Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway

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Key Words

antioxidant response element, oxidative stress, cytoprotection, gene expression, knockout mice

Abstract

Keap1-Nrf2-ARE signaling plays a significant role in protecting cells from endogenous and exogenous stresses. The development of *Nrf2* knockout mice has provided key insights into the toxicological importance of this pathway. These mice are more sensitive to the hepatic, pulmonary, ovarian, and neurotoxic consequences of acute exposures to environmental agents and drugs, inflammatory stresses, as well as chronic exposures to cigarette smoke and other carcinogens. Under quiescent conditions, the transcription factor Nrf2 interacts with the actin-anchored protein Keap1, largely localized in the cytoplasm. This quenching interaction maintains low basal expression of Nrf2-regulated genes. However, upon recognition of chemical signals imparted by oxidative and electrophilic molecules, Nrf2 is released from Keap1, escapes proteasomal degradation, translocates to the nucleus, and transactivates the expression of several dozen cytoprotective genes that enhance cell survival. This review highlights the key elements in this adaptive response to protection against acute and chronic cell injury provoked by environmental stresses.

INTRODUCTION

ARE: antioxidant response element

KEAP1: Kelch ECH Associating Protein 1

NRF2: nuclear factor erythroid 2-related factor 2

The relentless stresses imposed by electrophiles and oxidants exacerbate many chronic diseases. For example, oxidative stress contributes to aging and age-related diseases such as cancer, cardiovascular disease, chronic inflammation, and neurodegenerative diseases. Levels of oxidized proteins, phospholipids, and DNA increase in these processes. In addition, DNA-damaging electrophiles are often carcinogens. Cells have developed adaptive, dynamic programs to counteract environmental stresses imposed by intrinsic and extrinsic oxidants and electrophiles. These systems may be divided into four categories: (*a*) oxidations and reductions, which introduce or expose functional groups onto largely hydrophobic organic molecules and are often catalyzed by cytochrome P450 enzymes; (*b*) nucleophilic trapping processes that add glutathione or other cellular nucleophiles to electrophilic xenobiotics, such as those catalyzed by glutathione *S*-transferases (GSTs) and conjugations of electrophilic adenosine-containing cofactors with nucleophilic xenobiotics (e.g., UDP-glucuronosyl transferases), which often facilitate their excretion, as well as enzymes such as superoxide dismutases, glutathione peroxidase, and catalase, which inactivate reactive oxygen species; (*c*) efflux transporters that export toxic metabolites; and (*d*) thiol-containing molecules, such as glutathione and thioredoxin, which function to maintain reducing conditions within the cell. Often the outcome of the encounter of a cell with a potentially toxic agent is largely determined by the balance between the activities of the enzymes that activate substrates to reactive intermediates, and the activities of the enzymes that detoxify these reactive species. Normally, the conjugating and related enzymes are not expressed at their maximal capacity but are highly inducible by transcriptional activation, and they exert versatile, long-acting, and often catalytic protection against electrophile and oxidant damage.

As depicted in **Figure 1**, induction of this protective response requires at least three essential components: (*a*) antioxidant response elements (AREs) (1), upstream regulatory sequences present on each gene in either single or multiple copies; (*b*) Nrf2 (nuclear factor erythroid 2-related factor 2), the principal transcription factor that heterodimerizes with members of the small Maf family of transcription factors, binds to the ARE, and recruits the general transcriptional machinery for expression of ARE-regulated genes (2); and (*c*) Keap1 (Kelch ECH associating protein 1), a cytosolic repressor protein that binds to Nrf2, retains it in the cytoplasm, and promotes its proteasomal degradation. It is thought that several critical cysteine residues in Keap1 serve as the primary sensors for the stress signals and that their modification leads to conformational changes in Keap1, thereby producing liberated Nrf2. Since its initial discovery by Yamamoto and his colleagues (3), it has become increasingly clear that Keap1 plays a central role in regulating the protective response. It is also abundantly clear, that the downstream effector genes of this signaling pathway can affect responses to a variety of environmental and cellular stresses. Beyond the classical environmental stress response of catalyzing the detoxification of xenobiotics through conjugation and trapping processes, genomic analyses indicate that gene families affected by this transcription factor (*a*) provide direct antioxidants (4, 5), (*b*) encode enzymes that directly inactivate oxidants (6), (*c*) increase levels of glutathione

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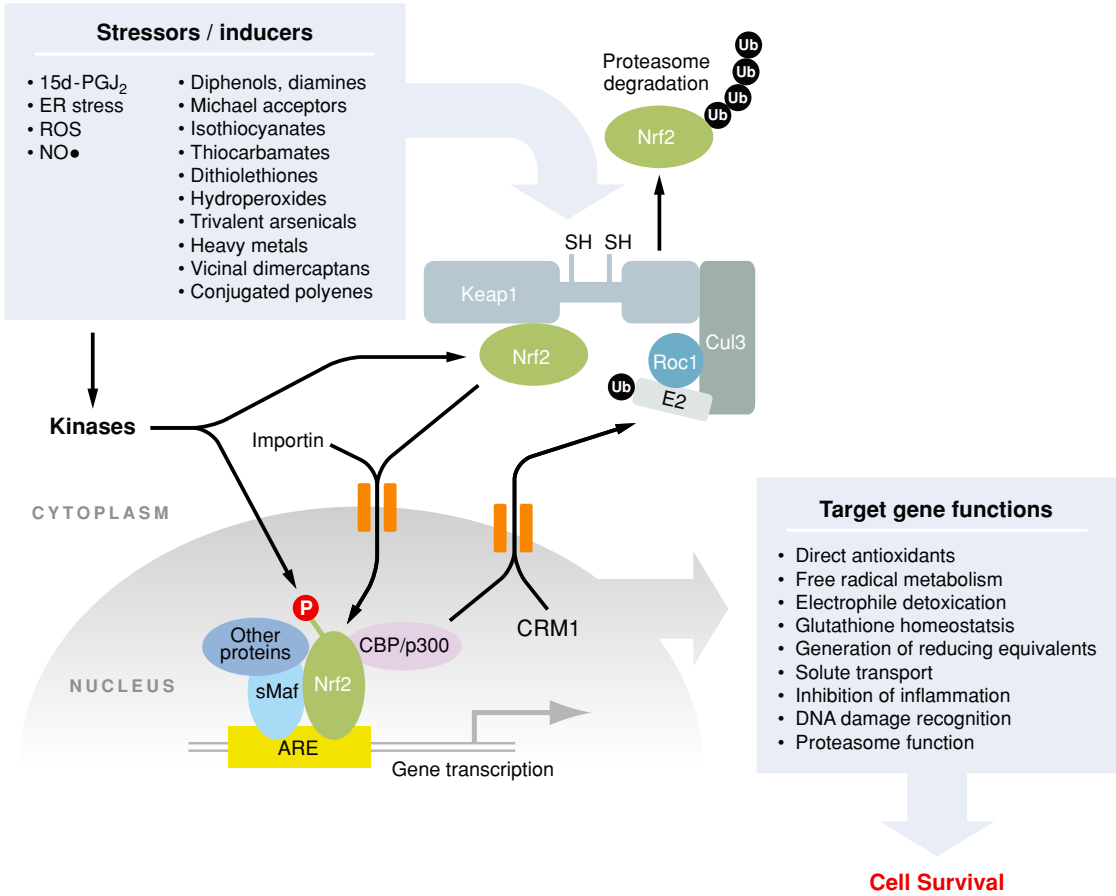

Figure 1

General scheme for the induction of gene expression through the Keap1-Nrf2-ARE signaling pathway. Small molecules of endogenous and exogenous origin lead to activation of Nrf2-regulated genes. These agents disrupt the association of Nrf2 with Keap1, leading to diminished rates of proteolysis of Nrf2 and enhanced nuclear accumulation. Phosphorylation of Nrf2 by a series of kinases also affects its fate and distribution. Interaction of Nrf2 with other transcription factors and proteins of the transcriptional complex allows for transactivation of ARE-responsive genes. Induction of these genes, which include prototypic conjugating and antioxidative genes, results in an adaptive response that enhances the resistance of cells to environmental stresses mediated by electrophiles and free radicals.

synthesis and regeneration (7), (*d*) stimulate NADPH synthesis (8, 9), (*e*) enhance toxin export via the multidrug response transporters (9), (*f*) inhibit cytokine-mediated inflammation (10), and (*g*) enhance the recognition, repair, and removal of damaged proteins (11). Although Nrf2 impacts the basal expression of many of these cytoprotective genes, the primary impact of this regulatory pathway lies on the control of their inducible expression. Consequently, of great relevance to disease

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prevention, this pathway can be enhanced by an expanding array of small-molecule drugs and natural products, some of which exhibit remarkable potency and specificity.

