



## Cytoprotection Against Oxidative Damage by Nrf2-regulated Genes

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Chronic oxidative stress produced by exposure to environmental chemicals or pathophysiological states can lead animals to aging, carcinogenesis and degenerative diseases. Indirect antioxidative mechanisms, in which natural or synthetic agents are used to coordinately induce the expression of cellular antioxidant capacity, have been shown to protect cells and organisms from oxidative damages. Electrophile and free radical detoxifying enzymes, which were originally identified as the products of genes induced by cancer chemopreventive agents, are members of this protective system. The NF-E2 family transcription factor Nrf2 was found to govern expression of these detoxifying enzymes, and screening for Nrf2-regulated genes has identified many gene categories involved in maintaining cellular redox potential and protection from oxidative damage as Nrf2 downstream genes. Further, studies using *nrf2*-deficient mice revealed that these mutant mice showed more susceptible phenotypes towards exposure to environmental chemicals/carcinogens and in oxidative stress related disease models. With the finding that cancer chemopreventive efficacy of indirect antioxidants (enzyme inducers) is lost in the absence of Nrf2, a central role of Nrf2 in the antioxidative protective system has been firmly established. Promising results from cancer prevention clinical trials using enzyme inducers propose that pharmacological interventions that modulate Nrf2 can be an effective strategy to protect tissues from oxidative damage.

**Key words:** Oxidative damage, Indirect antioxidants, Enzyme inducers, Nrf2, Keap1.

### INDIRECT ANTIOXIDANTS

It is recognized that oxidative stress is involved in the etiopathogenesis of a variety of human afflictions: aging, cancer, atherosclerosis, neurodegenerative diseases, and others, and there has been a long standing interest in the development of prophylactic measures to try to retard, block, or perhaps even reverse early and later stages in the progression of these different diseases. Traditionally, the approach has been to use the classical anti-oxidants - radical scavengers or interceptors - that typically function as electron or H-atom donors. However, there are some pharmacological barriers to this type of approach. Typically, these molecules have rather short pharmacodynamic half-lives unless they are recycled, such as the case with the vitamin E molecule. We need relatively high dosage, frequent adminis-

tration; we need to get the anti-oxidants at the right place at the right time and for extended periods of time.

But there is another approach - using indirect antioxidants that can induce the expression of genes that scavenge, detoxify, or otherwise attenuate free radicals and their damage products. These molecules appear to have very protracted pharmacodynamic half-lives. Thus, they act for a long period of time. We need fairly low concentrations and we only need infrequent administration because of their protracted pharmacodynamic action. Some questions that will be addressed about these indirect anti-oxidants are, "What kind of chemicals, in fact, are they?" "What is the sensor for these indirect antioxidants?" "What genes are coordinately regulated through this sensor and how might they contribute to protection against a variety of oxidative stress mediated diseases?"

Paul Talalay and colleagues have now defined nine different chemical classes of indirect antioxidants (Talalay *et al.*, 1988). These include isothiocyanates, which are constituents of a variety of different plants, dithiolth-

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iones, which are both natural and synthetic in origin, a variety of Michael reaction acceptors, arsenicals and heavy metals, hydroperoxides, vicinal dimercaptans, oxidized diphenols, phenylene diamines, quinones, as well as carotenoids. Overall, there is a wide diversity of agents that apparently do not share any common structural features. Some are oxidants, others are reductants and some appear to have no redox chemistry associated with them. Many of them are electrophiles; however, are a few nucleophiles, many are substrates for glutathione transferases, but the unifying feature appears to be that they are chemically reactive with sulfhydryl groups by oxidation or alkylation. This reactivity provides some key clues as to their molecular targets.

