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REVIEW

Active Defense under Oxidative Stress. The Antioxidant Responsive Element

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Abstract—This review considers the mechanisms and factors that stimulate transcription of genes regulated by the antioxidant responsive element (ARE). The latter is important for cell defense under conditions of oxidative stress and also for detoxification of electrophilic xenobiotics. There are differences in regulation of intracellular homeostasis involving Nrf2-mediated activation of ARE and other redox-sensitive factors (NF-κB and AP-1).

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It is believed that biosphere "contamination" with molecular oxygen produced by blue-green algae was one of the first global ecological catastrophes that occurred on the Earth. That not only wiped out major ancient anaerobic prokaryotes, but it also stimulated evolutionary development causing the initial appearance of aerobic eukaryotes and culminating in the subsequent diversity of animals and plants. They successfully overcame the problems related to appearance of atmospheric oxygen by developing an effective energy producing mechanism, oxidative phosphorylation, and universal mechanism of xenobiotic biotransformation, employing cytochromes P450, monooxygenases that introduce an oxygen atom in substrates [1]. Multicellular organisms had learned to use reactive products of incomplete oxygen reduction for self-protection against bacteria (e.g. about 90% of microbicidal potential of phagocytes is due to production of O_2^-, H_2O_2, $OH^\cdot$, $NO^\cdot$, etc. [2]) and also for inter- and intracellular signaling [2-4].

In mammals, ~20 so-called redox-sensitive regulatory elements have been described; they are responsible for changes in the ratio of reduced and oxidized SH groups in proteins or, more generally, in the ratio of pro- and antioxidants resulting in the development of oxidative or reductive stress [5]. Activation of redox-sensitive transcription factors Nrf2, NF-κB, and AP-1 influences expression of several hundred genes and therefore the activity of numerous metabolic processes [3]. These factors are key regulators of cell proliferation, differentiation, apoptosis, and also development of multidrug resistance. NF-κB and AP-1 are responsible for switch from one cell program to another "in the interests" of the whole organ or even the whole organism, and this is important under certain adaptive or pathological circumstances [6, 7]. In contrast to NF-κB and AP-1, regulation involving the antioxidant-responsive element (ARE) is important for maintenance of internal homeostasis under apoptosis-initiating [8, 9], carcinogenic [10-12], and stress [13, 14] exposures. This emphasizes the biological importance of ARE, because this element determines the functioning of other redox-sensitive factors and systems including those which respond to external signals [9].

In this review we have considered mechanisms involved in ARE regulation.

XENOBIOTIC ANTIOXIDANTS ACTIVATING ARE

The first experimental evidence for the existence of ARE was obtained in late 1980s during studies of xenobi-

Abbreviations: ARE) antioxidant responsive element; γ-GCS) γ-glutamylcysteine synthetase; GST) glutathione S-transferase; LPO) lipid peroxidation; $NO^\cdot$) nitric oxide; NQO) NAD(P)H:quinone oxidoreductase; NRH) dihydronicotinamide riboside; ROM) reactive oxygen metabolites; XRE) xenobiotic responsive element.

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otic metabolism. Some compounds turned out to induce phase I and II xenobiotic metabolizing enzymes. For example, the stimulation of cytochrome P4501A1 synthesis by polycyclic aromatic hydrocarbons realized via their interaction with an aromatic hydrocarbon receptor followed by subsequent activation of the xenobiotic responsive element (XRE) caused simultaneous induction of ~20 enzymes, including phase II xenobiotic metabolizing enzymes. Other compounds induced only phase II xenobiotic metabolizing enzymes such as glutathione S-transferase (GST), glucuronosyltransferase, NAD(P)H:quinone oxidoreductase (NQO), etc. Subsequent studies on the effects of electrophilic phenolic compounds on cells identified a new regulatory element, which differs from XRE. Initially it was denominated as β-naphthoflavone-inducible [15]; however, later it was renamed as the antioxidant responsive, because the major proportion of compounds inducing it belonged to the group of phenolic antioxidants [16]. Planar structure of a molecule was an important characteristic feature for aromatic compounds acting at aromatic hydrocarbon receptor, whereas redox properties were crucial for ARE activators [17].

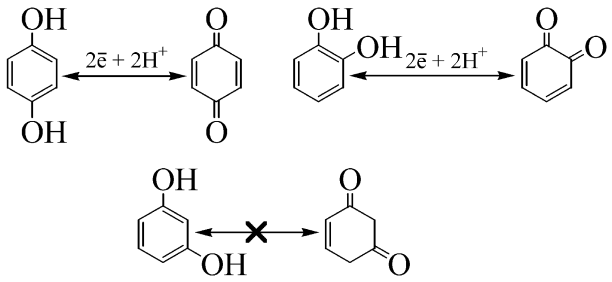
Many natural (ellagic acid, flavonoids, polyphenols of green tea extracts) and synthetic (*tert*-butylhydroquinone, butylated hydroxyanisole, probucol) phenols and also SH-containing compounds (isothiocyanates, dithiolthiones, dimercaptans) can increase transcription of genes regulated by ARE (Table 1). Besides aromatic and thiol-containing compounds, hydroperoxides, carotenoids, heavy metal atoms (Cd, Co, Cu, Au, Hg, Pb) and heme complexes also exhibit ARE-activating capacity [18-21]. Direct action of H_2O_2, $OH^{\cdot}$, $NO^{\cdot}$, ONOOH, O_3, and other reactive oxygen metabolites (ROM) and also radiation and short-wavelength UV light on cells is accompanied by activation of Nrf2 and expression of ARE-regulated genes [3, 8, 22]. Nitric oxide ($NO^{\cdot}$) generated by NO-synthases or produced during decomposition of S-nitroso-N-acetylpenicillamine did not stimulated GSTA2 expression in rat hepatoma cells, but its transformation into peroxynitrite resulted in stimulation of enzyme biosynthesis [22].

