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Advances in Mechanisms of Anti-oxidation

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Abstract

Reactive oxygen species (ROS) are a family of molecules that are continuously produced from oxygen consumption in aerobic cells. Controlled generation of ROS in normal cells serves useful purposes to regulate important cellular processes such as cell proliferation, inflammation, and immune response, but overproduction of ROS causes oxidative stress that contributes to the development of cancer, chronic disease, and aging. These hugely different consequences of ROS exposure demand a carefully balanced control of ROS production and disposition, which is largely achieved through the body's elaborate antioxidant system. The human antioxidant system consists of small antioxidants, antioxidant proteins, ROS-metabolizing enzymes, as well as many regulator proteins that mediate adaptive responses to oxidant stress. How such a complex system reacts with oxidants and achieves the required specificity and sensitivity for proper anti-oxidation is incompletely understood. In this respect, new advances in the understanding of the chemistry that determines the reaction of a given oxidant or antioxidant with a protein target provide considerable insights into these and related questions. The findings hold certain promise for new drug development for preventing and treating diseases associated with oxidant tissue damage.

Introduction: Oxidant Stress and Anti-oxidation Are Consequences of Oxygen Utilization

Eukaryotes are constantly exposed to reactive oxygen species (ROS) as a result of internal metabolism and external exposure (Balaban *et al.*, 2005). During the course of evolution, molecular oxygen (O₂) was selected as the terminal electron acceptor to generate biologically useful energy in the form of adenosine 5'-triphosphate (ATP) from carbon fuels, which reflects an adaptation of organismal life to the rising abundance of O₂ in the atmosphere as well as the favorable thermodynamic properties of O₂ (Berner *et al.*, 2007). In eukaryotes, controlled oxidation of NADH and FADH₂ generates an energy potential of protons across the mitochondrial inner membrane to drive the phosphorylation of ADP to ATP and the reduction of O₂ to H₂O. However, the electrons may leak from the respiratory

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chain to directly react with O₂ producing superoxide anion (O₂^{•-}) and hydrogen peroxide (H₂O₂), two major forms of ROS in the cell. In fact, most, if not all, enzymes that are capable of metabolizing oxygen can generate ROS, either purposely or as by-products. In addition, numerous exogenous agents, such as infectious microbes and environmental toxicants, stimulate ROS production in the body by interfering with the internal metabolism and utilization of O₂ (Ma, 2010).

ROS avidly react with cellular macromolecules such as nucleic acids, proteins, and membranous lipids. Uncontrolled production of ROS results in oxidative stress that potentially causes harm to the body and is implicated in aging, tumorigenesis, chronic inflammation, neurodegeneration, and chemical toxicity (Bossy-Wetzel *et al.*, 2004; Finkel, 2005). On the other hand, ROS function as physiologic signaling molecules in normal cells regulating critical cellular processes such as cell division, differentiation, inflammation, immune function, and stress response (Dickinson and Chang, 2011; Finkel, 2011). Therefore, it has been increasingly believed that reactive oxidants play important roles in the development of many physiological and pathophysiological outcomes. Accordingly, the production and disposition of ROS are tightly controlled in tissue, cell type, and time-specific fashions, such that cells can utilize the beneficial effects but avoid the toxicity of reactive oxidants.

The homeostasis of ROS in the body is achieved largely through anti-oxidation via an intricate antioxidant system. Human antioxidants consist of low-molecular-weight antioxidants such as reduced glutathione (GSH) and vitamins C and E; noncatalytic antioxidant proteins such as thioredoxin (Trx) and glutaredoxin (Grx); and ROS-metabolizing enzymes such as superoxide dismutase (SOD), catalase, peroxiredoxin (Prx), and glutathione peroxidase (GPx). Importantly, the antioxidants are regulated by a web of regulator proteins that mediate adaptive responses to oxidant stress and antioxidant inducers, which is best exemplified by the activation of the nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) to induce the expression of antioxidant response element (ARE)-controlled cytoprotective proteins including ROS-metabolizing enzymes (Kensler *et al.*, 2007; Ma, 2013). In addition, many plant-derived chemicals (phytochemicals and derivatives) exhibit antioxidant properties and protect cells from the damaging effects of reactive oxidants by means of chemical quenching and/or activation of adaptive responses. Anti-oxidation through exogenous antioxidants by way of dietary supplements, herbal medicine, and clinical drugs has been utilized as an effective and economic measure to prevent and treat cancer, chronic disease, and aging (Ma and He, 2012; Suzuki *et al.*, 2013a; Talalay *et al.*, 2003).

