration was significantly higher in $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ after 48 (7.6-fold) and 72 h (3.8-fold) exposure (Figure 1). The results therefore indicate that disruption of *Nrf2* significantly enhanced pulmonary sensitivity and responsivity to hyperoxic challenge.

Effect of Hyperoxia on Lung NRF2 mRNA Expression and DNA Binding Activity

Expression levels of NRF2 mRNA were measured by Northern blot analysis to determine whether hyperoxia exposure modulates mRNA levels of NRF2. NRF2 mRNA was not detectable in the lungs of air- or hyperoxia-exposed knockout mice (Figure 2A). In contrast, constitutive expression of NRF2 mRNA (2.38 Kb) was detected in the lungs of $Nrf2^{+/+}$ mice, and hyperoxia enhanced the steady-state level of NRF2 mRNA in the *wt* mice at 48 and 72 h (2- and 2.6-fold, respectively) compared with those in the air-exposed *wt* mice (Figure 2A).

NF-E2– and ARE-binding abilities of lung nuclear proteins were assessed by EMSA to determine whether hyperoxia enhances functional NRF2 activity of $Nrf2^{+/+}$ mice. The nuclear protein-DNA complex formation in the lung identified by shifted bands was greater in the mice exposed to 48 (2-fold) and 72 (3-fold) h of hyperoxia than in the air-exposed mice, regardless of DNA probe used (Figure 2B). Negligible protein binding to the DNA sequences was detected in the lungs of all $Nrf2^{-/-}$ mice (data not shown). To detect specific binding of NRF2 to these DNA sequences, we performed supershift analysis using the only commercially available anti-mouse NRF2 antibody (SC-722x; Santa Cruz Biotechnology, Santa Cruz, CA). However, we failed to obtain satisfactory supershifted bands representing antibody-NRF2-DNA complex in the lung of these mice, though the same antibody has yielded successful results when applied to liver tissue (42).

Differential Expression of Lung Antioxidant Defense Enzyme mRNAs between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ Mice

Expression levels of mRNA for selected antioxidant enzymes, phase 2 detoxifying enzymes, and HO-1 were compared between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice to identify downstream genes transcriptionally activated by NRF2 (Figure

TABLE 1
Primer sequences for RT-PCR

cDNAs		Primer sequences	Amplified size (bp)	Annealing temp (°C)	PCR cycle no.
NQO1	Forward	ATT GTA CTG GCC CAT TCA GA	1,164	60	30
	Reverse	GGC CAT TGT TTA CTT TGA GC			
GST-Ya	Forward	AAG CCA GGA CTC TCA CTA	408	55	28
	Reverse	AAG GCA GTC TTG GCT TCT			
GST-Yb_1	Forward	AAG ACC ACA GCA CCA GCA CCA T	301	55	30
	Reverse	CTC TCC TCC TCT GTC TCT CCA T			
GST-Yc	Forward	GGA AGC CAG TCC TTC ATT ACT	621	55	28
	Reverse	CGT CAT CAA AAG GCT TCC TCT			
GST-Yp_1	Forward	ATG CTG CTG GCT GAC CAG GGC	576	60	25
	Reverse	ATC TTG GGC CGG GCA CTG AGG			
UGT1a6	Forward	TGA TGC TCT GTT CAC AGA CC	669	55	25
	Reverse	AAT GCC CGA GTC TTT GGA TG			
GCLS	Forward	AGG AGC TTC GGG ACT GTA TT	598	55	25
	Reverse	TGG GCT TCA ATG TCA GGG AT			
HO-1	Forward	GAG CAG AAC CAG CCT GAA CTA	200	55	25
	Reverse	GGT ACA AGG AAG CCA TCA CCA			
SOD1	Forward	ATC CAC TTC GAG CAG AAG	340	55	30
	Reverse	TTC CAC CTT TGC CCA AGT			
SOD2	Forward	AGC GGT CGT GTA AAC CTC A	439	55	30
	Reverse	AGA CAT GGC TGT CAG CTT C			
SOD3	Forward	GGT GCA GAG AAC CTC AGC C	518	55	30
	Reverse	TGC GCA CCA CGA AGT TGC C			
GPx1	Forward	AGT ACG GAT TCC ACG TTT GA	533	55	35
	Reverse	GGA ACT TCT CAA AGT TCC AG			
GPx2	Forward	GAC TTC AAT ACG TTC AGA GG	390	55	35
	Reverse	GGA ACT TCT CAA AGT TCC AG			
GR	Forward	CTT CCT TCG ACT ACC TGG	400	55	35
	Reverse	ATG CCT GCG ATC TCC ACA			
Catalase	Forward	AAT CCT ACA CCA TGT CGG ACA	726	55	28
	Reverse	CGG TCT TGT AAT GGA ACT TGC			
β-Actin	Forward	GTG GGC CGC TCT AGG CAC CA	341	57	25
	Reverse	CGG TTG GCC TTA GGG TTC AGG			

