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Forum Review

Nrf2 Defends the Lung from Oxidative Stress

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ABSTRACT

Nuclear factor, erythroid 2 related factor 2 (Nrf2) belongs to the Cap'n'collar/basic region leucine zipper (CNC-bZIP) transcription factor family, and is activated by diverse oxidants, pro-oxidants, antioxidants, and chemopreventive agents. After phosphorylation and dissociation from the cytoplasmic inhibitor, Kelch-like ECH-associated protein 1 (Keap1), Nrf2 translocates to the nucleus and binds to an antioxidant response element (ARE). Through transcriptional induction of ARE-bearing genes that encode antioxidant-detoxifying proteins, Nrf2 activates cellular rescue pathways against oxidative injury, inflammation/immunity, apoptosis, and carcinogenesis. ARE-driven genes include direct antioxidants (e.g., GPx), thiol metabolism-associated detoxifying enzymes (e.g., GSTs), stress-response genes (e.g., HO-1), and others (e.g., PSMB5). Application of *nrf2* germ-line mutant mice elucidated protective roles for Nrf2 in various models of human disorders in the liver, lung, kidney, brain, and circulation. In the lung, deficiency of *nrf2* augmented injury caused by bleomycin and environmental oxidants including hyperoxia, diesel exhaust particles, and cigarette smoke. Microarray analyses of lungs from *nrf2*-deficient and -sufficient mice identified Nrf2-dependent genes that might be critical in pulmonary protection. Observations from these studies highlight the importance of the Nrf2-antioxidant pathway and may provide new therapeutic strategies for acute respiratory distress syndrome, idiopathic pulmonary fibrosis, cancer, and emphysema in which oxidative stress is implicated. *Antioxid. Redox Signal.* 8, 76–87.

OXIDATIVE STRESS AND ANTIOXIDANT RESCUE SYSTEMS

Molecular oxygen (O_2) and its homeostasis are essential for the survival of all aerobic organisms. Under normal physiological conditions, partially reduced oxygen metabolites including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{-\bullet}$), and hydroxyl radical ($OH^\bullet$) are generated as metabolic by-products. These reactive oxygen species (ROS) damage cellular macromolecules (i.e., DNA, lipids, proteins), and trigger generation of peroxidation products (e.g., organic and inorganic ROS, DNA adducts, lipid peroxides). To limit the potential toxicity of ROS, cellular and extracellular enzymatic or nonenzymatic antioxidant systems cope with the oxidative attacks. Excess ROS, however, can overwhelm antioxidant capacity to perturb the balance in this reduction-oxidation (redox) equilibrium, and eventually lead to oxidative stress of cells and tissues. Growing evidence has correlated increased oxidative stress with pathogenesis of various human diseases including cancer, atherosclerosis, ischemia-reperfusion injury, neurodegenerative disorders, and the aging process, though details of the molecular mechanisms and pathophysiology are not fully understood.

The endogenous cellular antioxidant defense system consists of a number of proteins (e.g., enzymes) and small molecules (e.g., vitamins C and E) that maintain the 'reducing' environment of the body. Among these, classical antioxidant enzymes inactivate ROS and prevent ROS-initiated reactions.

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These 'direct' antioxidants include superoxide dismutases (SODs), catalase, and glutathione peroxidase (GPx). Other traditional antioxidants include two biologically important small thiol-containing compounds, glutathione (GSH) and thioredoxin (Trx). Both participate in antioxidant defense by serving as substrates for antioxidant enzymes such as GPx and Trx peroxidase in redox cycles. They are easily oxidized and rapidly regenerated by *de novo* synthesis or replaced through enzymatic reduction of their disulfide. Due to the relative abundance of intracellular GSH (millimolar range) compared to Trx (micromolar range), the ratio of GSH to GSH disulfide (2GSH:GSSG) has often served as a parameter of cellular redox status. Trx is located in the inner mitochondrial membrane where it scavenges ROS and is also known to activate mitochondrial antioxidants such as SOD2 (39). Overexpression of *Trx1* or exogenous administration of Trx increases protection against oxidative stress and inflammation (73, 115).

Phase 2 detoxifying enzymes are classified as 'indirect' antioxidant enzymes due to their role in redox balance and thiol homeostasis. They contribute to biosynthesis/recycling of thiols or facilitate excretion of oxidized, reactive secondary metabolites (e.g., quinines, epoxides, aldehydes, peroxides) through reduction/conjugation reactions during xenobiotic detoxification. Phase 2 enzymes include glutathione-*S*-transferase (GST) isozymes, NADP(H):quinone oxidoreductase (NQO1), heavy (catalytic) and light (modifier) subunits of γ-glutamyl cysteine ligase (GCLc, GCLm), γ-glutamyltranspeptidase (GGT), UDP-glucuronyl transferase (UGT), Trx reductase (TXNRD), and Trx peroxidase (or peroxiredoxin, Prx). In addition, stress response proteins such as heme oxygenase (HO)-1 and ferritin (FTH and FTL, heavy and light chains) are cytoprotective against various oxidant or pro-oxidant insults (83, 104). HO-1 presumably protects through degradation of the pro-oxidant heme molecule, a process that also generates the antioxidative products, carbon monoxide and bilirubin, with release of iron (II). Ferritin is inducible by iron (II) and exerts its antioxidant function by sequestering iron from participation in free radical formation.