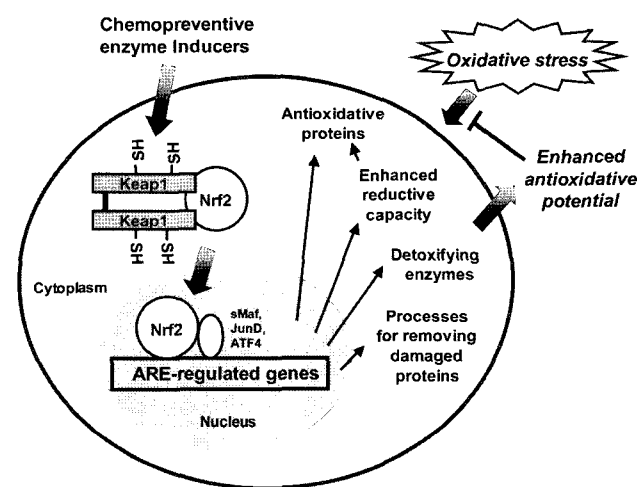
It has been our interests to identify both mechanisms and consequences of upregulation of genes involved in protection against chemical carcinogenesis using one of these indirect antioxidant classes of compounds, dithiolthiones (e.g., oltipraz and 1,2-dithiole-3-thione (D3T)). Using classical biochemical approaches as well as differential display, we recognized that there were several classes of genes that could be coordinately regulated, including those involved in carcinogen detoxication - glutathione S-transferases (GSTs) and UDP-glucuronosyl transferases; among many, but also recognizing that there are a number of antioxidative genes that are coordinately up-regulated, including quinone reductase (NQO1), the heavy and light chains of ferritin, which are involved in iron sequestration, heavy and light chains of  $\gamma$ -glutamyl cysteine ligase (the rate limiting enzyme in the biosynthesis of glutathione (GSH)), manganese superoxide dismutase, and catalase (Kensler, 1997; Otieno *et al.*, 2000; Primiano *et al.*, 1996). Also a number of inducible genes that appear to be anti-inflammatory in their activities, including one that we originally cloned as dithiolthione inducible gene 1, which now has a number of names including leukotriene B4 dehydrogenase or NADPH alkene/one oxidoreductase (Dick *et al.*, 2001; Primiano *et al.*, 1996; Primiano *et al.*, 1998).

### THE NRF2/KEAP1 SIGNALING PATHWAY REGULATES THE ACTIONS OF INDIRECT ANTIOXIDANTS

One feature that was common to all of these genes that we, and others in the field, were describing was that they appeared to share a common mode of regulation. The upstream sequences of these genes contained one or more copies of an "antioxidant response element" (ARE) with the core sequence of 5'-GTGAGnnnGCA-3' (Wasserman and Fahl, 1997). This finding provided

some key clues as to how these enzymes were regulated. There continues to be a lot of activity to understand which transcription factors interact with the ARE to drive the transcriptional activation of these genes by these many different classes of indirect antioxidants. A number of laboratories identified the transcription factor Nrf2 as a critical player in the upregulation of ARE-responsive genes. Nrf2 is a CNC (cap'n'Collar) basic leucine zipper (b-ZIP) transcription factor. There are a number of other members of b-ZIP transcription factor family; however, Nrf2 appears to be the most important member of this family in terms of regulating gene expression through the ARE.

Itoh *et al.* (1997) showed that homozygous disruption of *nrf2* in mice largely abrogated the inducible expression of GST and NQO1 by the phenolic antioxidant *tert*-butylhydroxyanisole (*t*-BHA) in liver and intestine. Other studies demonstrated that Nrf2 found to bind to the AREs of murine *GSTA1*, rat *GSTA2*, human *NQO1*, and human  $\gamma$ -glutamyl cysteine ligase genes (Itoh *et al.*, 1997; Kang *et al.*, 2000; Venugopal and Jaiswal, 1996; Wild *et al.*, 1999). As shown in Fig. 1, it is now recognized that for the binding to the ARE, Nrf2 needs to form a dimer with other leucine zipper proteins. Small Maf proteins and AP-1 complex including MafK, JunD,



**Fig. 1.** Cytoprotective role of Nrf2 against oxidative stress. Chemopreventive enzyme inducers trigger the release of Nrf2 from a cytoplasmic inhibitor Keap1 by altering the sulfhydryl bonds in Keap1 protein. Accumulated Nrf2 within nucleus transactivates the ARE-bearing genes and these genes involve in detoxification of environmental chemicals, enhancement of cellular reducing potential, and facilitation of the repair/removal of oxidatively damaged proteins. Collectively, these inducible genes by the *keap1*-Nrf2 pathway elevate cellular antioxidative potential and play an important role in cytoprotection against oxidative stress-associated tissue injuries.