3). β-Actin mRNA expression was not significantly different between genotypes or exposures (data not shown). Hyperoxia significantly increased the mRNA expression for NQO1 (48 and 72 h), GST-Ya (72 h), UGT (72 h), GPx2 (48 and 72 h), and HO-1 (48 and 72 h) in the $Nrf2^{+/+}$ mice over basal levels (Figure 3). The induced gene levels of all these enzymes as well as basal mRNA levels of NQO1 and UGT in the $Nrf2^{+/+}$ mice were significantly higher than those in the $Nrf2^{-/-}$ animals, although UGT and HO-1mRNAs were also inducible in the $Nrf2^{-/-}$ mice after hyperoxia (Figure 3). The steady-state expression level of GST-Yc mRNA was upregulated by hyperoxia exposure in the $Nrf2^{-/-}$ mice but not in the *wt* mice. However, both baseline and induced mRNA levels of GST-Yc in the *wt* mice were significantly greater than those in similarly exposed $Nrf2^{-/-}$ mice (Figure 3). Northern blot analyses were used to confirm small, but statistically significant, differences in enzyme gene expression as detected by reverse transcriptase polymerase chain reaction (RT-PCR) (data not shown).

The differential mRNA expression of all antioxidant defense enzymes assessed between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice

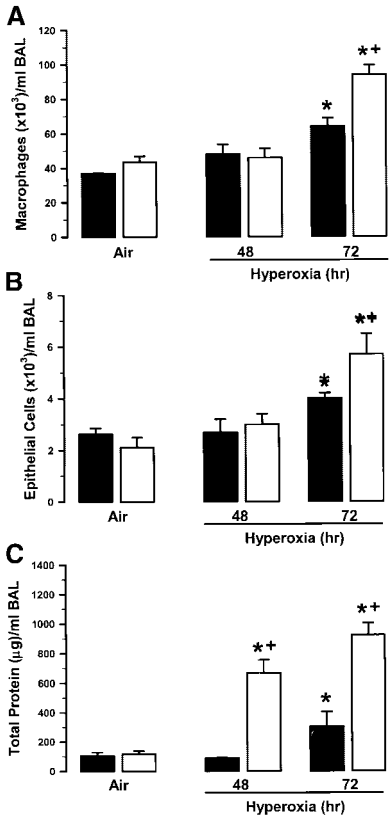

Figure 1. Effect of targeted gene disruption (knockout) of *Nrf2* on hyperoxia-induced changes in lung macrophages (*A*), epithelial cells (*B*), and total protein (*C*). Data are presented as means ± SEM ($n = 4$ per group). *Significantly different from genotype-matched air-control mice ($P < 0.05$). +Significantly greater than time-matched $Nrf2^{+/+}$ mice ($P < 0.05$). *Solid bars*, $Nrf2^{+/+}$; *open bars*, $Nrf2^{-/-}$.