The molecular mechanisms of how ROS elicit a specific response and by which the homeostasis of oxidants is controlled in the body remain largely unclear. In this respect, recent data obtained from molecular characterization of oxidant signaling and antioxidant-induced transcriptional regulation of oxidant defense provided new mechanistic insights into mammalian oxidant signaling and anti-oxidation (Dickinson and Chang, 2011; Ma, 2013). An emerging recurrent theme from both oxidant signaling and antioxidant response is reactive cysteine thiol-based redox reaction. In the former case, ROS induced by a proliferation signal such as the ligation of a growth factor receptor (GFR) oxidizes the thiol

group of critical cysteine residues of GFR to boost GFR activity and proliferation signaling (Paulsen *et al.*, 2012). In the latter case, an antioxidant, such as the phytochemical sulforaphane, covalently binds to selective cysteine thiols of Keap1 (Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1), a repressor of Nrf2, resulting in the activation of gene transcription through Nrf2 (Ma and He, 2012; Taguchi *et al.*, 2011). In both scenarios, the versatile and reversible reactions of reactive cysteine thiols appear to serve as the chemical basis to determine the specificity and sensitivity of these responses.

This review discusses recent progress in the understanding of the molecular mechanism that governs the interaction between reactive oxidants and mammalian cells and, in light of the apparent therapeutic and preventive potentials of anti-oxidation, will focus on the emerging role and mechanism of action of Nrf2 in the control of inducible anti-oxidation pertinent to medicine.

Biologically Relevant Reactive Oxidants

ROS

ROS encompass a family of molecules that represent key reactive oxidants in aerobic cells (Dickinson and Chang, 2011). Major biologically relevant ROS include hydroxyl radical ($\cdot\text{OH}$), singlet oxygen ($^1\text{O}_2$), lipid peroxides (ROOH), ozone (O_3), and hypochlorous acid (HOCl), in addition to $\text{O}_2^{\cdot-}$ and H_2O_2 described earlier. ROS are frequently generated at specific subcellular locations and associated with defined physiologic functions. Besides the mitochondrial respiratory chain, ROS are produced in phagosomes to kill pathogens and in peroxisomes for energy metabolism. Protein folding via disulfide bond formation in the endoplasmic reticulum (ER) by the glycoprotein Ero1 and the thioredoxin protein disulfide isomerase results in the release of H_2O_2 that may contribute to ER stress. A group of NADPH oxidases (Nox) localize in the plasma membrane and produce ROS to mediate redox signaling that regulates cell proliferation and immune response, and phagocytic killing to ward off invading microbes. Determining the chemical identity, subcellular localization, and local concentration of ROS is critical for elucidating the chemistry and function of ROS at a molecular level, which remains a formidable challenge for most investigators due to the lack of sensitive, specific, and easy-to-use probes and technology to detect reactive oxidants at the present time.

RNS

Reactive nitrogen species (RNS), such as nitric oxide ($\cdot\text{NO}$), are another group of reactive oxidants important in human biology (Pacher *et al.*, 2007). Nitric oxide is produced by NO synthase (NOS) and serves as a gaseous signaling molecule (gasotransmitter) for certain physiologic functions, such as blood vessel relaxation and neurotransmitter function. RNS react with ROS to form stronger oxidants, such as the potent peroxynitrite (ONOO^-) that can directly react with various biological molecules. Overproduction of RNS causes nitrosative stress that often accompanies oxidative stress and contributes to the overall oxidant stress-associated tissue damage. There is also a lack of specific markers to distinguish between nitrosative and oxidative stresses in a biological sample. For these

reasons, nitrosative stress is frequently discussed together with oxidative stress in the literature.

The Chemical Basis of Oxidant Signaling

Reactive cysteine thiols

Reactive cysteine residues are a small set of protein cysteines that have low pKa values in the range of 4 to 5 as a result of the influence from their surrounding amino acid microenvironment; on the contrast, most protein cysteines have pKa values of about 8.5. At pH 7.4, reactive cysteine thiols exist as thiolate anions ($-S^-$) that are more reactive than sulfhydryl groups ($-SH$) toward ROS, RNS, and electrophiles (Finkel, 2011).

ROS such as H_2O_2 easily oxidize reactive cysteine thiols to produce cysteine thiol oxidation products in the sequence of sulfenic acid ($-SOH$), sulfinic acid ($-SO_2H$), and sulfonic acid ($-SO_3H$) (Figure 1). Nitrosation of the cysteine thiols by *NO gives rise to S-nitrosothiol ($-SNO$) that is predisposed to further modifications. Sulfhydration of the cysteine thiols with hydrogen sulfide (H_2S), another gasotransmitter, forms sulfhydrate ($-SSH$). Reaction of the thiols with a protein $-SH$ group results in the formation of inter- and intra-protein disulfides, or S-glutathiolation if the other $-SH$ group comes from glutathione.

