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Targeting Nrf2 Signaling to Combat Chemoresistance

REVIEW

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Nuclear factor E2-related factor 2 (Nrf2) is a transcription factor that upregulates expression of a battery of genes to combat oxidative and electrophilic stress. Modification of Kelch-like ECH-associated protein 1 (Keap1) by reactive oxygen species stabilizes Nrf2 by escaping from degradation. Nrf2 then binds to antioxidant response elements (AREs) on the promoter region of various genes. Activation of the Keap1-Nrf2-ARE pathway plays critical roles in the chemopreventive effect of various phytochemicals. However, Nrf2 can protect cancer cells from oxidative stress and promote cell proliferation. Moreover, recent studies reveal that activation of the Nrf2 pathway is critical for resistance to chemotherapeutic agents. The aim of this review is to provide a molecular basis for the use of Nrf2 inhibitors in overcoming chemoresistance.

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Key Words: Nrf2, Keap1, Chemotherapy, Inhibitor

INTRODUCTION

Kelch-like ECH-associated protein 1 (Keap1)-Nuclear factor E2-related factor 2 (Nrf2) is a major cellular pathway that protect normal cells against oxidative and xenobiotic damage.[1] Nrf2 is an essential transcription factor for antioxidant and detoxification genes and is crucial for the chemopreventive effect of various phytochemicals against carcinogenesis. Representative chemopreventive agents that induce Nrf2 include carotenoids, curcumins, cyclic lactones, diterpenes, dithiolethiones, epithionitriles, flavonoids, indoles, isothiocyanates, organosulfides, and phenols.[2,3] Accumulating evidences have demonstrated that phytochemicals can protect cells from oxidative stress related diseases including inflammatory, cardiovascular, and neurodegenerative diseases and cancer, through Nrf2-dependent responses.

Paradoxically, recent researches demonstrated the dark side of Nrf2.[4] Cancer cells acquire a growth advantage by eliminating Keap1-mediated negative control of Nrf2, which leads to activation of the Nrf2-dependent defense response.[4] Constitutive upregulation of Nrf2 has been found in many types of cancers, including skin, breast, prostate, lung, head and neck, and endometrial cancer.[5] Overexpressed Nrf2 provides a growth advantage for cancer cells by protecting those cells from oxidative stress and anticancer agents, thus contributing to chemoresistance.[5] In this review, we summarize the therapeutic development of Nrf2 inhibitors that enhance the efficacy of the current cancer treatments.

REGULATION OF Nrf2 BY Keap1

Nrf2 is a basic leucine zipper transcription factor with 7 domains from Neh1 to Neh7. Neh1 has DNA binding motifs.[6] Neh3, Neh4, and Neh5 have domains involved in the transactivation of

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Nrf2 target genes by binding coactivators. Neh2, Neh6, and Neh7 regulate the stability of Nrf2. Neh2 has a major regulatory domain with 2 binding sites for Keap1 named ETGE and DLG (Fig. 1B).

Keap1, a 69 kDa protein, serves as a negative regulator of Nrf2. Keap1 has 5 functional domains including Kelch, intervening region (IVR), broad complex/tramtrack, Bric-a-Brac domain (BTB), an N-terminal region (NTR), and a C-terminal region (CTR).[3] Cysteine residues of IVR play as a sensor for reactive oxygen species (ROS). The Kelch domain binds to Nrf2 using 6 Kelch repeats. The BTB domain has a cys151 residue and is responsible for Nrf2 ubiquitination through binding to Cullin E3 ubiquitin ligase (Cul3)-based ubiquitin E3 ligase (Fig. 1A).