AP-1: activator protein 1

THE KEAP1-NRF2-ARE PATHWAY

Nrf2 as a Key Transcription Factor: A Brief History of Nrf2

There are several major protein-binding sites, including GATA and NF-E2/AP-1 sequences within DNase I hypersensitive sites of the α-and β-globin locus control region (12). Several proteins were identified in addition to AP-1 that activate transcription by binding to the NF-E2/AP-1 motif of hypersensitive site-2 in the β-globin locus control region. Nrf2 was cloned as one of such proteins and belongs to a subset of basic leucine-zipper (bZip) genes sharing a conserved structural domain, termed the CNC domain. First noted in the *Drosophila cap 'n' collar*, this gene is required for labial and mandibular development (13). In mammals, this CNC family is composed of four closely related proteins, p45-NFE2 (14), Nrf1 (15), Nrf2 (16, 17), and Nrf3 (18), as well as two distantly related proteins named Bach1 (19) and Bach2 (20). These proteins function as heterodimeric transcription factors by pairing with other bZip proteins, including the small-Mafs (21). Gene targeting studies have revealed critical roles for CNC-bZip genes in cell development and function. In the case of *p45-nfe2*-disrupted mice, there are severe defects in the formation of platelets from megakaryocytes (22, 23). *Nrf1*-disrupted mice show embryonic lethality at midgestation owing to anemia resulting from a fetal liver abnormality (23). Despite a broad expression pattern similar to that of Nrf1, there is no early phenotype observed in the several independently established lines of *Nrf2*-disrupted mice. The initial report from Kan and coworkers (24) concluded that Nrf2 is dispensable for growth and development. However, Jaiswal and colleagues (25) observed that the binding motif of Nrf1 and Nrf2 is very similar to the ARE found in the human *NQO1* [NAD(P)H:quinone oxidoreductase] gene regulatory region. Moreover, they demonstrated that this *cis*-element responded when HepG2 cells were transiently transfected with both Nrf1 and Nrf2. Thus, they proposed that Nrf2 may be a key transcription factor regulating expression of genes affecting xenobiotic metabolism. Independently, the Yamamoto group observed that the inducible expression of enzymes like GSTs and NQO1 was markedly attenuated in the liver and intestine of *Nrf2*-disrupted mice (26). As discussed below, the impact of *Nrf2* disruption on the transcriptosome has been reported for several tissues and with a variety of inducers. As a consequence of an altered inducible response, these mice are very sensitive to exogenous challenges that result from oxidative or electrophilic stresses.

The Discovery of Keap1

Examination of Nrf2 sequences across various species indicated six well-conserved domains (17), termed Neh (Nrf2-ECH <chicken Nrf2> homologous domain). It was hypothesized that those conserved domains were most likely to support the molecular functions of Nrf2. The most homologous domain, Neh2, which is located in

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the N terminus of Nrf2, was identified by molecular dissection as the negative regulatory domain for the *trans*-activating activity of Nrf2. *Trans*-activation activity was upregulated by deletion of the Neh2 domain, suggesting that the Neh2 domain must be the site of interaction for binding to a repressor in the cell. Keap1 cDNA was isolated as the direct binding molecule to Nrf2 by yeast two-hybridization using Neh2 as bait (3). Surprisingly, Keap1 represented 80% of the independent clones isolated, indicating that this molecular interaction between Keap1 and Nrf2 was quite specific. This interaction was confirmed in vitro and in mammalian cell systems. Keap1 showed a broad pattern of expression at the transcriptional level, one very similar to that observed for Nrf2. The primary structure predicted from Keap1 cDNA revealed that murine Keap1 consisted of 624 amino acids and shared ~95% identity between mouse and human. There are two canonical domains in Keap1. One is the BTB/POZ (Bric-a-brac, tramtrack, broad-complex/poxvirus zinc finger) domain, which has been shown to form homomeric as well as heteromeric multimers (27). The other, the Kelch domain, is named after the *Drosophila* egg-chamber regulatory protein Kelch, where it was first identified (28). This motif appears six times in Kelch and in Keap1, leading to the classification of Keap1 as a Kelch-repeat super family protein. A growing array of proteins containing the Kelch domain in combination with a BTB domain is being described. The BTB/POZ domain was previously shown to mediate protein dimerization. A key distinguishing feature of Keap1 is the high number of cysteine residues in this molecule. Murine and rat Keap1 have 25 cysteine residues, whereas human Keap1 has 27 cysteine residues. Nine of these cysteines are predicted to be reactive by virtue of their location adjacent to basic amino acids, a phenomenon known to decrease their pKa values, thus increasing their reactivity.

Mechanism of Induction of ARE-Regulated Genes by Nrf2

Studies on the Keap1-Nrf2 regulation of oxidative- and xenobiotic-stress responses have revealed a unique mode of nuclear/cytoplasmic collaboration. Keap1 interacts with both Nrf2 and the actin cytoskeleton to retain Nrf2 in the cytoplasm. This tethering promotes low basal gene expression of the cytoprotective enzymes and proteins in quiescent cells under normal physiological conditions. When oxidative and/or electrophilic stimuli liberate Nrf2 from its Keap1-mediated cytoplasmic entrapment, Nrf2 quickly translocates to the nucleus with the assistance of a nuclear localization sequence where it functions as a strong transcriptional activator of ARE-responsive genes in partnership with other transcription factors (**Figure 1**).

Nrf2 mainly functions as a transcription activator in the nucleus in partnership with small Mafs. However, immunohistochemical, living color-Tag analyses indicate that Keap1 is mainly localized in the cytoplasm. Through the Kelch domain, Keap1 interacts with the Neh2 domain of Nrf2 in the cytoplasm (3). This interaction alone might be insufficient to keep Nrf2 localized to the cytoplasm, but confocal laser microscopic immunohistochemical analyses revealed that Keap1 associates with the actin-cytoskelton (29). The actin adhesion surface has been linked to the Kelch domain. Recent crystal structure analysis of the Kelch domain in human Keap1 reveals

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that it contains six structurally similar β-propeller blades (30). Extensive inter- and intrablade hydrogen bonds maintain the structural integrity and proper association of Keap1 with Nrf2. A phylogenetically conserved hairpin motif (ETGE) in the C terminus of the Neh2 domain of Nrf2 binds to the β-propeller of Keap1 at the entrance of the central cavity on the bottom side with conserved arginine residues (31). A second sequence in the N terminus of Neh2, called the DLG motif, is conserved among members of the CNC-bZip family members. It is thought that the binding of Keap1 to the ETGE motif of Neh2 occurs first followed by binding of the DLG motif and these cooperative interactions contribute collectively to the overall stability of the Keap1-Nrf2 complex. Presumably, Keap1 in turn forms a multi-higher degree structural complex sharing its β-sheets with Nrf2 and actin filaments. Collectively, these observations indicate that the Keap1-Nrf2 complex is formed and retained in the cytoplasm through interactions with the cytoskeletal network. Nrf2 is then poised to be released from this complex when the cell senses stresses, allowing for a rapid defensive response through transcriptional activation of protective genes.