Reactive cysteine thiols are also modified by a variety of chemicals through alkylation, thiol-ether and thiol-amide bond formation, and metal chelation. Recent evidence reveals that these modifications provide an important means of sensing and distinguishing various endogenous and environmental chemicals. Pertinent to this review, many antioxidants were found to covalently bind to selective cysteine thiols of target proteins to induce adaptive responses for anti-oxidation (Ma, 2013).

Oxidant signaling through reactive cysteines

Many proteins undergo reversible cysteine thiol oxidation and the list is rapidly growing covering a wide range of biological functions. Oxidant signaling through reactive cysteine thiols is best illustrated by epidermal growth factor (EGF)-induced signaling through EGF receptor (EGFR) (Finkel, 2011; Paulsen *et al.*, 2012). Ligand binding to EGFR induces the production of $O_2^{\bullet-}$ and H_2O_2 through membrane-bound Nox. In turn, H_2O_2 augments EGFR signaling by two mechanisms: (a) oxidization of the active site cysteine thiols of local protein tyrosine phosphatases (PTP) resulting in PTP inactivation and reduction of protein dephosphorylation; (b) oxidization of EGFR at Cys⁷⁹⁷ to increase the EGFR kinase activity. Both actions increase the local protein tyrosine phosphorylation near the plasma membrane and, consequently, enhance EGFR signaling.

In addition to cysteine residues, other amino acid residues, such as lysine, arginine, proline, and histidine can be oxidized at their side chains by ROS/RNS, some of which are reversible and potentially serve as signaling mechanisms for oxidants. However, examples of redox signaling via amino acids other than cysteine are currently scarce in the literature.

The Evolving Concepts of Antioxidant and Anti-oxidation

Plants and animals produce various antioxidants to cope with the damaging effects of ROS generated from oxygen consumption. The term antioxidant initially refers to a chemical that prevents the consumption of O₂. The study on how vitamin E prevents lipid peroxidation led to the realization that antioxidants are reducing agents that prevent oxidative reactions by scavenging ROS. Further research revealed that many antioxidants also elicit adaptive responses such as the induction of enzymes that detoxify ROS and ROS-promoting chemicals besides directly quenching ROS. Thus, mammalian anti-oxidation is achieved through both direct (scavenging) and indirect (adaptive) modes of action of antioxidants.

The recognition that controlled production of oxidants in normal cells serves useful purposes to regulate critical cellular functions has also changed the concept of biological anti-oxidation in several ways.

First, it implies that the function of anti-oxidation in cells is not to remove ROS entirely, but instead to keep them at an optimum level. In this view, induction of adaptive anti-oxidation increases oxidant defense quickly in response to inducing signals, but induction subsides as the inducers are eliminated, avoiding sustained elevation of anti-oxidation once a homeostatic state of ROS is achieved. Therefore, adaptive (inducible) anti-oxidation is perhaps better fitted for the control of oxidant homeostasis in the body than non-inducible antioxidant mechanisms, especially when oxidant levels exceed the buffering capacity of existing antioxidants.

Second, many physiologic functions are accompanied with the production of ROS and/or RNS that, in turn, impact the functions either positively or negatively. Consistent with the notion, many antioxidants exhibit activities other than anti-oxidation such as anti-cancer, anti-viral, anti-inflammatory, and anti-immune functions, indicating that anti-oxidation generally does not occur alone. Therefore, anti-oxidation by an antioxidant should be analyzed in the context of the overall biological system and include other functions such as inflammation and immune response.

Third, anti-oxidation is boosted by many endogenous signals, which is likely through reactive cysteine thiols of target proteins. Physiologic adaptations, such as physical exercise and micronutrient control, enhance body's anti-oxidation and, accordingly, the overall health and resistance to disease (del Valle and Hernandez, 2013; Nkenggack *et al.*, 2013). In physical exercise, the stimulus for adaptive anti-oxidation is likely ROS themselves, because acute and chronic exercise is associated with increased production of ROS from increased muscular O₂ consumption and activation of the endothelial xanthine oxidase that converts hypoxanthine and xanthine to uric acid and H₂O₂. In this scenario, continued presence of low concentrations of ROS induces the expression of antioxidant enzymes and other defense mechanisms. This phenomenon can be explained by the concept of hormesis in which the interaction between the stimulus and response follows a particular dose-response relationship: at high doses, ROS is inhibitory and harmful to the body, but at low doses, ROS stimulate anti-oxidation adaptation to induce beneficial effects.

The Nrf2/ARE Paradigm as a Model of Inducible Anti-oxidation

The interest on inducible anti-oxidation for human health has an origin in the observation that phenolic antioxidants, such as butylated hydroxyanisole (BHA), protected animals from benzo[a]pyrene (BaP)-induced tumor formation, which correlated with inhibition of the formation of BaP mutagenic metabolites and induction of GSTs and NQO1, drug-metabolizing enzymes that detoxify many carcinogens and toxicants (Talalay *et al.*, 1978; 1988; Watternberg, 1985). These findings led to the concept of “chemoprevention” in which pre- or co-treatment with antioxidants prevents against chemical carcinogenesis. Induction of detoxifying enzymes has since become a mechanistic model for analyzing antioxidant biological effects. Subsequent molecular studies of the induction events revealed an inducible, xenobiotic-activated receptor (XAR)-mediated transcriptional response to small chemicals that consists of several major components (Figure 2) (Ma and He, 2012; Nguyen *et al.*, 2003).