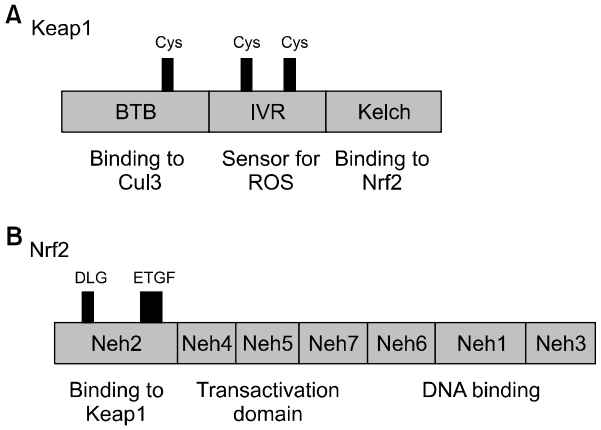

Figure 1. Structures of Kelch-like ECH-associated protein 1 (Keap1) and nuclear factor E2-related factor 2 (Nrf2). BTB, Bric-a-Brac domain; IVR, intervening region; Cul3, Cullin E3 ubiquitin ligase; ROS, reactive oxygen species; DLG and ETGF, binding sites for Keap1.

Under unstressed conditions, Nrf2 forms a complex with Keap1, leading to polyubiquitination and subsequent degradation by the 26S proteasome (Fig. 2A).[7] Upon exposure to a variety of stressors, including ROS, toxicants, and carcinogens, Nrf2 is released due to modification of the cysteine residues of Keap1 (Fig. 2B).[8] Two mechanistic models have been proposed for the regulation of Nrf2: the hinge and latch model and the Keap-Cul3 dissociation model.[8-10] In the hinge and latch model, 2 binding motifs of the Neh2 domain in Nrf2 have different affinities.[9] For the ubiquitination, both the DLG and ETGF motifs should be occupied by Keap1 proteins. ROS modify the cysteine residues of Keap1, leading to the release of the DLG motif (latch) without changing the ETGF motif (hinge) on Neh2. Release of the DLG motif inhibits ubiquitin ligase. Another model is the Keap-Cul3 dissociation model in which oxidative stress disrupts the Keap1-Cul3 E3 ligase interaction without changing the conformation of Keap1.[8]

Nrf2 stabilization with de novo synthesis increases the cytoplasmic level of Nrf2. Nrf2 translocates into the nucleus and binds to an antioxidant response element (ARE) as a heterodimer with musculo-aponeurotic fibrosarcoma (Maf) to recruit a transcriptional coactivator and promote thetranscription of various cytoprotective genes that are associated with antioxidant and detoxification enzymes. Upon recovery of cellular redox homeostasis, Keap1 translocates to the nucleus to dissociate Nrf2 from the ARE resulting in degradation of Nrf2.[11]

