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REVIEW

Mechanism of the Nrf2/Keap1/ARE Signaling System

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Abstract—Nrf2 regulates expression of genes containing antioxidant-respons(iv)e element (ARE) in their promoters and plays a pivotal role among all redox-sensitive transcription factors. Nrf2 is constitutively controlled by repressor protein Keap1, which acts as a molecular sensor of disturbances in cellular homeostasis. These molecular patterns are in close interconnection and function as parts of the integrated redox-sensitive signaling system Nrf2/Keap1/ARE. Depending on cellular redox balance, activity of this signaling system changes at the levels of transcription, translation, posttranslational modification, nuclear translocation of transcription factor, and its binding to ARE-driven gene promoters. This review summarizes current conceptions of Nrf2/Keap1/ARE induction and inactivation.

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ARE was discovered in the late 1980s when xenobiotics that are ligands of aromatic hydrocarbon receptor (AhR) and act via xenobiotic responsive element (XRE) were investigated. Some xenobiotics were able to activate enzymes involved in phases I and II of xenobiotic metabolism (biphasic inducers) or – independent from XRE – only enzymes of the second phase (single-phase inducers) [1]. Subsequent studies of the impact of electrophilic compounds on cells revealed new regulatory structure that was called antioxidant responsive element (ARE) since most compounds inducing the structure belonged to the group of phenolic antioxidants [2]. Fundamental differences in structure and chemical properties of XRE and ARE inducers were found: while AhR activators are characterized by planar structure, redox properties of a molecule are important for the second ones [3]. Later the action of biphasic inducers was explained by their ability to initiate the synthesis of monooxygenase enzymes via the XRE pathway and then to be metabolized in cells generating electrophilic compounds that activate ARE [1]. ARE motives have now been found in promoter regions of many genes. In addition, among transcription factors such as leucine zipper, which bind and activate these genes, the leading role of Nrf2 (NF-E2-related factor 2) belonging to the Cap'n'Collar family (CNC) was shown [4].

Abbreviations: AhR, aromatic hydrocarbon receptor; ARE, antioxidant-responsive element; CNC, Cap'n'Collar family; CTR, C-terminal region; Cul3-E3-ligase, cullin-3-containing ubiquitin–ligase E3 complex; 15d-PGJ_2, 15-deoxy-prostaglandin J_2; GCLC, glutamate-cysteine ligase catalytic subunit; HO-1, heme oxygenase-1; IRES, internal ribosome entry site; Keap1, Kelch-like ECH-associated protein 1; MAPK, mitogen-activated protein kinase; Neh, Nrf2-ECH homolog; NES, nuclear export signal; NLS, nuclear localization signal; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, NF-E2-bound factor 2; NTR, N-terminal region; PGAM5, phosphoglycerate mutase 5; ROS, reactive oxygen species; tBHQ, *tert*-butylhydroquinone; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element; XRE, xenobiotic-responsive element.

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Nrf2/Keap1/ARE INDUCERS

A vast number of Nrf2/Keap1/ARE signal system inducers were discovered among natural and synthetic xenobiotics and products of the organism's metabolism. The inducers can be divided into 10 main categories [5, 6]: diphenols, phenylenediamines, and quinones; Michael acceptors; isothiocyanates, thiocarbamates, and related sulfur-containing compounds; 1,2-dithiol-3-

thiones, oxathiolene oxides, alk(en)yl (poly)sulfides; hydroperoxides; compounds of trivalent arsenic; heavy metal ions (Cd, Co, Cu, Au, Hg, Pb); vicinal dimercaptans; carotenoids and their analogs; selenium-containing compounds (particularly diselenides and selenols). In addition, the following factors are able to activate expression of ARE-regulated genes: heme complexes, oxidized lipoproteins; direct action of $OH^{\cdot}$, CO, $NO^{\cdot}$, $ONOO^{-}$/ONOOH, O_3, HOCl, short wavelength UV, radiation, ischemia/reperfusion, hyperoxia and hypoxia, and shear stress [7].

In spite of differences in ARE inducer structures, they share common properties of electrophilicity (for this reason ARE is often called EpRE, electrophile response element) and ability to modify SH-groups in proteins via alkylation or oxidation [8]. Thus, Nrf2 activation by phenol antioxidants (compounds containing two OH-groups in *ortho*- or *para*-position only have this ability [2]) requires their primary transformation into corresponding quinones, which, in turn, oxidize or electrophilically bind to sulfhydryl groups of repressor protein Keap1 (Kelch-like ECH associating protein 1) [9]. Fishbein and Holland [10] proposed their classification of modifying agents/Nrf2 inducers on the basis of the mechanism of their reaction with SH-groups of cysteine residues in target proteins. They divided agents into six classes: monofunctional electrophiles alkylating Keap1 by a mechanism of nucleophilic substitution S_N2; bifunctional molecules possessing epoxide group (S_N2-alkylating agent) and Michael-acceptor structure; monofunctional Michael acceptors; inducers modifying sulfhydryl groups and forming thiocarbamates (isothiocyanates); thiol-oxidizing compounds and reagents containing chemical elements from the fourth and lower periods. The main members and mechanisms of action of different Nrf2 inducer classes are presented in Table 1.

Table 1. Members and mechanism of action of different Nrf2 inducer classes

Group of agents	Members		Mechanism of action
	Xenobiotics and their metabolites	Endogenous compounds	
Diphenols, quinones, and phenylenediamines	tBHQ, BHT, BHA, curcumin, resveratrol, quercetin, ethoxyquin, probucol, epigallocatechin-3-gallate	dopamine, 4-hydroxyestrol, 2-hydroxyestradiol, 4-hydroxyestradiol, estradiol-3,4-quinone	forming of quinones that oxidize or bind to SH-groups in Keap1 and increase of intracellular H_2O_2 production [9]
Michael acceptors	EPA, DHA, crotonic aldehyde, methyl acrylate, methyl propionate, methyl vinyl sulfone	acrolein, 4-hydroxy-2,3-nonenal, PGA_2, 15d-PGJ_2, J_2-isoprostane	binding to SH-groups of Keap1 [11]
Isothiocyanates	sulforaphane, 3-morpholinopropyl isothiocyanate	–	binding to SH-groups of Keap1 [12]
1,2-Dithiol-3-thiones	1,2-dithiolthione, oltipraz, 5-(*para*-methoxyphenyl)-1,2-dithiol-3-thione	–	increase of H_2O_2 intracellular production [13]
Hydroperoxides	*tert*-butyl hydroperoxide, cumol hydroperoxide, H_2O_2	H_2O_2, lipid hydroperoxides	oxidation of SH-groups in Keap1 [14]
Compounds of trivalent arsenic	As_2O_3, AsO_2^-, As^{3+}, phenylarsine oxide, $CH_3As(OH)_2$	–	binding to SH-groups of Keap1 [15], increase of intracellular H_2O_2 production [16]
Heavy metal ions	Cd^{2+}, Co^{2+}, Cu^{2+}, Au^{1+}, Hg^{2+}, Pb^{2+}	–	increase of intracellular H_2O_2 production [17]
Vicinal dimercaptans	(±)-2,3-dimercapto-1-propanol, 1,2-ethane dithiol	–	not determined
Carotenoids	3-hydroxy-β-damascone, lycopene	–	not determined, preliminary oxidation of compounds is required [18]
Selenium-containing compounds	ebselen, dialkyl diselenides, seleninic acids, phenyl selenol	–	not determined

Note: tBHQ, *tert*-butylhydroquinone; BHT, butylhydroxytoluene; BHA, butylhydroxyanisole; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PGA_2, prostaglandin A_2; 15d-PGJ_2, 15-deoxy-prostaglandin J_2.