Studies using functional ARE reporter assays together with Keap1 and Nrf2 expression vectors revealed that coexpression of Keap1 with Nrf2 in cells or zebrafish repressed the transcriptional activation seen with Nrf2 alone (3, 32). Inducer treatment of cells with dual expression of Keap1 and Nrf2 abrogated the repressing activity of Keap1. Additional evidence that Keap1 is the negative regulator of Nrf2 comes from observations in *Nrf2*-disrupted mice. *Nrf2* knockout mice were generated by switching *Nrf2* exon V (encoding DNA binding and dimerization domains) to NLS (nuclear localization signal)-LacZ (coding β-galactosidase); these mice still produce the N terminus of Nrf2, including the Neh2 domain fused to β-galactosidase. Presumably, this fused protein can still interact with endogenous Keap1. This fusion protein could not be detected in tissues of control knockout animals. However, when animals were fed an inducer, immunohistochemical staining (using anti-β-galactosidase antibody) could be readily observed in nuclei of cells throughout the crypts of the intestine (33). This observation confirmed that Keap1 plays a significant negative control role through interaction with the Neh2 domain of Nrf2. Moreover, *Keap1*-disrupted mice showed an exaggerated accumulation of Nrf2 in the nucleus, leading to very high constitutive expression of Nrf2 target genes (34). This phenotype was completely reversed in *Keap1::Nrf2* double knockout mice. Collectively, these findings in single and compound knockout mice provide strong in vivo evidence that Keap1 acts as the specific negative regulator of Nrf2. Zipper & Mulcahy (35) noted that homodimerization of Keap1 through its BTB domain was essential for retaining Nrf2 in the cytoplasm. Ser-104 is highly conserved in a comparison of BTB/POZ domains from various Kelch proteins with that of Keap1. Mutation of Ser-104 in the BTB/POZ domain disrupted Keap1 dimerization and eliminated the ability of Keap1 to sequester Nrf2 in the cytoplasm. Finally, this mutant Keap1 protein could not repress Nrf2 transactivation of the ARE in the promoter region of the γ-glutamylcysteine ligase regulatory subunit gene. These results suggest that disruption of the Keap1 dimer is associated with the release of Nrf2 in vivo. In vitro binding assays using purified recombinant

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murine Keap1 and the Neh2 domain of Nrf2 indicated that the Keap1-Nrf2 complex was formed at a 2:1 ratio of Keap1 to Nrf2 (36).

A key question is how does Keap1 regulate Nrf2 homeostasis? The Keap1-Nrf2 complex appears to facilitate the degradation of Nrf2. Constitutive levels of Nrf2 mRNA can be detected easily by RNA blot analyses, although protein levels are hard to detect, suggesting that there is rapid degradation of Nrf2 in cells. Nrf2 has a short half-life that is estimated to be less than 20 min in macrophages and various cell lines (33, 37, 38). Use of proteasome inhibitors first indicated that degradation of Nrf2 occurs via the ubiquitin-proteasome pathway. Several lines of evidence indicate that degradation is associated with the Neh2 domain that, in turn, interfaces with Keap1—what might be termed Keap1-dependent degradation. Keap1 may be one molecule constituting a novel ubiquitin E3 ligase complex. This idea suggests that there is a Nrf2-specific E3 ligase that controls constitutive levels of Nrf2 protein (39). There is also Keap1-independent degradation that occurs under inducible conditions. In the case of human Keap1, it is reported that Keap1 inhibits Nrf2 degradation by the ubiquitin proteasome pathway in the HepG2 cell line (40). This finding is opposite to enhanced instability of Nrf2 by formation of a complex with Keap1. More detailed studies using reasonable in vivo or cellular systems will be required to elucidate the mechanisms controlling Nrf2 stability.

The high cysteine content of Keap1 suggested that it would be an excellent candidate as the sensor for inducers. As described by Talalay and coworkers, inducers of environmental stress response genes belong to 10 chemically distinct classes: (*a*) oxidizable diphenols, phenylenediamines, and quinones; (*b*) Michael acceptors (olefins or acetylenes conjugated to electron-withdrawing groups); (*c*) isothiocyanates; (*d*) thiocarbamates; (*e*) trivalent arsenicals; (*f*) dithiolethiones; (*g*) hydroperoxides; (*h*) vicinal dimercaptans; (*i*) heavy metals; and (*j*) polyenes (41, 42). Within the class of Michael acceptors, the order of inducer potency parallels the order of reactivity with nucleophiles in the Michael reaction. Similarly, the order of inducer potency in the class of heavy metals parallels the order of their affinity for sulfhydryl groups ($Hg^{2+} > Cd^{2+} > Zn^{2+}$). Moreover, the inducer potency was closely correlated with the electron withdrawing power of the substituents at the para position within a series of methyl cinnamate analogues and with the rate of reactivity with sulfhydryl reagents within a large series of phenolic mono and double Michael reaction acceptors. Thus, compelling evidence has accumulated to support the idea that the cellular sensor for inducers must be endowed with highly reactive sulfhydryl groups, most likely two cysteine residues in close spacial proximity to account for the extremely high inducer potency of trivalent arsenicals that are classically known to react with vicinal thiols leading to the formation of stable cyclic thioarsenites. Use of radiolabeled or biotinylated inducers indicates that multiple cysteines in Keap1 can be modified in similar but not completely uniform patterns (43–45). Moreover, electrophiles that do not induce cytoprotective genes labeled a completely different set of cysteines. As we recently reviewed, it is quite likely that sites of sulfhydryl adduction or modification may vary among different classes of inducers and across phylogenic species (42). Such flexibility in sensing inducing signals is in agreement with the fact that many different endogenous and xenobiotic molecules can activate this pathway.

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NRF2-REGULATED GENES

Nrf2 is truly a transcription factor of the postgenomic era. Through the study of its target genes and pathways in mouse models, a greater appreciation has been gained of Nrf2 as a pleiotropic transcription factor involved in regulating the susceptibility to diseases, ranging from neurodegenerative disorders to endotoxin-mediated septic shock. Since the first report by the Yamamoto group in 1997 that the inducible expression of GST and NQO1 is markedly attenuated in the liver and intestine of *Nrf2*-disrupted mice (26), many microarray analyses have been performed using different tissues in mice in response to different stressors and disease triggers. Clustering of the Nrf2-dependent upregulated genes in response to stressors into physiologic networks has revealed pathways involved in xenobiotic detoxification, antioxidative response, and proteome maintenance. The broad spectrum of genes and pathways regulated by Nrf2 in response to xenobiotics had led to the consensus that this transcription factor is involved in several key survival pathways. Less clear is the extent to which the expression and inducibility of these genes are regulated by Nrf2 in human cells (46).

Electrophile and Oxidant Metabolism

The first clue to the ability of Nrf2 to regulate a broad spectrum of enzymes and proteins affecting the disposition of electrophiles and oxidants came from studies by Thimulappa et al. (8). A transcriptional profile of the small intestine was generated using an oligonucleotide array of wild-type and *Nrf2*-disrupted mice treated with vehicle or sulforaphane, an anticarcinogen known to function, in part, through induction of xenobiotic metabolizing enzymes. Comparative analysis of gene expression changes between different treatment groups of wild-type and *Nrf2*-disrupted mice facilitated the identification of numerous genes regulated by Nrf2. These genes included *Nqo1*, multiple *Gsts* and *Ugts*, gamma-glutamylcysteine synthetase (*Gcs*), and epoxide hydrolase; genes well recognized as sulforaphane inducible in earlier biochemical studies. Also identified were genes encoding cellular NADPH-regenerating enzymes (glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and malic enzyme), antioxidants (glutathione peroxidase, glutathione reductase, ferritin, and haptoglobin), and biosynthetic enzymes of the glutathione and glucuronidation conjugation pathways. In toto, this investigation expanded the horizon of Nrf2-regulated genes, highlighted the cross-talk between various metabolic pathways, and divulged the pivotal and broad role played by Nrf2 in regulating cellular defenses against carcinogens and other toxins.