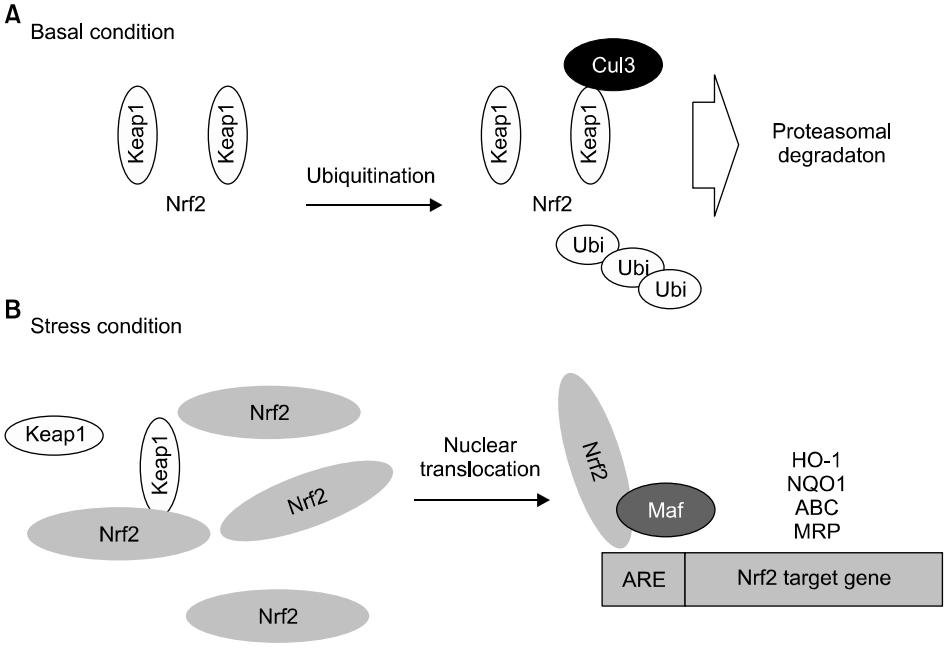

Figure 2. The Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor E2-related factor 2 (Nrf2)-antioxidant response elements (ARE) signaling pathway. Cul3, Cullin E3 ubiquitin ligase; Ubi, uniquitin; Maf, musculo-aponeurotic fibrosarcoma; HO-1, heme oxygenase-1; NQO1, NAD(P)H: quinone oxidoreductase 1; ABC, ATP binding cassette; MRP, multidrug resistance-associated protein.

Nrf2 TARGET GENES

Nrf2 binds to AREs in promoter regions of various genes that regulate the cellular response to oxidative and xenobiotic stress. Nrf2 target genes include antioxidant genes and phase II enzymes such as heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1), glutathione S-transferase (GST), and glutathione peroxidases.

The ARE-bearing genes that are regulated by Nrf2 in humans have been studied using microarray analysis of genes induced by chemopreventive agents or knockout of Keap1 in human cell lines.[2] These genes can be categorized into 5 groups: antioxidant genes (GCLC, GCLM, GPX2, GSR, SLC7A11, SRXN1, and TXND1), NADPH-generating enzymes (G6PD, ME1, and PGD), metal-binding proteins (FTH1, FTL, MT1, and MT2), drug-metabolizing enzymes and drug transporters (AKR1B1, AKR1B10, AKR1C1, AKR1C2, AKR1C3, AKR1C4, CBR1, GSTM3, MRP2, NQ1, and PTGR1), and stress response proteins (GADD45, HMOX1, HSP40, and HSP70).[2] Knockdown of Keap1 in human keratinocytes resulted in upregulation of 23 mRNAs while AKR1B1, AKR1B10, and AKR1C1 were induced to the greatest extent, showing increases of between 12- and 16-fold indicating that aldo-keto reductases could be useful biomarkers for Nrf2 activation.[12] As Keap1 is responsible for inhibitor kappa B (IκB) kinase beta (IKK-β) ubiquitination, upregulation of some of these genes could be attributable to the increase of nuclear factor-kappa B (NF-κB) activity.[13]

Expression profiling in mice has revealed that Nrf2 regulates approximately 100 genes and that two-thirds of Nrf2-target genes are not involved in detoxication or antioxidation. Instead, many of the target genes are associated with inflammation and immunity proteins.[2]

CROSS-TALK BETWEEN Nrf2 AND OTHER SIGNALING PATHWAYS

The aryl hydrocarbon receptor (Ahr) is a transcription factors that mediates the biological effects of its xenobiotic ligands including dioxin.[14] The Ahr activates the transcription of NQO1 and other genes through the xenobiotic-responsive element (XRE).[15] Ahr induces the direct binding of Nrf2 to promoters or indirectly produces reactive intermediates that trigger the Nrf2 signaling pathway.[16]