ANTIOXIDANT RESPONSE ELEMENT (ARE) AND Nrf2

Phagocytic cells (e.g., neutrophils, macrophages) and a variety of nonphagocytic cells produce ROS via NADPH oxidase, a membrane-bound enzyme complex, or other oxidases including cytochrome P450 and lipoxygenase (102, 103). H_2O_2 and $O_2^{-\bullet}$ activate signal transduction through receptor protein tyrosine kinase and protein tyrosine phosphatase (23, 107), although the precise mechanisms of ROS-mediated signaling are not fully understood. The activator protein (AP)-1 and nuclear factor (NF)-κB families of transcription factors are generally considered as the most critical downstream components of redox-sensitive signal transduction. AP-1 members, including Jun, Fos, and Fra proteins, are basic region/leucine zipper (bZIP) transcription factors that bind to 12-*O*-tetradecanoyphorbol-13-acetate (TPA) responsive elements (TRE, TGACTCA). Various AP-1 proteins are known to regulate antioxidant enzymes including GCL, SOD2, and HO-1 (68, 90).

Rushmore *et al*. (95) identified a TRE-like, but distinct, *cis*-acting sequence in the rat GST-A2 gene promoter, and named it the antioxidant response element (ARE, GTGACNNNGC). A 41-base pair enhancer sequence named the electrophile response element (EpRE) was simultaneously discovered in the mouse GST-Ya gene (32); the EpRE is now referred to as ARE due to homology of their core sequences. Because AREs from multiple antioxidant genes contained two or more TRE and TRE-like elements (70, 87), AP-1 proteins had been predicted to be the ARE-binding transcription factors.

Although AP-1 proteins do, in part, mediate ARE activity, the principal ARE-binding proteins are nuclear factor-erythroid 2 (NF-E2) related factors 1 and 2 (Nrf1, Nrf2) which belong to the Cap'n'collar (CNC)-bZIP transcription factor family. Unlike NF-E2, the expression of which is restricted to hematopoietic tissues (i.e., erythroid cells, megakaryocytes), Nrf1 and Nrf2 are expressed in multiple tissues (48, 84); murine Nrf2 is most abundant in the intestine, lung, and kidney where detoxification reactions occur routinely (47). Due to high similarity of the NF-E2 binding motif (TGCTGAG/CTCAT/C) to ARE, studies have associated CNC-bZIP transcription factors in ARE-mediated detoxification responses. Nrf1 seems to be essential for development and cell survival, probably by maintaining redox balance (13), but embryonic lethality of *nrf1* knockout (*nrf1*$^{-/-}$) mice precluded further functional analyses. Investigations with *nrf2*$^{-/-}$ mice found that Nrf2 is critical in regulation of ARE-mediated antioxidant and detoxifying enzyme expression (15, 47). Nrf3 is a newly cloned CNC family transcription factor (61) and a negative regulatory role for Nrf3 was reported for the NQO1 ARE response (96).

MOLECULAR ASPECTS OF Nrf2 ACTIVATION FOR ARE RESPONSE

Kelch-like ECH-associated protein 1 (Keap1) is a cytoplasmic, cysteine-rich, actin-bound protein that represses Nrf2 in mouse, human, and zebrafish (49, 62); the rat homologue is termed INrf2 (25). In unstressed cells, Keap1 sequesters Nrf2 in the cytoplasm by binding to the N-terminal Neh2 domain, and thus prevents nuclear accumulation, analogous to the IκB-NF-κB regulatory system. The role of Keap1 has been elucidated in *keap1*$^{-/-}$ mice in which constitutive transactivation of Nrf2 and overproduction of its target genes lead to esophagus/forestomach hyperkeratosis (109). Because the Keap1•Nrf2 complex is considered a key cellular sensor of oxidative stress, the regulatory mechanisms of Keap1-dependent turnover and translocation of Nrf2 have been investigated with great interest. The Kelch repeat domain (Kelch1-Kelch6) of Keap1 binds directly to Nrf2 and actin filaments for cytoplasmic sequestration of Nrf2 (50, 56). The N-terminal BTB/POZ domain of Keap1 may mediate homomeric or heteromeric dimerization of Keap1 (120). In addition, cysteine residues of Keap1 (e.g., C259, C273, C288, C297) in the intervening region between BTB and Kelch repeat domains are known to have a role in Keap1•Nrf2 complex formation (26, 117). Keap1 may also regulate rapid proteasomal

degradation of Nrf2 (51, 77). Moreover, it is suggested that disruption of actin cytoskeleton is required to dysregulate Keap1 for nuclear accumulation of Nrf2 (56), and phosphatidylinositol 3-kinase contributes to depolymerization of actin in response to a pro-oxidant tBHQ (55). Indeed, accumulating evidence indicates that Nrf2 is activated by phosphorylational modification via several protein kinase pathways, which eventually leads to Keap1•Nrf2 dissociation, nuclear Nrf2 translocation, and ARE responsiveness. This topic is discussed in more detail by Jeong, Jun, and Kong (see p. 99, this issue).

Nrf2 must form a heterodimer with other bZIP transcription factors for ARE binding and target gene expression. In complex with Nrf2, c-Jun (54) and activating transcription factor (ATF)-4 (38) positively regulate ARE-mediated induction, while c-Fos and Fra-1 (108) are negative regulators. The direction of ARE regulation by small Nrf2•Maf heterodimers, however, varies depending on cell type, stimuli, or target gene (24, 47, 60). Furthermore, coactivator proteins CREB binding protein (CBP)/p300 and ARE-binding protein-1 are also presumed to interact with Nrf2•Maf to regulate ARE–dependent gene expression (119). More recent observations suggest that Nrf2 interacts with other transcription factors such as peroxisome proliferator-activated receptor (PPAR)-gamma and the retinoic acid X receptor (RXR) on ARE and PPAR-response element (PPRE), respectively, for downstream antioxidant gene regulation (45, 86).

Identification of an ARE-like motif within the murine Nrf2 promoter suggests autoregulation of Nrf2 (63), a mechanism supported by the observed transcriptional modulation of Nrf2 in some models (16, 64). Similarly, certain small Maf genes (e.g., *mafG*, *mafF*) are known to have Nrf2•maf-responsive AREs in their promoters (57, 93), also suggestive of feedback regulation of Nrf2-ARE pathways.