GENES WITH ARE-CONTROLLED EXPRESSION

Mammalian cells contain several hundreds of ARE-driven genes. Sixty-three genes (0.6% of determined genes) whose expression reliably increased under the action of *tert*-butylhydroquinone were detected by oligonucleotide microanalysis in human neuroblastoma cells [19]. Comparable analysis of changes in gene expression caused by 1,2-dithiol-3-thiones in liver of wild type mice, knockouts in Nrf2 or double knockouts in Nrf2 and Keap1 revealed 231 Nrf2/ARE-induced genes and 31 genes with inhibited transcription under Nrf2 activation (1.8% and 0.25% among 12,400 analyzed genes, respectively) [20]. Similar studies of action of effective ARE inducer sulforaphane in murine small intestine cells with genotype $NRF2^{+/+}$ and $NRF2^{-/-}$ revealed 77 genes regulated by Nrf2 (1.3% among 6000 analyzed genes) [21], while there are 562 genes (11%) in liver cells [22]. After different periods of hyperoxia (more than 95% O_2), 175 genes with increased transcription and approximately 100 repressible genes were detected. Notably, the expression in wild type mice increased more intensively than in Nrf2 knockouts [23]. Therefore, the redox-sensitive Nrf2/Keap1/ARE system regulates from 1 up to 10% of genes.

Nrf2/ARE-regulated genes encode enzymes and regulatory and structural proteins that can be divided into several groups according to their main function: enzymes of detoxication and export of xenobiotics and products of cellular metabolism from the cell as well as enzymes providing reparation (utilization) of damaged macromolecules; enzymes controlling redox status of the cell – possessing direct antioxidant activity or synthesizing endogenous reducing agents (first of all, glutathione); regulators of apoptosis, cell cycle, and differentiation; chaperones and heat shock proteins; proteins of intercellular adhesion, cytoskeleton, and intracellular transport; regulators of ribosomal protein synthesis; regulators of immune response and inflammation; and a wide group of enzymes involved in cellular metabolism [7, 20, 24, 25]. The study of the functions of proteins that are encoded by Nrf2-induced genes showed that the main biological action of the Nrf2/Keap1/ARE signaling system is maintenance of intracellular homeostasis, cellular defense against potentially dangerous chemical agents and physical exposure, identification of damaged macromolecules and their repair/utilization depending on possibility of their normal structure recovery, and, under significant degree of damage –initiation of apoptosis. Due to this, the Nrf2/Keap1/ARE signaling system prevents neoplastic transformation of cells and arrests processes of cancerogenesis [24].

At the same time, effects of Nrf2 activation can be determined by its ability to inhibit transcription of various genes. Thus, Nrf2 inducers suppress the activation of transcription factor NF-κB, depressing expression of a wide group of proinflammatory genes (in human monocytes stimulated by lipopolysaccharide, among 88 induced genes of early response 90% are NF-κB-dependent [26]). Thus, the Nrf2/Keap1/ARE signaling system reduces intensity of acute inflammatory response and intensifies its resolution preventing transition of the pathological process into chronic form [27, 28]. Nrf2 activation in liver cells under the action of 1,2-dithiol-3-thione decreases expression of genes encoding enzymes of lipid and cholesterol biosynthesis and metabolism [20].

Nrf2, Keap1, AND ARE STRUCTURES

The DNA region containing nucleotide sequence 5′-A$^{/G}$TGAC$^{/T}$nnnGCA$^{/G}$-3′ (“core”) is an ARE regulatory *cis*-activating element [29]; nuclear transcription factor Nrf2, which belongs to CNC family, binds with this region. Six factors from this family typical for mammals (forming the NF-E2 subfamily) are now known: p45, Nrf1, Nrf2, Nrf3, Bach1, and Bach2, as well as SKN-1 found in *Caenorhabditis elegans* and CNC found in *Drosophila melanogaster*. All factors from the NF-E2 subfamily can form regulatory active dimers, but depending on ability to bind transcription cofactor they enhance or inhibit expression of ARE-driven genes; therefore, their shutdown or hyperproduction leads to different effects at cellular or organismic levels [30].

Mutational analysis clarified the presence of ARE structures located in promoters of different genes. Thus, it has been found that the presence of the TA$^{/C}$A sequence located at the 5′-end at a distance of 2 base pairs from the “core” is important for induction of gene transcription [29]. Therefore, the length of functionally active ARE is 16 nucleotides, and five of them are variable, which makes additional conditions for the diversity of ARE in the genome (Fig. 1a). Some ARE contain a binding site for AP-1 factor (5′-TGACTCA-3′; 12-O-tetradecanoylforbol-13-acetate-responsive element, TRE) and for this reason Jun- and Fos-proteins can take part in ARE-controlled gene transcription [31]. According to discovered differences in nucleotide sequence, all ARE can be divided into four structural–functional classes: 1) containing 5′-triplet TA$^{/C}$A and TRE in the “core” (genes *NQO1*, *ACR1c2*, and a gene of human ferritin light chain); 2) containing only TA$^{/C}$A sequence (genes *GSTA1* and *GSTA2* in mouse and rat, respectively); 3) containing only TRE (gene of mouse metallothionein-II), and 4) containing neither of these structures (mouse gene of multidrug resistance protein-2). Genes with promoters containing ARE of the first and the second classes exhibit more prominent induction in response to Nrf2 activation and are under less negative control of other transcription factors from the bZIP family. Genes of ARE of the first and the third classes also respond to activation of transcription factor AP-1 [32]. It is important to note