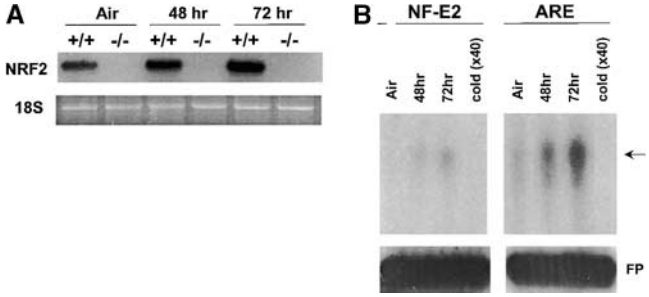

Figure 2. (*A*) The expression of NRF2 mRNA in the lungs of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice after 48 and 72 h exposure to either air or hyperoxia. Aliquots of total-lung RNA isolated from each mouse were pooled for each group ($n = 4$ mice/group), and 2.38-kb NRF2 mRNA was detected by Northern blot analysis. (*B*) EMSA of lung nuclear proteins for NF-E2- and ARE-binding activity in $Nrf2^{+/+}$ mice exposed to either air or hyperoxia (48 and 72 h). Nuclear protein extracts from pooled lung homogenates (2 μg) of mice exposed to room air or hyperoxia ($n = 4$ mice per group) were incubated with an end-labeled oligonucleotide probe containing an NF-E2 or ARE consensus sequence. A reaction mixture was also incubated with 40-fold excess amount of each unlabeled probe to determine the specificity of NF-E2 or ARE binding proteins in the nuclear extracts, which resulted in an elimination of the protein-DNA binding activities (cold lane). *Arrow* indicates shifted bands (NF-E2– or ARE-protein complex). FP indicates free probes.

exposed to either hyperoxia or air are summarized in Figure 4. In addition to those discussed above, the steady-state levels of mRNAs for GCLS (48 and 72 h), GPx1 (72 h), and GR (72 h) were markedly enhanced in the $Nrf2^{+/+}$ mice by hyperoxia, whereas the mRNA level for SOD2 (48 and 72 h) was significantly elevated only in the $Nrf2^{-/-}$ mice (*see* Figure 4). However, no significant differences in the abundance of these enzyme mRNAs were detected between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice. No statistically significant effects of either hyperoxia or genotype were found on the mRNA levels of GST-Yb_1 and SODs 1 and 3 (*see* Figure 4).

Differential Activities of Lung Phase 2 Enzymes Between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ Mice

Basal NQO1 activity was 50% greater in the lungs of $Nrf2^{+/+}$ mice compared with $Nrf2^{-/-}$ mice (Figure 5A). Hyperoxia significantly enhanced NQO1 activity in lung cytosol of $Nrf2^{+/+}$ mice at 48 and 72 h (55 and 74%, respectively). Hyperoxia did not change lung NQO1 activity in $Nrf2^{-/-}$ mice. Furthermore, NQO1 activity in hyperoxia-exposed $Nrf2^{-/-}$ mice was significantly lower than that in similarly exposed $Nrf2^{+/+}$ mice.

The total GST activity measured in *wt* mice exposed to either air or hyperoxia (72 h) was significantly higher (1.6- to ∼ 2.2-fold) than that measured in the corresponding $Nrf2^{-/-}$ mice (Figure 5B). No exposure-induced changes were observed in the total GST activity in the lungs of both genotypes of mice.

Discussion

We have demonstrated that NRF2 contributes to the protection against hyperoxic lung injury in mice. Compared with *wt* mice, mice lacking NRF2 expression and activity had significantly enhanced lung damage characterized by increased protein permeability, macrophage inflammation, and epithelial injury after hyperoxia exposure. Upregulation of NRF2 mRNA and increased DNA binding of nuclear NRF2 was found in the lungs of *wt* mice in response to hyperoxia. Furthermore, significant attenuation in basal and/or hyperoxia-induced mRNA expression of NQO1, GST-Ya and -Yc (which compose the class α GST in rodents), UGT, HO-1, and GPx2 was observed in the lungs of mice deficient in *Nrf2*, relative to the *wt* mice. This suggests that these enzyme genes are downstream effector molecules transcriptionally activated by NRF2 in the lungs of mice. NRF2-mediated pulmonary protection against hyperoxia may be attributed at least in part to these enzymes.