FUNCTIONAL ROLE OF Nrf2-ARE RESPONSES FOR CYTOPROTECTION

Fahey and Talalay (29) described nine classes of chemical agents that induce thiols and phase 2 defense enzymes *in vivo* (primarily in liver) and *in vitro*. These are mainly electrophiles (e.g., trivalent arsenicals, divalent heavy metals such as cadmium), pro-oxidants/oxidants (e.g., H_2O_2), chemoprotectors (e.g., isothiocyanates including sulforaphane from broccoli, 1,2,-dithiole-3-thiones [D3T], oltipraz), or phenolic antioxidants (e.g., β-naphthoflavone [β-NF], butylated hydroxyanisole [BHA], *t*BHQ, green tea polyphenols), and have been widely applied for functional analysis of the Nrf2-ARE pathway.

In vitro analyses with these putative Nrf2 inducers identified functional AREs in many antioxidant genes including those encoding GSTs, NQO1, HO-1, GCLc, GCLm, UGT 1a6, and SODs 1 and 2. Nrf2 overexpression studies (78, 98) or use of primary cells from *nrf2*$^{+/+}$ and *nrf2*$^{-/-}$ mice (12, 37, 45, 46) further validated a role for Nrf2 in ARE-mediated activation of these antioxidant-defense genes. In addition to these well-documented antioxidant proteins, Trx, TXNRD1, MSP 23 (mouse Prx1), A170 (ubiquitin-and PK-Cζ-binding protein), MRP1/ABCC1 (multidrug resistance-associated protein), CD36 (scavenger receptor for uptaking oxidized low-density lipoproteins), xCT (cysteine/glutamate exchange transporter), ferritin, epoxide hydrolase, GPx2, and PSMB5 (a proteasome subunit) have been suggested as Nrf2-ARE responsive gene products (45, 46, 65). Complementary DNA microarray analyses using *nrf2*$^{-/-}$ mice or cells have profiled novel tissue (cell)-specific genes regulated by Nrf2 in the liver (by D3T), small intestine (by sulforaphane), lung (by cigarette smoke, hyperoxia), and neuronal cells (by BHQ) (18, 42, 66, 93) which are potentially important in Nrf2-mediated cytoprotection.

Exogenous or metabolically generated electrophiles and ROS are believed to be principal carcinogens. Overexpression of antioxidant enzymes (e.g., GSTs, NQO1, UGT, HO-1) has been reported in variety of cancers (99). In addition, a large body of evidence indicates that polymorphisms in these antioxidant genes are associated with risk of cancers in multiple tissues including bladder, lung, gastrointestinal tract, and prostate (71, 75, 81). These observations suggest that activation of antioxidant and detoxification protein may be a highly effective strategy for reducing susceptibility to carcinogens. In support of this concept, a relationship between consumption of cruciferous vegetables, a source of multiple, potent Nrf2 inducers, and decreased cancer risk was reported in prostate, lung, bladder, and breast cancer (74, 100). In rodent models, sulforaphane and a number of other isothiocyanates have effectively blocked chemical carcinogenesis (34, 118). A chemopreventive role for Nrf2 has been identified using *nrf2*$^{-/-}$ mice (91, 92). For example, pretreatment with the phase 2 enzyme inducer oltipraz significantly attenuated (>50%) multiplicity of benzo[a]pyrene-induced gastric (forestomach) tumor and DNA-adduct formation in *nrf2*$^{+/+}$ mice while there was no effect of oltipraz on tumorigenesis in *nrf2*$^{-/-}$ mice. In addition, these knockout mice are intrinsically more susceptible to carcinogens, and have suppressed induction of phase 2 enzymes. Similarly, sulforaphane blocked benzo[a]pyrene-induced forestomach cancer formation in *nrf2*$^{+/+}$ mice but not in *nrf2*$^{-/-}$ mice (28). Compared with wild-type mice, *nrf2*$^{-/-}$ mice are also more susceptible to urinary bladder cancer induced by *N*-nitrosobutyl(4-hydroxylbutyl)amine (42). Further, oltipraz decreases tumor incidence in *nrf2*$^{+/+}$ mice, but not in *nrf2*$^{-/-}$ mice. Collectively, these studies suggest that identification and administration of dietary chemoprotectors, including various constituents of cruciferous plants, have important implications in carcinogenic risk (see article by Jeong and Kong).

PROTECTIVE ROLE OF Nrf2 IN THE LUNG

Airway antioxidants and their therapeutic use

Redox balance is particularly important in the airways because they are the first points of contact with airborne oxidants such as environmental ozone, particles, and cigarette smoke. Inhaled oxidants interact primarily with the epithelial lining fluid (ELF), which contains surfactant, antioxidants, other proteins and unsaturated lipids. In addition to ELF, abundant cellular and extracellular antioxidants exist throughout the airway tissues to counteract oxidative burden.

ROS have been implicated in the pathogenesis of many acute and chronic principal pulmonary diseases including idiopathic pulmonary fibrosis (IPF), asthma, emphysema, cystic fibrosis, bronchopulmonary dysplasia (BPD), acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), and lung malignancies (31, 36, 97). Depletion of lung GSH and alteration of other antioxidants have been observed often in specimens from individuals inflicted with such diseases (69). These patients have been subjected to a variety of antioxidant therapies including supplementation with vitamins C and E or *N*-acetylcysteine (a GSH precursor), or intravenous treatment of polyethylene glycol-conjugated SOD and catalase (5, 97). To date, however, the benefits of these therapies have been equivocal at best.