Inducers

The chemicals that induce GSTs and NQO1 include phytochemicals and derivatives such as sulforaphane and triterpenoids; therapeutics such as oltipraz, auranofin, and dimethyl fumarate; environmental agents such as paraquat and arsenic; and endogenous inducers such as 15-deoxy-^{12,14}-prostaglandin J₂ (15d-PGJ₂), nitro-oleic acid, and 4-hydroxynonenal (4-HNE) (Kensler *et al.*, 2007; Ma and He, 2012; Talalay *et al.*, 1988). The inducers differ considerably in structure but share certain chemical property in that many inducers are electrophiles or redox-active compounds capable of modifying protein cysteine thiols by oxidation, reduction, alkylation, and metal chelation. For instance, sulforaphane and triterpenoids are Michael reaction acceptors that covalently bind to specific Keap1 cysteine thiols to activate Nrf2. Recent studies also identified a list of endogenous inducers, among which 15d-PGJ₂, nitro-fatty acids, 4-HNE, acrolein, and nitric oxide directly modify the sensor motifs of Keap1. A few protein factors such as autophagy cargo receptor protein p62 and cell cycle inhibitor p21 activate Nrf2 by interfering Nrf2-Keap1 binding through their structural motifs that bind Keap1.

Antioxidant response element (ARE) and ARE controlled genes

A core DNA sequence, 5'-TGACnnGC-3' (n=any base) was identified from rat Gsta2 and later from mouse Gsta1 and rat and human NQO1 gene promoters as the antioxidant response element necessary for induction of the genes by phenolic antioxidants (Nguyen *et al.*, 2003). The core ARE was later expanded into a 16-base-pair consensus sequence, 5'-TMA_nRTGAYnnGCR-3' (M=A or C, R=A, Y=C or T, W=A or T) (Hayes *et al.*, 2010). In recent years, genome-wide search for Nrf2 target genes has led to the identification of an array of ARE-regulated genes (Malhotra *et al.*, 2010). Three notable groups of ARE-controlled genes are closely related to the cellular response to oxidants and electrophiles, including drug metabolizing enzymes and transporters such as UGT1A1, ALDH3A1, CYP2A5, and MRP2 besides GSTs and NQO1; antioxidant defense enzymes such as GPx2, Prx1, and TXNIP; and oxidant signaling molecules such as p62, DJ-1, and PTP1 (Ma, 2013). In addition, the Nrf2/ARE pathway regulates proteasomal protein degradation (Kwak *et al.*, 2003), cell proliferation (Malhotra *et al.*, 2010), and metabolic reprogramming

(Mitsuishi *et al.*, 2012). The presence of ARE in a broad range of genes provides a rational explanation for the multiple functions of Nrf2 and the involvement of anti-oxidation defense in various distinct physiologic and pathophysiologic processes.

Nrf2 and small Mafs

Nrf2 belongs to the cap 'n' collar (CNC) subfamily of basic leucine zipper transcription factors. Nrf2 was identified by way of its binding to the NFE2-binding motif, a *cis*-DNA sequence that controls β -globin expression critical for erythropoiesis and platelet development (Moi *et al.*, 1994). Nrf2 does not seem to be essential for hematopoietic functions, but was instead identified as a transcription factor to mediate antioxidant induction of GSTs and NQO1 through ARE (Itoh *et al.*, 1997). Nrf2 was later found to be involved in the cellular defense against a wide range of toxic and disease conditions that are characteristically associated with oxidative pathology (Kensler *et al.*, 2007; Ma, 2013). Induction via Nrf2 increases the detoxification and elimination of many exogenous and some endogenous toxic chemicals including ROS. In this role, Nrf2 functions as an XAR to regulate the adaptive response to oxidants and electrophiles. Nrf2 hetero-dimerizes with a small Maf (musculoaponeurotic fibrosarcoma) protein (MafG, MafK) that has a bZip domain but lacks a transactivation domain in the nucleus (Motohashi *et al.*, 1997). The Nrf2/small Maf dimer binds to ARE where Nrf2 binds to the ARE core and provides the transactivation activity, and MafG/K recognizes the GC dinucleotide of ARE core to increase binding affinity and specificity.