Previous studies using *Nrf2*-knockout mice and *Nrf2*-transfected or -deficient cell lines demonstrated that NRF2, in association with other transcription factors such as c-Jun and small Maf, plays an essential role in preventing carcinogenesis of cells or tissues (e.g., liver) (17, 21, 30, 31). This activity is thought to occur via ARE-mediated induction of phase 2 detoxifying enzymes including NQO1, GST, or GCLS. Chan and Kan have suggested a protective role of NRF2 against butylated hydroxytoluene (BHT) through the

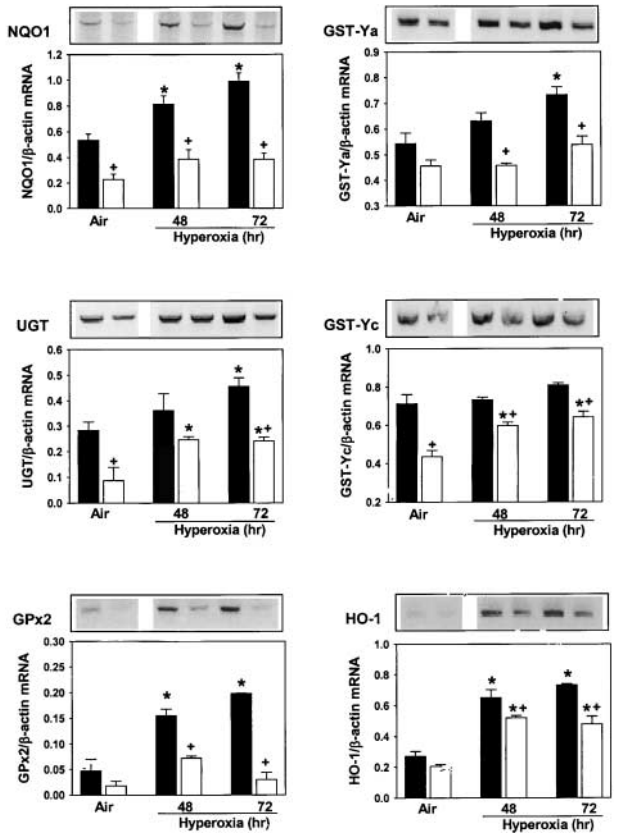

Figure 3. Differential expression of antioxidant defense enzymes in the lungs of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice exposed to either air or hyperoxia (48 and 72 h). Total-lung RNA was isolated from each mouse, and each enzyme cDNA was amplified by RT-PCR using specific primers as indicated in Table 1 and separated on ethidium bromide-stained 1.2 to ~ 2% agarose gel. Digitized images of cDNA bands from each mouse were quantitated using a Gel Doc Analysis System and normalized to β-actin cDNA (an internal control). All data are presented as the group means ± SEM (n = 4 mice per group). Representative agarose gel images for each enzyme are shown on top of the graphs, and the order of individual cDNA bands corresponds to that of graph bars. *Significantly different from genotype-matched air-control mice ($P < 0.05$). +Significantly different from exposure and time-matched $Nrf2^{+/+}$ mice ($P < 0.05$). *Solid bars*, $Nrf2^{+/+}$; *open bars*, $Nrf2^{-/-}$.

activation of pulmonary antioxidant defense enzymes (19). These investigators demonstrated that *Nrf2*-knockout mice exposed to BHT had more severe acute lung injury and lower levels of lung mRNA transcripts for antioxidant defense enzymes including NQO1, UGT, catalase, and SOD1 than similarly exposed *wt* mice. The potential contribution of NRF2 in oxidative tissue injury has been demonstrated in a study by Ishii and colleagues (22), who reported that peritoneal macrophages isolated from electrophile-susceptible *Nrf2*-knockout mice had impaired mRNA induction of HO-1, A170, and peroxiredoxin MSP23. They concluded that NRF2 is a key transcription factor for oxidative stress-inducible proteins. The present study, to our knowledge, is the first to demonstrate a protective role of NRF2 in oxidative tissue injury of the lungs.