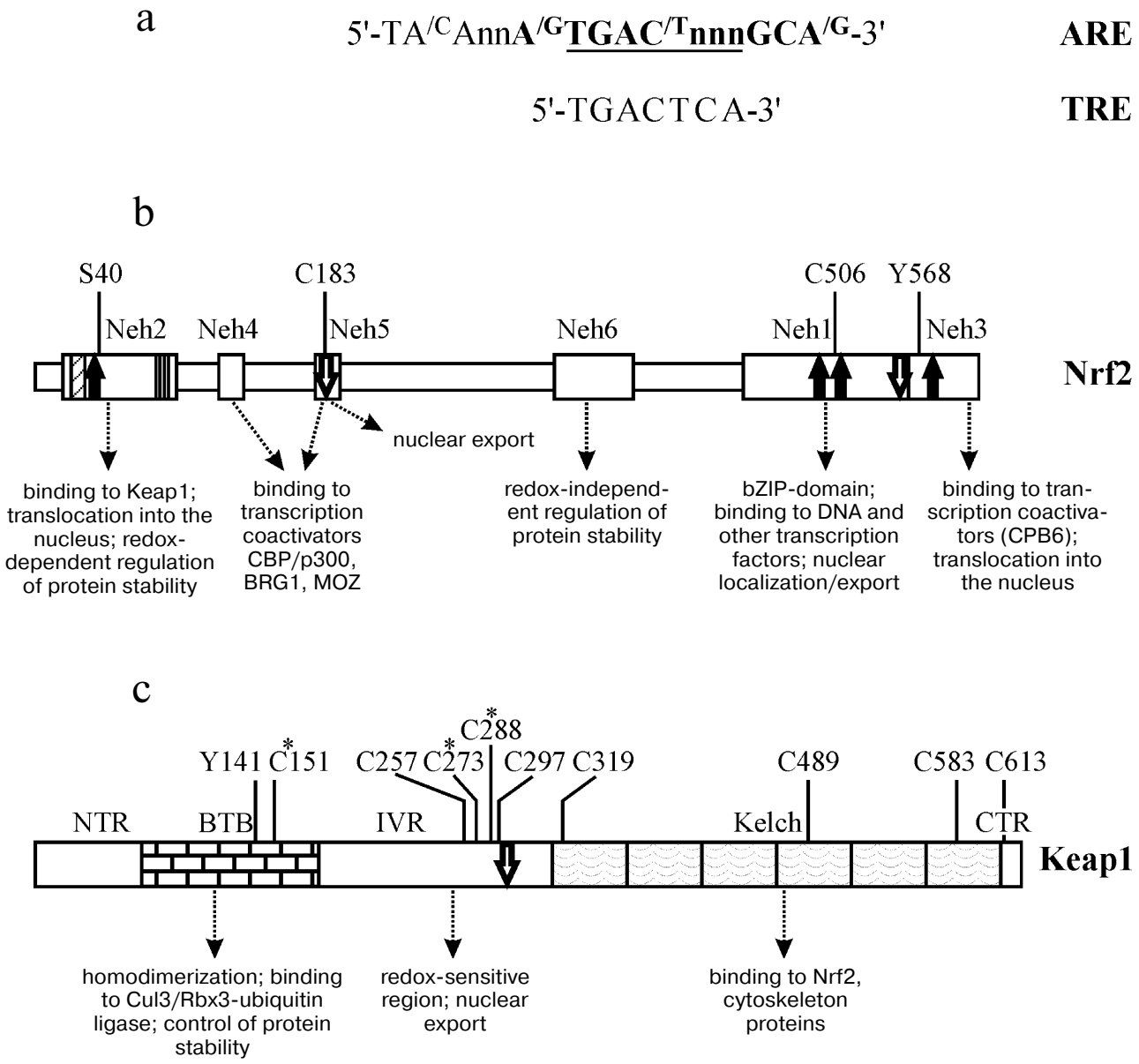

Fig. 1. Structure of antioxidant-responsive element (a), human proteins Nrf2 (b) and Keap1 (c). a) Nucleotide sequences ARE and TRE are presented; nucleotide sequence absolutely essential for Nrf2 binding is bolded in ARE, the region potentially containing site for transcription factor AP-1 binding (TRE), whose structure is presented below, is underlined; b, c) domain position and functions, localization of cysteine amino acid residues sensitive to oxidation/modification, and key phosphorylation sites are depicted. Physiologically relevant cysteine residues are bolded (explanations are in the text).

that certain genes, such as human and rodent heme oxygenase genes, contain several ARE sequences in their promoters that belong to different classes. This allows precise regulation of antioxidant enzyme expression in response to stresses [32].

Mouse protein Nrf2 has molecular weight 66.9 kDa and contains 597 amino acid residues (in human – 67.8 kDa and 605 amino acid residues) that form six highly conservative domains Neh1-Neh6 (Nrf2-ECH homology; ECH – chicken analog of Nrf2) (Fig. 1b) [25, 26]. The Neh2 N-terminal domain takes part in redox-dependent regulation of protein stability due to its attachment to Keap1 (Kelch-like ECH associating protein 1, see below) and conjugation with ubiquitin [25]. Domains Neh3, Neh4, and Neh5 mediate Nrf2 transactivating effect by binding to histone acetyltransferases [33-35]. Domain Neh6 contains redox-independent degron limiting the time of molecule existence under stress conditions when Keap1 repressor function is impaired [36]. Neh1 is the hydrophobic domain of the type of bZIP leucine zipper (basic region and leucine zipper) responsible for dimerization and binding to ARE [25]. Due to Nrf2

bZIP-domain structural features, which do not allow forming homodimers, bZIP-containing partner proteins are necessary for Nrf2 binding to ARE. Among them, participation of transcription factors from the family of small Maf (musculoaponeurotic fibrosarcoma) and Jun proteins in ARE-dependent gene induction is the most studied [32]. In addition, Nrf2 carries several signals of nuclear localization NLS and signals of nuclear export NES responsible for the transcription factor translocation to nucleus or cytoplasm, respectively [37, 38]. Nrf2 molecules contain a number of cysteine amino acid residues (six in human and seven in mouse). Some of them are conservative and are present in unrelated species: C119, C191, C235, and C506 are common for bird, rat, mouse, and human proteins, C316 and C414 – for mammalian Nrf2, C311 – for rodents. This gives a reason to believe that Nrf2 activation can also depend on modifications of its own sulfhydryl groups.

Mouse Keap1 with molecular weight 69.5 kDa contains 624 amino acid residues including 25 cysteine residues (in human 69.7 kDa, 625 amino acid residues, 27 cysteine residues, respectively), which are sensors for a wide range of compounds activating Nrf2 [39]. The protein consists of five domains (Fig. 1c): N-terminal region (NTR); BTB domain responsible for dimerization and interaction with Cul3-E3-ligase [40-42]; intermediate domain IVR (intervening region) containing cysteine residues sensitive to oxidation and NES motif [43]; Kelch-domain consisting of six repeats (KR1-KR6) and possessing the structure of a six-bladed β-propeller that mediates association of Keap1 with Nrf2 and cytoskeleton proteins actin and/or myosin VIIa [25, 44] (though some authors did not discover Keap1/Nrf2 complexes and actin colocalization [45]); and C-terminal region (CTR).

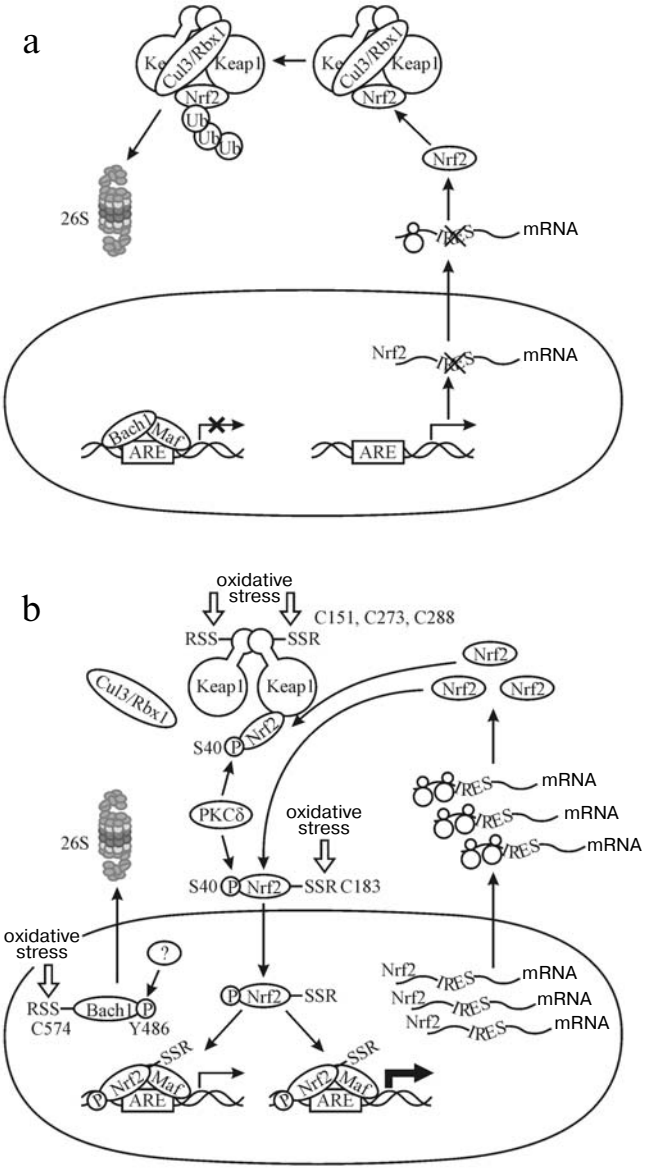

Fig. 2. The mechanism of Nrf2 activation (explanation in the text). a) Regulation of Nrf2/Keap1/ARE activity under physiological conditions; b) Nrf2/Keap1/ARE activation in response to oxidative stress.