Hyperoxic lung injury and protection by Nrf2

ALI and its most severe form, ARDS, are common adult pulmonary diseases, with reported mortality rates of 35–65% in the United States (112). ARDS is defined by noncardiogenic pulmonary edema, diffuse alveolar inflammation, and respiratory failure in seriously ill patients. Because of similar pathologic features, hyperoxic lung injury induced by >95% O_2 exposure has been studied as an experimental model of ARDS. Hyperoxia causes extensive pulmonary damage characterized by inflammation and death of capillary endothelial and alveolar epithelial cells that result in pulmonary edema and severe impairment of respiratory functions (20). Sufficiently long exposure (>3 days) to hyperoxia is lethal.

Although the precise molecular mechanisms by which hyperoxia produces lung injury remain unresolved, hyperoxic conditions trigger excess production of ROS and induce expression of numerous antioxidant proteins in the lung. SODs, catalase, GPx1, and GSH reductase are the most widely examined classical enzymatic antioxidants in hyperoxia models. A pivotal protective role for several classical antioxidant and defense enzymes has been well documented in hyperoxia-injured lungs by application of genetically-engineered mice. For example, relative to wild-type mice, lung inflammation and damage was attenuated in mice that overexpressed either SOD2 or SOD3 (30, 40). Conversely, SOD2 deficiency rendered adult and developing lung more sensitive to O_2 toxicity (4, 105). Exogenous transfer of SOD1 and catalase genes also provided enhanced lung antioxidant levels and protection from hyperoxic lung toxicity of rats (21). Further, increased lung HO-1 by gene transfer or by a specific inducer (hemoglobin) protected rats from hyperoxic lung injury (82, 101). Although GSH was known to protect the lung from oxidative injury (88), only limited information was available for the roles of thiol-related phase 2 antioxidant enzymes in oxidative lung injury until the identity and function of Nrf2 was clarified. Enhanced mRNA expression and enzyme activity of NQO1 and GCL were elicited by oxidant exposure (e.g., hyperoxia, hydrogen peroxide) in pulmonary tissues or cells (89, 113). However, increased pulmonary NQO1 activity in response to 3-methylcholanthrene or BHT did not significantly improve the survival rate of rats after hyperoxia (113).

To determine the influence of genetic background and characterize the molecular mechanisms underlying hyperoxic lung injury, Hudak *et al.* (41) screened inbred strains of mice for injury in response to >95% O_2 and found that C57BL/6J (B6) and C3H/HeJ (C3) mice were the most susceptible and resistant, respectively. Two independent genome-wide linkage analyses were then carried out using a B6C3F_2 cohort (n = 126) and BXH recombinant inbred strains to determine susceptibility gene(s) (Fig. 1). Both analyses identified a significant quantitative trait locus (QTL), hyperoxia susceptibility locus 1 (*Hsl1*), spanning about 2 cM (45–47 cM) on chromosome 2 (17). The genes in this mouse chromosome 2 QTL and the human homolog (2q31–32) are conserved (Fig. 2A). Candidate susceptibility genes in *Hsl1* include *nrf2* (Fig. 2B). Comparative sequencing analysis of the 5' upstream promoter (about 1 kb) and coding regions of *nrf2* between B6 and C3 mice identified an essential promoter polymorphism at –336 (T or to C substitution), which generated a Sp1 transcription factor binding site on the B6 promoter relative to the C3 promoter (17). Significantly, this polymorphism correlates with attenuated lung expression of Nrf2 mRNA in susceptible B6 mice relative to C3 mice after hyperoxia. Restriction fragment length polymorphism genotypes of T–336C in the B6C3F_2 cohort cosegregated with informative simple sequence length polymorphism genotypes at *Hsl1* QTL, and strongly supported *nrf2* as the susceptibility gene (17).

Proof of concept for *nrf2* as a candidate susceptibility gene in hyperoxic lung injury was determined by comparing responses to hyperoxia in *nrf2*$^{+/+}$ and *nrf2*$^{-/-}$ mice. Relative to

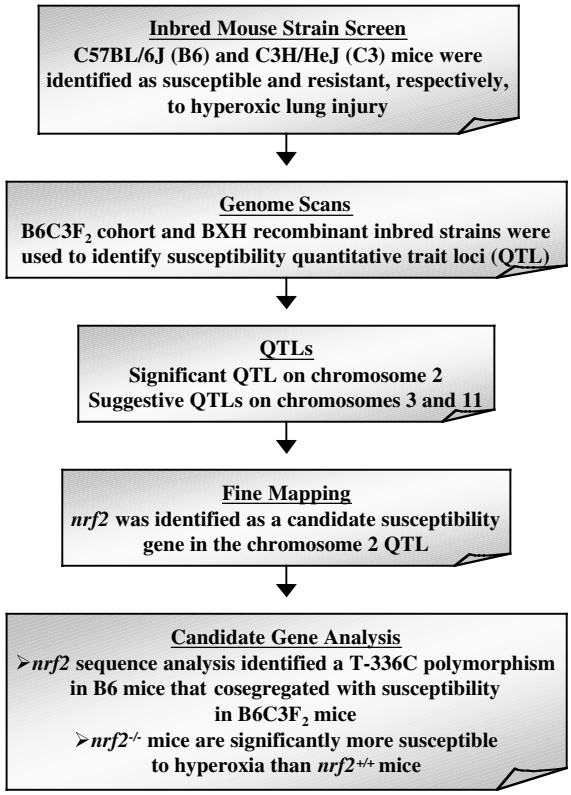

FIG. 1. Procedures used to determine *nrf2* as a candidate susceptibility gene for hyperoxic lung injury in mice.