The cross-talk between NF-κB and Nrf2 is more complex. Several chemopreventive agents, such as sulforaphane, epigallocatechin-3-gallate, and curcumin, induce Nrf2 signaling with concomitant repression of NF-κB.[14] The NF-κB p65 subunit represses the Nrf2-ARE pathway by preventing CREB binding protein (CBP) from binding Nrf2 through competitive interaction or by promoting the recruitment of histone deacetylase 3 (HDAC3), a corepressor, to the ARE.[17] In chondrocyte apoptosis, shear-induced cyclooxygenase-2, an NK-κB target gene, can suppress phosphatidyl inositol 3-kinase (PI3K) activity, causing a reduction in theNrf2-mediated transcriptional response.[18] In contrast, Nrf2 inhibits the activity of NF-κB by attenuating phosphorylated IκB. The loss of Nrf2 increases ROS, leading to activation of NF-κB. Nrf-2 target genes, including HO-1 have been reported to negatively affect NF-κB activation.[19,20]

p53 has been reported to suppress the Nrf2-dependent transcription of antioxidant response genes by directly interacting with ARE-containing promoters. Previous studies have suggested that the function of this negative regulation is to prevent the formation of antioxidant environment, which could hinder the induction of apoptosis by p53.[21] In contrast, direct targets of p53 could upregulate Nrf2 activity. p21 is a potent cyclin-dependent kinase inhibitor that regulates cell cycle progression. Studies have shown that p21 inhibits the degradation of Nrf2 by interacting with the DLG motif, leading to upregulation of antioxidant genes.[22] These dual functions of p53 toward Nrf2 indicate that p53 may decide the fate of damaged cells whether to repair the damaged cells or apoptosis.

Nrf2 is also involved in autophagy by inducing p62 which is a receptor for autophagic degradation. As p62 could block binding between Nrf2 and Keap1, p62 contributes to the activation of Nrf2 target genes in response to oxidative stress creating a positive feedback loop.[23]

DEREGULATION OF Nrf2 IN CANCER

The association of Nrf2 overexpression and chemoresistance has been reported in many cancers including non-small cell lung cancer, stomach cancer, endometrial cancer, and osteosarcoma.[24-27] Overexpression of Nrf2 target genes was also found in a variety of solid tumors.[28] HO-1 overexpression was found in brain cancer, prostate cancer, and renal cancer.[29,30] NQO1 is known to be overexpressed in hepatoblastoma, colon cancer, breast cancer, and non-small cell lung cancer.[31]

Several mechanisms have been proposed for the activation of Keap1-Nrf2 signaling in cancer. Mutation of Keap1 leads to the overexpression of Nrf2 and its target genes. Mutation of Keap1 can result in accumulation of Nrf2 in nucleus through decreased degradation or inhibition of nuclear export.[28] In non-small cell lung cancer, heterozygous mutation of Keap1 (8% of patients) was

reported to be sufficient for Nrf2 overexpression.[32] In addition to lung cancer, mutations in the *Keap1* gene were found in various cancers including breast (2%), colon (8%), gastric (11%), liver (9%), ovary (19%), and prostate (8%).[33-36] Mutations in the Neh2 domain of *Nrf2* were found in lung (11%), cancer (6%), and head and neck cancers (25%).[37,38] All of these mutations were somatic mutations.

Upregulation of Nrf2 in cancer can occur without *Keap1* or *Nrf2* mutations. Mutation of *EGFR*, *Kras*, *Braf*, *Myc*, and the *Bcr-Abl* fusion can activate Nrf2, resulting in enhancement of ROS detoxification and other oncogenic roles of Nrf2, including chemoresistance.[39-41] Furthermore, posttranslational modifications can activate the Keap1-Nrf2 signaling pathway. Hypermethylation of the Keap1 promoter was found in 47% of lung cancer patients and this feature was associated with poor outcome.[42] Epigenetic regulation of Keap1 was also found in malignant glioma, colon cancer, and breast cancer.[43-45]

ONCOGENIC ROLE OF Nrf2 IN CANCER

In addition to the upregulation of cytoprotective genes, constitutive expression of Nrf2 may confer a survival advantage to cancer cells by promotion of cell proliferation, chemoresistance and inhibition of apoptosis.