Nrf2/Keap/ARE ACTIVATION MECHANISMS

Transcription and translation control of Nrf2/Keap1/ARE signaling system activity. Originally, it was assumed that *NRF2* transcription did not change in response to the impact of specific inducers [46]; however, later the presence of ARE sequence in promoter of this gene was discovered. Moreover, the transcription factor is able to activate the synthesis of its own mRNA [47], which leads to autoregulatory increase in the process of activation of this signaling system in response to exposure of inducers (Fig. 2b). In addition, *NRF2* promoter includes three XRE sequences providing an increase in its transcription in response to dioxins through activation of aryl hydrocarbon receptor and its binding to DNA [48]. Data proving the participation of epigenetic mechanisms in regulation of activation of the Nrf2/Keap1/ARE signaling system were obtained in recent years. For example, hypermethylation of CpG-rich islands in *NRF2* promoter in cells of mouse adenocarcinoma TRAMP (Transgenic Adenocarcinoma of Mouse Prostate) is accompanied with failures in RNA-polymerase II binding to DNA and with repression of corresponding mRNA synthesis [49]. In this case the treatment of these cells with DNA-methyltransferase and histone deacetylase inhibitors restores expression of Nrf2 and ARE-dependent genes. Interestingly, *KEAP1* expression also depends on degree of methylation of its promoter [50].

An increase in the amount of Nrf2 in the cytoplasm was also the result of enhancement of its mRNA translation induced by prooxidants [51] and due to the activation of corresponding mRNA cap-independent transla-

tion in addition to constitutive cap-dependent translation occurring in cells [52]. This process is connected with the presence of redox-sensitive internal ribosomal entry site (IRES) in the untranslated 5′-end of Nrf2 mRNA [52]. $IRES_{Nrf2}$ consists of two rRNA binding regions, one of which is highly conservative and is located near to inhibitor element, which has hairpin structure and prevents the interaction of $IRES_{Nrf2}$ with ribosomes under normal physiological conditions. Under oxidative stress (200 μH H_2O_2) or after treatment with ARE inducers (50 μM sulforaphane) the suppression removes, $IRES_{Nrf2}$ activity rises, leading to enhancement of Nrf2 translation (Fig. 2b).

Keap1 as a molecular sensor of electrophiles and prooxidants. It is assumed that Nrf2 activation is mainly controlled at posttranslational level through changing its stability. Nrf2 is a short-lived protein: life time ($t_{1/2}$) in mouse hepatoma Hepa cells is 13 min [53], in human HepG2 cells – 15 min [54], in African green monkey Cos-7 cells – 7.5 min [40], in mouse peritoneal macrophages – 18.5 min [55]. Nrf2 stability is regulated by Keap1-dependent ubiquitination and degradation in 26S proteasomes (Fig. 2): Keap1 functions as an adaptor protein mediating interaction between Nrf2 and Cullin-3-containing ubiquitin-ligase E3 complex (referred to as Cul3-E3-ligase) [40]. In HepG2 cells Nrf2 concentration and its stimulatory effect increased after inhibition of proteasomes activity [56]. It has been shown that Nrf2 activation by prooxidants or electrophilic compounds is associated with suppression of Keap1 ubiquitin-ligase activity [57].

It has been argued that Keap1 binds to actin cytoskeleton in the cell [7, 58]. But recently Lo and Hannink at the University of Missouri discovered that the complex consisting of Nrf2, Keap1, and phosphoglycerate mutase 5 (PGAM5) was localized at the outer mitochondrial membrane [59]. The formation of such complex results in Nrf2 inhibition since expression of ARE-dependent genes is enhanced in *PGAM5*$^{-/-}$ cells. It has been shown that the same Keap1 amino acid residues take part in its interaction with Nrf2 and PGAM5. But, though phosphoglycerate mutase can act as a substrate for Cul3-E3 ligase, ubiquitination of this protein anchored in mitochondrial membrane with its N-terminal region does not occur. In cells only a small part of Nrf2 molecules has mitochondrial localization, but existence of Nrf2/Keap1/PGAM5 complex can play an important biological role transmitting the signal to the cell nucleus under failures of mitochondrial functions [59]. This hypothesis is proved by data indicating that changes in redox balance in extracellular medium [60], tBHQ [61], or curcumine [62] impact, and inhibition of respiratory chain enzymes by rotenone [63] activate Nrf2 through an increase in mitochondrial ROS production.

The processes of oxidative/electrophilic modification of sulfhydryl groups of cysteine residues in the inhibitor protein play a key role in Keap1-dependent repression of Nrf2 transcription factor. It has been found from experiments that cysteine residues in the Keap1 molecule interact with alkylating agents and prooxidants with different rates. This is caused both by pK_a value depending on their amino acid environment and by inducer nature (Table 2). In addition, differing physiological relevance of individual cysteine residues has been shown. The replacement of C273 and C288 with serine or alanine does not influence Keap1 stability and intracellular distribution and does not change the constant of its interaction with Cul3-E3-ligase and Nrf2 [40, 57, 77] but breaks degradation of the transcription factor molecule during transfection of cells with a mutant protein. Synchronous transfection of *KEAP1*$^{-/-}$ cells with two plasmids carrying *KEAP1* with C273A and C288A mutations in the ratio 1 : 1 restores repressor activity of the corresponding protein [55]. On this basis a model of Nrf2 activation that implicates formation of intermolecular disulfide interaction between C273 and C288 in Keap1 homodimer was proposed [78]. Transgenic mice synchronously expressing mutant proteins Keap1 (C273A) and Keap1 (C288A) are characterized by a high basal level of Nrf2 activation. Therefore, Keap1 should contain both cysteine residues for providing of the repression function under physiological conditions [79].

C151 situated in the BTB domain of the repressor protein is crucial for normal functioning of the Nrf2/Keap1/ARE signaling system. Under oxidative stress and electrophile action, different low molecular weight compounds covalently bind to this residue. As a result, Keap1 modifications resistant to reducing agents [80] are formed or a disulfide bond between Keap1 molecules in homodimer complex appears [14]. C151 residue loss or modification does not affect the ability of Keap1 to suppress the expression of Nrf2/ARE-dependent genes under basal conditions but leads to the reduction of Nrf2 activation caused by *tert*-butylhydroquinone or sulforaphane [64, 79] due to conformational changes in the repressor protein region (amino acids 125-127) and failures in Cul3-E3-ligase interactions [80, 81]. In addition, C151 mutations promote and intensify Keap1 degradation after treatment of cells with tBHQ [82]. These data indicate that C151, which is modified under oxidative stress, can act as a "sensor" of redox balance and is responsible for Nrf2/ARE-dependent gene activation. At the same time, As(III) derivatives inhibit transfer of ubiquitin residues to the Nrf2 molecule without Cul3-E3-ligase dissociation from the repressor protein (by contrast, their interaction straightens). They are also able to activate transcription factor affecting mutant Keap1 with C151 replaced by serine [83]. Notably, sulforaphane, a classical Nrf2 inducer, does not alkylate cysteine residues C151, C273, and C288 in human recombinant Keap1 [84]. In addition, it has been found that Nrf2 activation under oxidative stress is accompanied by formation of an intramolecular disulfide bond between amino acid