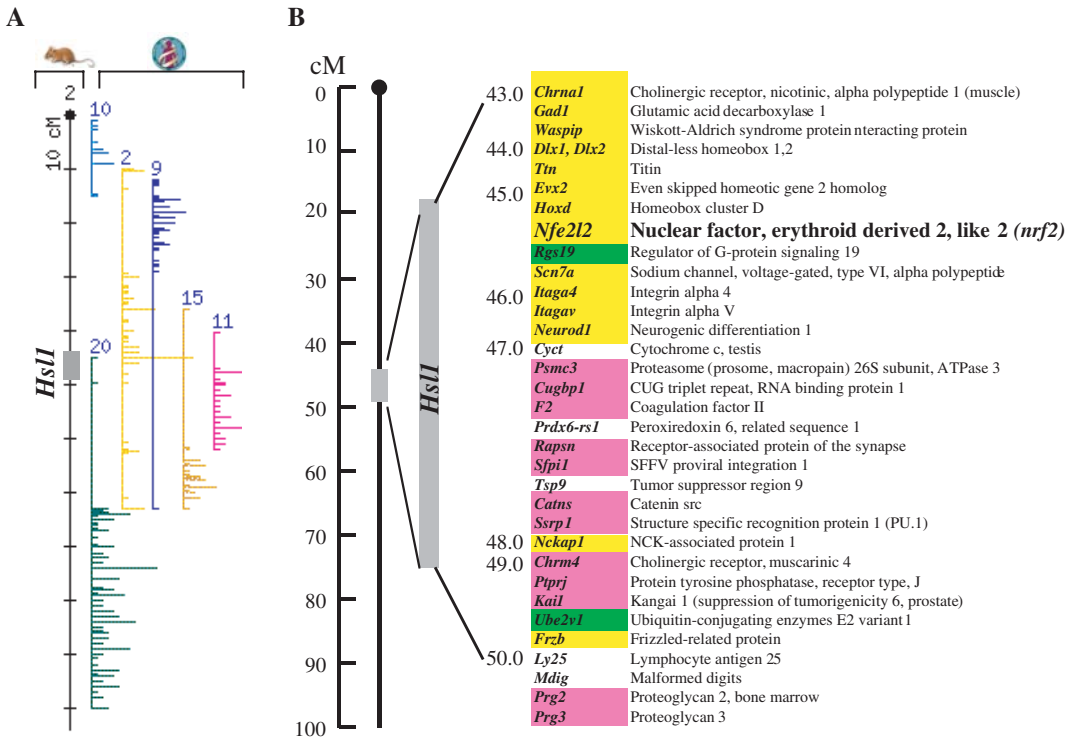

FIG. 2. (**A**) Orthology map indicates homologous regions between mouse chromosome 2 (*black*) and human chromosomes 2, 9, 10, 11, 15, and 20 (http://www.informatics.jax.org/menus/homology_menu.shtml). Each bar in the histogram of human genome is positioned at the location of a nonrecombinant bin in the mouse map. The length of the bar indicates the number of human/mouse orthology found in that bin. (**B**) Genes within and adjacent to *Hsl1* on mouse chromosome 2. Color of the gene name matches with that of human chromosome (A) on which the human homologue is mapped.

wild-type mice, lung hyperpermeability, inflammation, and epithelial cell injury were enhanced in *nrf2*$^{-/-}$ mice (16). Consistent with a protective role for the Nrf2-ARE pathway during lung toxicity by hyperoxia, Nrf2-DNA binding activity and expression of multiple antioxidant enzymes (e.g., GSTs, NQO1, UGT, GCL, HO-1, GPx2) were markedly suppressed in *nrf2*$^{-/-}$ mice. Additional support for the functional association between Nrf2 and antioxidants in the pulmonary airways is provided by the observation that, in lung tissues, Nrf2 exists predominantly throughout the epithelial cells lining large and small airways and alveolar macrophages where many ARE-dependent antioxidant enzymes (e.g., GPx2, GSTs) are localized (18).

Pulmonary fibrosis and protection by Nrf2

Tissue fibrosis is characterized by uncontrolled deposition of extracellular matrix molecules, represented by collagen and elastin, with persistence of fibroblasts/myofibroblasts. It is an irreversible end-state process manifested in many chronic diseases of kidney, liver, and lung. IPF in humans is an example of a disease with unknown etiology and progressively deteriorating respiratory function leading ultimately to death from respiratory failure. It is becoming clear that ROS play a critical role in the pathogenesis of IPF as supported by enhanced alveolar oxidant burden and decreased antioxidants in patients with IPF (9, 11). However, little evidence is available for effectiveness of antioxidant therapy for IPF (22).

Bleomycin has been used as a combined therapy for carcinomas and lymphomas, but bleomycin also causes IPF-like lung fibrosis in susceptible patients. As a model of IPF to investigate underlying molecular mechanisms, numerous investigators have determined protective or injurious effects of cytokines (e.g., interferon-γ, interleukin-10), matrix metalloproteinases (MMPs), and antifibrinolytic system (e.g., tissue inhibitors of metalloproteinases, TIMPs) on bleomycin-induced pulmonary fibrosis. Bleomycin triggers excess production of ROS and DNA damage in the lung due to its mechanism of action as an anticancer drug (44). Pulmonary fibrogenic effects of bleomycin have been antagonized by antioxidant enzymes (e.g., SODs) in rodents (10, 67). Based on these findings, we investigated the role of Nrf2 in lung fibrosis induced by bleomycin administered by intratracheal instillation. Compared to *nrf2*$^{+/+}$ controls, *nrf2*$^{-/-}$ mice were more susceptible to lung inflammation, injury, edema, and fibrogenesis induced by bleomycin (19). Attenuated antioxidant gene expression and augmented fibrosis indices (hydroxyproline content, collagen accumulation, cell proliferation, fibrotic score) and fibrotic marker expression (e.g., transforming growth factors [TGF-βs], tenascin-C) in *nrf2*$^{-/-}$ mice suggested that the Nrf2-mediated antioxidant pathway is essential in limiting bleomycin-induced fibrosis. Interestingly, TGF-β is known to

engage in ROS generation (72), and suppresses transcription of antioxidant genes such as GST, SOD, and GCLm (3, 58) by mediating interaction of Smad3-ATF3 with Nrf2 (7, 52). It is tempting to speculate that the lack of Nrf2 and overproduction of TGF-β synergistically suppress ARE-dependent responses in *nrf2*$^{-/-}$ mice.