Among polyene carotenoids, lycopene and to a lesser extent β-carotene induced NAD(P)H:quinone oxidoreductase (NQO) and γ-glutamylcysteine synthetase (γ-GCS), whereas phytoene and astaxanthin did not demonstrate such property [19]. Analysis of effects of various prostaglandins (PG) revealed that PGJ_2 and its metabolite, 15-deoxy-$\Delta^{12,14}$-PGJ_2 induced synthesis of GSTP1 and heme oxygenase-1 [23]. Uptake of oxidized low-density lipoproteins by peritoneal macrophages and macrophagal RAW 264.7 cell line was accompanied by increased synthesis of mRNAs encoding catalytic and modifying subunits of γ-GCS; oxidized low-density lipoproteins also increased binding of transcription factors Nrf1, Nrf2, and c-Jun with ARE sequences in promoter sites of genes encoding both these subunits [24]. Induction of ARE-regulated genes was also observed in response to ischemia/reperfusion [25], hyperoxia [26], hypoxia (1% O_2) [20], and shear stress [27].

Although ARE activators differ structurally, all of them are electrophilic (and so they are also known as electrophile responsive element) and may modify SH groups in proteins by means of alkylation or oxidation/reduction [10]. Benzene and phenol as well as butylated hydroxytoluene did not demonstrate ARE-inducing activity, and simple di- and triphenols were active but only in the presence of OH groups at *ortho*- (catechol) or *para*-positions (hydroquinone), whereas aromatic compounds with OH groups at *meta*-positions (resorcinol and 1,3,5-trihydroxybenzene) did not cause expression of ARE-regulated genes [16, 28]. Similar dependence was noticed when studying phenylenediamines in which hydroxyl groups were substituted for amino groups: 1,2- and 1,4-phenylenediamines, but not 1,3-diamines induced biosynthesis of ARE-regulated NQO1 [29]. Such dependence of biological activity on location of oxidizable groups suggests that ARE activation occurs due to two-electron oxidation-reduction of Nrf2–Keap1 complex, which may involve polyphenols with *ortho*- and *para*- but not *meta*-hydroxyl groups (Scheme 1).

Oxidation of ascorbic acid (vitamin C) to dehydroascorbate can also involve this vitamin in two-electron redox transformations. In contrast to hydroxylated phenolic acids (sinapic, caffeic, ferulic, protocatechuic) lacking ARE-inducing activity, ascorbic acid stimulates expression of ARE-regulated genes encoding thioredoxin reductase and NQO1 [30]. Since hydroxylated phenolic acids exhibit marked antioxidant effect in various model systems [5], it is clear that the ARE-inducing effect of phenols is not directly related to its antiradical effect. Studies of a large group of plant phenylpropenoids and their synthetic analogs revealed the existence of reverse correlation between NQO1-inducing capacity of the compounds studied and their calculated reductive potential [31].

It is rather difficult to find a relationship between structure of phenols and their activating effect on ARE



Redox conversion of diphenols

Scheme 1

because in cells aromatic compounds are effectively hydroxylated by intracellular monooxygenases, and it is therefore often impossible to discriminate the effect of initial compound and its metabolites. The other difficulty consists in existence of regulatory sites in promoters of many genes for binding of different transcription factors. For example, promoter of the human gene encoding copper-zinc superoxide dismutase contains ARE and XRE elements, which can be independently involved in activation of transcription of this gene [32]. It should also be noted that the existence of an ARE site in a promoter does not necessarily mean that it is functionally active and is involved in regulation of transcription of this gene. For example, promoter of human keratinocyte and melanocyte *GSTP1-1* contains three ARE sites and one ARE-like site, but classic ARE activators (sulforaphane, *tert*-butyl hydroquinone) did not influence gene transcription [33]. In mouse hepatocytes phenolic antioxidants increased synthesis of metallothionein 1; although promoter of the gene encoding this protein contains ARE [34], some evidence exists that regulation of this gene expression involves metal-activated transcription factors [35].

Compounds containing Michael acceptors in their structure (usually a double bond coupled to an electron