Table 2. Modification of cysteine residues in Keap1

Domain	Cys	Modifying agent																		
		BMCC	BMCC	*DEM*	*DEX*	DEX	GSSG	IAB	IAB	IAB	ISO	LIQ	*NAPQ*	8-nitro-cGMP	*PGA_2*	*15d-PGJ_2*	*15d-PGJ_2*	QE	SHO	SULF	XAN
NTR	12																		+	++	
	13																		+	++	
	23	+					°°°		+		++			+					+		
	38	+					°°°		+		±			+					+	+	+
BTB	77	+	+++			++	+++			++	++		+	+				+		++	±
	151	+++		+				+++	+++		+++	+		+				+	+++		+++
	171													+							
IVR	196		+++			++				+++	++			+							
	226	++				++	+		++	++	+++		+	+					+	++	
	241					+	°		±	+++									++		
	249		+			+++	°			±	±								++	++	
	257	++			+++		°°°	+	++	++			+	+		++		+	+++	++	
	273	+			+++			+	++			+	+	+	+	++	+	+	+		
	288	+++		+	+++		+	+++	+++	+++		+	+	+			+	+			
	297	++			+++		+++/°°°	+++	++						+						
KR1	319	+++		+			+++/°°	++	+++	++	++	+							++		+++
KR2	368		+++	+			+++		±	++	+			+				+	+++	+	
	395										++			+							
	406													+							
KR3	434	+++		+			+++		++		+		+	+				+	++		++
KR4	489		+++	+		+++				++	+		+	+	+			+		+++	+
KR5	513									±	++		±	+				+		++	±
	518										++		±	+				+		++	
KR6	583									++	++		+							+++	
CTR	613	+++		+	+++			+	++		+		+	+					++		+++
	622					+	+/°		±	++									++		
	624						+/°		±	++									++	+++	
Compound class (according to Fishbein and Holland [10])		C	C	C	A	A	E	A	A	A	C	B	C	A	C	C	C	C	C	D	C
Reference		[64]	[65]	[66]	[67]	[68]	[69]	[70]	[71]	[65]	[64]	[72]	[73]	[74]	[66]	[73]	[66]	[75]	[64]	[76]	[64]

Note: Relative chemical activity of cysteine residues during their modification by alkylating agents in Keap1 is presented: +++, high; ++, moderate; +, low (±, modification of these sites was not observed in some experiments), or during oxidation with formation of intramolecular disulfide bond (°°°, high; °°, moderate; °, low). Experiments where human recombinant Keap1 was used are marked with upright type, and those where mouse polypeptide was used are italicized. Physiologically relevant cysteine residues are bolded (see text). BMCC, 1-biotinamido-4-(4′-[maleimidoethyl-cyclohexane]-carboxamido)butane); DEM, diethylmaleate; DEX, dexamethasone; GSSG, oxidized glutathione; IAB, *N*-iodoacetyl-*N*-biotinylhexylenediamine; ISO, isoliquiritigenin; LIQ, liquistid; NAPQ, *N*-acetyl-*para*-benzoquinoneimine; 8-nitro-cGMP, 8-nitroguanosine monophosphate (cyclic); PGA_2, prostaglandin A_2; 15d-PGJ_2, 15-deoxy-prostaglandin J_2; QE, estradiol-3,4-quinone; SHO, 10-shogaol; SULF, sulforaphane; XAN, xanthohumol.

residues C226 and C613 in Keap1, though replacement of these cysteines by serine does not affect repressor capacity of the mutant protein [14]. Cysteine residue C23 forming an intramolecular disulfide bond with C38 after treatment with oxidized glutathione is another physiologically relevant site of Keap1 oxidative modification. Replacement of C23 by tyrosine was discovered in human cancer tumor cells with impaired Keap1-dependent Nrf2 ubiquitination [85].

It seems that the interaction of Keap1 with Nrf2 is regulated not only by direct modification of cysteine residues: for example, it was shown that zinc atoms are incorporated in Keap1. Mouse Keap1 contains 0.9 Zn^{2+} ion per subunit; metal cations interact with sulfur atoms in C273 and C288 residues and their removal lead to dissociation of Nrf2–Keap1 complexes [86].

Until recently a model of dissociation of cytoplasmically anchored Nrf2/Keap1 complex via oxidative modification and conformational changes in repressor protein was considered as the conventional mechanism of activation of the Nrf2/Keap1/ARE signaling pathway. As a result, released transcription factor Nrf2 translocates into the nucleus where it interacts with ARE through accessory proteins Maf and Jun and transcription coactivators and thus activates gene expression; and Keap1 is ubiquitinated, transferred to 26S proteasome by actin, and degraded. But a number of experimental facts conflicts with the dissociation model: sulforaphane activates ARE in micromolar concentrations (20-50 μM), while millimolar concentrations of isothiocyanate are required for breaking the bind between Keap1 and Nrf2 (this is 2000 times larger than the Keap1 concentration in the cell) [43]. It has been shown in many studies that a significant number of Nrf2/Keap1 complexes remain associated after induction of antioxidant-responsive element by sulforaphane, *tert*-butylhydroquinone, and other electrophiles [39, 44, 78, 91].

Currently model of constitutive degradation of Nrf2 complex is widely discussed. According to this model a mobile pool of transcription factor molecules is constitutively present in the cell; the number of molecules in this pool is regulated by Keap1. Detailed studies of the polypeptide functional structure of Nrf2 and Keap1 permitted Japanese scientists to propose a "hinge and latch" model [42, 44], which is based on the presence in Neh2 (Keap1-binding domain of transcription factor Nrf2) two sequences with high ($K_a = 20 \cdot 10^7$ M^{-1}; ETGE: Leu-Asp-Glu-Glu-Thr-Gly-Glu) and low ($K_a = 0.1 \cdot 10^7$ M^{-1}; DLG: Leu-Trp-Arg-Gln-Asp-Ile-Asp-Leu-Gly) affinity to inhibitor, "hinge" and "latch", respectively. Negatively charged glutamate residues cause effective interaction of the ETGE motif. These residues promote the interaction of the ETGE motif with positively charged high conservative arginine triplet (R380, R415, R483) situated at the bottom of the β-propeller Kelch-domain of Keap1. Interestingly, among seven lysine residues forming nine turns of α-helix that binds together motifs ETGE and DLG, six are situated at one side [42]. This facilitates the ubiquitination of Nrf2 by these residues in the "latch" "locking" position (i.e. when low affinity DLG motif is associated with Keap1). Studies conducted using high resolution electron microscopy revealed the distance between Nrf2-binding regions of Keap1 homodimers, which is equal to 80 Å and accurately complies with calculated distance between ETGE and DLG sequences in the transcription factor [87].

According to the "hinge and latch" model, there is constitutive synthesis of new Nrf2 molecules in the cell. Newly synthesized molecules bind to Keap1 and are constantly ubiquitinated and degraded in proteasomes, so the concentration of free Nrf2 is low in homeostasis (Fig. 2a). As a result of oxidative stress, Keap1 is modified mainly due to oxidation of redox-sensitive cysteine residues in its IVR and BTB domains. Such protein with modified conformation loses its affinity to DLG motif (the "latch" jumps off and the Nrf2 hangs down on the "hinge" – high affinity ETGE motif). It is possible that an intermolecular disulfide bond between Keap1 monomers is formed after oxidation of C273 and C288. As a result, Nrf2 ubiquitination and, therefore, its proteasome degradation (but not the association with Keap1) is broken, the molecule pool become saturated, and this leads to an increase in the number of free Ntf2 molecules. In addition, Nrf2 transcription and translation activation under oxidative stress explain seeming inefficiency in cellular resources when the cell constantly synthesizes Nrf2 that is immediately degraded in proteasomes. Actually constitutive synthesis is characterized by a moderate level that is essential for maintaining basal antioxidant activity. When redox balance is broken, the mechanisms of Nrf2 additional synthesis are activated. Together with the mechanism of retardation of transcription factor degradation they rapidly and effectively activate ARE (Fig. 2b).

Discovery of *NRF2* mutations in cells of some tumor cells and in cells of primary human carcinomas sufficiently confirms this model. These mutations change amino acid sequences in DLG or ETGE motifs [88, 89] and as a result the stability of the transcription factor molecule and expression of ARE-dependent genes are anomalously increased. The cells with such mutations possess stability toward oxidative stress and chemotherapeutic agents, and the blocking of Nrf2 production by corresponding siRNA reverses these undesirable advantages [88].