Keap1

Keap1 was identified as a cytoplasmic Nrf2-binding protein by using yeast two-hybrid screening with the Nrf2 N terminal inhibitory motif Neh2 as bait (Itoh *et al.*, 1999). Keap1 contains two notable protein-protein interaction domains. The BTB (bric-a-brac, tramtrack, broad-complex) domain in the N terminal region mediates homodimerization of Keap1 and binding of Keap1 to Cullin (Cul) 3, a scaffold protein of the Nrf2 ubiquitin ligase (E3). The Kelch repeat (or double glycine repeat, DGR) domain in the C terminal region is responsible for binding to actin and Nrf2. An intervening region (IVR) or linker region (LR) locates between BTB and DGR and is rich in cysteine residues. Keap1 binds to the Neh2 domain of Nrf2 through its Kelch repeat to inhibit Nrf2 in the cytoplasm (Taguchi *et al.*, 2011).

Molecular Mechanism of Chemical Sensing by Nrf2/Keap1

The mechanism by which Nrf2 is activated to mediate gene induction is two-fold: suppression of Nrf2 under a basal condition and activation of Nrf2 by an inducer.

Ubiquitination/proteasomal degradation of cytoplasmic Nrf2 through Keap1

Nrf2 mRNA is expressed broadly and independently of inducers; on the other hand, inhibition of protein synthesis completely blocks the basal and induced expression of ARE genes, suggesting a post-transcriptional regulation through protein turnover as a major mechanism of Nrf2 regulation (Ma and Kinner De-Fede, 2001). Indeed, in unstimulated cells, Nrf2 is rapidly ubiquitinated and degraded with a half-life of ~20 minutes (He *et al.*, 2006). Ubiquitination is mediated through the Keap1/Cul3 ubiquitin ligase complex in

which Cul3 serves as a scaffold protein, Keap1 as an adaptor to bring Nrf2 into the E3 by binding to Cul3 via its BTB domain, and RING box protein 1 as a recruiter for ubiquitin-conjugating enzyme (E2) that catalyzes the polyubiquitination of Nrf2 to signal proteasomal degradation.

Structural studies reveal that Keap1 forms a homo-dimer that resembles a cherry-bob (Figure 2). Each monomer forms a large globular sphere (representing Keap1 IVR, Kelch repeats, and the C terminal end) that is linked to the other monomer through short linker arms to the sides of a small forked-stem structure (representing the dimerization interphase of two BTB domains) (Li *et al.*, 2004; Ogura *et al.*, 2010). The Kelch repeats fold into a drum-shaped, six-bladed β -propeller structure with an inner cavity buried in the central core and open to the top and bottom of the sphere. The overall structure of Nrf2 is not available at the present time, but the Neh2 domain was found to be intrinsically disordered with certain secondary structures. A α -helix locates at the center of rod-shaped Neh2 and contains seven lysine residues. Juxtaposed to the carboxyl end of the α -helix is a mini anti-parallel β -sheet with a conserved ETGE sequence between the two sheets. A β -hairpin structure containing a conserved DLG sequence flanks the α -helix at its amino side. Nrf2 binds to Keap1 in a 1:2 ratio through its Neh2 domain where the ETGE and DLG motifs each binds to one globular sphere of Keap1 at the binding cleft near the bottom opening of the central tunnel. The α -helix between DLG and ETGE is exposed to E2 with six lysine residues facing the enzyme for ubiquitination. The ETGE-Keap1 binding is two orders of magnitude stronger than the DLG-Keap1 binding, suggesting a hinge-and-latch-like model of binding where the Nrf2-Keap1 binding occurs first through ETGE, followed by binding at DLG.

Activation of Nrf2 through inducer-cysteine thiol binding

Many ARE inducers are electrophiles suggesting an inducer-cysteine thiol interaction as the initial chemical reaction for Nrf2 activation (Dinkova-Kostova *et al.*, 2004). Evidence for binding of inducers to Keap1 cysteine thiols was provided using labelled inducers, stoichiometry analysis, mass spectrometry, and mutational studies (Dinkova-Kostova *et al.*, 2001; Eggler *et al.*, 2005; He and Ma, 2010; Hong *et al.*, 2005; Zhang *et al.*, 2004). Notably, the Keap1 IVR domain is rich in cysteine residues, among which Cys²⁷³, Cys²⁸⁸, and Cys²⁹⁷ were frequently labeled by inducers. Reconstitution in Keap1 knockout (KO) mice revealed that Cys¹⁵¹ is required for activation of Nrf2 by electrophiles, and Cys²⁷³ and Cys²⁸⁸ for suppression of Nrf2 under a basal condition (Yamamoto *et al.*, 2008). Cumulative evidence indicates that inducers bind to Keap1 and/or Nrf2 through distinctive sets of cysteine residues, coined “cysteine codes,” to activate Nrf2. For instance, Keap1 Cys²⁸⁸ is a sensor for alkenals such as 4-HNE, and Cys¹⁵¹ together with a cluster of neighboring basic amino acids, namely His¹²⁹, Lys¹³¹, Arg¹³⁵, Lys¹⁵⁰, and His¹⁵⁴, forms an NO sensor (McMahon *et al.*, 2010). In contrast to Keap1 that has 25 or more cysteine residues, Nrf2 has only 6 (human) or 7 (mouse and rat) cysteine residues that are highly conserved and were shown to have multiple effects on Nrf2 activation and function (He and Ma, 2009). The structural basis for the selectivity of inducer-cysteine code interaction remains to be elucidated for most inducers.