Table 1. Main classes of ARE activators

ARE activators	ARE activators
Phenols and quinones	Isothiocyanates
p-Benzoquinone	4-Methylsulfinylbutyl isothiocyanate (sulforaphane)
tert-Butylhydroquinone	6-Methylsulfinylhexyl isothiocyanate
4-*tert*-Butylcatechol	8-Methylsulfinyloctyl isothiocyanate
3,5-di-*tert*-Butylcatechol	4-Methylsulfonylbutyl isothiocyanate
Butylated hydroxyanisole	6-Methylsulfonylhexyl isothiocyanate
Hydroquinone	8-Methylsulfonyloctyl isothiocyanate
Catechol	4-Methylthiobutyl isothiocyanate
Quercetin	6-Methylthiohexyl isothiocyanate
Curcumin	
Probucol	Michael acceptors
1,2,3-Trihydroxybenzene	
Caffeic acid phenylethyl ester	4-Hydroxy-2,3-nonenal
Fisetin	Crotonic aldehyde
Chlorogenic acid	Methylacrylate
Ellagic acid	Methyl vinyl sulfone
Ethoxyquin	
	Aromatic diamines and aminophenols
Peroxides	
	4-Aminophenol
Hydrogen peroxide	1,2-Phenylenediamine
tert-Butyl hydroperoxide	1,4-Phenylenediamine
Cumene hydroperoxide	
	Dithiolthiones
Polycyclic hydrocarbons	
	1,2-Dithiolthione
4′-Bromoflavone	5-(2-Pyrazinyl)-4-methyl-1,2-dithiol-3-thione (oltipraz)
α-Naphthoflavone	5-(*p*-Methoxyphenyl)-1,2-dithiol-3-thione
β-Naphthoflavone	
	Dimercaptans
	(±)-2,3-Dimercapto-1-propanol
	1,2-Ethanedithiol

acceptor group Z: CH_2=CH–Z) exhibited high activity in expression of ARE-regulated genes (Table 1). Many plant aromatic and heterocyclic compounds possess Michael acceptor groups. For example, stimulation of synthesis of the antioxidant enzymes by triterpenoids was determined by the presence of Michael acceptor groups in these molecules [29]. Polyunsaturated aldehydes and ketones formed during lipid peroxidation (LPO) also are Michael acceptors. Hydroxyalkenals including one of the main products of free radical oxidation of arachidonic acid, 4-hydroxy-2,3-nonenal, are important regulators of cell functions during adaptation and development of pathological processes [4, 36]. For example, 4-hydroxy-2,3-nonenal can cause dissociation of the complex of transcription factor Nrf2 and its inhibitor Keap1 directly [37], or act via activation of atypical protein kinase C and other kinases [38, 39]. Induction of γ-GCS in rat epithelial type II cells by 4-hydroxy-2,3-nonenal depended on activity of ERK and p38MAPK kinases [40]. 4-Hydroxy-2,3-nonenal also mediated ARE-activating effect of oxidized lipoproteins [41]. Thus, a wide spectrum of biological effects of 4-hydroxy-2,3-alkenals can be realized via activation of Nrf2 and changes in intracellular environment.

There is an interesting direction of studies of ARE-inducing activity of a large group of phenolic compounds (cinnamates, chalcones, curcuminoids, coumarins, cyclopentanes, and cyclohexanes) containing both hydroxyl groups and Michael acceptors (-CH=CH–CO-) coupled to an aromatic ring. The presence of an OH group at *ortho*-position to the acceptor group significantly increased potency of flavonoids, curcuminoids, and cinnamates to induce NQO1 in mouse hepatoma cells [29]. In the case of cinnamates and coumarins such mutual positioning of reactive groups caused 100-fold increase in the biological activity of such compounds compared with their analogs containing OH groups in *para*- and *meta*-positions. This shows that LPO products exhibiting properties of Michael acceptors during interactions with endogenous phenolic antioxidants, hormones or proteins can form effective ARE regulators.

Cruciferous vegetables (cauliflower, cabbage, broccoli, horseradish, radish, etc.) contain large amounts of isothiocyanates and glucosinolates; glucosinolate hydrolysis is accompanied by formation of sulfur-containing products including isothiocyanates [42]. These compounds are effective in prophylaxis of oncological and cardiovascular diseases. High ARE activating effect of sulforaphane has been demonstrated using various *in vitro* and *in vivo* experimental models [43, 44]. Induction of GST in rat liver epitheliocytes by sulforaphane analogs was influenced by the presence of isothiocyanate group (–N=C=S), and also by the number of methylene groups (n) in the structures CH_3–S–$(CH_2)_n$–N=C=S, CH_3–SO–$(CH_2)_n$–N=C=S, and CH_3–SO_2–$(CH_2)_n$–N=C=S [45]. *In vivo* thiol-containing compounds exhibit complex action, and their effects may be mediated not only by induction of ARE-regulated gene transcription but also by other mechanisms.

ARE-REGULATED GENE EXPRESSION

In mammalian cells, there are several hundred ARE-regulated genes. Oligonucleotide microarray analysis revealed that in human neuroblastoma cells *tert*-butyl hydroquinone influenced expression of 137 genes (1.2% of total number of detectable genes) [46]. Hepatic gene expression profiles examined in 1,2-dithiol-3-thione treated wild-type mice, as well as in *nrf2* single and *keap1-nrf2* double knockout mice revealed 231 ARE-activated genes and 31 genes responding by repression to the treatment with this compound [47]. The system employed for analysis could evaluate 12,400 genes and so the total number of ARE-regulated genes represented about 2%, and this value was similar to *nrf2* single and *keap1-nrf2* double knockout mice. Similar study on the effect of ARE activator sulforaphane in small intestine of wild type and *nrf2* knockout mice revealed 77 Nrf2-regulated genes (1.3% of 6000 genes analyzed) [48]. Under hyperoxic conditions (>95% O_2), transcription increased in 175 genes and decreased in 100 genes; the hyperoxia-induced up-regulation of gene expression was more pronounced in wild type than in *nrf2* knockout mice [49]. Thus, a significant part of the mouse genome was sensitive to ARE-mediated regulation.