Since the initial study with sulforaphane, elaborate microarray analyses have been performed of Nrf2-activating chemopreventive agents, such as 3*H*-1,2-dithiole-3-thione and the triterpenoid 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole, both of which effectively inhibit aflatoxin-induced tumorigenesis in rodent liver (47, 48). These studies pointed to a role of Nrf2 in regulation of an even larger number of genes that interact with several other cytoprotective pathways. Moreover, they collectively suggested that while Nrf2 genotype can effect basal expression of many of these genes, it is often to a limited extent and quite variable

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between tissues. However, Nrf2 plays a dominant role in regulating the inducible expression of these genes.

As discussed in the next section, Nrf2 is an important modifier of several pulmonary inflammatory diseases that involve oxidative stress. The studies by Rangasamy et al. (49) of wild-type and *Nrf2*-deficient mice exposed to cigarette smoke provided evidence of a clear link between defects in the adaptive response regulated by Nrf2 and excessive oxidative stress, increased apoptosis, inflammation, and exacerbated emphysema. Cigarette smoke is a complex mixture containing thousands of toxicants, many of which can be potent activators of Nrf2, either directly as electrophiles or indirectly by causing redox imbalance and oxidative stress. In response to cigarette smoke, Nrf2 regulates genes involved in two major redox systems: the glutathione (GSH) and the thioredoxin systems. Enzymes involved in GSH synthesis (Gclc and Gclm), members of the glutathione reductase and peroxidase families (GSR, GPx2, and GPx3), and genes that constitute the thioredoxin system (*TrxR* and *Prx1*) are all induced in the lungs of wild-type mice in response to cigarette smoke. The members of these redox systems interact with various transducer and effector molecules to bring about antioxidant-specific responses. The regeneration of reduced Trx and GSH by TrxR and GSR, respectively, utilizes NADPH as a reducing equivalent generated by glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase, both of which are also induced in lungs. Prx 1 and GPx reduce hydroperoxides by utilizing two electrons provided by Trx and GSH, respectively. In addition, GPx and peroxiredoxins have been shown to play a role in protecting against peroxynitrite, a potent oxidant generated from the reaction of superoxide and nitrous oxide present in cigarette smoke. Furthermore, the oxidized forms of GPx and peroxiredoxins are reduced back to their functional forms by Trx. These results suggest interdependence between the thioredoxin and GSH redox systems and the NADPH-regenerating system.

Several GSTs, as well as UGTs and NQO1, are selectively induced only in wild-type but not knockout mice in response to cigarette smoke. These enzymes play important roles in the detoxification of tobacco smoke carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, benzo(a)pyrene, and other polycyclic aromatic hydrocarbons that act as electrophiles and cause DNA damage and cytotoxicity. Various enzymes, including aldehyde dehydrogenase and aldo-keto reductase, that are involved in the detoxification of reactive aldehydes, such as acetaldehyde and acrolein, are also selectively induced in the lungs of cigarette smoke–exposed mice. HO-1, a critical enzyme that protects against oxidant-mediated cellular injury and inflammation, and the iron-sequestering protein ferritin light chain 1, which prevents uncontrolled surges in the intracellular free concentration of the highly reactive, yet poorly soluble, ferric iron, are also induced. The reduction of ferric ion by superoxide can generate reactive hydroxyl radicals via the Fenton reaction. Superoxide dismutase, the antioxidant enzyme in the lungs that attenuates reactive oxygen species–mediated lung cell injury and inflammation, is also selectively upregulated.

Other Nrf2 targets induced in response to cigarette smoke include multiple drug resistance associated protein (MRP), carboxyl esterase, esterase D (sid478p), retinal oxidase/aldehyde oxidase, and carbonic anhydrase–like sequence. MRP is a

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founding member of a subfamily of ATP binding cassette (ABC) and is a primary active transporter of GSH, glucuronate, lipid peroxidation products, sulfate-conjugated and -unconjugated organic anions, heavy metals, and carcinogenic nitrosamines. Carboxyl esterases are a large family of broad-specificity esterases that hydrolyze xenobiotics containing ester, thioester, or amino groups. Aldehyde oxides are involved in the metabolism of many xenobiotics, including aldehydes, nitropolycyclic aromatic hydrocarbons, and nitrosamines. Carbonic anhydrase can protect cells from oxidative damage by scavenging oxygen radicals. Coordinate transcriptional induction of these enzymes along with antioxidants can effectively provide a greater defense against the complex mixture of toxicants that damage lungs.

Similarly, exposure of mice to hypoxia resulted in Nrf2-dependent transcription of a broad spectrum of antioxidative and conjugating enzymes in the lungs (50). Microarray analysis of astrocytes isolated from wild-type and knockout mice treated with tert-butyl-hydroquinone have confirmed the role of Nrf2 as a multiorgan protector against xenobiotic stress (51). The antioxidant and xenobiotic metabolic pathways regulated by Nrf2 are summarized in **Figure 2**.

Other Cell Survival Genes

Nrf2 also regulates a class of proteins that constitute the proteasomal pathway. Accumulation of abnormal proteins as a result of oxidative modification or interaction with electrophilic toxicants in cells impedes cellular function and can lead to apoptosis (52). 26S proteasomes are responsible for the degradation of damaged or misfolded proteins and control levels of key regulatory molecules (53). The proteasome is a large multisubunit complex that contains a proteolytic active 20S core complex consisting of a cylindrical stack of four rings. Two inner rings formed with seven β-subunits have proteolytic activity, whereas the two outer rings of α-subunits maintain structure. Access to the inner facet of the cylinder is controlled through gating by a 19S regulatory subunit attached to one or both ends. The 19S proteasome participates in the recognition and processing of substrates before their translocation and degradation by the catalytic core. The 20S proteasome can directly degrade oxidized proteins, whereas ubiquitination marks many proteins for recognition and turnover by the entire 26S complex (54). A decreased capacity for protein degradation is related to several neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis, in which accumulation of abnormal polypeptides within cells leads to death of neurons. This mechanism is also a factor in diabetes and atherosclerosis. An altered ubiquitin-proteasome system and reduced proteasome activity are associated with some of these diseases (55–57).

The expression of most subunits of 20S and 19S proteasomes, which collectively assemble the 26S proteasome, is transcriptionally induced in livers of mice in a Nrf2-dependent manner with dithiolethiones. Kwak et al. (11) showed that multiple AREs in the promoter of the catalytic subunit PSMB5 of the 20S proteasome bind Nrf2. Chemopreventive inducers have also been shown to induce heat shock proteins and ubiquitin/26S proteasome subunits (47). The induction of heat shock proteins, such as HSP40 and mitochondrial stress-70 protein, and of sequestosome 1 and ubiquitin