We have determined that both basal and hyperoxia-inducible NQO1 expression are NRF2 dependent in the murine lungs. In addition, transcriptional regulation of GST isozyme α and UGT was also at least in part mediated through NRF2 in this model. Consistent with their NRF2-dependent gene expression patterns, enzyme activities for lung NQO1 were significantly higher in *wt* mice than in $Nrf2^{-/-}$ mice. Total GST activity, which is attributed to all isoenzymes (e.g., α, μ, π, and θ), was also significantly higher in *wt* mice than in $Nrf2^{-/-}$ mice following air or hyperoxia exposure. Although the total GST activity does not discriminate between the contributions of the various isoenzymes, results largely reflected the mRNA expression pattern of α GST (composed of Ya and Yc subunits; *see* Figure 3). The antioxidant role of phase 2 detoxifying enzymes has been widely examined in cells and several tissues due to their protection against toxic and neoplastic effects of electrophilic metabolites or ROS generated by chemical carcinogenes or xenobiotics (14, 15). Moreover, as a component of the glutathione redox system, GST has been postulated to provide protection to the lung from oxidative injury induced by toxicants (32). However, only one previous study has investigated the contribution of phase 2 enzymes to the hyperoxic lung injury in laboratory animals (33). In that study, increased pulmonary NQO1 activity by pretreatment with 3-methylcholanthrene and BHT did not significantly improve the survival rate of rats exposed to hyperoxia. Our observations suggest that in addition to conjugating reactive electrophilis or xenobiotics, phase 2 detoxifying enzymes may also exert indirect antioxidant functions in the hyperoxic lungs of mice. However, functional analyses are necessary to establish their importance in the pathogenesis of oxidative lung injury.

Accumulating evidence has suggested that the microsomal enzyme HO-1 is highly inducible as a protective mechanism by various oxidative stresses, including hyperoxia (13, 34) and electrophiles that induce phase 2 enzymes (35). Recent *in vitro* studies (20, 36) and an *in vivo* study using *Nrf2* gene-knockout mice (22) have determined that NRF2 upregulates ARE-mediated HO-1 expression. The present study demonstrated that hyperoxia-inducible lung HO-1 expression is partially mediated through NRF2. Interestingly, we also observed a significant induction of HO-1 mRNA in the $Nrf2^{-/-}$ mice by hyperoxia challenge, which could be explained by evidence indicating that either NF-κB (37) or AP-1 (38) plays a role in the transcriptional regulation of HO-1.

Among lung classical antioxidant enzymes, regulation of mRNA for GPx2, a recently identified isoform of cellular GPxs in the gastrointestine of rodents (39), was largely NRF2-dependent in the lungs of hyperoxia-exposed mice. To date, only GPx1 has been widely investigated as the representative isoform of cellular GPx in the lungs of laboratory animals. However, a study using a mouse model with targeted disruption of GPx1 demonstrated that the hyperoxic survival rate was not increased in GPx1-deficient mice (40). The results from this and our current study suggest an important role of GPx2 as a critical component of pulmonary antioxidant defense system. We found that catalase and SOD (1, 2, and 3) mRNA expression were not dependent on NRF2 in the hyperoxic lungs. It is likely