Among many protein products of genes regulated by ARE, there are two groups of enzymes involved in maintenance of redox balance and the increase in cell antioxidant defense and also detoxification of electrophilic xenobiotics and their elimination from cells (Table 2). Phase II biotransformation enzymes (GST, UDP-glucuronosyl transferase, NQO-1, heme oxygenase-1, etc.) are involved in inactivation and removal of toxic endogenous oxidation products (hydroperoxides, quinones, hemes) and xenobiotics. Most of these enzymes exhibit certain antioxidant properties and the increase in their synthesis under conditions of oxidative stress exhibits protective action on cells.

Easily oxidizable proteins and peptides containing cysteine and methionine residues play an important role in the antioxidant defense and maintenance of redox balance. Glutathione concentrations in the millimolar range are the highest ones in eukaryotic cells and so this tripeptide is often considered as the major water-soluble antioxidant [4]. In animals and in man glutathione exists in the oxidized (GSSG, 10% of total) and reduced (GSH) form. Under conditions of oxidative stress, increased content of GSH protects cell structures and proteins and also increases inactivation of hydroperoxides and other toxic oxidation products. Mammalian cell membranes are poorly permeable for glutathione and so it is synthesized

Table 2. Proteins that are expressed in ARE-driven manner

Xenobiotic detoxification enzymes	Antioxidants
Mouse GSTA1	Mouse and human heme oxygenase-1
Mouse, rat, and human GSTA2	Light and heavy chains of mouse and human γ-GCS
Mouse GSTA4	Heavy chain of human γ-GCS
Rat and human GSTA5	Human glutathione peroxidase 2
Mouse GSTM1	Mouse and human glutathione reductase
Mouse GSTM2	Human thromboxane A_2 synthase
Human and mouse GSTM3	H- and L-subunits of ferritin
Mouse GSTM4	Mouse and rat metallothionein-1
Rat GSTP1	Rat metallothionein-2
Mouse GSTT2	Rat inducible NO synthase
Mouse and human NQO1	Mouse, bovine, and human thioredoxin
Human NRH:quinone oxidoreductase-2	Bovine and human thioredoxin reductase
Glucuronosyltransferase-1a6	Mouse and human peroxiredoxin 1
	Human superoxide dismutase 1
	Mouse superoxide dismutase 3

endogenously. Glutathione synthesis involves two enzymes, γ-GCS and glutathione synthase. Formation of γ-glutamyl cysteine from corresponding acids catalyzed by γ-GCS is the rate limiting and controllable step [50]; this reaction depends on the presence of L-cysteine and its oxidation to L-cystine is negatively regulated by reduced glutathione.

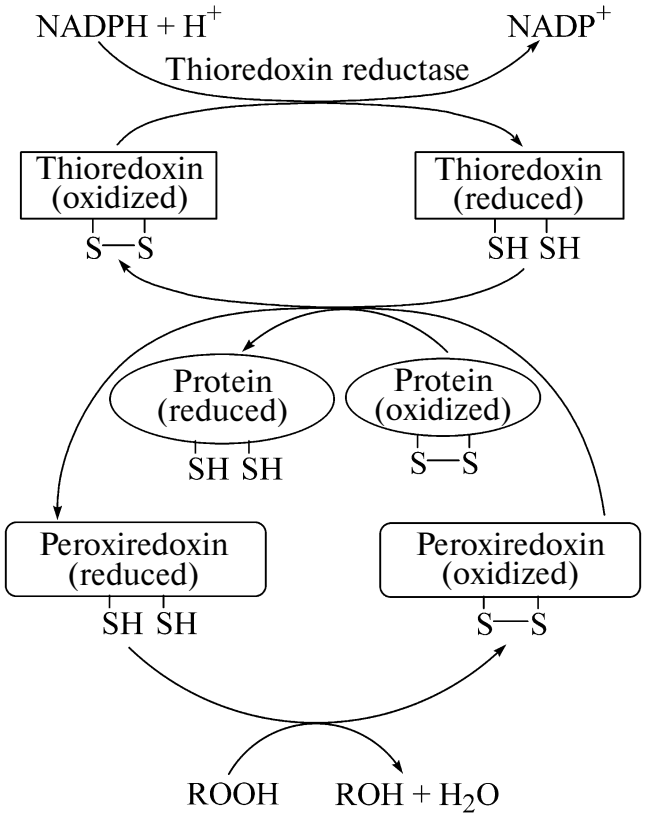
Mammalian γ-GCS is a heterodimer that consists of catalytic heavy chain and regulatory light chain. In humans, the molecular masses of heavy and light chains are 73 and 28 kD, respectively. All catalytic activity as well as feedback regulation by glutathione is associated with the heavy subunit. Under physiological conditions, the light subunit determines kinetic properties possibly due to formation of redox sensitive disulfide bond between subunits. ARE regulates synthesis of both γ-GCS subunits in wild type mice; in *nrf2* knockout animals γ-GCS expression is greatly decreased [51, 52]. The promoter of the gene encoding the heavy subunit of human γ-GCS includes four ARE sites; two of them are critical for synthesis of corresponding mRNA in hepatoma HepG2 cells [50, 53]. In contrast to heavy subunit, no evidence exists for ARE involvement in regulation of transcription of light chain gene in human hepatocarcinoma cells [54]. Besides ARE sites, promoters of mouse and human genes encoding heavy chain of γ-GCS contain XRE- and metal-sensitive elements as well as domains binding transcription factors NF-κB, AP-1, and AP-2, and so synthesis of this enzyme (and consequently synthesis of glutathione) can be increased by various treatments [50, 53]. For example, the effect of curcumin may be realized by combined AP-1 induction and ARE activation [53]. Although the promoter site of the gene encoding catalytic subunit of γ-GCS lacks ARE, synthesis of this protein depends on transcription factors Nrf1 and Nrf2, which may act by increasing expression of the other factors, AP-1 and NF-κB [55].