Modification of cysteine residues in the Nrf2 molecule is another mechanism of regulation of its stability. It has been found that transcription factor molecules with mutations C506A, C235A, and C191A have shorter time of existence in cells (39, 66, and 58% compared with normal protein $t_{1/2}$, respectively) due to intensive interaction with Keap1 and ubiquitin, which leads to decrease in their ability to activate expression of target genes [90]. ARE activators tBHQ, As(III), and phenylarsine oxide

bind to cysteine sulfhydryl groups in Nrf2 resulting in stabilization of Nrf2 [90].

Together with Keap1, other regulators of Nrf2 stability are known. DJ-1 (product of gene *PARK7*) inhibits the ubiquitination of the transcription factor increasing its lifetime and enhancing spontaneous and stimulated expression of ARE-dependent genes in mouse embryonic fibroblasts [91]. In mesencephalon of DJ-1-deficient mice the amounts of Nrf2 and β5-subunit of 20S proteasome were decreased after administration of paraquat, a well-known inducer of oxidative stress [92]. The severity of clinical implications in the case of obstructive pulmonary disease in smokers has an inverse relationship with intracellular level of Nrf2 (though the amount of appropriate mRNA does not change) and DJ-1. And treatment of Beas2B human lung epithelium cells with cigarette smoke results in oxidative modification and accelerates proteasome degradation of DJ-1. The suppression of DJ-1 expression leads to impairment in the stability of Nrf2 and disturbs the transcription of its target genes induced by cigarette smoke in mouse lung cells, mouse embryonic fibroblasts, and in Beas2B cells [93]. Interestingly, preliminary treatment of DJ-1-deficient cells with sulforaphane or suppression of Keap1 expression restores Nrf2-dependent expression of antioxidant enzymes [93]. Though, on the other hand, it has been shown that activation of the Nrf2/Keap1/ARE signaling system in cortical neurons, striatum nerve cells, and mouse astrocytes is DJ-1-independent [94].

Oncosuppressor protein $p21^{Cip1/WAF1}$ stabilizes Nrf2, competing with Keap1 for interaction with DLG- and ETGE-sequences in the transcription factor. Therefore, basal and induced levels of ARE-dependent gene expression are lowered in mice with $p21^{-/-}$ genotype *in vivo* [95]. Due to such cooperation among signal systems, p21 induction in response to DNA damage (induced by UV-irradiation, ROS, genotoxic compounds) results, on one side, in cell cycle arrest and initiation of repair processes, and, on the other side, in Nrf2-dependent activation of antioxidant defense and detoxication systems, which neutralize damaging agents [96].

Adapter protein p62 (sequestosome 1) performs a range of important functions in cells: it facilitates protein–protein interactions due to many protein-binding regions present in its structure; aggregates damaged and unfolded proteins; transports polyubiquitinated proteins to proteasomes and their aggregates to lysosomes, thus participating in autophagy [97]. Like Nrf2, p62 contains in its structure an ETGE sequence that mediates interaction with an arginine triplet of the Keap1 Kelch-domain, thus inhibiting enzymatic activity of ubiquitin-ligase complex and increasing stability of the transcription factor [98]. Such protein–protein interaction leads to Keap1 aggregation, enhances its ubiquitination and proteasome degradation [99, 100]; therefore, an increase in endogenous (as a result of failure in autophagy) or ectopic p62 expression lowers the number of Keap1 molecules. Thus, authors of these studies postulate the existence of a non-conventional (not connected with modifications of cysteine residues) mechanism of Nrf2 de-repression.

CRIF1 (CR6-interacting factor 1) is another recently discovered regulator of Nrf2 stability. It (like Keap1) enhances ubiquitination and proteasome degradation of the transcription factor molecule. But, in contrast to Keap1, this regulatory protein except for the Neh2 domain interacts with C-terminal bZIP region, is able to bind mutant Nrf2 with modified ETGE sequence, and, what is most important, repression mediated by this protein persists under oxidative stress conditions. Therefore, CRIF1 is redox-independent negative regulator of ARE-dependent gene expression that acts at the stage of Nrf2 posttranslational modification [101].

Role of protein kinase in Nrf2 activation. An important mechanism regulating expression of ARE-dependent genes is phosphorylation of Nrf2 and Keap1 molecules, which is catalyzed by protein kinases from various families.

One of the main kinases involved in ARE regulation is protein kinase C, which phosphorylates a serine residue in the Neh2 fragment of Nrf2 (S40 situated between motifs ETGE and DLG) and it leads to disruption of its interaction with Keap1 (Fig. 2b). Phosphorylation of S40 does not influence Nrf2 translocation to the nucleus or its interaction with DNA regulatory region, but it increases Nrf2 stability and lifetime in cells [102]. The main isoform of this enzyme phosphorylating Nrf2 is protein kinase C-δ, which is classified as an atypical protein kinase by the structure of its regulatory domain [82]. Both phosphorylation of the transcription factor and modification of cysteines in Keap1 repressor are necessary for full activation of the Nrf2/Keap1/ARE signaling system.

Nrf2 is a substrate for PERK kinase (pancreatic eIF-2α-related endoplasmic reticulum kinase), which is activated in response to production of unfolded proteins. In addition, PERK-dependent phosphorylation leads to stabilization of the transcription factor, inducing dissociation of the Nrf2/Keap1 complex and preventing its reassociation, and increases expression of ARE-driven genes [103, 104].

Protein kinase cascade PI3K/Akt triggers Nrf2 activation and expression of ARE-dependent genes in different cells under the action of sulforaphane [105], phenol antioxidant curcumine [106], triterpenoids [107], 15d-PGJ_2 [108], lipopolysaccharide [109], and peroxynitrite [110]. PI3K/Akt-mediated Nrf2 activation regulates redox status of aortal endothelial cells in mice *in vivo* and prevents development of atherosclerotic plaques [111]. Nevertheless, in spite of a large number of experimental data, the mechanism of PI3K/Akt-dependent Nrf2 activation remains unknown.

Mitogen-activated protein kinases ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal pro-

tein kinase), and p38 take part in the regulation of Nrf2 activity. An inhibition in their activity in different ways leads to various effects – from full abrogation of Nrf2 activation to its multiple enhancement, according to experimental conditions (the results are summarized in the study of Sun et al. [112]). Nrf2 inducers – sulforaphane, tBHQ, and pyrrolidine thiocarbamate – activated ERK2 kinase in human HepG2 and mouse Hepa1c1c7 hepatoma cells, and pharmacological inhibition or expression of functionally inactive mutant enzyme decreased expression of ARE-dependent genes and Nrf2 binding to DNA [113]. In mouse keratinocytes ERK1/2 are the main enzymes mediating activation of *NQO1* expression in response to Nrf2 inducers – 1,2-dithiol-3-thione, sulforaphane, oltipraz, and H_2O_2 [114]. The activity of the chemoprotective agent phenethyl isothiocyanate in PC-3 prostate cancer cells increased expression of *HO-1* through activation of JNK1/2 and ERK1/2. In addition, it has been shown that these enzymes colocalize with Nrf2 in the nucleus and are able to phosphorylate transcription factor molecules *in vitro* [115].