Alternative mechanisms of Nrf2 activation

Activation of Nrf2 by some inducers involves mechanisms other than inducer-cysteine thiol binding. Some modifications serve as part of the signaling mechanism triggered by inducer-cysteine binding. For example, human Nrf2 is phosphorylated by casein kinase 2 in response to ARE inducers and the phosphorylation is necessary for nuclear translocation of Nrf2 (Apopa *et al.*, 2008). Several protein factors including p62, p21, PGAM5, and DJ-1 can compete with Keap1-Nrf2 binding through their structural motifs that resemble the Nrf2 ETGE or DLG motif and, thereby, persistently activate Nrf2 under certain physiological and disease conditions (Chen *et al.*, 2009; Clements *et al.*, 2006; Komatsu *et al.*, 2010; Lo and Hannink, 2008). The Nrf2 Neh6 region contains several serine residues that can be phosphorylated by GSK-3 to form a recognition motif for β -TrCP (β -transducin repeats-containing proteins) leading to Nrf2 ubiquitination through the β -TrCP/Cul1 E3 complex and proteasomal degradation (Rada *et al.*, 2011).

Translational Aspects of the Nrf2/Keap1/ARE Pathway

The mechanistic elucidation of the Nrf2/Keap1 pathway for induction of ARE-controlled genes has invigorated the enthusiasm toward application of inducible anti-oxidation in human health. In supporting the notion, cumulative evidence has shown that activation of Nrf2 by either pharmacological means (treatment with inducers) or genetic manipulation (knockout of Keap1) protects animals from damages associated with oxidant stress and pathology, whereas knockout of Nrf2 markedly reduces the expression of cellular defense genes leading to increased sensitivity to a range of disease pathology including cancer, chronic obstructive pulmonary disease (COPD), and neuro-degenerative disease, as well as chemical toxicity such as acetaminophen hepatotoxicity (drug-induced liver injury, DILI) and alcohol-induced liver disease (AILD) (Kensler *et al.*, 2007; Ma and He, 2012). For example, Nrf2 KO mice had higher incidence of BaP-induced gastric tumor and BaP-DNA adducts than wild type, whereas Nrf2 inducers (oltipraz, sulforaphane) protected mice from BaP-induced gastric tumor formation in a Nrf2-dependent manner (Fahey *et al.*, 2002; Ramos-Gomez *et al.*, 2003; 2001). In humans, a single nucleotide polymorphism in the *NRF2* upstream promoter region (rs6721961) was associated with reduced expression of Nrf2 and increased risk of lung cancer (Suzuki *et al.*, 2013b). On the other hand, persistently elevated expression of ARE genes in tumors was found to be advantageous for tumor cells to proliferate and resist to drug therapy (Kensler and Wakabayashi, 2010). These findings suggest that translation of mechanistic and animal studies to medicine require careful weighing of the pros and cons of Nrf2 activation.

Nrf2 in cancer

Somatic mutations in the genes of the *KEAP1* gene were found in human tumors; the mutations are mostly located at the DC region followed by IVR and BTB, suggesting that the mutations affect Keap1-Nrf2 binding and/or Nrf2 ubiquitination (Takahashi *et al.*, 2010). Similarly, mutations were found in the ETGE and DLG motifs of Nrf2 in certain cancers that disrupt Keap1-Nrf2 binding leading to stabilization of Nrf2 in tumor cells. Some tumors have increased methylation in the *KEAP1* gene that suppresses Keap1 expression and activates Nrf2 (Wang *et al.*, 2008). In certain renal tumors, fumarate hydratase inactivation

leads to accumulation of fumarate that modifies Keap1 cysteine thiols to activate Nrf2 (Adam *et al.*, 2011). In these cases, the tumors have elevated Nrf2 levels and increased expression of ARE genes. Elevated Nrf2 function is advantageous for tumor cells in at least three ways: (a) boosting tumor resistance to anti-cancer drugs and endogenous tumor killing chemicals; (b) enhancing proliferation by reducing ROS associated with cell proliferation and by promoting metabolic reprogramming of tumor cells; and (c) increasing Notch1 expression.