Role of Nrf2 in other pro-oxidant-induced lung toxicity

In laboratory animals, the cytoprotective capacity of Nrf2 in other acute pulmonary injury models has been described. In a model of BHT-induced ALI, *nrf2*$^{-/-}$ mice were more susceptible to lung inflammation and toxicity compared with *nrf2*$^{+/+}$ mice (14). *nrf2*$^{-/-}$ mice also had suppressed expression of pulmonary antioxidants including catalase, SOD1, NQO1, and GCLc at baseline and after treatment, and had more severe lung injury and inflammation, relative to similarly treated wild-type mice.

Cigarette smoke is the major factor in the pathogenesis of chronic obstructive pulmonary disease (COPD), and has been considered as the dominant risk factor for lung cancer. A microarray analysis on airway epithelium obtained by bronchoscopy determined significant upregulation of 16 out of 44 tested antioxidant-related genes in smokers compared to nonsmokers (35). In addition, Nrf2 and several detoxifying enzyme genes encoding HO-1, NQO1, and GCLc were found to be involved in nasal and pulmonary pathogenesis of rats exposed to acute (3 h) and subacute (3 h/day, 5 days/week for 3 weeks) mainstream cigarette smoke by microarray analysis (33). *In vitro* exposure to acrolein, a potential electrophilic carcinogen in cigarette smoke, also stimulated Nrf2 activation and transcriptional induction of phase 2 enzymes (NQO1, GCL) in type II lung cells (106). These results suggest a potential involvement of antioxidants and Nrf2 in smoking-induced pulmonary symptoms. In support of these findings, Rangasamy and colleagues (93) recently demonstrated that chronic exposure to cigarette smoke (6 months) causes more severe emphysema symptoms (with oxidative DNA adduct formation, apoptosis, and suppressed antioxidant enzymes) in lungs of *nrf2*$^{-/-}$ mice, compared to those in *nrf2*$^{+/+}$ controls.

Diesel exhaust particles (DEP) are environmental pollutants that contain various pro-oxidants and carcinogens including quinones and polycyclic aromatic hydrocarbons and have been implicated in the pathogenesis of asthma (76). The significance of the Nrf2-ARE signaling pathway and its clinical application to this disease are addressed in the article by Li and Nel (see p. 88, this issue).

Identification of Nrf2-dependent genes in the lung

Comprehensive expression profiles of mRNAs extracted from lungs of *nrf2*$^{+/+}$ and *nrf2*$^{-/-}$ mice identified novel Nrf2-dependent genes modulated by oxidants. Although it is necessary to investigate functional AREs in their promoters, these results provide new and important insight into the molecular basis of Nrf2-mediated protection against oxidative lung injury. For example, exposure to hyperoxia or cigarette smoke caused transcriptional induction of many genes encoding phase 2 defense enzymes such as GSH synthetase (GS), GGT, glucose-6-phosphate dehydrogenase (G6PD), TXNRD1, and Prx in a Nrf2-dependent manner (18, 93). These results suggest a critical contribution of thiol-mediated redox cycle components in pulmonary protection against oxidative stress. The functional relevance of individual Nrf2-inducible antioxidant genes to pulmonary protection has been recently demonstrated in hyperoxia models. For example, targeted disruption of GGT caused more diffuse lung injury and lower survival rate after hyperoxia exposure, compared with wild-type mice (8, 53). Prx is a recently described superfamily of cysteine-bearing nonseleno-peroxidases that catalyze the reduction of a broad spectrum of peroxides using thiols as reductants, and abundantly expressed in the lung (59). The lungs of Prx-deficient mice (*Prx6*$^{-/-}$) exhibited more severe protein edema, epithelial cell injury, and higher level of ROS after hyperoxia (110). Conversely, overexpression of *Prx6* via adenoviral administration protected against hyperoxic lung injury (111). Microarray analysis also revealed that induction of mRNAs for FTL is attenuated in *nrf2*$^{-/-}$ mice, which supports the previous finding that ferritin protects against hyperoxic lung injury (116).

Expression of specific cytochrome P450s (e.g., CYP2D) and non-P450 oxidative enzymes (e.g., aldehyde oxidase-1, the p40^{phox} subunit of NADPH-oxidase, carboxylesterase, alcohol dehydrogenase-3, aldehyde dehydrogenase) was also transcriptionally attenuated in the lungs of *nrf2*$^{-/-}$ mice after hyperoxia or cigarette smoke. Cytochrome P450s are phase I drug (or xenobiotic) metabolizing enzymes with monooxygenase or dehydrogenase function. The CYP1A family contains *cis*-acting xenobiotic response element (XRE, 5'-TGCGTG-3') for their transcriptional activation though aryl hydrocarbon receptor and aryl hydrocarbon nuclear translocator binding while promoters of CYP2B, CYP3A, or CYP4A genes are transcriptionally activated by heterodimers of RXR with the steroid family of orphan receptors (e.g., constitutive androstane receptor) or nuclear hormone receptors (e.g., PPAR-gamma) (94). In addition to hyperoxia, other Nrf2 activators (e.g., oltipraz, Cd, BHA) caused upregulation of CYP1A or CYP2A transcription parallel with ARE-driven defense induction (1, 6, 80). Interestingly, several antioxidants such as NQO1, UGT1a6, and SOD1 possess functional XREs as well as AREs in their promoters, and their XREs have a transcriptional role in response to xenobiotics (6, 79). Lack of Cd-mediated induction in CYP2A5 mRNA in the liver and kidney of *nrf2*$^{-/-}$ mice also demonstrated a role for Nrf2 in CYP expressions (1). Taken together, it is postulated that communication (interaction) or feedback regulation exists between ARE and XRE responses in antioxidant gene induction.