cJun, cFos and ATF4 have been recognized as partner proteins of Nrf2 (Itoh *et al.*, 1997; Venugopal and Jaiswal, 1996, 1998; Wild *et al.*, 1999). Further studies on the gene regulation by chemopreventive, indirect antioxidants in *nrf2*-disrupted mice have expanded the list of genes regulated by Nrf2. It has been shown that *nrf2*-disrupted mice lost inducibility of GSTs (GSTA1-5, GSTM1/5 and GSTP1/2) and NQO1 in small intestine following treatment with indole-3-carbinol, *t*-BHA and ethoxyquin (McMahon *et al.*, 2001). Also, inducible expression of GSTA1, NQO1, microsomal epoxide hydrolase,  $\gamma$ -glutamyl cysteine ligase, UDP-glucuronosyl transferase 1A6 and HO-1 by 1,2-dithiole-3-thione was largely attenuated in livers of *nrf2*-disrupted mice (Kwak *et al.*, 2001b). Abrogation of inducibility of antioxidative genes in *nrf2*-disrupted mice was also detected in mouse liver, lung, small intestine and forestomach following oltipraz treatment (Ramos-Gomez *et al.*, 2003). Other antioxidative genes including peroxiredoxin MSP32 and cysteine membrane transporter A170 were identified as Nrf2-dependent genes in peritoneal macrophages (Ishii *et al.*, 2000).

These results collectively support the essential role of Nrf2 in the induction of multiple antioxidative genes. In addition, the cytoprotective role of antioxidative genes in chemical-induced carcinogenesis was further supported by the studies Ramos-Gomez *et al.* (2001) and Fahey *et al.* (2002). Pretreatment with oltipraz or sulforaphane significantly inhibited gastric tumor formation induced by benzo[a]pyrene in wild-type mice, but these agents did not show any protective effect in *nrf2*-disrupted mice (Ramos-Gomez *et al.*, 2001).

## REGULATION OF NRF2

The cytoskeletal-binding protein Keap1 plays a central role in the regulation of Nrf2 activity. Keap1 (Kelch-like ECH-associated protein1) was isolated as an inhibitor protein of Nrf2 by yeast two-hybrid screening (Itoh *et al.*, 1999). Keap1, which associates with F-actin in cells, anchors Nrf2 in the cytoplasm through binding to the Neh2 domain of Nrf2. Targeted disruption of the *keap1* gene in mice clearly demonstrated the crucial role of Keap1 in the regulation of Nrf2 (Wakabayashi *et al.*, 2003). Levels of proteins for GSTs and NQO1 in livers of young *keap1*-disrupted mice were substantially higher than those of age-matched wild-type mice. Consistent with this observation, constitutive nuclear levels of Nrf2 and transcript levels for NQO1 in livers and fibroblasts from *keap1*-disrupted mice were high compared to tissues and cells from wild-type mice (Kwak *et al.*, 2004; Wakabayashi *et al.*, 2003). However, exact molecular

mechanism of repressive role of Keap1 on Nrf2 is not fully understood at this time. Repressive role of Keap1 in Nrf2 function has been accounted for by two observations; i) Dissociation of Nrf2 protein from Keap1 can be triggered by treatment with sulfhydryl-reactive chemicals such as di-ethylmaleate (Itoh *et al.*, 1999). A conformational switch through changes in sulfhydryl bonds of cysteine residues in Keap1 protein following treatment with chemicals has been hypothesized as a molecular mechanism of Nrf2 activation. Wakabayashi *et al.* have demonstrated that C273 and C288 of Keap1 are the critical sensors modified by chemopreventive agents that lead to the disruption of the Keap1-Nrf2 complex (Wakabayashi *et al.*, 2004). ii) Keap1 is also involved in facilitation of Nrf2 degradation by acting as an adaptor protein of E3 ubiquitin ligase (Kobayashi *et al.*, 2004; McMahon *et al.*, 2003; Zhang and Hannink, 2003). Therefore, when tethered to Keap1, Nrf2 is targeted for proteasomal degradation, thereby keeping cellular concentrations low (Itoh *et al.*, 2003; Zhang and Hannink, 2003).