Nrf2 overexpression promotes a hyperproliferative phenotype through the PI3K-Akt signaling pathway. An active PI3K-Akt pathway augments the nuclear accumulation of Nrf2 which then redirects glucose into the anabolic pathway to increase metabolism, indicating the reinforcement of metabolic reprogramming by Nrf2.[46] Nrf2 could mediate cell proliferation with dual regulation through epidermal growth factor receptor (EGFR) signaling and Keap1 interactions.[39] In cells with the *Keap1* gene mutation (A549 cells), activated Nrf2 promotes cell proliferation independent of EGFR signaling. Therefore, EGFR tyrosine-kinase inhibitors are intrinsically ineffective in these types of non-small cell lung cancer.[39] Cancer metastasis and tumor progression requires the epithelial-mesenchymal transition (EMT) and the loss of E-cadherin is considered to be a main event in EMT. In HEK293 cells, complex of E-cadherin and beta-catenin could bind to C-terminus of Nrf2 preventing nuclear translocation of Nrf2.[47] As E-cadherin inhibits Nrf2-mediated transcription, loss of E-cadherin could promote Nrf2 translocation and confer an additional survival advantage to cancer cells.[47]

The Keap1-Nrf2 pathway is involved in the inhibition of apoptosis by interacting with p53 and B cell lymphoma-2 (Bcl-2). p53 inhibits the activation of Nrf2 target genes by direct interacting with ARE-containing promoters or activating p21.[21] Considering that p53-induced apoptosis requires the accumulation of ROS, increased activity of antioxidant genes by Nrf2 in cancer cells can inhibit p53 dependent apoptosis. Bcl-2 can repress cell death by dimerization with Bcl-2-associated X protein (Bax) and the Bcl-2 homology 2 (BH2) domain of Bcl-2 is required for this heterodimerization.[48] It was found that Keap1 binds to the BH2 domain and facilitates the ubiquitination of Bcl-2 leading, to Bax accumulation and enhanced apoptosis. Antioxidants could antagonize the interaction between Keap1 and Bcl-2 to inhibit apoptotic cell death. Thus, mutations in the Keap1 binding site for Bcl2 are responsible for the anti-apoptotic effect as well as overexpression of Nrf2.[49] Moreover, it has been reported that Nrf2 can directly activate the transcription of Bcl-2 and Bcl-XL.[50,51]

Many studies have reported the association between Nrf2 upregulation and chemoresistance in various cancers, including gastric cancer, osteosarcoma, non-small cell lung cancer, endometrial cancer, bladder cancer, and neuroblastoma.[24-27,52]

Platinum drugs generate electrophilic molecules that damage cancer cells. Doxorubicin and etoposide can produce free radicals that interact with cancer DNA. Chemoresistance to these drugs can be explained by high expression of antioxidant Nrf2 target genes.[4] Another mechanism of chemoresistance is the induction of the drug efflux pump family, which includes the MDR, by Nrf2.[53] Some drugs, including HDAC inhibitors, oxaliplatin, and proteasome inhibitors can induce Nrf2 and thus decrease the ef-

Table. Summary of studies using Nrf2 inhibitor

Nrf2 inhibitor	Concentration	Cell line	Therapeutic agents used with Nrf2 inhibitor
Alkaloids			
Trigonelline[57]	0.1-0.5 μM	Panc1, Colo357	Etoposide
Vitamin C			
Ascorbic acid[61]	0.125 mM	KCL22	Imatinib
Quassinoid			
Brusatol[60]	40 nM	A549	Cisplatin Carboplatin, 5-fluorouracil, Etoposide, Paclitaxel
Flavonoid			
Chrysin[58]	10-20 μM	BEL-7402	Doxorubicin
Apigenin[59]	10-20 μM	BEL-7402	Doxorubicin
Luteolin[62]	1-20 μM	MCF7, Caco2, A549	Actinomycin D, Oxaliplatin, Bleomycin, Doxorubicin

Nrf2, nuclear factor E2-related factor 2.

fectiveness of chemotherapy.[54-56]

INHIBITORS OF THE Nrf2

In light of the data presented in this review, Nrf2 is an attractive molecular target for the inhibition of cancer. In contrast to Nrf2 activators, including numerous phytochemicals, only a small number of Nrf2 inhibitors have been identified (Table).