In addition to the antioxidant-defense genes, many novel genes with little known antioxidant function were also modulated by Nrf2 in mouse lungs after oxygen exposure (18). Among these, PPAR-gamma is a transcriptional regulator of cellular differentiation and inflammation against peroxisome proliferators. Importantly, PPAR-gamma is involved in Nrf2 activation and in antioxidant gene (e.g., GST-A2, CD36) induction through dimerization with Nrf2 or RXR on AREs (45, 86). Collectively, these observations are suggestive of feedback regulation between PPAR-gamma and Nrf2. Bacteria binding macrophage receptor with collagenous structure (MARCO, Ly112) contributed to lung defense by binding to unopsonized

inhaled particles (TiO2) and bacteria in mice (2). Nrf2-dependent elevation of MARCO (18) and CD36 (45) by oxidants supports involvement of scavenger receptors in Nrf2-mediated protection against oxidative injury. Nrf2 deficiency also suppressed several tyrosine phosphatase genes (e.g., *Ptpb2, C62, Ptp1*) in the lung after oxidative insults (18, 93), and ARE type sequences from a few of these genes were identified (93). In contrast, induced expression of genes encoding heat shock proteins (e.g., HSP70) and matrix proteins such as collagens (specifically type 1, 4, and 6 alpha, tropoelastin, and vinculin) was potentiated in the absence of Nrf2 after hyperoxia exposure (18). As participation of HSP70 has been previously investigated in lung stress responses against hyperoxia (114), and enhanced matrix production is a marker of airway regeneration and remodeling after injury, elevated expression of these genes in *nrf2*$^{-/-}$ mice is probably a secondary effect caused by more severe injury of *nrf2*$^{-/-}$ mice relative to *nrf2*$^{+/+}$ controls. However, it is also possible that interaction of Nrf2 with other transcriptional corepressors may suppress induction of these genes in oxygen-exposed lungs, as seen by the negative regulation of thromboxane synthase expression by the PPAR-gamma·Nrf2 complex (43).

Upstream molecular mechanism for pulmonary Nrf2 activation

The signaling pathway leading to hyperoxia-induced Nrf2 activation has been partially elucidated. In a murine alveolar epithelial cell line (C10), inhibition of NADPH oxidase or the extracellular signal regulated kinase (ERK) pathway prevented hyperoxia-induced Nrf2 translocation and ARE-mediated transcriptional activation (85). Consistent with this observation, Nrf2 phosphorylation after hyperoxic insult was not observed in ERK1-deficient cells. These results indicate that ROS gen-