The major antioxidant effect of glutathione is realized by its involvement in reactions catalyzed by the antioxidant enzymes. Being the substrate for glutathione peroxidases, glutathione is a hydrogen donor for reduction of H_2O_2 and lipoperoxides. Biological regeneration of oxidized glutathione involves glutathione reductase and NADPH. Among seven known isoforms of glutathione peroxidases, ARE regulates synthesis of selenium glutathione peroxidase 2, which can reduce hydrogen peroxide and fatty acid hydroperoxides but not phospholipid hydroperoxides [56]. In rats, this enzyme is mainly localized in the gastrointestinal tract, where it serves as a barrier for ingested hydroperoxides. ARE is also involved in regulation of glutathione reductase reducing oxidized glutathione [57]. In DYR21 cells from kidneys of Syrian hamster, ARE regulates synthesis of xCT (cystine/glutamate transporter), which is crucial for glutathione synthesis [58]. Thus, ARE controls key elements of synthesis and metabolism of glutathione.

Besides glutathione, thioredoxins, peroxiredoxins, and glutaredoxins play an important role in reduction of disulfide bonds of proteins and maintenance of redox balance in cells. The family of mammalian thioredoxins includes more than 10 low molecular weight proteins containing in their structure active sites with two cysteine

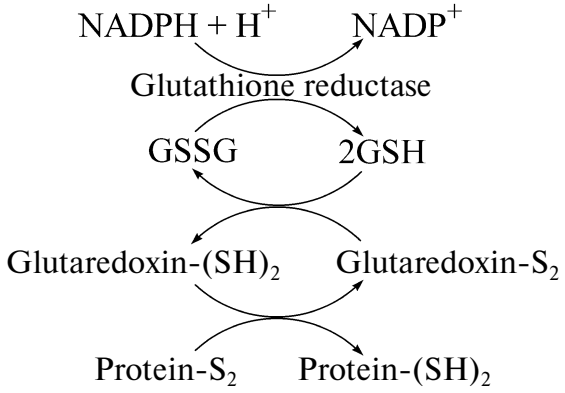
residues (usually -Cys-Gly-Pro-Cys-); they exhibit oxidoreductase activity. Thioredoxins can reduce intra- and intermolecular disulfide bonds in proteins including peroxiredoxins; oxidized thioredoxin then undergoes NADPH dependent reduction catalyzed by thioredoxin reductase (Scheme 2). Active sites of major glutaredoxins (thiol transferases) also contain two cysteine residues (usually as -Cys-Pro-Tyr-Cys-), and so they are often pooled in one group. However, in contrast to thioredoxins reduced by specialized enzymes, thioredoxin reductases, glutaredoxins are reduced by glutathione and employing the glutathione system, they act as hydrogen carriers from NADPH to oxidized proteins (Scheme 3).

In erythroleukemic K562 cells hemin and *tert*-butyl hydroquinone induced expression of thioredoxin gene mediated by ARE activation [59]. Stimulation of thioredoxin synthesis in mouse retinal cells by sulforaphane was also accompanied by ARE activation and the decrease in toxic effect of light [44]. It should be noted that thioredoxin is regulated by ARE and the latter is influenced by thioredoxin by increased binding of Nrf2-transcription factor with its promoter [60]. It is suggested that such mechanism of self-potentiation of thioredoxin action provides faster cell response to oxidative treatments and effective maintenance of redox balance. Among three isoforms of thioredoxin reductases identified in mammalian cells, only biosynthesis of cytoplasmic selenium containing thioredoxin reductase 1 is regulated at the transcription level by ARE. Treatment of human and mouse hepatoma cells (Hepa1c1c7 and Hep2) with classic ARE activators (sulforaphane, *tert*-butylhydroquinone, and β-naphthoflavone) was accompanied by increased synthesis of mRNA of this thioredoxin reductase as well as an increase in its catalytic activity [61]. In bovine vascular endothelial cells cadmium ions, diethyl maleate, and arsenite induced synthesis of thioredoxin and thioredoxin reductase 1 by stimulation of ARE as Nrf-2 regulatory element [62].



Redox conversions of thio- and peroxiredoxins

Scheme 2



Glutaredoxin-dependent chain of proton transfer

Scheme 3

ARE is the key element in the regulation of transcription of the peroxiredoxin-1 gene. In mouse peritoneal macrophages various ARE activators (paraquat, H_2O_2, *tert*-butylhydroquinone, $CdCl_2$, etc.) increased synthesis of peroxiredoxin-1; this was accompanied by translocation of Nrf2 into the nucleus; in cells isolated from *nrf2*-knockout animals such translocation phenomenon was not detected [41]. Treatment of murine peritoneal macrophages and aortic smooth muscle cells with oxidized low-density lipoproteins and with 4-hydroxy-2,3-nonenal caused simultaneous increase in the stress proteins A170, heme oxygenase-1, and peroxiredoxin-1 [41]. However, studies on rat hepatic macrophages and monocyte derived RAW264.7 cells revealed that induction of peroxiredoxin-1 by phorbol ester depended on protein kinase C, Ras, MEKK1, and p38 kinases but it did not depend on Nrf2 expression [63].

