Nrf2-DEPENDENT GENE EXPRESSIONS: A MOLECULAR TOXICOLOGICAL ASPECT

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ABSTRACT — Although NF-E2 related factor 2 (Nrf2) was found to be a transcriptional regulator that controls an expression of the β-globin gene, the notion is now widely accepted that this transfactor serves as a master regulator for the gene expression of a battery of proteins acting on anti-oxidative stress and detoxification of electrophiles. The function of Nrf2 that bears transcriptional activation depends solely on its nuclear localization, which is regulated by interaction with the cytosolic anchor protein Keap1 and its own turnover rate. In the present mini-review, we focus on the regulation of Nrf2 function and discuss the physiological and toxicological aspects of this transcriptional factor.

KEY WORDS: Nrf2, Oxidative stress, Bach1, Keap1, Phase 2 proteins, Chemoprevention

INTRODUCTION

It has been known since the early seventies that phenolic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyldihydroquinone (tBHQ) exert a protective effect against chemical carcinogens (Wattenberg, 1972, 1973). These observations have been interpreted as a consequence that the antioxidants induce Phase II drug-metabolizing enzymes such as glutathione S-transferases (GST) (Benson et al., 1978), UDP-glucuronosyltransferases (Cha and Heine, 1982) and NAD(P)H-quinone oxidoreductase 1 (NQO1) (Benson et al., 1980), which detoxify the electrophilic metabolites of the carcinogens, in addition to Phase I enzymes (cytochrome P450s) (Wattenberg, 1975). On the other hand, it is also known that experimental conditions that commit animals to oxidative stress including depletion of reduced glutathione (GSH) and administration of electrophilic chemicals induce stress proteins such as γ-glutamyl cysteine synthetase (γGCS) (Meister, 1984), cystine-glutamate transporter (xCT) (Bridges et al., 2001; Kim et al., 2001), an inducible isoform of heme oxygenase (HO-1) (Keyse et al., 1990), epoxide hydrolase (Benson et al., 1979) and dihydrodiol dehydrogenase (Ciaccio et al., 1994). These enzymes protect tissues from oxidative insults even though those functions differ. Genomic analysis of these inducible proteins revealed an antioxidant response element (ARE) (Rushmore et al., 1990, 1991) and electrophile response element (EpRE) (Firling et al., 1990) that were identified as cis-acting elements required for gene expression induced by the antioxidants and the electrophiles, respectively. Because of complete overlapping of these sequences, it was considered that an identical transcriptional factor governs these elements and thus induces Phase II enzymes and stress proteins simultaneously. Therefore, the gene battery, including classical Phase II enzymes and stress proteins, has been labeled as Phase 2 proteins (Talalay et al., 2003), as the ARE/EpRE promoter controls these genes. A striking sequence similarity of ARE/EpRE, Maf recognition element (MARE) and TPA response element allows an extensive investigation of the transcription factor that binds to and controls positively the elements in response to the antioxidants and/or electrophiles (Li and Jaiswal, 1992; Jaiswal, 1994; Oguro et al., 1996). These investigations suggest activator protein-1 (AP-1), which has been implicated in cell growth and stress-responsive signals, is a convincing candidate. While, at the same time, there have been numerous inexplicable phenomena observed if AP-1 is major trans-factor

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responsible for ARE/EpRE-mediated gene expression (Numazawa et al., 1997). A breakthrough emerged from a diverse scientific field. Yamamoto and his colleagues, whose research interests had been on gene regulation during erythroid differentiation, generated germline MARE binding protein Nrf2-deficient mice (Itoh et al., 1997). Nrf2 is involved in regulation of globin gene transcription, acting through locus control regions upstream of the α- and β-globin gene clusters (Moi et al., 1994). These animals show little if any defect in erythropoiesis but complete disruption of the gene expression of Phase 2 proteins in response to electrophiles (Itoh et al., 1997). In addition, the Nrf2-deficient mice are much more susceptible to hepatic toxicities of acetaminophen (Chan et al., 2001; Enomoto et al., 2001), pulmonary toxicities of hyperoxia (Cho et al., 2002) and stomach carcinogenesis by benzo[a]pyrene (Ramos-Gomez et al., 2001; Fahey et al., 2002). These observations indicate clearly that Nrf2 acts in a critical role in ARE/EpRE-mediated gene expression, thus protecting tissues from oxidative and xenobiotic stresses. The next focus of interest moved on to molecular mechanism for Nrf2 activation. This mini-review focuses and introduces recent observations in terms of intracellular signals leading to Nrf2 and ARE/EpRE activation which could be molecular targets for cancer and oxidative stress-mediated diseases.