Since MEK-kinases inhibitors reduce Nrf2 content in HepG2 cells and phosphatase inhibitor okadaic acid has an opposite effect, it is supposed that ERK-dependent phosphorylation results in transcription factor stabilization, weakening its interaction with Keap1 [4]. The ubiquitous plant phenol curcumin [116] increases the level of heme oxygenase-1 in epithelium cells via activation of p38 kinase. On the other hand, in study [117] where macrophages RAW264.7 and embryonic mice fibroblasts with $p38^{-/-}$ genotype were used, it was shown that suppression of p38 protein kinase activity, its deficiency, or hyperexpression of functionally inactive mutant enzyme intensify Nrf2-dependent expression of heme-oxygenase-1 gene. This may be associated with an increase in ROS production and stimulation of phosphorylation of ERK-kinases. Moreover, p38-mediated phosphorylation of the transcription factor molecule promotes stabilization of Nrf2/Keap1 complex, and induction of ARE-dependent genes in response to sulforaphane is associated with inhibition of MKK3/6, which is a direct activator of p38 [118]. Provocative data about an interaction between MAPK and Nrf2/ARE were obtained by Li et al. They showed that the impact of tumor necrosis factor α or high glucose concentrations on vessel smooth muscle cells in old (24-month-old) rats leads to predominant activation of ERK1/2 (while JNKs are activated under the same conditions in cells of six-month-old rats), and inhibition of these enzymes is accompanied by an increase in the number of Nrf2 molecules in the nucleus, its binding to ARE, and *GCLC* expression [119]. Therefore, effects of different MAPK activations can, in addition, depend on animal age.

At the same time, it has been shown that under Nrf2 activation in HEK293T human embryonic kidney cells the protein is multiply phosphorylated by MAPK-family enzymes at residues S215, S408, S558, T559, and S577. This phosphorylation does not influence the interaction of the transcription factor with Keap1 and its stability, but it increases import into the nucleus. Only replacement of all five of these amino acids by alanine leads to moderate depression of ARE-dependent gene expression. Therefore, the authors of this study believe hat MAPK play a restricted part in regulation of the Nrf2/Keap1/ARE signaling system under physiological conditions [112].

Certain protein kinases participate in negative regulation of signal transduction in the Nrf2/Keap1/ARE system. Phosphorylation of serine/threonine residues in Nrf2 transcription factor by calmodulin-dependent casein kinase 2 decreases its stability and depresses ability to bind to ARE [120]. Keap1 synthesized *de novo* is phosphorylated constitutively by tyrosine kinase at BTB-domain Y141 position. Dephosphorylation of this amino acid residue under oxidative stress promotes degradation of the repressor protein. Mutant Keap1 (Y141A) also has shorter lifetime and, even though it can form dimers, it is functionally inactive [121].

Therefore, phosphorylation is one of the key steps in Nrf2 activation, but the role of individual protein kinases and phosphatases in the Nrf2/Keap1/ARE signal system mainly depends on cell type as well as on inducer origin.

Regulation of Nrf2 transport into and out of the nucleus. Nrf2 contains three signals of nuclear localization (NLS) in its structure and two signals of nuclear transport (NES), which compensate each other under physiological conditions. When homeostasis is disturbed the relative activity of NLS prevails and due to it the transcription factor accumulates in the nucleus. The NLS_{TA} region was identified as a redox-sensitive regulatory element. This region contains amino acid residue Cys183, which is extremely reactive and is modified by low doses of alkylating agents [44] (Fig. 2b). Modification of C183 in response to oxidants (H_2O_2, sulforaphane, and tBHQ) dose-dependently leads to Nrf2 accumulation in nuclei of cells with suppressed synthesis of Keap1. And mutations resulting in replacement of cysteine by alanine in these positions disturb induced Nrf2 translocation into the nucleus and expression of target genes [37]. Therefore, the action of Keap1 as a molecular sensor of oxidative stress can play a permissive role in Nrf2 activation, while direct oxidative modification of the transcription factor regulates strength, speed, and duration of ARE-dependent response in relation to intensity of the stress.

Nrf2 binding to Maf proteins forming heterodimer complex via their interaction with bZIP-domains leads to NES_{Zip} masking and decreases transport of the factor from nucleus to cytoplasm [122]. Nuclear matrix protein NRP/B (nuclear restricted protein/brain), which belongs to the same family as Keap1, is able to bind to Nrf2. This protein–protein interaction is intensified by H_2O_2 and leads to increase in ARE-dependent gene expression

[123] through prevention of the transcription factor transport to the cytoplasm and its accumulation in the nucleus. NRP/B mutations altering its translocation from nucleus to cytoplasm disturb induced expression of ARE-dependent genes [124].

Posttranslational modification of exportin Crm1 suppresses reverse transport of Nrf2 to the cytoplasm in response to oxidative/nitrative stress. S-Nitrosylation of cysteine amino acid residues C528 and C585 in Crm1 arrests transport of target proteins to the cytoplasm through failure of the interaction of Crm1 with NES sequences. As a result of this process, nitric oxide donors promote ARE-dependent gene expression via Nrf2 accumulation in the nucleus [125].

Regulation of Nrf2 binding to DNA and transcription coactivators. Nrf2 binding to DNA is controlled through changing redox balance inside nucleus since transcription factor effectively interacts with ARE when C506 in its DNA-binding Neh1 domain is in reduced state (mutant protein Nrf2C506S is functionally less active) [126]. As C506 is modified in the cytoplasm under oxidative stress, reduction of this amino acid residue is essential for normal Nrf2/Keap1/ARE signaling system functioning. It has been shown that C506 is reduced by thioredoxin-1 [127], which is thus a coactivator of ARE-dependent gene expression [128]. Apurine/apyrimidine endonuclease APE/Ref-1 takes part in this process, acting as unique "redox-chaperone" and enhancing Nrf2 binding to thioredoxin [129]. C119 and C235 are other cysteine residues participating in the interaction between Nrf2 and ARE since their replacement by alanine (as well as replacement of C506) significantly decreases the ability of the mutant proteins to bind to *NQO1* enhancer under suppression of proteinase activity in comparison with the wild type protein [90]. The same amino acid residues were found to be necessary for interaction with transcription coactivator p300/CBP [90]. DNA-binding ability of Nrf2 is also regulated by acetylation of lysine residues in the Neh1 domain. Under oxidative stress residues are acetylated by histone acetyltransferase p300/CBP. Acetylated Nrf2 increases expression of *NQO1*, *TRXR*, and *GSTA1* but not *HO-1*, therefore selectively interacting with different promoters [130].

Under normal physiological conditions, ARE sequences are associated with Bach1, which forms dimers with Maf proteins. They do not possess transactivating ability and prevent Nrf2 binding to DNA (Fig. 2a). Bach1 is a negative regulator of ARE-dependent gene expression [131-133], and it has high affinity to *HO-1* promoter [133]. Induction of *HO-1* expression by heme is accompanied by dissociation of Bach1/Maf complexes [134], Crm1-dependent export of Bach1 to the cytoplasm, its ubiquitination, and proteasomal degradation [135]. In response to tBHQ, amino acid residue Y486 in Bach1 is rapidly phosphorylated by an unknown tyrosine kinase. This leads to dissociation of the protein from DNA and its relocalization to the cytoplasm, and as a result, interaction of Nrf2 with ARE becomes possible [136]. In addition, oxidation of amino acid residue C574 in the DNA-binding domain of Bach1 is essential for switching off redox-dependent Bach1 repressor action. The replacement of this residue with serine results in loss of sensitivity to oxidative stress [137] (Fig. 2b). Bach2 and Nrf3 from subfamily NF-E2, short isoform p65 of Nrf1 factor (in contrast to "full-sized" protein), as well as C-terminal Nrf2 fragment formed after the transcription factor molecule degradation by caspase-3 also bind ARE but do not have transactivating activity, and therefore these proteins function as repressors of gene transcription [30, 138, 139]. Moreover, c-Fos, Fra1 (in complex with Jun proteins), small Maf proteins (which form homodimers with repressor properties), and c-Maf (in the form of heterodimers with small Maf) compete with Nrf2 for binding sites [140-142].