Quinone oxidoreductases (NQO1 and NRH: quinone oxidoreductase-2) are flavoproteins that catalyze quinone reduction by hydrogen transfer from NAD(P)H or dihydronicotinamide riboside (NRH). Human NQO1, initially described as DT-diaphorase, is a homodimer of 54 kD; this enzyme catalyzes two- or four-electron reduction of a wide spectrum of endogenous and exogenous quinones, quinone imines, and some other nitrogenous compounds [64]. The main antioxidant effect of

NQO1 is realized by inhibiting cyclic redox transformations of quinones accompanied by ROM generation. The enzyme maintains ubiquinone (coenzyme Q) and tocopherylquinone (oxidized vitamin E) in the reduced state; this effect is especially important for structural integrity of mitochondria under conditions of impaired functioning of the electron transport chain. Irrespectively to catalytic activity, NQO1 can bind to chaperones Hsp70 and Hsp40 and also to oncosuppressor p53 and stabilize this suppressor in the cytoplasm. Coumarins (dicumarol and curcumin) and some flavones inhibit NQO1, increase p53 degradation, and therefore decrease p53-induced apoptosis in thymocytes and myeloid leukemic cells [64].

The human NQO1 gene has been localized to chromosome 16 (16q2.2); it is characterized by high polymorphism. Homozygous and heterozygous mutants have been found in all ethnic groups. The homozygous null genotype is carried by 4% of Caucasians, 5% African Americans, 16% of Mexican Americans, and 22% of Asians [65]. These people were characterized by twofold increase in incidences of stomach cancer and also by increased risk of the development of myeloid leucosis, lung and urothelial tumors, cutaneous basal cell carcinoma, colon cancer, and esophageal squamous cell carcinoma [64]. The existence of functionally active ARE sites determining basal and inducible (by various electrophilic compounds) level of enzymes has been demonstrated for promoter sites of mouse, rat, and human NQO1 genes [57, 66]. Besides ARE promoters of NQO1 genes contain XRE, TRE, and AP-2 binding sites, and so the enzyme is induced by many xenobiotics and other treatments [64, 67]. ARE, XRE, and SP1 sites have also been found in the regulatory site of the NRH:quinone oxidoreductase-2 gene [68].

Microsomal heme oxygenase-1 catalyzes the rate-limiting step in heme catabolism; this enzyme catalyzes the conversion of heme into biliverdin-IXa followed by release of the iron atom and CO. The necessity of such cleavage in cells can be attributed to the fact that at concentrations exceeding 1 μM protein unbound heme becomes an effective generator of ROM [69]. Biliverdin formed during cleavage of the heme moiety is rapidly converted by biliverdin reductase into bilirubin. The latter has effective antiradical activity against superoxide and peroxyl radicals. Thus, heme oxygenases may be referred to the phase II xenobiotic transformation enzymes (as they effectively remove reactive heme complexes) and also to antioxidant enzymes [70]. In mammalian cells, three isoforms of heme oxygenase have been characterized. These include constitutive heme oxygenase-2 (the major form under physiological conditions), inducible heme oxygenase-1 (classified as heat shock protein Hsp32), and less studied heme oxygenase-3. (The latter was found only in rats.) Transcription of heme oxygenase-1 is regulated by ARE and is also stimulated by various prooxidant and inflammatory stimuli: H_2O_2, UV light, heme and transition metal ions, dopamine, prostaglandins, bacterial polysaccharides, and T-helper cytokines [18, 21, 71].

In contrast to other ARE regulated enzymes, heme oxygenase-1 is characterized by the highest stimulation index in response to activators. This can be attributed to the fact that under normal conditions the basal level of enzyme production is inhibited by transcription factor Bach1. The latter as well as Nrf2 belong to the family of leucine zipper transcription factors; it forms heterodimers with additional Maf-proteins [72]. It is suggested that in cells free heme regulates synthesis of heme oxygenase-1 by Bach1 derepression [69]. Similarly to Nrf2, transcription factor Bach1 is detected in cytoplasm; *tert*-butylhydroquinone caused rapid translocation of both proteins into the nucleus, but translocation of Nrf2 occurs faster and so initial increase in synthesis of ARE-regulated enzymes was followed by subsequent reduction to the normal level [73]. Although the inhibitory effect of Bach1 was also found for other ARE-regulated genes (e.g., NQO1), its manifestation was significantly weaker [73]. In cultures of various human cells, hypoxia, interferon-γ, and iron ion chelator desferrioxamine stimulated expression of Bach1 and decreased production of heme oxygenase-1, whereas $CoCl_2$ caused the opposite effect [74]. Cd^{2+} ions inhibited Bach1 translocation into the nucleus and induced heme oxygenase-1 mRNA transcription [75].