**CYTOSOLIC ANCHOR OF Nrf2 BY KEAP1**

It has been considered that a primary control of Nrf2 function lies on subcellular distribution rather than induction of the protein, as Nrf2 mRNA levels are invariable during the treatment of electrophiles. From this point of view, Itoh et al. (1999) demonstrated that an N-terminal domain of Nrf2, the so-called Neh2 domain, inhibited its own transactivation and cloned the Nrf2-binding protein Kelch-like ECH-associated protein 1 (Keap1) by a two-hybrid screen using Neh2 domain as a bait. As similar to the Drosophila actin binding protein Kelch, Keap1 possesses Broad complex, Tramtrack, and Bric a brac (BTB) domain on its N-terminal and the Kelch repeat domain on its C-terminal regions, both of which are involved in protein-protein interaction (Adams et al., 2000). A recent approach using transgenic animal-derived cells revealed that Keap1 actuary binds and co-localizes to the actin filament (Kang et al., 2004). In addition, they demonstrated that direct interaction between the Neh2 domain of Nrf2 and Kelch repeat domain of Keap1 make it possible for Nrf2 to retain the cytoplasmic compartment. Electrophiles stimulate the Keap1 release from the Neh2 domain of Nrf2, thereby inducing nuclear translocation of the transcription factor. Therefore, it is proven that the complex composed of Nrf2 and Keap1 is a major target of phenolic antioxidants and oxidative stress in the induction machinery of Phase 2 proteins.

The query is how Keap1 releases Nrf2 in response to the electrophiles. In this respect, Keap1 likely acts as a direct sensor of the oxidants (Dinkova-Kostova et al., 2002). Murine Keap1 contains 25 cysteine residues that are conserved between species. Dinkova-Kostova et al. (2002) paid attention to the reactivity of these cysteine residues and found that the 4 residues, residing in the linker region between the BTB domain and Kelch repeat domain, were highly reactive in vitro. It has recently been demonstrated by several different groups that reduced situations of C273 and C288 are necessary for interaction with the Neh2 domain of Nrf2 in cells (Zhang and Hannink, 2003; Wakabayashi et al., 2004). Consequently, it became eventually clear that Keap1 functions as a redox sensor in the process for induction of Phase 2 proteins.

**PROTEASOMAL DEGRADATION OF Nrf2**

The report (Sekhar et al., 2000), showing that a gene encoded the γ-GCS catalytic subunit is induced by 26S proteasome inhibitor lactacystin, became the beginning of evidence, indicating that changes in the stability of Nrf2 during the course of ARE/EpRE activation (Nguyen et al., 2003; Sekhar et al., 2002; Stewart et al., 2003; Itoh et al., 2003; McMahon et al., 2003; Zhang and Hannink, 2003). In unstimulated cells, a turnover rate of endogenous Nrf2 protein present in the cytosolic compartment is very fast (t1/2 ranging from 10 to 30 min) (Nguyen et al., 2003; Stewart et al., 2003). Such a rapid turnover of Nrf2 was explained by the ubiquitin-dependent proteasomal degradation as observed in other transcription factors, such as c-Jun, c-Fos, NFkB, STAT1 and p53 (Pahl and Baeuerle, 1996; Salvat et al., 1999). The PEST-like sequence (Rogers et al., 1986) that resides in Nrf2 protein might be involved in the ubiquitination signals (Stewart et al., 2003) as in other labile proteins, such as ornithine decarboxylase and 1xB. Furthermore, Keap1 actively and efficiently sequesters de novo synthesized Nrf2, causing its ubiquitination and degradation (McMahon et al., 2003; Zhang and Hannink, 2003). In
stimulated cells, Nrf2, released from and/or liberated from sequestration by Keap1, also undergoes ubiquitin-dependent proteolysis; however, the degradation rate is significantly delayed in comparison with that in unstimulated cells. Consequently, Keap1 is a key regulator that controls not only the subcellular localization but also the stabilization of Nrf2. Keap1-deficient mice develop normally but die soon after birth, probably from malnutrition resulting from hyperkeratosis in the esophagus and forestomach (Wakabayashi et al., 2003). These phenotypes of the Keap1 deficiency could be reversed by breeding to Nrf2-deficient mice, confirming that Keap1 acts upstream of Nrf2 and governs ARE/EpRE-dependent cellular response (Wakabayashi et al., 2003).