Retinoic acids enhance translocation of RARα (retinoic acid receptor α) to the nucleus where it forms complexes with Nrf2, disrupting its interaction with DNA. As a result ARE-dependent gene expression is increased in cells of small intestine in mice with $NRF2^{+/+}$ (but not $NRF2^{-/-}$) genotype, which were kept on vitamin A-poor diet [143].

NF-κB subunit p65 activated via phosphorylation of its S276 residue competes with Nrf2 for binding transcription coactivator CBP and facilitates interaction of histone deacetylase 3 with Nrf2/Maf complex. As a result, chromatin structure becomes more compact and expression of ARE-dependent genes is complicated [144]. Orphan receptor ERRβ (estrogen-related receptor β), estrogen receptor α, and transcription factor ATF3 (activating transcription factor 3) containing bZIP-domain form complexes with Nrf2 and thus diminish its interaction with transcription coactivators [145-147]. This explains repression of ARE-dependent gene expression by 17β-estradiol (natural ligand of estrogen receptor α) [145] and transforming growth factor β (activator of ATF3 production) [148].

MECHANISMS OF Nrf2/Keap1/ARE INACTIVATION

In spite of active study of Nrf2/Keap1 signaling pathway activation mechanisms, its deactivation during the post-induction period when redox balance is recovered in the cells is poorly understood. In one of several studies dedicated to investigation of downward Nrf2/Keap1-mediated cellular response, it was shown that Nrf2 export from nucleus to cytoplasm is directed by Keap1 due to the NES motif in its structure [45, 149]. The authors suppose that Keap1 contains an unconventional redox-sensitive NLS motif that is activated during redox-homeostasis recovery and promotes protein mov-

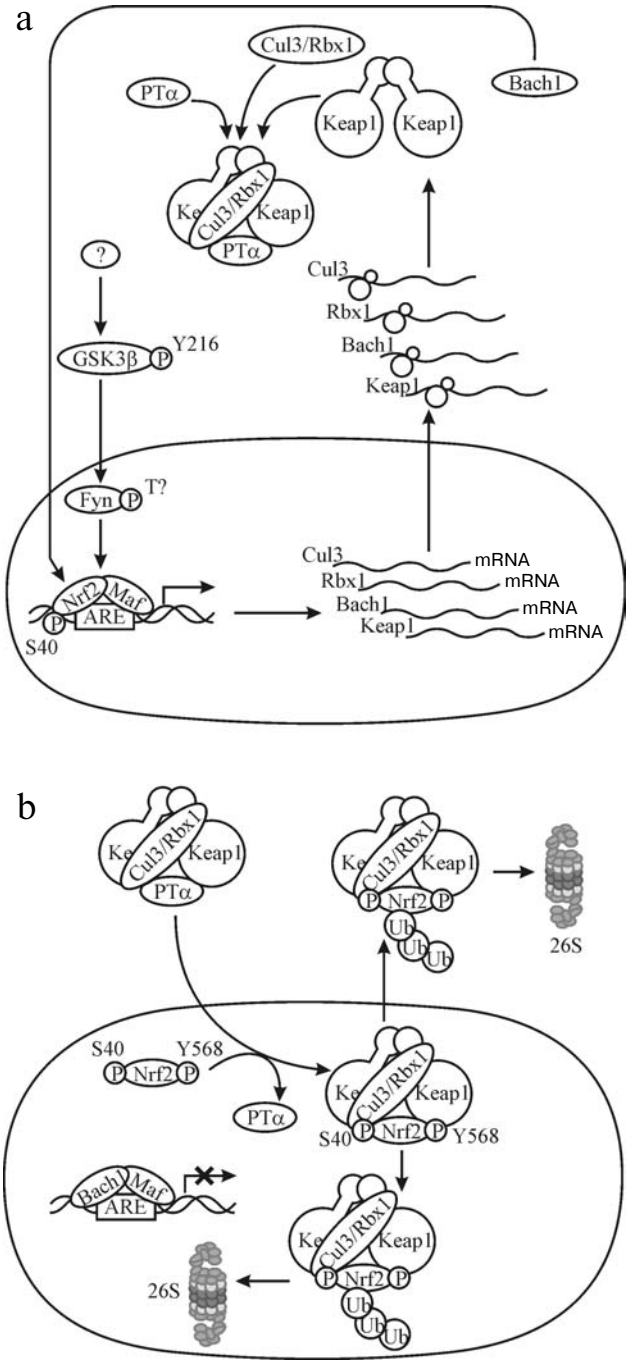

Fig. 3. Nrf2 inactivation mechanism (explanations in the text). Early (a) and late (b) stages of switching off of Nrf2 signaling action.

ing to the nucleus, where it binds to Nrf2 and transports it back to the cytoplasm. However, it remains unclear how Keap1 is released from interaction with actin. In addition, another mechanism was proposed. While previously it was assumed that the Nrf2 molecule is ubiquitinated only in the cytoplasm, it was then discovered that ubiquitin–ligase complex associated with Keap1 is able to transport to the nucleus together with prothymosin-α, which competes with Nrf2 for binding to Kelch-domain and is not exposed to Keap1-dependent ubiquitination [150]. In the nucleus prothymosin-α is replaced with Nrf2, which is accompanied by intranuclear ubiquitination of the transcription factor with subsequent degradation in proteasomes [151]. It has been supposed that this mechanism mediates the end of Nrf2-dependent activation and "switching off" of ARE-driven genes (Fig. 3, a and b). As(III) compounds, which are strong Nrf2 inducers, increase the life-time of the transcription factor from 21 to 200 min in mouse hepatoma cells Hepa1c1c7, disturbing the forming and initiating dissociation of already formed Keap1/Nrf2 complexes in the nucleus [152]. Therefore, this class of ARE-inducers acts at the "switching off" stage of the Nrf2 signaling effect.

Tyrosine kinase Fyn is another significant enzyme that inactivates Nrf2. It phosphorylates the Y568 residue in Nrf2 in the nucleus, and as a result its export to the cytoplasm and Keap1-mediated degradation are intensified. Under oxidative stress Fyn is activated after phosphorylation of an unknown threonine residue by GSK-3β kinase and thereby ARE-dependent gene expression is negatively controlled (Fig. 3, a and b) [153].

Nrf2 shutdown is promoted by increase in expression of *BACH1*, *KEAP1*, *RBX1*, and *CUL3*, as well as by increase in expression of ubiquitin and components of the 26S proteasome that contain ARE sequences in their promoters and regulate transcription factor activity by a feedback mechanism [20, 136, 154, 155]. Thus, for example, it has been shown that tBHQ inducing rapid Bach1 degradation also initiates synthesis of new repressor protein molecules. For this reason the level of the protein in the nucleus recovers to the basal level already in 4 h after Nrf2 inducer impact (Fig. 3a) [136].

More than 20 redox-sensitive transcription factors containing easily oxidized amino acid residues have been found in mammalian cells [156]. Several phosphatases and kinases participating in intracellular signal transfer also contain in their structure elements sensitive to oxidation. Therefore, we face a complicated system of intracellular redox regulation. A large number of cysteine residues exposed to oxidative modifications, which specifically response to different stimuli [66] and possibility of Nrf2 redox regulation at various stages, from mRNA synthesis to binding to ARE sequences are differential characteristics of the Nrf2/Keap1/ARE system. On one hand, it makes the system extremely sensitive to different inducers and, on the other, highly flexible and able to respond to diverse stimuli. For this reason the Nrf2/Keap1/ARE signaling system, though it is not vital, determines the physiological level of changes in redox balance whose increase leads to pathological changes and induces "preventive" cellular response to disturbance of homeostasis.

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