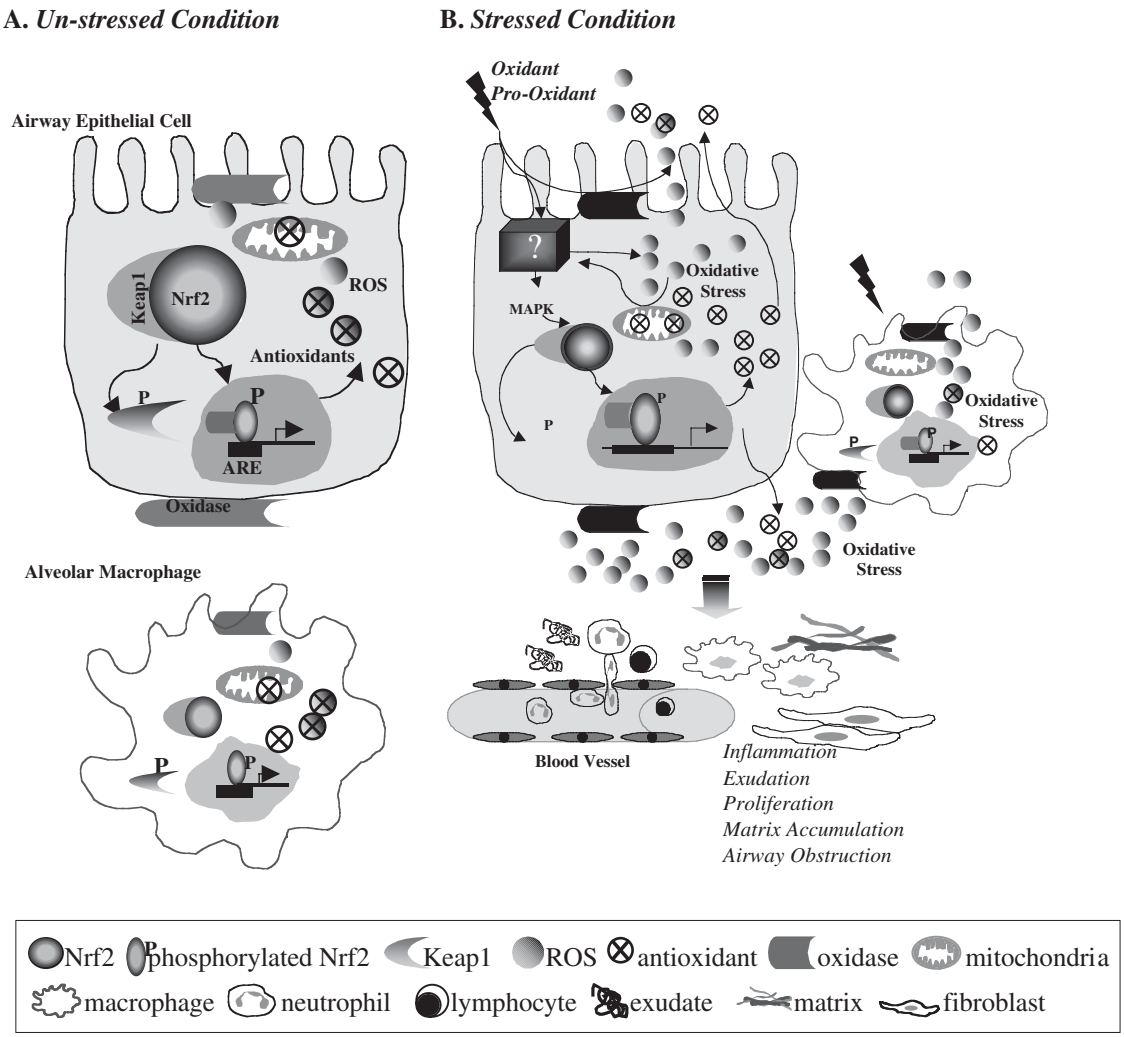

FIG. 3. A hypothetical diagram depicting Nrf2-antioxidant defense system in the airway cells. In normal physiologic condition (**A**), a balance between antioxidants and oxidants maintains cellular redox equilibrium. Under stressed condition (**B**), oxidative stimuli could accelerate ROS production, directly or indirectly, and activate Nrf2 for production of ARE-driven antioxidants, while overwhelming ROS over antioxidant capacity may cause oxidative injury leading to pulmonary pathologic symptoms.

eration and Nrf2 activation are dependent on NADPH oxidase and ERK1, respectively, in pulmonary epithelial cells. Interestingly, microarray analysis determined that hyperoxia induces expression of the major pulmonary PK-C isozyme, PK-Cα , in a Nrf2-dependent manner (18). Furthermore, previous genetic linkage analysis had identified the gene encoding PK-Cα as a candidate protective gene in asthma and pulmonary adenocarcinoma (27). Collectively, these observations suggest that PK-Cα may be involved in modulation of the Nrf2-Keap1 sensor for ARE activation during oxidative lung injury.

CONCLUSION

Oxidative stress causes various pulmonary symptoms including inflammation, protein edema, cell proliferation, matrix accumulation, and airway obstruction. Nrf2 is a transcription factor that is ubiquitously expressed throughout the lung, but is predominantly found in epithelium and alveolar macrophages. Recent investigations have clearly demonstrated that Nrf2 protects the lung from oxidative insults such as high oxygen tension and environmental particulates. The mechanism of Nrf2-mediated protection likely involves activation of ARE-regulated antioxidant genes whose products function directly or indirectly to limit ROS-mediated pulmonary pathogenesis (Fig. 3). Utilization of *nrf2*$^{-/-}$ mice and gene expression array analyses have provided significant insight into the role of Nrf2 in, and identified novel Nrf2 target genes that may contribute to, the pathogenesis of human pulmonary disorders such as ARDS, IPF, and emphysema. However, upstream events during activation of the Keap1-Nrf2-ARE pathway by oxidants are not fully understood. Similarly, the functional roles of Nrf2-dependent downstream effectors and their potential cross-talk or feedback regulation in the pathogenesis of pulmonary disorders also require further examination. A more complete understanding of Nrf2 function in the lung may provide opportunities to prevent oxidant-induced injury and disease pathogenesis.

ABBREVIATIONS

ALI, acute lung injury; AP-1, activator protein-1; ARDS, acute respiratory distress syndrome; ARE, antioxidant response element; ATF, activating transcription factor; BHA, butylated hydroxyanisole; β-NF, beta-naphthoflavone; BPD, bronchopulmonary dysplasia; CBP, CREB binding protein; CNC-bZIP, Cap'n'collar-basic region leucine zipper; COPD, chronic obstructive pulmonary disease; DEP, diesel exhaust particle; D3T, 1,2-dithiole-3-thiones; ELF, epithelial lining fluid; EpRE, electrophile response element; ERK, extracellular signal-regulated kinase; FTH, ferritin heavy chain; FTL, ferritin light chain; GCLc, gamma glutamyl cysteine ligase, catalytic subunit; GCLm, gamma glutamyl cysteine ligase, modifier subunit; GGT, gamma glutamyltranspeptidase; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GS, glutathione synthetase; GSH, glutathione; GST, glutathione-*S*-transferase; HO-1, heme oxygenase-1; H_2O_2, hydrogen peroxide; *Hsl1*, hyperoxia susceptibility locus 1; IκB, inhibitor of kappa B; IPF, idiopathic pulmonary fibrosis; Keap1, kelch-like ECH-associated protein 1; MARCO, macrophage receptor with collagenous structure; MMP, matrix metalloproteinase; NF-E2, nuclear factor-erythroid 2; NF-κB, nuclear factor-kappa B; NQO1, NADP(H):quinone oxidoreductase 1; Nrf2, nuclear factor-erythroid 2 related factor 2; $O_2^{-\bullet}$, superoxide anion; $OH^{\bullet}$, hydroxyl radical; PK-C, protein kinase-C; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor response element; Prx, thioredoxin peroxidase; QTL, quantitative trait locus; ROS, reactive oxygen species; RXR, retinoic acid X receptor; SOD, superoxide dismutase; tBHQ, tetra-butylated hydroxyquinone; TGF-β, transforming growth factor-beta; TIMP, tissue inhibitor of metalloproteinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, 12-*O*-tetradecanoylphorbol-13-acetate responsive element; Trx, thioredoxin; TXNRD, thioredoxin reductase; UGT, UDP-glucuronyl transferase; XRE, xenobiotic response element.

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