OTHER POST-TRANSLATIONAL MODIFICATIONS OF Nrf2

Because PKC inhibitors significantly suppressed ARE/EpRE-mediated gene expressions, Huang et al. (2000) demonstrated by using a cell-free system that PKC directly phosphorylates Nrf2. Among the potential phosphorylation sites of Nrf2 by PKC, serine 40 (S40) residing in the Neh2 domain plays a central role in the interaction between Nrf2 and Keap1 (Huang et al., 2002). Later, it was confirmed by several groups including us (Numazawa et al., 2003; Bloom and Jaiswal, 2003) that phosphorylation of S40 by PKC actually occurs in cells treated with phenolic antioxidants and electrophiles, causing the Nrf2 escape from Keap1 sequestration. It seems that the phosphorylation of S40 is not directly involved in nuclear accumulation and transactivation of Nrf2 (Bloom and Jaiswal, 2003). These findings, anyhow, indicate that the interaction between Keap1 and Nrf2 is regulated simultaneously by post-translational modification of Nrf2 and redox control of Keap1. We demonstrated that atypical protein kinase C (aPKC), a PKC subgroup independent of calcium and diacylglycerol for the enzyme activity, is responsible for the Nrf2 S40 phosphorylation under oxidative stress condition (Numazawa et al., 2003). Several reports indicate that the phosphatidylinositol-3-kinase (PI3K) pathway is involved in Nrf2 activation in cells exposed to tBHQ (Kang et al., 2002b; Lee et al., 2001), peroxynitrite (Kang et al., 2002a) and hemin (Nakaso et al., 2003). Concerning the fact that aPKC acts downstream of PI3K (Izumi et al., 1998; Uberall et al., 1999; Wootten et al., 2000), it assumes that certain stimuli utilize a pathway PI3K→aPKC→Nrf2 for ARE/EpRE transactivation.

Meantime, there are reports showing that mitogen-activated protein (MAP) kinases, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAP kinase, are involved in the induction of Phase 2 proteins (Numazawa et al., 1997; Oguro et al., 1998; Elbirt et al., 1998; Yu et al., 1999; Alam et al., 2000; Otterbein et al., 2000; Zipper and Mulcahy, 2000). However, it is still obscure how MAP kinases induce ARE/EpRE-dependent transcription because there is no direct evidence indicating that activation of the kinases affects subcellular localization of Nrf2. Based on the observation that a cAMP response element binding protein binding protein (CBP) and p300 act as a co-activator for Nrf2-mediated ARE/EpRE transactivation (Katoh et al., 2001; Zhu and Fahl, 2001), a recent study shows that ERK and JNK pathways induce the recruitment of the co-activator to the transcription factor complex (Shen et al., 2004). Accordingly, these studies revealed that ARE/EpRE-dependent transactivation is a harmonized consequence of diverse signaling pathways.