Several groups have identified multiple signaling pathways that may affect Nrf2 activity (Chen and Kong, 2004; Nguyen *et al.*, 2003). The MAPK cascade including ERK2, JNK1 and p38 MAPK is involved in the regulation of Nrf2 activity positively or negatively depends on cell types and inducers. The phosphatidylinositol 3-kinase (PI3K) pathway was also known to affect Nrf2 activity and a pharmacological inhibitor of PI3K largely attenuates inducible expression of antioxidative genes in response to *t*-BHQ treatment (Kang *et al.*, 2001; Lee *et al.*, 2001). In addition, phosphorylation of the Ser 40 residue of Nrf2 by protein kinase C in response to *t*-BHQ treatment has been directly demonstrated *in vivo* (Huang *et al.*, 2002). As a upstream event of MAPK cascades for activation of Nrf2, a very recent study by Cho *et al.* have proposed that members of the G $\alpha$ 12 family involves in regulation of PI3K-dependent Nrf2 activation through modulation of PKC and JNK cascades (Cho *et al.*, 2007). Taken together, these results indicate that Keap1-Nrf2, which is a critical complex for the regulation of the expression of antioxidative genes, is coordinately controlled by diverse, but intersecting, signaling pathways.

Transcriptional regulation of Nrf2 also has been suggested as one of molecular mechanisms to activate the Nrf2 pathway. Cho *et al.* have demonstrated increased transcripts for Nrf2 in mouse lung exposed to hyperoxia. They have identified a point mutation in the promoter region of the *nrf2* gene in hyperoxia-sensitive mouse strains and found that this point mutation is associated with differential activation levels of Nrf2 in

response to hyperoxia (Cho *et al.*, 2002a, b). The ARE-like elements were identified in the proximal promoter region of murine *nrf2* gene and these elements were shown to mediate the activation of the *nrf2* promoter in response to dithiolethione treatment and Nrf2 overexpression in murine keratinocytes (Kwak *et al.*, 2002). Furthermore, a very recent study by Marzec *et al.* identified a single nucleotide polymorphism (SNP) in the ARE-similar sequences of the human *nrf2*, and found that this polymorphism is strongly associated with the high risk of development of acute lung injury in human (2007).

### GENES REGULATED BY THE NRF2 SIGNALING PATHWAY

Several groups have performed genome-wide screening using comparisons between wild-type and *nrf2*-disrupted mice to identify genes regulated by the Keap1-Nrf2 pathway. These reports have consistently confirmed that genes promoting the antioxidative capacity of cells are increased by Nrf2 activation. First, Thimmulappa *et al.* (2002) have identified fifty genes as Nrf2-dependent, chemopreventive sulforaphane-inducible genes in the small intestine following daily treatment of sulforaphane for one week. Most of these genes are associated with metabolism of electrophiles and free radicals, and proteins and enzymes enhancing cellular reducing potential such as glucose-6-phosphate dehydrogenase. Second, Kwak *et al.* (2003b) have reported that expression of three hundred genes was elevated in the liver isolated from D3T-treated wild-type mice and over 80% of these genes were not induced in *nrf2*-disrupted mice. These genes can be classified into several categories including typical xenobiotic metabolizing enzymes, antioxidative genes associated with maintaining redox potential, molecular chaperones and 26S proteasomes. Antioxidative genes induced by D3T in an Nrf2-dependent manner included thioredoxin, peroxiredoxin and enzymes involved in maintenance of homeostasis of antioxidants, including GSH-biosynthetic enzymes, GSH reductase, and thioredoxin reductase. Major novel gene categories found in this study were molecular chaperones and the 26S proteasome. Over 70% of the subunit genes that collectively form the 26S proteasome were shown to be regulated in an Nrf2-dependent manner. Moreover, disruption of the Nrf2 gene abrogated their inducibility. In further studies, it was demonstrated that induction of the catalytic subunit of the 20S proteasome, PSMB5, was regulated by Nrf2 through interactions with AREs located in its proximal promoter region (Kwak *et al.*, 2003a). These results implicate that possible protective

role of the 26S proteasome against cytotoxicity mediated by oxidatively damaged proteins. Recently, it has been demonstrated that coordinated induction of the proteasome subunits by sulforaphane resulted in protection against hydrogen peroxide-mediated cytotoxicity and accumulation of oxidized proteins (Kwak *et al.*, 2007).

A third study carried out by Shih *et al.* (Ishii *et al.*, 2000) has defined the transcription network of Nrf2 in rat brain cells. They focused on identifying genes elevated by Nrf2 itself in neuronal and glial cells from rat embryo by forcing overexpression of Nrf2 protein with a replication-deficient adenovirus. Their results also support the primary role of Nrf2 in the transcriptional activation of ARE-bearing genes such as detoxifying genes and antioxidants. This report, taken together with that of Lee *et al.* (2003), which used primary cultured cortical astrocytes, strongly suggests a possible neuroprotective role of Nrf2 downstream genes against oxidative/electrophilic stress.

### PHENOTYPES OF NRF2-DISRUPTED MICE

Based on the different gene expression patterns in the absence of Nrf2, *nrf2*-disrupted mice were hypothesized to be a good model to prove the role of antioxidative defense systems in protection against oxidative damage. Many studies are showing that constitutive expression of several antioxidative genes is reduced in the absence of Nrf2. Levels of transcripts for subunits of GSTA1 and GSTP1, microsomal epoxide hydrolase and ferritin heavy chain were significantly lower in *nrf2*-disrupted mice than wild-type mice (Kwak *et al.*, 2001a; Ramos-Gomez *et al.*, 2001). Intestinal enzymatic activities for NQO1 and GST were also suppressed 30~70% in *nrf2*-disrupted mice compared to wild-type mice (McMahon *et al.*, 2001). Due to this repressed expression of detoxifying enzymes, *nrf2*-disrupted mice have been predicted to be more susceptible to toxicities evoked by environmental chemicals and stress. Now there is much data to support this hypothesis. Table 1 summarizes published results examining the susceptibility of *nrf2*-disrupted mice to oxidative/electrophilic stresses; additional studies are in progress related to aging and neurodegenerative diseases. Several studies have employed chemicals to induce stress. First, administration of the pulmonary toxicant BHT to *nrf2*-disrupted mice showed a higher mortality than in wild-type mice (Chan and Kan, 1999). Second, *nrf2*-deficient mice were more sensitive to hepatotoxicity induced by acetaminophen intake than wild-type mice (Chan *et al.*, 2001; Enomoto *et al.*, 2001). Third, formation of gastric tumors by benzo[a]pyrene

**Table 1.** Phenotypes affected by the *nrf2* genotype in chemical exposure and oxidative damage-associated disease models

	Type of stressor	Measured phenotype	Reference
Chemical exposures	BHT	Pulmonary toxicity	Lee <i>et al.</i> , 2003
	Acetaminophen	Liver toxicity	Chan <i>et al.</i> , 1999, 2001
	Benzo[a]pyrene	Tumor formation in forestomach/	Ramos-Gomez <i>et al.</i> , 2001, 2003
	Diesel exhaust	DNA adduct formation	Aoki <i>et al.</i> , 2001
	Aflatoxin B <sub>1</sub>	DNA adduct formation	Kwak <i>et al.</i> , 2001b
Disease models	Bleomycin	Pulmonary fibrosis	Cho <i>et al.</i> , 2004
	Hyperoxia	Pulmonary damage	Cho <i>et al.</i> , 2002a
	Smoking	Emphysema	Rangasamy <i>et al.</i> , 2004
	Carageenan	Systemic inflammation	Itoh <i>et al.</i> , 2004
	Autoimmune nephritis in aged female mice	Nephritis and increased level of autoantibodies	Yoh <i>et al.</i> , 2001
	Hemolytic anemia	Hemolysis and increased level of autoantibodies	Lee <i>et al.</i> , 2004
	Systemic autoimmune disease in aged female mice	SLE-like syndrome in multiple organ	Li <i>et al.</i> , 2004

treatment was significantly increased in *nrf2*-disrupted mice compared to wild-type mice (Ramos-Gomez *et al.*, 2001). Fourth, levels of DNA adducts in target tissues are elevated in these knockout mice following exposure to the carcinogens aflatoxin B<sub>1</sub>, benzo[a]pyrene and diesel exhaust (Aoki *et al.*, 2001; Kwak *et al.*, 2001a; Ramos-Gomez *et al.*, 2003). Fifth, indices of lung fibrosis, including hydroxyproline content, collagen accumulation, fibrotic score and cell proliferation, were elevated to a greater extent in bleomycin-treated *nrf2*-disrupted mice than in wild-type mice (Cho *et al.*, 2004).

In addition, phenotypes of *nrf2*-disrupted mice have been tested using oxidative damage-related disease models (Table 1). First, *nrf2*-deficient mice were found to be very susceptible to hyperoxic damage of the lung compared to wild-type mice (Cho *et al.*, 2002a). Second, exposure to tobacco smoke, a well-known inducer of oxidative stress, produced emphysema in *nrf2*-disrupted mice but not in wild-type mice (Rangasamy *et al.*, 2004). Third, Nrf2 has a role in protection from inflammation-induced damage. Carageenan treatment produced significantly higher systemic inflammatory response in the *nrf2*-disrupted mice than in wild-type mice (Itoh *et al.*, 2004). Fourth, deficiency of Nrf2 is strongly associated with development of autoimmune diseases. Enhanced autoimmunity is also known to be caused by increased protein degradation in response to oxidative damage. Therefore, Nrf2 might have a role in protection against oxidative damage-induced autoimmunity. Targeted disruption of *nrf2* leads to development of lupus-like autoimmune nephritis accompanied by higher levels of IgG, IgM, C3, and anti-double strand DNA antibody in female mice over 60 weeks of age (Yoh *et al.*, 2001). Li *et al.* (2004) have also demonstrated that aged *nrf2*-deficient female mice developed multiple organ pathologies resembling human systemic lupus erythe-

matusus (SLE) and high levels of autoimmune antibodies were detected in kidney, liver, heart and brain. Also, these mice developed a regenerative immune-mediated hemolytic anemia due to decreased antioxidant potential and increased oxidative stress in erythrocytes (Lee *et al.*, 2004). Taken together, these different phenotypes of *nrf2*-disrupted mice can be explained by their reduced antioxidant potential and increased oxidative damage in various tissues.

## CONCLUDING REMARKS

Many recent studies demonstrate the pivotal role of Nrf2 in the regulation of antioxidative potential in mammalian cells. Successful development of *nrf2*-disrupted mice led to new insights into the gene categories regulated by this transcription factor, although descriptions of the Nrf2-regulated networks are far from complete. These mice are also useful in determining critical susceptibility pathways in chemical-mediated toxicities and in the development of murine models of human diseases. Important classes of cancer preventive agents activate the Nrf2 pathway and these models facilitate the identification and molecular study of such compounds. Translation of Nrf2 as a molecular target in humans is underway. Clinical trials using oltipraz in high-risk groups have shown that induction of antioxidative and electrophile detoxication genes by oral administration of oltipraz was an effective regimen to enhance the excretion of the potent human hepatocarcinogen aflatoxin B<sub>1</sub> (Wang *et al.*, 1999). It was also demonstrated that anethole dithiolethione (5-(*p*-methoxyphenyl)-1,2-dithiole-3-thione) reduced the extent of bronchial dysplasia in smokers in clinical trials, further implicating a role of induction of antioxidative genes in humans (Lam *et al.*, 2002). Current work seeks to identify natu-

ral products found in foods that activate the Keap1-Nrf2 pathway, as these items may be particularly useful in achieving protection in humans. Clearly, the transcriptional activity of Nrf2 may be a critical determinant in development of toxicity and carcinogenicity following exposure to environmental chemicals.

Modulation of Nrf2 could be also effective in preventing tissue damage, especially in the brain, that is produced by oxidative stress during the process of aging. Decreased transcriptional activity of Nrf2 has been observed in aged rats and this decline appears to play a central role in age-related loss of GSH and a reduced capacity to control cellular homeostasis (Suh *et al.*, 2004). Studies using neuronal cells from animals and humans have highlighted the prospect that Nrf2 controls regulation of antioxidative genes in response to typical enzyme inducers in these cells. Therefore, potential neuroprotective roles of the Nrf2 pathway against aging-associated tissue injuries could be attractive targets. Our continuing challenge will be to identify better inducers - better indirect antioxidative agents - that we can apply in a broad spectrum of chronic disease states.

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