Targeting Nrf2 for drug development

Because Nrf2 is implicated in an increasing list of disease processes, there are substantial interests in developing Nrf2 activators as therapeutic drugs. A series of triterpenoids were derived from oleanolic acid, a phytoantioxidant, and were shown to be among the most potent inducers of Nrf2 genes (Liby *et al.*, 2007). For comparison, the induction potency for NQO1 expression is 2 nM for CDDO-Im (an imidazolide of triterpenoid), 100 nM for sulforaphane, 10 mM for oltipraz, and 45 mM for butylated hydroxytoluene. Triterpenoid inducers have been shown effective in protection against cancer and various chronic diseases. Bardoxolone methyl (CDDO-Me) was effective for treating chronic kidney disease associated with type 2 diabetes, but was withdrawn from a phase III clinical trials due to adverse events (Pergola *et al.*, 2011). In another example, dimethyl fumarate, a Michael reaction acceptor and ARE inducer, is safe and highly efficacious for the treatment of multiple sclerosis. BF-12 (Tecfidera), an oral drug containing dimethyl fumarate, was recently approved by FDA for treatment for multiple sclerosis. These examples illustrate the increasing demand for, in addition to high efficacy, a good safety profile for new Nrf2 activators. Because tumor cells can hijack the protective functions of Nrf2 by persistently activating Nrf2 genes, there is also an interest in developing Nrf2 inhibitors for the treatment of cancers that exhibit elevated Nrf2 functions. Specific and efficacious Nrf2 inhibitors for drug development are currently lacking.

Potential drug targets associated with Nrf2 in chronic diseases

Nrf2 was associated with chronic disease through molecules that are likely important for disease, providing new therapeutic targets for treating chronic disease. For example, a recent study implicates Nrf2 in the resistance to severe malaria among carriers of the Sickle cell anemia mutation who are infected with malaria (Ferreira *et al.*, 2011). In this scenario, the carriers have elevated levels of free heme in the blood. Free heme is converted by heme oxygenase-1 (HO-1) to iron that binds to ferritin and the antioxidant molecules carbon monoxide (CO) and biliverdin. CO and biliverdin prevent oxidative tissue damage and inhibit pathogenic CD8⁺ T cell immune responses, both of which are associated with severe malaria. Because Nrf2 is a major regulator of HO-1 induction, modulating Nrf2 function potentially impacts the resistance to malaria through induction of HO-1 expression. As discussed earlier, some protein factors such as p62 in autophagy, p21 for cell cycle inhibition, and DJ-1 associated with Parkinson's disease activate Nrf2 by interfering with Keap1-Nrf2 binding, whereas the ER stress protein PERK activates Nrf2 by phosphorylating Nrf2 at Thr⁸⁰. The levels of these proteins are elevated in certain chronic diseases or disease processes. Therefore, it is tempting to speculate that modulating the levels of these proteins and/or the Nrf2/ARE pathway may influence the development of the disease.

Conclusion

There is growing appreciation of biological anti-oxidation as it controls the homeostasis of reactive oxidants in normal cells and copes with oxidant stress associated with cancer and chronic disease. The molecular mechanism of mammalian anti-oxidation is not well understood. Some recent studies provided new insights into the interaction between an oxidant or an inducing signal and a protein target, as demonstrated for oxidant signaling and adaptive anti-oxidation. In both scenarios, the reaction of the oxidant/inducer with a reactive cysteine thiol of the target protein triggers the molecular cascade for downstream functions. Inducible anti-oxidation is best exemplified by antioxidant activation of the Nrf2/Keap1 pathway for induction of ARE-controlled cytoprotective genes. Mechanistically, Nrf2 is suppressed under basal conditions through Keap1-controlled ubiquitination-proteasomal degradation, and is activated by oxidants and electrophilic inducers via modification of the critical cysteine thiols of Keap1 and Nrf2 to induce gene expression for anti-oxidation. The potentials of the Nrf2 pathway for drug development and medicine are intriguing as animal studies have provided a large body of evidence supporting that activation of Nrf2 protects against a variety of diseases. On the other hand, sustained activation of Nrf2 has been found to link to increased tumor growth and resistance to chemotherapy. Certain potent Nrf2 activators exhibited multiple effects, some of which induce undesirable events. A careful balance of both the protective and deleterious effects of Nrf2 activation is warranted in future translation of the Nrf2 pathway for human health.