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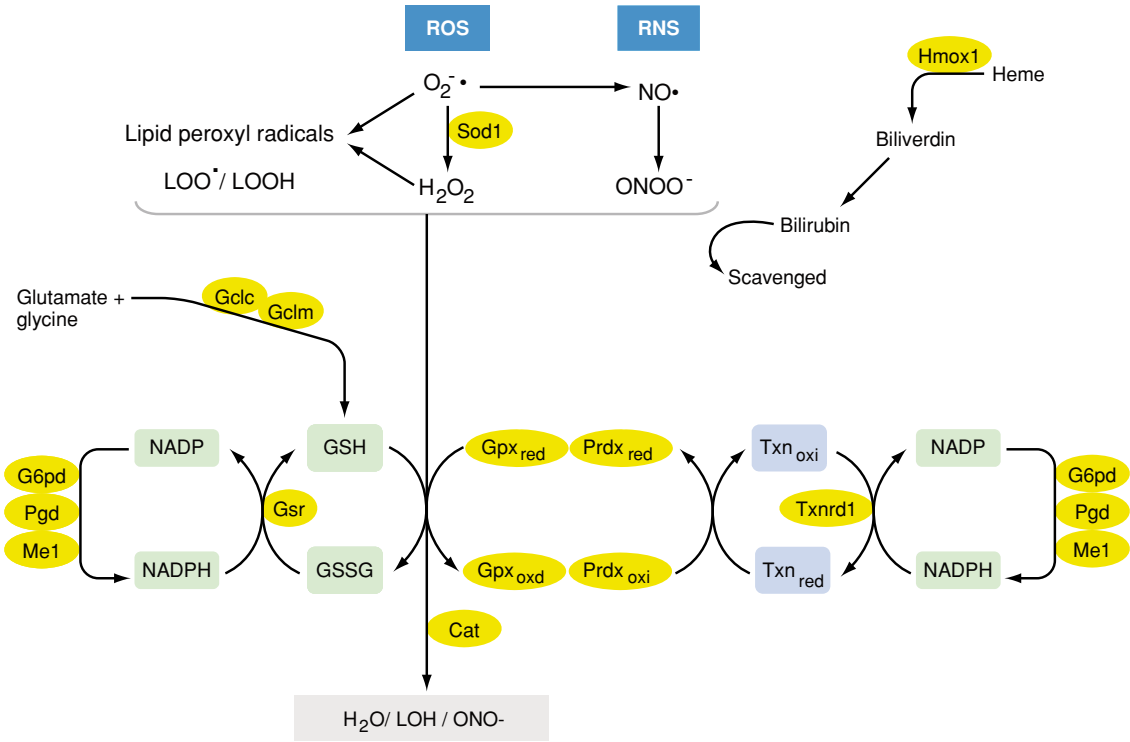

Figure 2

Schematic diagram representing Nrf2-regulated antioxidants and xenobiotic detoxification enzymes. In response to oxidative or electrophilic stress, Nrf2 has been demonstrated to coordinately upregulate expression of (*a*) antioxidants (SOD1, heme oxygenase) and genes associated with glutathione pathway (glutathione peroxidase, glutathione reductase, Gclc, and Gclm), thioredoxin pathway (thioredoxin reductase, peroxiredoxin), as well as NADPH-regenerating enzymes (glucose 6-phosphate dehydrogenase, phosphogluconate dehydrogenase, and maleic enzyme 1) and (*b*) xenobiotic detoxification enzymes (glutathione S-transferase, UGT1A1, epoxide hydrolase, carbonyl reductase, LTB4dh, NQO1, and MRP). These antioxidants and xenobiotic detoxification enzymes act in a concerted fashion to attenuate pathological damage caused by ROS, RNS, and electrophiles.

C, which are involved in the degradation of oxidized proteins resulting from cigarette smoke exposure, is also dependent on Nrf2(49).

Cross-Talk Between Signal Transduction Pathways

Major signaling pathways affect the posttranscriptional regulation of ARE-responsive genes by interacting with the Keap1/Nrf2 complex and include the MAPK (mitogen-activated protein kinase) cascade, phosphatidylinositol 3-kinase, and protein kinase C. Phosphorylation of Nrf2 in response to treatment with the inducer tert-butylhydroquinone has been demonstrated in vivo and the Ser 40 residue in the

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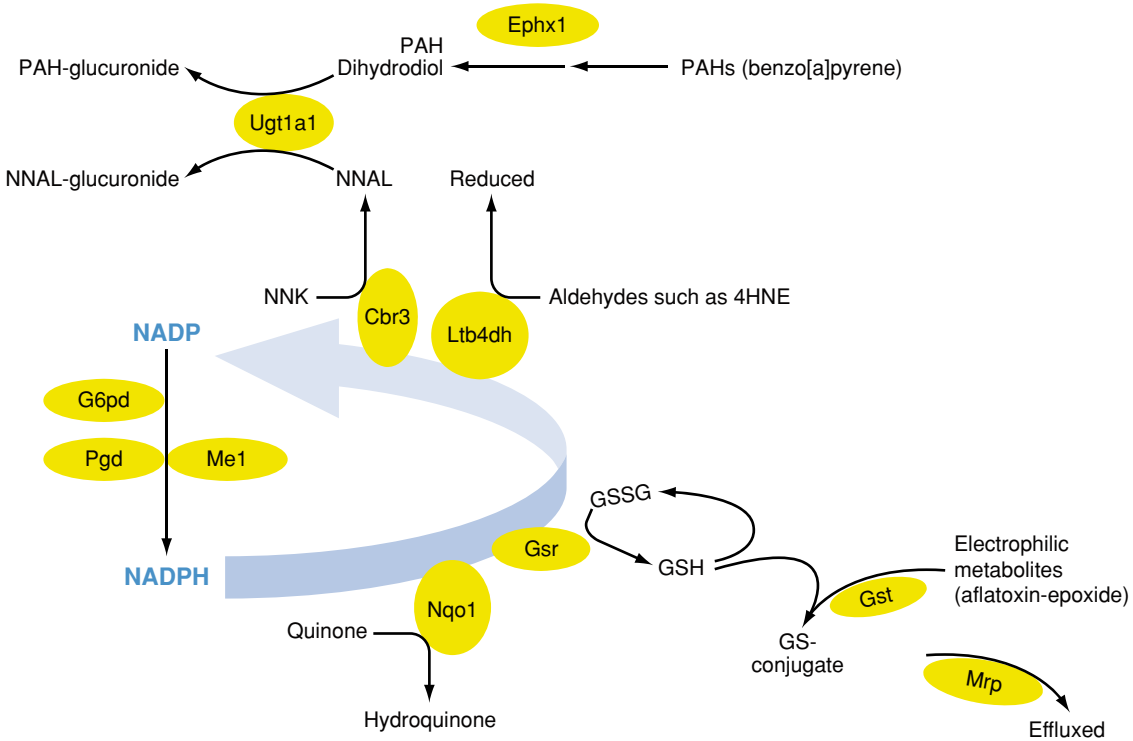

Figure 2

(*Continued*)

Neh2 domain has been identified as a phosphorylation site by protein kinase C (58). Phosphorylation of Nrf2 facilitated its dissociation from Keap1, whereas mutant Nrf2 at Ser 40 remained bound to Keap1. Bloom and Jaiswal also confirmed phosphorylation of Nrf2 on Ser 40 (59). While they found phosphorylation is required for Nrf2 release from Keap1, it was not necessary for Nrf2 stabilization/accumulation in the nucleus. Interestingly, studies conducted in vitro indicate that the affinity of Nrf2 heterodimeric complexes with small Mafs to ARE sequences is similar, regardless of the phosphorylation state of Nrf2. Nrf2 is a substrate of the protein kinase associated with the MAPK/ERK (extracellular signal-regulated kinase) signaling cascade (60) or PERK [endoplasmic reticulum (ER)-resident kinase] relating ER stress to PERK-dependent cell survival (61). The isolation of the cDNA of Nrf2 from a yeast two-hybrid system using PERK catalytic domain as the bait, coupled with the observation of the cellular distribution of Nrf2 in *Perk*-deficient mouse embryonal fibroblasts under conditions of ER stress suggests an additional regulatory mechanism. In *Perk*-deficient cells, the nuclear translocation of Nrf2 was markedly reduced following treatment with the ER stress inducer, tunicamycin. This model suggests that ER stress stimulates Keap1-Nrf2 complex formation by enhancing the

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phosphorylation of Nrf2 by PERK, or that phosphorylation by PERK enhances Nrf2 degradation.