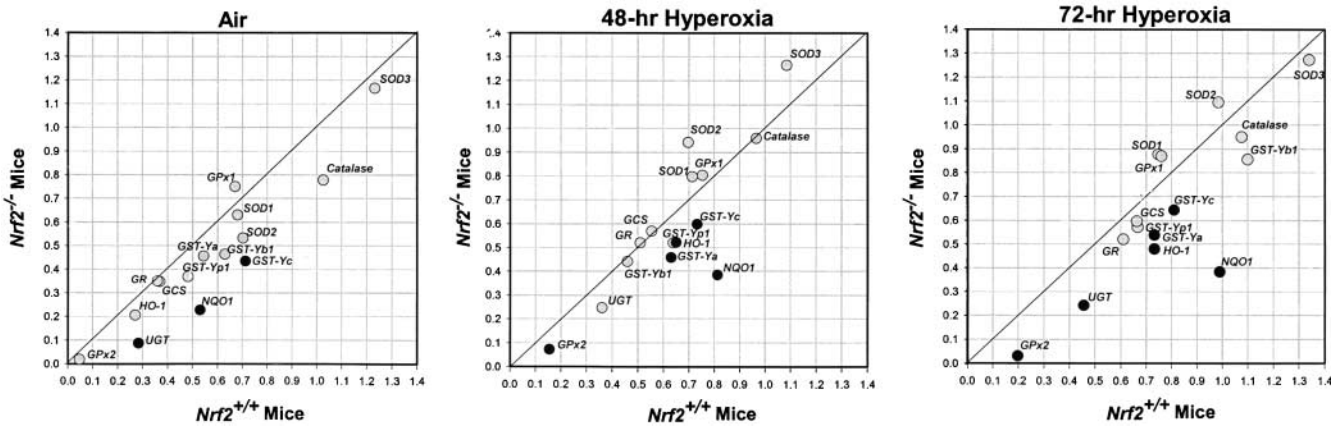

Figure 4. Ratio of mean ($n = 4$ per group) mRNA levels for lung antioxidant defense enzymes determined by semi-quantitative RT-PCR to elucidate genotype ($Nrf2^{+/+}$, $Nrf2^{-/-}$) effects on each enzyme gene expression after air or hyperoxia (48 and 72 h) exposure. *Black circles* show enzymes of which mRNA levels vary significantly between two genotypes, and *gray circles* show others.

that the contribution of NRF2 to the induction of these lung antioxidant enzymes may be very limited in the protection against oxygen toxicity. These observations are inconsistent with the results from a previous study in which BHT treatment induced mRNA expression of SOD1 and catalase via NRF2 (19).

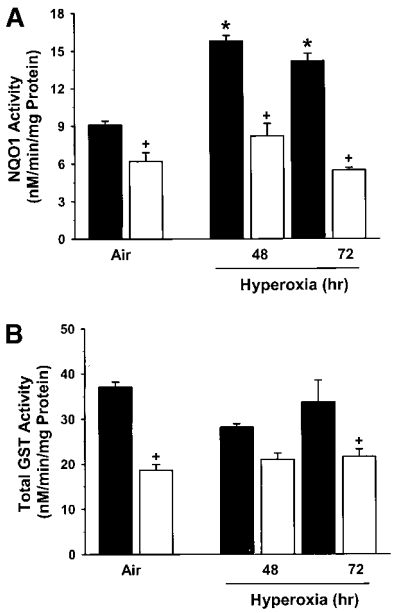

Figure 5. Differential enzyme activity of NQO1 and total GST in the lungs of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice exposed to either air or hyperoxia (48 and 72 h). Cytosolic protein was prepared from pooled lung homogenates of each group. Enzymatic activity of NQO1 in 30-μg protein was measured spectrophotometrically using 2,6-dichloroindophenol as the electron acceptor in the presence or absence of dicoumarol (*A*). Enzymatic activity of total GST in 45-μg protein was measured spectrophotometrically using CDNB as the enzyme substrate (*B*). Data are presented as the group means ± SEM from three separate measurements. *Significantly higher than genotype-matched air-control mice ($P < 0.05$). +Significantly lower than exposure and time-matched $Nrf2^{+/+}$ mice ($P < 0.05$). *Solid bars*, $Nrf2^{+/+}$; *open bars*, $Nrf2^{-/-}$.

The present study also demonstrates that hyperoxia exposure enhances expression of NRF2 mRNA and functionally activated nuclear NRF2 in the lungs of normal (*wt*) mice. The regulatory mechanisms of NRF2 have been largely unknown with the exception of Keap1, a cytoplasmic chaperone that suppresses NRF2 transcriptional activity by specific binding to the N-terminal regulatory domain (Neh2) of NRF2 (41). Ishii and colleagues (22) postulated that NRF2 may be activated at the posttranslation level, probably by deactivation of Keap1 and, in turn, induction of NRF2 nuclear translocation. However, upregulation of the liver NRF2 mRNA level and a subsequent increase of nuclear NRF2 translocation has been reported in a recent *in vivo* study with mice treated with a cancer chemoprotective agent (42). This recent investigation and our present observation provide new understanding of the regulatory mechanisms of NRF2.

In conclusion, we determined that NRF2 plays a significant role in the protection against hyperoxic pulmonary injury in mice possibly by transcriptional activation of lung antioxidant defense enzymes. The results from our study add a potential protective mechanism through NRF2 and a putative role of phase 2 detoxifying enzymes as indirect antioxidants in oxidative lung injury.

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