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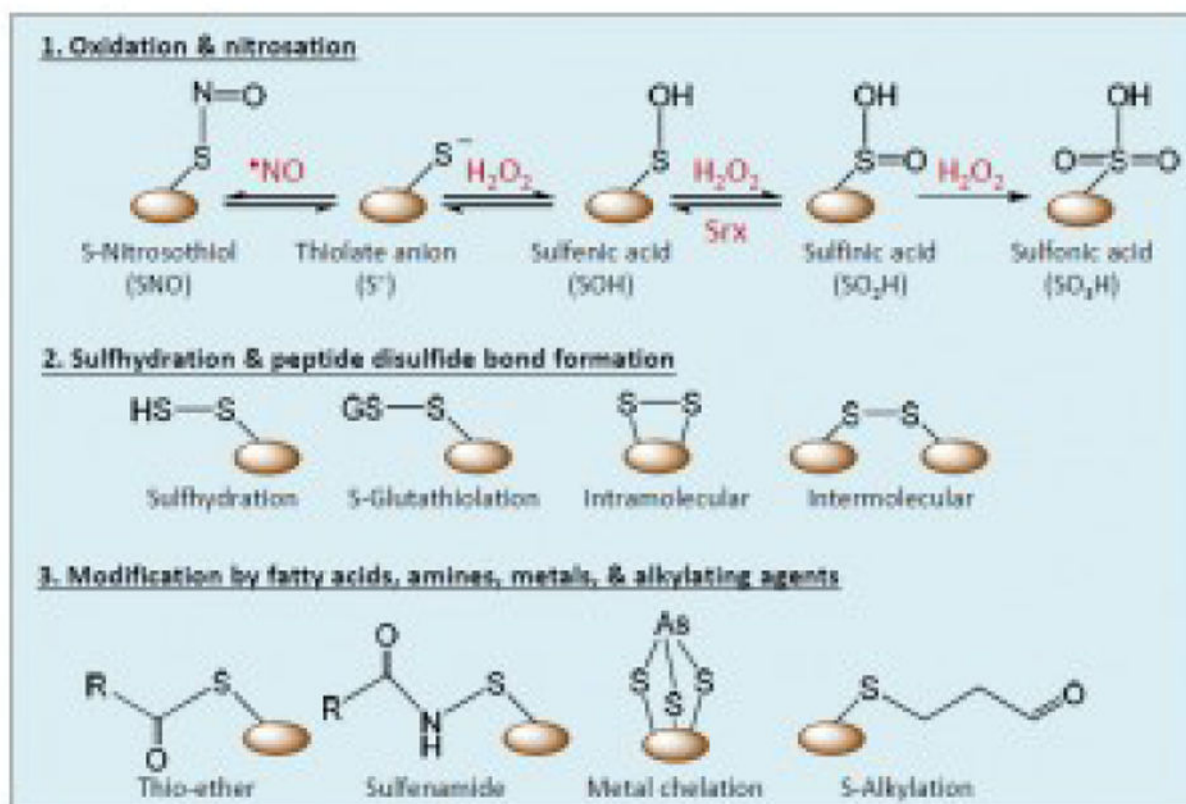


Figure 1. Reactive cysteine thiol reactions. Reactive cysteine thiols exist as thiolate anions and are more reactive toward ROS, RNS, and electrophiles than sulfhydryl groups. See text for details. Srx, sulfiredoxin.

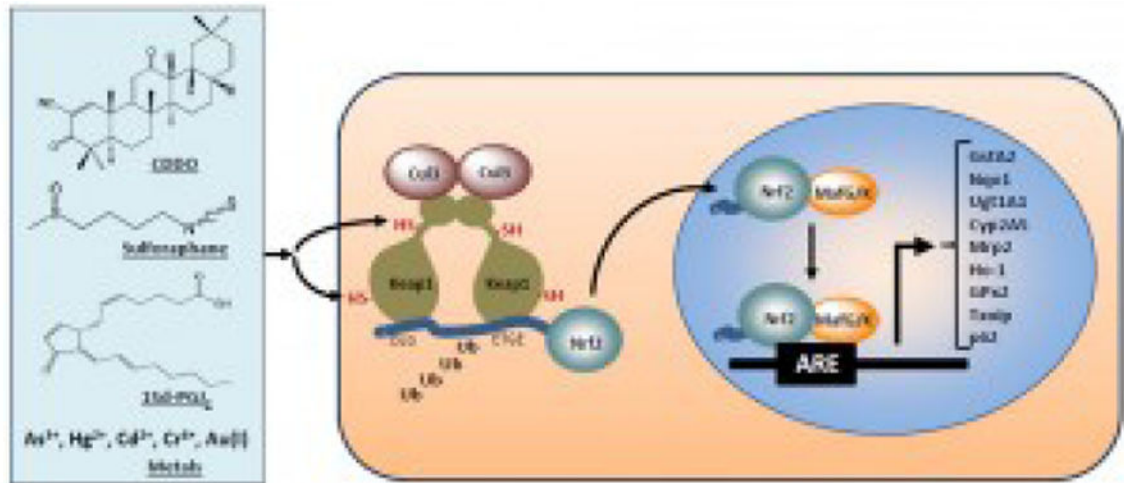


Figure 2.
Model for activation of Nrf2 by ARE inducers. See text for details. CDDO, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid.