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Review

An Important Role of Nrf2-ARE Pathway in the Cellular Defense Mechanism

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The antioxidant responsive element (ARE) is a *cis*-acting regulatory element of genes encoding phase II detoxification enzymes and antioxidant proteins, such as NAD(P)H: quinone oxidoreductase 1, glutathione *S*-transferases, and glutamate-cysteine ligase. Interestingly, it has been reported that Nrf2 (NF-E2-related factor 2) regulates a wide array of ARE-driven genes in various cell types. Nrf2 is a basic leucine zipper transcription factor, which was originally identified as a binding protein of locus control region of ß-globin gene. The DNA binding sequence of Nrf2 and ARE sequence are very similar, and many studies demonstrated that Nrf2 binds to the ARE sites leading to up-regulation of downstream genes. The function of Nrf2 and its downstream target genes suggests that the Nrf2-ARE pathway is important in the cellular antioxidant defense system. In support of this, many studies showed a critical role of Nrf2 in cellular protection and anti-carcinogenicity, implying that the Nrf2-ARE pathway may serve as a therapeutic target for neurodegenerative diseases and cancers, in which oxidative stress is closely implicated.

Keywords: Antioxidant responsive element, Nrf2

Introduction

Reactive oxygen species and electrophiles cause DNA damage and neuronal cell death resulting in development of malignancy and neurodegenerative disease, respectively. To counteract these insults, higher animals have developed elaborate defense mechanism, including phase II detoxification enzymes and antioxidant proteins (Ishii *et al*., 2002). Studies designed to identify the regulatory element for the phase II detoxification and antioxidant genes revealed a central role of the antioxidant responsive element.

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Antioxidant Responsive Element

The antioxidant responsive element (ARE) is a *cis*-acting regulatory element or enhancer sequence, which is found in promoter regions of genes encoding phase II detoxification enzymes and antioxidant proteins. Okuda and coworkers (1989) first described an enhancer element, which is similar to TPA-responsive element (TRE) or AP-1 site in the rat glutathione *S*-transferase (GST)-P gene. This was followed by the characterization of a similar *cis*-acting regulatory element or enhancer sequence in rat GST Ya (Rushmore *et al*., 1990), mouse GST Ya (Friling *et al*., 1990), and human NAD(P)H:quinone oxidoreductase-1 (NQO1) (Li and Jaiswal, 1992). Collectively, these studies provided a common mechanism for phase II detoxification gene induction.

The core ARE sequence was defined as 5'-TGACnnnGCA-3' based on mutational analysis of the rat GST A1 promoter sequence (Rushmore *et al*., 1991). In addition, many studies have demonstrated that the ARE sequence is found in numerous genes, and plays an important role in down stream gene expression. The list of ARE-driven genes includes rat GST A1, mouse GST A1, rat GST P1, rat NQO1, human NQO1, human glutamate-cysteine ligase (GCL), mouse ferritin-L, mouse metallothionein-1, and mouse UDP-glucuronyl transferase (UGT). It has been well described that the Ah receptor mediates the gene expression of phase I drug metabolizing enzymes through XRE. However, little was known about the transacting factor or binding protein for the ARE sequence until Nrf2 was identified.

Identification of ARE Binding Transcription Factors

In order to understand the molecular mechanism of ARE-driven gene expression, many studies have focused on the identification of transcription factor or binding protein for the ARE site.

First, the role of AP-1 transcription factor in the ARE activation has been extensively studied due to its binding

sequence similarity to the ARE sequence. Binding of c-Fos to the ARE sequence of mGST A1 and hNQO1 has been demonstrated by electrophoretic mobility shift assay (Li and Jaiswal, 1992). Also, Friling and coworkers (1992) demonstrated that phorbol-12-myristate-13-acetate (PMA) and *tert*-butylhydroquinone (tBHQ) increase GST Ya gene expression through AP-1 protein, and AP-1-mediated-ARE activation is protein kinase C (PKC)-independent. In contrast, Nguyen and coworkers (1994) reported that c-Fos does not bind to the ARE sequence in rabbit reticulocytes, and protein binding to the ARE sequence was not inhibited by c-Fos antibody. Based on these observations, the authors concluded that although AP-1 binding site can resemble an ARE site in its response to various ARE inducers, the ARE sequence is not a high-affinity binding site for the Jun/Fos heterodimer. AP-1 is also not believed to be involved in transcriptional machinery because the GC nucleotide outside the core TRE site is essential for the ARE-driven gene expression.

In addition to AP-1, unidentified proteins have been suggested to bind to the ARE sequence. For example, Nguyen and Pickett (1992) demonstrated that an unidentified heterodimer of 28 kDa and 45 kDa protein binds to the ARE sequence using a photochemical cross-linking approach. Also, Wang and Williamson (1994) detected 160 kDa protein(s) (ARE-binding protein, ARE-BP) from the nuclear extracts of HeLa cells. This protein specifically binds to the ARE of NQO1, not to the TRE. Furthermore, this 160 kDa protein does not contain Fos or Jun (Wang and Williamson, 1994). The identities of these proteins, however, still remain to be characterized.

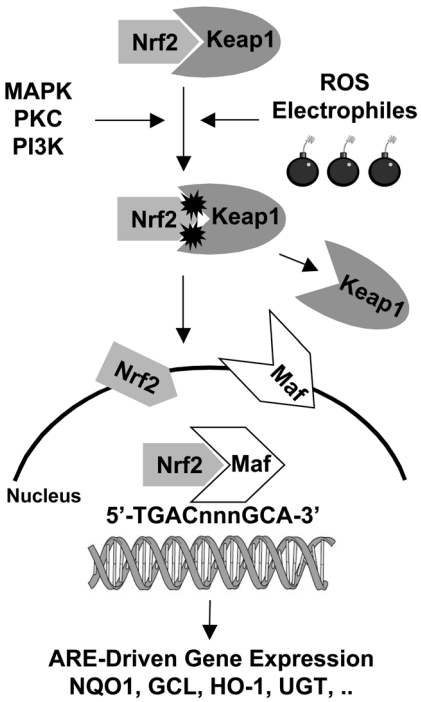

Fig. 1. ARE-driven gene expression by Nrf2. ARE activation signals dissociate the Nrf2-Keap1 complex allowing Nrf2 to translocate into the nucleus where it binds to the ARE and transcriptionally activates downstream target genes.

Nrf2 and ARE

Many proteins have been suggested to regulate the ARE, but the underlying ARE activation mechanism began to be elucidated with the identification of Nrf2.

Nrf2 was identified during attempts to isolate the transcription factor for the locus control region of ß-globin gene. Using the tandem repeat of NF-E2/AP-1 as a recognition site probe in yeast expression systems, two NF-E2 related proteins, Nrf1 (Chan *et al*., 1993) and Nrf2 (Moi *et al*., 1994) were identified. Moi and coworkers (1994) have reported that Nrf2 has a C-terminal basic leucine zipper containing basic region and heptad repeats of leucine, and N-terminal acidic domain (rich in glutamic and aspartic acid), which can potentially function as a acidic type of transactivation domain. They have also found that the expression pattern of Nrf2 is not restricted to the hematopoietic cells in contrast to that of NF-E2.

Venugopal and Jaiswal (1996) first reported an important role of Nrf2 in the ARE activation. The authors reasoned that the transcription factor binding to the ARE might be an AP-1-related protein because both human NQO1-ARE and rat NQO1-ARE contain AP-1 and AP-1-like element. They also noticed that the tissue specific expression of the Nrf2 transcription factor was identical to that of NQO1, and the DNA binding sequence for Nrf2 was similar to the ARE sequence. Based on these facts, they tested several basic leucine zipper proteins, and found that Nrf1 and Nrf2 positively while c-Fos and Fra1 negatively regulate the ARE of hNQO1 using EMSA and an ARE-reporter gene activity assay.

Similarly, Itoh and coworkers (1997) hypothesized that certain members of the Cap'n'collar (CNC) protein and/or Maf family transcription factor may bind to ARE sequence based on the fact that the ARE consensus sequence is strikingly similar to a binding site of Maf recognition element (MARE) or NF-E2 binding site (5'-GCTGAGTCA-3'). Also, they speculated that the complex of Nrf2 and small maf protein is the most likely candidate to function in this context. Nrf2 knockout mice were generated to test their hypothesis, and they found decreased expression levels of GSTs and NQO1 in $Nrf2^{+/-}$ mice compared to wild-type counter part (Itoh *et al*., 1997).

Subsequently, it has been demonstrated that Nrf2 is sequestered in the cytoplasm by Keap1 and ARE activation signals (i.e., protein kinase pathways and electrophiles) disrupt the Nrf2-Keap1 complex leading to nuclear translocation of Nrf2 and transcriptional activation of ARE-driven genes (Fig. 1).

Nrf2 Confers Cytoprotection

The function of Nrf2 and its downstream target genes have been shown to be important for protection against oxidative stress- or chemical-induced cellular damage. In support of this idea, numerous studies showed that the decreased levels of phase II detoxification enzymes and antioxidant proteins make $Nrf2^{-/-}$ (knockout) mice highly sensitive to cytotoxic electrophiles compared with $Nrf2^{+/+}$ (wild-type) mice. First, Chan and Kan (1999) demonstrated an important role of Nrf2 in pulmonary toxicity. They showed that $Nrf2^{-/-}$ mice are extremely susceptible to the antioxidant butylated hydroxytoluene (BHT). With doses of BHT that were tolerated by wild-type mice, $Nrf2^{-/-}$ mice succumbed from acute respiratory disease syndrome (Chan and Kan, 1999). Similarly, Cho and coworkers (2002) demonstrated a protective role of Nrf2 against pulmonary hyperoxic injury. Liver is another organ where Nrf2 plays an important role for protection. Chan and coworkers (2001) showed that $Nrf2^{-/-}$ mice are more sensitive to acetaminophen-induced hepatotoxicity than $Nrf2^{+/+}$ mice. This differential sensitivity was attributed to decreased gene expression levels of GCL, UGT 1A6, and GST pi in $Nrf2^{-/-}$ mice. Similarly, Enomoto and coworkers (2001) showed that administration of acetaminophen induced more severe centrilobular hepatocellular necrosis in $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice. The authors suggested that decreased expression levels of UGT and GCL result in lower levels of UGT activity and hepatic non-protein sulfhydryl contents leading to higher sensitivity of $Nrf2^{-/-}$ liver to electrophiles (Enomoto *et al*., 2001). Although it appears that Nrf2-dependent ARE-driven detoxification and antioxidant proteins are major contributing factors for Nrf2-conferred cellular protection, Nrf2 also might be involved in apoptosis signaling pathways. For example, Ohtsubo and coworkers showed that caspase-3 (-like) proteases cleave Nrf2. In addition, overexpression of cleaved Nrf2 (C-terminal fragment) induced apoptosis in HeLa cells, implying cleaved Nrf2 might have some role in the induction of apoptosis (Ohtsubo *et al*., 1999). Recently, Kotlo reported that Nrf2 is an inhibitor of the Fas pathway using the "Achilles' Heel Method". The authors transfected HeLa cells were with an antisense cDNA expression library in an episomal vector followed by selection with a suboptimal dose of the apoptosis inducer, and found that antisense cDNA of Nrf2 sensitizes cells. They also found that overexpression of Nrf2 protects cells from Fas-induced apoptosis suggesting an important role of Nrf2 in anti-apoptotic pathways (Kotlo *et al*., 2003). Furthermore, Nrf2 is an important effector of PERK-mediated cell survival (Cullinan *et al*., 2003), and Nrf2 regulates the sensitivity of death receptor signals (Morito *et al*., 2003). Collectively, these observations suggest that Nrf2-ARE pathway play an important role in cellular survival by modulating both cellular antioxidant potential and apoptosis pathway.

In addition to cytoprotection, several studies showed anti-carcinogenic effect of Nrf2. For example, Ramos-Gomez demonstrated that $Nrf2^{-/-}$ mice have a significantly higher burden of gastric neoplasia after treatment with benzo[a]pyrene than $Nrf2^{+/+}$ mice. They also showed that oltipraz, which is currently in clinical trials, significantly reduced multiplicity of gastric neoplasm only in $Nrf2^{+/+}$ mice suggesting that constitutive and inducible expression of phase II enzymes through the Nrf2-ARE pathway affect the susceptibility to carcinogenesis (Ramos-Gomez *et al*., 2001). Aoki and coworkers reported that diesel exhaust induces more DNA adducts in $Nrf2^{-/-}$ mice compared with $Nrf2^{+/-}$ mice due to suppressed activity of phase II detoxification enzymes in $Nrf2^{-/-}$ mice (Aoki *et al*., 2001). Finally, Fahey and coworkers showed that an ARE inducer, sulforaphane, which is abundant in broccoli, is a potent bacteriostatic agent against *Helicobacter pylori* and blocks benzo[a]pyrene-evoked forestomach tumors in ICR mice suggesting the dual actions of sulforaphane may function synergistically to provide a diet-based protection against gastric cancer (Fahey *et al*., 2002).