Mammalian glutathione S-transferases are the large group of enzymes that includes seven cytosolic families (α, μ, π, σ, θ, ω, ζ), the mitochondrial family κ, and also four microsomal families involved in biosynthesis of eicosanoids. In human tissues, there are four main cytosolic classes of GST (α, μ, π, and θ). They are dimers, which consist of subunits with molecular masses of 22 to 26 kD. In liver, kidneys, and ovaries GST class α dominates, whereas GST class π is the major form in lungs, muscles, brain, pancreas, erythrocytes, and skin. GSTs exhibit board substrate specificity; these enzymes metabolize many endogenous and exogenous electrophilic compounds by conjugating them with glutathione; this increases their water solubility and thus facilitates removal of the glutathione conjugates from cells. Members of mouse and human GST class α family exhibit glutathione peroxidase activity, but in contrast to classic Se-containing glutathione peroxidases GSTA1-1 and GSTA2-2 reduce fatty acid and phospholipid hydroperoxides, but do not interact with H_2O_2 [36, 76]. In human liver and ovary cells, GSTA1-1 and GSTA2-2 reduce more than 50% of fatty acid and phospholipid hydroperoxides [36]. Thus, GSTs are important components of antioxidant defense against endogenous reactive metabolites formed during oxidative stress [76]. Redox-dependent protein–protein interactions involve GST in cell regulation. For example, GSTP1 binds to JNK protein kinase, and H_2O_2 causes dissociation of this complex and activation of JNK [13].

The first experimental evidence for the existence of ARE came from the study of GST-Ya expression in rats [15]. LPO products (4-hydroxy-2,3-nonenal, *trans*-2-hexenal, 2-propenal, and ethacrynic acid) caused induction of GSTA1-1 and GSTA4-4 synthesis in rat hepatoma cells [77]. Promoter of mouse *GSTP1* contains three ARE sequences and at least seven androgen receptor binding sites [78] and so basal level of *GSTP1* expression in male liver is about 10 times higher than in the female, and significant reduction of its expression after castration is attributed to altered level of androgens [79]. Study of the effect of butylated hydroxyanisole on mRNA of various classes of GSTs revealed higher augmentation of the rate of GST mRNA synthesis in the liver of female mice than in males [80]. *nrf2*-Knockout mice had lower basal level of liver GST classes α and μ than wild type animals, expression of π-GST was the same in both groups of mice but induction of these GSTs by xenobiotics was significantly lower in the mutants [81, 82]. In rat liver, basal expression of *GSTP1* was lower than in mice, but stimulation index in response to inducers was higher in rats. ARE activators did not increase *GSTP1* transcription in humans; the latter might be explained by inhibitory effect of the other transcription factor, NF-κB [33].

ARE also regulate expression of genes encoding metallothioneins. Low molecular weight (6-7 kD) metallothioneins located in cytosol and nucleus of eukaryotic cells are cysteine-rich proteins (cysteine constitutes more than 30% of all amino acid residues). They exhibit high chelation capacity with respect to heavy metal ions (Cu, Zn, Cd, Hg, etc.). Each metallothionein molecule can bind up to seven metal ions. Metallothioneins are involved in regulation of homeostasis of Cu and Zn ions; in the case of some intoxications, they are responsible for removal of heavy metal atoms (Cd, Hg, Pb). High content of SH groups also underlines metallothionein capacity as effective radical inhibitors [83]. Metallothionein expressed in molar concentration was 340 times more effective inhibitor of OH radicals, 800 times more potent inhibitor of $OH^{\cdot}$-induced DNA damage, and 10 times more effective inhibitor of microsomal LPO than glutathione. In vertebrates promoters of metallothionein genes contain metal- and glucocorticoid-responsive elements, and also binding sites for transcription factors Sp, AP-1, and Nrf2 [83]. In low nontoxic concentrations (<20 μM) sulforaphane stimulated synthesis of metallothioneins MT-1 and MT-2 in human hepatoma HepG2 cells, and this induction was accompanied by an increase in Nrf2 content in cell nuclei [84].

MECHANISMS OF ARE ACTIVATION

A DNA site containing the nucleotide sequence 5′-$A^{/G}TGAC^{/T}$nnn$GCA^{/G}$-3′ is the *cis*-activating ARE element [85]. This site binds nuclear transcription factor Nrf2, which belongs to the NF-E2 family. Four members of this family, p45, Nrf1, Nrf2, and Nrf3, have originally been described as the factors activating NF-E2/AP-1 motif located in the promoter of β-globin gene (and determining its expression). The Nrf2 DNA binding site represents bZip domain, which is responsible for dimerization with other proteins and interaction with DNA regulatory site. Structural features of Nrf2 bZip domain do not allow forming homodimers and so Nrf2 binding to DNA requires additional protein partners containing a suitable bZip domain. These include members of so-called small Maf-protein family: MafF, MafG, and MafK [11].

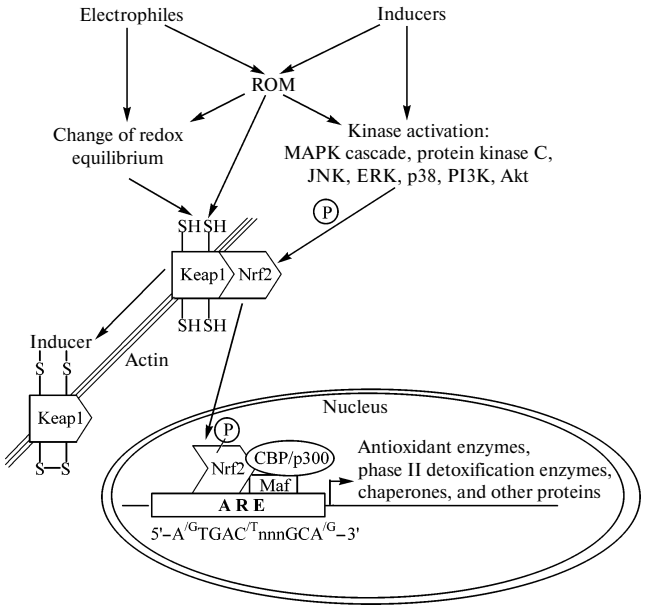
The ARE regulatory site shares similarity with AP-1 (5′-TGACTCA) and so activation of transcription of ARE-regulated genes may also involve Jun-proteins (c-Jun, Jun-B, Jun-D) and also c-Fos [86]. Other factors (e.g., ATF4, Fra1, YABP, ARE-BP1, Ah, hMAF) can interact with ARE promoter site and stimulate or inhibit transcription of ARE-regulated genes [87, 88]. Promoters of some genes (e.g., human *NQO1*) contain nucleotide sequences 5′-TGACTCAGCA-3′ identical to TRE (5′-TGAG$^{/C}$TCA-3′) and ARE and so expression of such genes by xenobiotics may be realized via different mechanisms [67].