Trigonelline, a coffee alkaloid, efficiently decreased basal and tertiary butylhydroquinone (tBHQ)-induced Nrf2 activity in chemoresistant pancreatic carcinoma cell lines (Panc1, Colo357, and MiaPaca2) which have high Nrf2 activity. Along with inhibiting Nrf2, trigonelline blocked the Nrf2-dependent expression of proteasomal genes. The sensitivity of all cell lines to anticancer drugs and tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis was enhanced by trigonelline.[57]

Chrysin (5,7-dihydroxyflavone) is a natural flavonoid that is found in many plant extracts. In doxorubicin resistant hepatocellular carcinoma cell line (BEL-7402/ADM), chrysin significantly reduced Nrf2 expression by downregulating the PI3K-Akt and ERK pathways.[58] In that study, chrysin restored chemosensitivity by downregulating the Nrf2-downstream genes such as HO-1, AKR1B10, and MRP5.

Apigenin (4',5,7-trihydroxyflavone) is a natural flavone that is present in many fruits and vegetables and has various biological activities, such as anti-inflammatory and antioxidant properties. In a study using BEL-7402/ADM cell lines, apigenin reduced Nrf2 expression as well as the expression of Nrf2-downstream genes. When cells were treated with doxorubicin, apigenin showed a synergistic anti-tumor effect on BEL-7402/ADM cell lines, resulting in reduced cell proliferation and more substantially induced apoptosis.[59]

Brusatol is a quassinoid that is extracted from *Brucea javanica*. Brusatol reduced Nrf-2 protein level without changing the Keap1 level and sensitized various cells including HeLa, MDA-MB-231, and A549 to chemotherapeutic agents such as carboplatin, 5-fluorouracil, etoposide, and paclitaxel.[60] The anticancer effect of brustalol mediated by Nrf2 was confirmed in a xenograft model indicating that brusatol could be used to combat intrinsic resistance.

Ascorbic acid is an antioxidant that can suppress the level of Nrf2. In the imatinib-resistant chronic myelogenous leukemia cell line KCL22/SR, binding of Nrf2 to the ARE of the gamma-glutamylcysteine synthetase gene promoter was much stronger than in the parental imatinib-sensitive cell line KCL22.[61] Furthermore, addition of ascorbic acid to KCL22/SR cells resulted in a decrease in Nrf2-DNA binding and partial restoration of imatinib sensitivity to KCL22/SR.[61]

Luteolin (3',4',5,7-tetrahydroxyflavone) is a polyphenolic flavonoid found in high concentrations in celery, green pepper, and parsley. In a study using a cell-based ARE-reporter assay, luteolin was found to be a potent Nrf2 inhibitor.[62] In that study, luteolin significantly sensitized A549 cells to the anticancer drugs oxaliplatin, bleomycin, and doxorubicin indicating the potential application of luteolin as a natural sensitizer in chemotherapy.

CONCLUSION

In normal cells, Nrf2 is under tight regulation by Keap1. Constitutively expressed Nrf2 can promote cancer cell proliferation and protect cells against oxidative stress and therapeutic agents. It must be kept in mind that chemopreventive agents including various phytochemicals can induce chemoresistance and tumor progression by activating the Keap1-Nrf2 pathway. Evidence indicates that Nrf2-targeting agents can be used to overcome this chemoresistance. However, it will be necessary to understand the molecular regulation of Nrf2 and identifying the individualized status of Nrf2 expression.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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