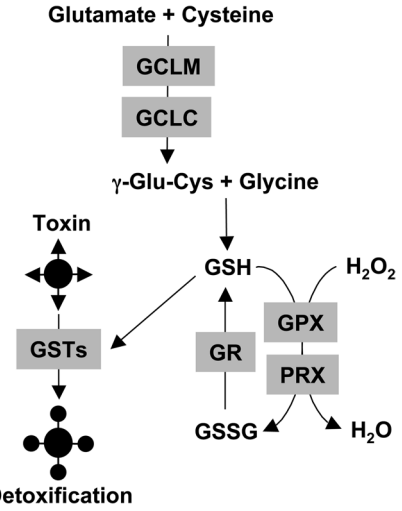

Fig. 2. An orchestrated gene expression by Nrf2. Nrf2 coordinately up-regulates genes which are involved in maintenance and utilization of GSH. GCLM, glutamate-cysteine ligase modulatory subunit; GCLC, glutamate-cysteine catalytic subunit; GR, glutathione reductase; GSTs, glutathione *S*-transferases; GPX, glutathione peroxidase; and PRX, peroxiredoxin. Adapted from reference Lee *et al*., 2003a.

Neuroprotection Conferred by Nrf2-ARE Pathway

Many chronic neurodegenerative diseases (i.e. Parkinson's disease and Alzheimer's disease) are thought to involve oxidative stress as a component contributing to the progression of the disease, and oxidative stress can be relieved by the Nrf2-ARE pathway as demonstrated previously. One of the Nrf2-dependent ARE-driven genes, NQO1, has been demonstrated to play an important role in protecting cells

against oxidative stress (Murphy *et al*., 1991). Interestingly, overexpression of NQO1 and one GST isoenzyme did not protect N18-RE-105 rodent neuroblastoma cells from free radical-mediated toxicity (Duffy *et al*., 1998), although tBHQ treatment, which upregulates a battery of ARE-driven genes, protected N18-RE-105 cells from glutamate toxicity (Murphy *et al*., 1991). These observations imply that the coordinate upregulation of ARE-driven genes, not one or two genes, is more efficient in protecting cells from oxidative damage. A recent study identified the ARE-driven genes including NQO1 that were responsible for protecting IMR-32 human neuroblastoma cells from H_2O_2-induced apoptosis (Li *et al*., 2002). In addition, neural cells from $Nrf2^{-/-}$ mice were more sensitive to oxidative stress compared with those from $Nrf2^{+/+}$ mice (Lee *et al*., 2003a, 2003b), and overexpression of Nrf2 dramatically increased the resistance of neurons to oxidative cell death (Shih *et al*., 2003). Recently, oligonucleotide microarray analysis revealed that Nrf2 regulates the orchestrated gene expression of detoxification enzymes, antioxidant proteins, anti-inflammatroy proteins, calcium homeostasis protein, and signaling molecules (Lee *et al*., 2003a, 2003b). For example, Nrf2 coordinately up-regulates genes which are involved in maintenance (i.e. synthesis and regeneration) and utilization of glutathione (Fig. 2). This orchestrated up-regulation of ARE-driven genes by Nrf2 appeared to be very efficient in increasing cellular detoxification and antioxidant capacity, implying an important role for Nrf2-ARE pathway as a cellular antioxidant defense system.

Conclusions

Numerous studies have addressed a pivotal role of Nrf2 in protecting cells from oxidative stress. One unique feature about the Nrf2-ARE pathway (namely programmed cell life pathway) (Li *et al*., 2001) is that Nrf2-ARE pathway coordinately up-regulates many protective detoxification and antioxidant genes, which can synergistically increase the efficiency of cellular defense system. Since the Nrf2-ARE pathway acts as a master regulator of many protective genes, the programmed cell life pathway may serve as a therapeutic target for neurodegenerative diseases and carcinogenesis, in which oxidative stress is involved.

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