REGULATION OF ARE/EpRE ACTIVITY BY BACH1

HO-1 shows the most significant induction among the ARE/EpRE-dependent genes. This is at least partly because of its low expression levels under a homeostatic condition. One possible mechanism explaining such a low basal expression is that a transcription factor BTB and CNC homology 1 (Bach1) functions as a repressor for the HO-1 gene. Bach1 found as a small Maf binding protein possesses the BTB domain on its N-terminal region as Keap1 and bZip DNA binding domain on the C-terminal region (Oyake et al., 1996). Bach1 heterodimerizes with small Maf proteins, such as MafK and MafG, and the complex interacts through the BTB domain, which in turn binds and regulates multiple MAREs in the locus control region of the β-globin gene (Igarashi et al., 1998) and clusters of ARE/EpRE (Sun et al., 2002) on the 5'-region of the HO-1 gene. Under homeostatic conditions, it is assumed that the Bach1 complex occupies the ARE/EpRE and recruits a co-repressor-histone deacetylase complex including SMRT or N-CoR as similar to other BTB co-repressors such as BCL-6 (for B cell lymphomas 6) (Dhordain et al., 1997) and HIC-1 (for hypermethylation in cancer-1) (Deltour et al., 1999), thereby silencing the transcription of the HO-1 gene. Nrf2 translocated to the nucleus in response to the stimuli might be exchanged for Bach1 on the target
gene and recruit the co-activator, causing the transactivation. Therefore, this model advocates that the balance between Nrf2 and Bach1 on ARE/EpRE determines the level of HO-1 gene expression. Using gene-targeting strategy, it was confirmed that Bach1 actively suppresses transcription of mouse ho-1 gene (Sun et al., 2002). On the other hand, other genes encoding phase 2 proteins, such as NQO1 and GST A2, possess a single ARE/EpRE in the promoter region, resulting in higher basal expression and lower

![Diagram of signaling pathways](image)

**Fig. 1.** Proposed scheme of signaling pathways for ARE/EpRE activation. Nrf2 sequestered by Keap1 is subjected to polyubiquitination and degradation under unstimulated condition. Oxidative stress conditions and electrophiles induce oxidation of cysteine residue of Keap1, resulting in release of Nrf2, increased turnover rate and its nuclear translocation. The stimuli also cause Nrf2 phosphorylation via a PKC pathway, which accelerates liberation from the Keap1 sequestration. Furthermore, MAP kinase pathways boost transactivation of the target gene by phosphorylation of a co-activator complex. On the other hand, repressor function of Bach1, which binds multivalent ARE/EpREs and silences HO-1 gene, is reversed by binding an HO-1 substrate heme and a redox change induced by oxidative stress.
Nrf2-dependent gene expressions.

inducibility compared to HO-1.

How is the target gene liberated from the Bach1-mediated transcrip
tional repression? In this regard, it has been reported that heme, a substrate and an inducer of HO-1, modulates Bach1 function by binding to the repressor (Sun et al., 2002, 2004). In addition, Cd\(^{2+}\) induces nuclear export of Bach1 by a mechanism depending on the nuclear export carrier Crm1 (Suzuki et al., 2003). Our preliminary results, however, indicate that oxidative stress conditions do not induce the nuclear export of Bach1 (Ishikawa, Numazawa and Yoshida, unpublished data), suggesting that the active nuclear export is a phenomenon specific to Cd\(^{2+}\) exposure. We noted the point that Bach1 was rich in cysteine and found that oxidation of the residue in the bZip domain significantly reversed its repressor activity. These observations suggest that Bach1 function, as well as Keap1, is controlled by redox circumstance. A detailed study is currently under way to elucidate redox control of the repressor function in our laboratory.

CONCLUSION

Evidence is increasingly accumulating that ARE/EpRE activation by Nrf2 plays a central role in the molecular mechanism governing the protective function of Phase 2 proteins against chemical carcinogens and oxidative stress. Based on these detailed in vitro and in vivo studies, chemoprevention trials of ARE/EpRE activator olitipraz, a substituted 1,2-dithiole-3-thione originally developed as an antischistosomal agent, is currently under way in China (Kwak et al., 2001) and the United States (Pendyala et al., 2001). Anticarcinogenic action of sulforaphane, a naturally occurring isothiocyanate that induces Phase 2 proteins (Zhang et al., 1992), also depends on the Nrf2 (Ramos-Gomez et al., 2001). In addition to these anticarcinogenic agents, the cellular system involving ARE/EpRE activation possibly will be a molecular target for distinct drugs treating ischemia-reperfusion disorders and inflammatory diseases.

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