Characterization of the patterns of gene expression up- and downregulated by inducers showed that these compounds lead to increases in the steady-state levels of Nrf2 transcripts in cells and animals. Two putative AREs in the proximal region of the Nrf2 promoter were identified; promoter deletion and mutation analyses indicated that they were functional (62). Thus, Nrf2 appears to autoregulate its own expression, leading to persistent nuclear accumulation of Nrf2 and protracted induction of protective genes in response to inducers. Subsequent studies indicate that Nrf2 may also regulate the expression of other transcription factors as well as be regulated by them.

AhR: aryl hydrocarbon receptor

NF-κB: nuclear factor kappaB

Nrf2 and the aryl hydrocarbon receptor (AhR) are two distinct transcription factors involved in the regulation of xenobiotic-metabolizing enzymes. Increasing evidence from several studies implies that AhR and Nrf2 have direct links. The AhR pathway has been well characterized and is known to mediate its effects through interaction with xenobiotic response elements (XRE) in the promoters of target genes. Several xenobiotic metabolism genes contain XREs and AREs, which have been thought to function independently. However, genetic experiments in using *AhR*-, *Arnt* (aryl hydrocarbon receptor nuclear translocator)- or *Nrf2*-deficient cells reveal that, while induction of NQO1 by TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) depends on the presence of AhR and Arnt, the basal and inducible expression of NQO1 by TCDD requires functional Nrf2 (63). Miao et al. (64) recently demonstrated that Nrf2 gene transcription is directly modulated by AhR activation. DNA sequence analyses of the mouse *Nrf2* promoter revealed the presence of several functional XRE-like elements. Direct binding of AhR to the *Nrf2* promoter was observed. Cells with silenced AhR expression using siRNA also lost Nrf2 mRNA induction by TCDD. Positioned downstream of the AhR-XRE pathway, this direct regulation of Nrf2 by AhR could facilitate an integrated regulation of genes facilitating xenobiotic metabolism. *AhR* and *Nrf2* compound null mutant mice provide a useful model to examine the integrated function of AhR- and Nrf2-regulated enzymes in detoxification. As predicted, the compound mutant mice respond only weakly to AhR ligand or Nrf2 inducer (65).

Interplay between Nrf2 and nuclear factor kappaB (NF-κB) has been reported in the regulation of cell adhesion molecules (66) and glutathione homeostasis (67). Studies in *Nrf2* knockout mice also suggest possible interactions. As discussed in a subsequent section, Nrf2 is shown to be a modifier of the innate immune response. *Nrf2*-deficient mice are also more sensitive to inflammatory challenges, including carrageenan-induced pleurisy and endotoxin-induced sepsis (68). In these models, Nrf2 functions as a firefighter to quench the inflammatory responses. In addition, *Nrf2*-deficient mouse embryonic fibroblasts show greater activation of NF-κB and interferon regulatory factor 3 in response to lipopolysaccharide and polyinosinic-polycytidylic acid stimuli (69). Other studies show that Nrf2 inducers block NF-κB transactivation and DNA binding, and that these effects are accompanied by changes in the levels of phosphoinhibitor of NF-κB (70). However, the full dynamics of Nrf2–NF-κB interactions remains to be resolved.

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ENHANCED TOXICITY IN NRF2-DEFICIENT MICE

Nrf2-deficient mice develop normally under standard laboratory conditions, although aged female *Nrf2*-deficient mice display a shortened lifespan, exhibit multiorgan autoimmune inflammation and develop severe glomerulonephritis at 60 weeks of age (71–73). However, as summarized in **Table 1**, and discussed in subsequent sections, *Nrf2* knockout mice exhibit profound sensitivity to a variety of acute and chronic chemical and biological stresses in multiple target organs. As such, they provide valuable tools for dissecting out the key determinants of susceptibility to a number of pathological states.

Hepatotoxicity

Among the first experiments to demonstrate a role of Keap1-Nrf2 signaling as a stress response pathway in vivo were studies from the laboratories of Yamamoto and Kan (74, 75), both of which developed *Nrf2*-disrupted mice. Both groups then described the

Table 1 Enhanced sensitivity of *Nrf2*-disrupted compared with wild-type mice following acute and chronic toxicological challenges

Challenge	Site	Outcome	Reference
Acetaminophen	Liver	Increased lethality and greater severity in hepatic damage, as demonstrated by increased plasma alanine aminotransferase activity, and centrilobular hepatocellular necrosis	(74, 75)
Butylated hydroxytoluene	Lung	Lethality coupled with extensive lung injury: enlarged and hemorrhagic, with pulmonary infiltrates and destruction of the alveolar architecture	(97)
Bleomycin	Lung	Increased lung weight, epithelial cell death, inflammation, and pulmonary fibrosis	(98)
Hyperoxia (>95% O_2)	Lung	Enhanced pulmonary damage characterized by increased protein permeability, macrophage inflammation, and epithelial injury	(50)
Tobacco smoke	Lung	Earlier onset and more extensive emphysema with pronounced bronchoalveolar inflammation	(49, 105)
Elastase	Lung	Enhanced inflammation and emphysema	(106)
Ovalbumin	Lung	Enhanced asthmatic response	(94)
Bacterial endotoxin	Lung	Increased pulmonary inflammation, edema, septic shock	(69)
Diesel particles	Lung	Severe hyperplasia and accumulation of the oxidative DNA adduct 8-hydroxy-deoxyguanosine in bronchial epidermis	(107)
Carrageenan	Pleural cavity	Enhanced inflammation coupled with persistent invasion by neutrophils and delayed recruitment of macrophages	(68)
Benzo[a]pyrene	Stomach	Increased multiplicity and tumor volume in neoplasia of the forestomach coupled with higher levels of carcinogen-DNA adducts	(78, 108)
Hydroxybutyl-nitrosamine	Bladder	Increased incidence of transitional cell carcinomas	(80)
Malonate, 3-Nitropropionic acid	Brain	Increased sensitivity to morphological and behavioral neurotoxicities by inhibition of mitochondrial complex II	(100, 109)

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enhanced sensitivity of *Nrf2* knockout mice to the hepatotoxicity of acetaminophen (APAP) and related this outcome to lowered cellular thiol levels and diminished capacity for conjugation reactions. Doses of 300 mg/kg APAP or greater caused death in the homozygous knockout mice only, and those that survived showed a greater severity in hepatic damage than the wild-type mice, as demonstrated by increased plasma alanine aminotransferase activity, decreased hepatic nonprotein sulfhydryl content, and centrilobular hepatocellular necrosis. Increased anti-APAP immunoreactivity was also noted in the livers of the knockout mice shortly after treatment. A subsequent study observed that *Nrf2*-disrupted mice were considerably more sensitive to pentachlorophenol, a mouse hepatocarcinogen. Oxidative stress biomarkers, cell proliferation, and histopathological evidence of hepatoxicity were all elevated following treatment with several dose levels of this environmental pollutant.

Taking an opposite experimental approach, Okawa et al. (76) have recently developed mice with hepatocyte-specific disruption of the *Keap1* gene using the Cre-loxP recombinase system regulated by the albumin promoter. These mice exhibited high levels of Nrf2 in hepatic nuclei, high constitutive expression of hepatic NQO1 and GST and striking resistance to the mortality of high doses of APAP. Consistent with these genetic models, it has been long established that pharmacological inducers (e.g., oltipraz, BHA) of GSTs are effective inhibitors of APAP hepatotoxicity in mice (77).

Chemical Carcinogenesis

Induction of conjugating enzymes and nucleophilic trapping processes appears to be an effective means for achieving protection against a variety of carcinogens in animals and humans. These enzymes can accelerate the rate of detoxication and excretion of proximate and ultimate carcinogens, leading to reduced burdens of DNA and other critical biomolecular damage. The collateral induction of antioxidative and antiinflammatory genes likely also contributes to attenuation of carcinogenesis inasmuch as oxidants and inflammation play significant roles in its etiopathogenesis. In this light, *Nrf2*-deficient mice had a significantly higher burden of gastric neoplasia after treatment with benzo[a]pyrene than did wild-type mice (78). Oltipraz significantly reduced multiplicity of gastric neoplasia in wild-type mice, but had no effect on tumor burden in *Nrf2*-deficient mice. A similar result was observed when the isothiocyanate sulforaphane was used as the chemopreventive agent in this model (79). The effect of *Nrf2* genotype on susceptibility to the urinary bladder-specific carcinogen N-nitrosobutyl(4-hydroxybutyl)amine (BBN) and the chemopreventive efficacy of oltipraz has been examined by Itoh et al. (80). The incidence of urinary bladder carcinoma by BBN was significantly higher in *Nrf2* knockout mice than in wild-type mice; invasive carcinoma was found in 24.0% and 38.5% of wild-type and *Nrf2*-disrupted mice, respectively. Oltipraz induced enzymes responsible for BBN detoxification in the liver and urinary bladder in an Nrf2-dependent manner. As expected, therefore, oltipraz decreased the incidence of urinary bladder carcinoma by BBN in wild-type mice but had little effect in knockout mice. In wild-type mouse liver, oltipraz significantly induced BBN glucuronidation and decreased the urinary concentration of N-nitrosobutyl(3-carboxypropyl)amine, a proximate carcinogen of

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BBN. The total loss of anticarcinogenic efficacy of oltipraz and sulforaphane in *Nrf2*-disrupted mice in several models highlights the prime importance of elevated gene expression in chemoprotection by these and similar enzyme inducers.

Pulmonary Inflammatory Diseases

Many studies in recent years have clearly shown that *Nrf2*-disrupted mice have increased sensitivity to several pulmonary inflammatory diseases. These findings have reinvigorated the study of oxidative stress in lung diseases, and oxidative stress is now considered to be a major factor in their etiopathogenesis.

Emphysema. Pulmonary emphysema associated with chronic obstructive pulmonary disorder (COPD) is a complex disease characterized by abnormal inflammation, air space enlargement, and the loss of alveolar structures. Chronic exposure to cigarette smoke leads to the loss of lung maintenance and causes emphysema. The oxidative burden in the lungs of smokers has been estimated to be very high: 1×10^{14} free radicals per puff (81). Markers of oxidative stress (e.g., hydrogen peroxide and the end products of lipid peroxidation, such as ethane, pentane, and 8-isoprostane) are elevated in the breath and serum of patients with COPD (82). Oxidative stress has been suspected to enhance inflammation in smokers (83). Evidence of the interplay between inflammation and oxidative stress in abetting lung destruction is highlighted by measures of significant release of reactive oxygen species from macrophages and neutrophils in smokers (84, 85). The oxidants of cigarette smoke can activate alveolar macrophages to produce both reactive oxygen species and several inflammatory mediators that attract neutrophils, macrophages, and other inflammatory cells into lungs. Susceptibility of the lung to oxidative injury, such as that originating from inhalation of cigarette smoke, depends largely on the upregulation of antioxidant systems. Disruption of the *Nrf2* gene in mice leads to earlier-onset and more extensive cigarette smoke–induced emphysema than is found in wild-type littermates (49). Emphysema in *Nrf2*-deficient mice exposed to cigarette smoke for 6 months is associated with more pronounced bronchoalveolar inflammation; enhanced alveolar expression of 8-oxo-7,8-dihydro-2'-deoxyguanosine, a marker of oxidative stress; and with an increased number of apoptotic alveolar septal cells—predominantly endothelial and type II epithelial cells—as compared with wild-type mice. As described earlier, microarray analysis has identified the expression of nearly 50 Nrf2-dependent antioxidant and cytoprotective genes in the lungs that may work in concert to counteract cigarette smoke–induced oxidative stress and inflammation. The responsiveness of the Nrf2 pathway may be a major determinant of resistance to tobacco smoke–induced emphysema by upregulating antioxidant defenses and decreasing lung inflammation and alveolar cell apoptosis. Increased emphysema in *Nrf2*-deficient mice after direct administration of elastase to lungs also supports a role of Nrf2 in maintaining the balance between proteases and antiproteinases (106).

Allergic asthma. Several studies have shown that increases in reactive oxygen species that occur during asthma are associated with damage to a wide range of biological

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molecules in the lung (86, 87). Inflammatory cells in the airways and alveolar spaces can release reactive oxygen species and reactive nitrogen species after phagocytosis of inhaled particles or after their functional activation by various stimuli. Eosinophils in lungs are the major source of reactive oxygen species after antigen challenge in allergic subjects (88). Reactive oxygen species–mediated activation of NF-*k*B can induce the expression of proinflammatory factors. Individuals with asthma have depressed levels of ascorbate and α-tocopherol in bronchiolar-alveolar lavage fluid, diminished activities of superoxide dismutase, and elevated oxidized-reduced glutathione (GSSG/GSH) ratios, suggesting increases in both reactive oxygen species and reactive nitrogen species and decreased antioxidant capacity (89). Decrease in SOD activity owing to oxidative modification has been strongly linked to allergic asthma (90–92). More recently, pollen allergens were shown to possess intrinsic NADPH activity that is involved in causing oxidative stress in airway epithelium (93).

Although a defect in antioxidant responses was speculated to exacerbate asthma severity, this was difficult to demonstrate with certainty until the recent studies utilizing the *Nrf2* knockout mouse model. Disruption of the *Nrf2* gene in mice leads to severe allergen-driven airway inflammation and airway hyper-responsiveness (94). The enhanced asthmatic response as a result of ovalbumin sensitization and challenge in *Nrf2*-disrupted mice is associated with more pronounced mucus cell hyperplasia and infiltration of eosinophils into the lungs than seen in wild-type littermates. *Nrf2* disruption results in an increased expression of the T helper type 2 cytokines in bronchoalveolar lavage fluid and in splenocytes after allergen challenge. Thus, the enhanced severity of the asthmatic response is a result of a lowered antioxidant status of the lungs caused by both lower basal and adaptive expression of multiple antioxidant genes. Responsiveness of Nrf2-directed antioxidant pathways may play a major role in susceptibility to allergen-mediated asthma.

Acute lung injury. Acute lung injury, which is manifested by edema and inflammation in the lungs, is a complex disorder with involvement of oxidative stress (95). Hyperoxia (>95% oxygen) generates reactive oxygen species and causes extensive pulmonary damage and has been used as a model for studying acute lung injury. Genome-wide linkage analyses of hyperoxia susceptibility genes in C57BL/6J (susceptible) and C3H/HeJ (resistant) mice by intercross (F2) and recombinant inbred cohorts suggested a quantitative trait locus on chromosome 2 that contained the Nrf2 gene (96). Variation in lung *Nrf2* messenger RNA expression in specific strains and a T → C substitution in the B6 *Nrf2* promoter that cosegregated with susceptibility phenotypes in F2 animals revealed *Nrf2* as a candidate gene. Hyperoxia caused greater pulmonary hyperpermeability, macrophage inflammation, and epithelial injury in *Nrf2*-disrupted mice than in wild-type mice. Other evidence for the role of Nrf2 in acute lung injury comes from studies in mice treated with butylated hydroxytoluene (97). At doses of butylated hydroxytoluene that are well tolerated by wild-type mice, *Nrf2*-disrupted mice suffer from acute lung injury. In both of these models, lower expression of pulmonary antioxidant genes contributes to the increased susceptibility of *Nrf2*-deficient mice to acute lung injury. Interestingly, Nrf2 also protects against acute lung injury in a mouse model in which oxidative stress does not play an important

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role. Intratracheal instillation of carrageenan rapidly produces alveolitis and edema within a day, which resolves itself in few days (68). In the carrageenan-induced pleurisy model, inhibition of Cox-2 reduces the production of 15-delta-Δ^{12-14}-prostaglandin J_2 (15d-PGJ_2) and delays the resolution of inflammation, whereas intervention with 15d-PGJ_2 inhibits the inflammation. One of the molecular targets of 15d-PGJ_2 is Nrf2 (68). Prolonged inflammation in *Nrf2*-disrupted mice as well as failure of 15d-PGJ_2 to resolve inflammation in these mice demonstrate the important role of Nrf2. Because oxidative stress does not drive the carrageenan-dependent inflammation, genes other than the antioxidants regulated by Nrf2 have been speculated to play a role in the resolution of inflammation.

Pulmonary fibrosis. The molecular mechanisms of pulmonary fibrosis are poorly understood, although reactive oxygen species are thought to have an important role. Whether Nrf2 regulates TGF-β in lungs, which is involved in the tissue remodeling that causes fibrosis, is not clear. However, Nrf2 protects lungs from injury and fibrosis induced by bleomycin, an antineoplastic agent that causes pulmonary fibrosis in susceptible patients. Bleomycin induces greater increases in lung weight, epithelial cell death, and inflammation in *Nrf2* knockout mice than in wild-type mice (98). In wild-type mice, bleomycin causes the activation of Nrf2, which leads to the transcriptional induction of several Nrf2-inducible antioxidant enzyme genes and protein products. The lack of an Nrf2 response causes greater lung fibrosis as reflected by higher hydroxyproline content, collagen accumulation, fibrotic score, and cell proliferation in the bleomycin-treated knockout mice.

Inflammation and Innate Immune Response

Host genetic factors that regulate innate immunity determine susceptibility to sepsis. Recent studies have shown that Nrf2 may be one such host factor. Disruption of *Nrf2* dramatically increases the susceptibility of mice to endotoxin-induced septic shock and results in early death (69). Increased mortality of *Nrf2*-deficient mice is also induced by acute septic peritonitis. Nonlethal systemic and local delivery of lipopolysaccharide results in greater lung inflammation and injury in *Nrf2*-deficient mice. TNF-α stimulus also induces enhanced lung inflammation in *Nrf2*-deficient mice. Temporal analysis of global pulmonary gene expression after lipopolysaccharide challenge shows that the expression of several proinflammatory genes is augmented in association with the innate immune response at as early as 30 min in knockout mice. The lack of an Nrf2 response leads to an uncontrolled surge of inflammation as a result of greater activation of the Myd88-dependent signaling pathway, which leads to NF-κB activation, and the Myd88-independent signaling (TRIF/IRF3/5) pathway, which results in the expression of interferon-regulated genes. Nrf2-regulated cellular GSH and antioxidant levels are indispensable in bringing about optimal NF-κB activation in response to lipopolysaccharide and TNF-α. Modulation of this pathway is likely to have enormous impact in the area of immunotoxicology and several lung diseases such as asthma and COPD, for which exacerbation due to pulmonary infection leads to worsened outcome. Correspondingly, recent studies also indicate that rats can

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be protected from the lethality of endotoxin-induced sepsis by pretreatment with activators of the Nrf2 signaling pathway such as 3*H*-1,2-dithiole-3-thione (70).

Neurodegenerative Diseases

Oxidative stress in the brain contributes greatly toward several neurodegenerative diseases, including amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease. Increased neuronal degeneration in these diseases has been shown to be associated with several markers of oxidative damage to lipids, nucleic acids, and proteins in affected brain regions. Oxidative stress contributes to cell death, which is responsible for disease progression. Primary cortical astrocytes from *Nrf2*-disrupted mice are more sensitive to oxidative stress caused by treatment with tert-butylhydroquinone than are cells derived from wild-type animals (51). Nrf2 has been shown to be activated in these cells as a result of oxidative stress, leading to increased induction of the target antioxidants and enzymes that protect against apoptosis. Similar results of a protective role of Nrf2 against oxidative stress have been shown with primary cortical neurons (99). Treatment of *Nrf2* knockout mice with mitochondrial complex II inhibitor 3-nitropropionic acid (3-NP) causes greater motor deficits and striatal lesions on a more rapid timescale than seen in identically treated *Nrf2* wild-type mice (100). Increasing Nrf2 activity in vivo either by direct intrastriatal adenovirus-mediated Nrf2 overexpression or dietary intervention with the Nrf2 inducer tert-butylhydroquinone attenuates 3-NP toxicity and significantly reduces lesion size.

Several lines of investigation point at the role of Nrf2 in protecting against stroke damage induced by cerebral ischemia, which causes oxidative stress and cell death. Acute intracerebroventricular or intraperitoneal pretreatment with tert-butylhydroquinone activates Nrf2 and reduces cortical damage and sensorimotor deficits at 24 h and even 1 month after ischemia-reperfusion in rats. *Nrf2*-disrupted mice have a larger infarct volume as the result of increased cell death (101). Similarly, systemic administration of high dose of sulforaphane has been shown to decrease cerebral infarct volume after focal ischemia in rats (102). More recently, protection of stroke in a cerebral/ischemic mouse model by electrophilic neurite outgrowth-promoting prostaglandin compounds has been reported to occur through a Nrf2-dependent mechanism (103).

Aging

The decline in antioxidant levels as a result of aging has been a subject of considerable interest because oxidative stress is linked with increased damage to cellular macromolecules. Suh et al. (104) determined that a decrease in Nrf2 is responsible for the significant decline in GSH in the liver of aging rats. With age both the catalytic and the modulatory subunits of gamma-glutamylcysteine ligase, the rate-controlling enzyme in GSH synthesis, decrease by half. Concomitant with lower subunit levels, ligase activity and total and nuclear Nrf2 levels drop by approximately 50%. It remains to be determined whether the age-related decline in Nrf2 occurs in other critical organs such as lungs and brain, and whether it contributes to increased

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susceptibility to various diseases in which this transcription factor appears to play an important role.

SUMMARY POINTS

1. The Keap1-Nrf2 molecular complex contributes to the regulation of cellular defense mechanisms that enhance survival.
2. Basal and especially inducible expression of cytoprotective genes are regulated by the transcription factor Nrf2.
3. Classes of genes regulated by Nrf2 affect electrophile detoxication, free radical metabolism, glutathione homeostasis, generation of reducing equivalents, and solute transport, as well as enhance proteasome function and dampen inflammation.
4. Nrf2 bidirectionally intersects other signaling pathways, including AhR and NF-κB.
5. Disruption of *Nrf2* in mice leads to enhanced sensitivity to hepato-, pulmonary-, ovary- and neurotoxicants; carcinogens; allergens; and endotoxins. Conversely, conditional hepatic overexpression of Keap1 leads to enhanced protection.
6. Expression of Nrf2-regulated genes can be modulated by many classes of compounds that share a common feature of sulfhydryl reactivity, presumably with cysteines in Keap1. Such molecules exert potent protective effects against toxicants in wild-type but not *Nrf2*-disrupted mice.
7. Elements of this Keap1-Nrf2 regulatory pathway for cell survival hold strong promise as targets for interventions.

UNRESOLVED ISSUES

1. To what extent is the murine Nrf2-Keap1-ARE signaling pathway recapitulated in humans?
2. Are there functional polymorphisms in *Keap1* and/or *Nrf2* and to what extent do they alter susceptibility to environmental stresses or diseases? Are there other components of the pathway that also serve as susceptibility determinants?
3. Does Nrf2 serve as a critical node and what is the extent of cross-talk with other signaling networks?
4. To what extent does Nrf2 control either the basal or inducible expression of its effector genes, and how much does this vary between genes and target tissues? Does Nrf2 signaling vary with age and during the development of pathological states?

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