According to the generally accepted viewpoint, transcription factor Nrf2 translocated into the nucleus forms dimers with Maf- or Jun-proteins, which bind to ARE and induce transcription of corresponding genes [11]. There is experimental evidence that all factors of NF-E2 family (p45, Nrf1, Nrf2, and Nrf3) can form regulatory active dimers; however, knockout or overproduction of each of them causes development of different effects in cells and the whole organisms [14, 89]. Nrf1 can also bind to Jun-proteins (c-Jun, Jun-B, and Jun-D) or Maf-proteins (MafG and MafK) and thus activate transcription of genes encoding phase II xenobiotic detoxification enzymes [86]. However, Nrf1 is significantly less active in stimulation of ARE-regulated gene expression than Nrf2 [13]. Increase in Nrf3 content in HepG2 decreased basal and *tert*-butylhydroquinone-induced NQO1 activity; the latter suggests that Nrf3 causes a negative effect on ARE-regulated genes [89]. The complexes c-Jun–c-Fos or c-Jun–Fra1 competed with Nrf2 for binding with ARE regulatory site and inhibited transcription of ARE-regulated gene expression [88]. It is suggested that small Maf proteins form homodimers that can inhibit the inducing effect of Nrf2 by competing with ARE [90]. Such interrelationships become even more complex due to feedback regulation: for example, the gene encoding MafG has the ARE-active site and so dimer complexes MafG–Nrf2 increase *mafG* transcription [91].

Human Nrf2 is a protein of 67 kD; it consists of 605 amino acid residues that form six highly conservative domains: Neh1-Neh6 [92]. Neh1 is a bZip domain; Neh2 is the domain responsible for negative regulation of Nrf2

functioning via binding with Keap1 (cytoskeleton actin binding protein); the domains Neh4 and Neh5 mediate transactivating effect of Nrf2 (they bind to the transcription coactivator CBP/p300) [93]. The complex CBP/p300 exhibits histone acetyl transferase activity; it also attracts additional histone acetyl transferases. CBP/p300 can also interact with components of RNA polymerase II complex, thus increasing enzyme concentration in the transcriptionally active zone. Although Nrf2 binds CBP/p300 using two trans-activating domains, each of them can bind the trans-activator separately. Their simultaneous presence in the Nrf2 structure causes mutual augmentation of CBP/p300 interaction, i.e., binding exhibits a cooperative mode.

Antioxidants and xenobiotics do not influence transcription of *nrf2*; this suggests posttranslational regulation of Nrf2 activity [41]. Under normal conditions, the nuclear content of Nrf2 is rather small and its major proportion is bound to Keap1 on nuclear membrane or cytoskeleton [92]. It is suggested that Keap1 operates as a shuttle protein between nucleus and cytoplasm [94]. Mouse Keap1 is a protein of 69.5 kD. It contains 624 amino acid residues, including 25 cysteine residues, which possibly act as sensors for many compounds inducing dissociation of Nrf2–Keap1 complex [95]. The Keap1 IVR domain, which anchors Nrf proteins, is particularly rich in cysteine residues (8 of 102 amino acids constituting this domain). Four cysteine residues (Cys257, Cys273, Cys288, and Cys297) sensitive to oxidative modification can form disulfide bonds [10]. In human Keap1, there are two additional cysteine residues; their oxidation caused dissociation of the Nrf2–Keap1 complex [96]. Mouse Nrf2 (66.9 kD), which consists of 597 amino acid residues, also contains seven cysteine residues; they are more resistant to oxidation and have minor influence (if any) on dissociation of the Nrf2–Keap1 complex [60]. It has recently been demonstrated that the Nrf2 binding site of Keap1 can contain zinc atoms. Recombinant mouse Keap1 contained 0.9 Zn atom per subunit. The Zn^{2+} ions interacted with active sulfur atoms of cysteine residues; removal of Zn^{2+} ions was accompanied by dissociation of Nrf2–Keap1 complexes [97]. Thus, Nrf2–Keap1 complex is a redox-sensitive metalloprotein; its activity depends on the ratio of oxidized and reduced thiols (Scheme 4).



Putative mechanisms of dissociation of the Nrf2–Keap1 complex and activation of transcription of ARE regulated genes

Scheme 4

The half-lifetime of free Nrf2 in cells is rather short: $T_{1/2} = 13$ and 15 min in rat hepatoma Hepa-cells [98] and human HepG2 cells [99], respectively. So induction of ARE regulated gene expression mainly depends on release and stability of the transcription factor Nrf2. Inhibition of proteasome activity is accompanied by the increase in nuclear concentration of Nrf2 and its stimulatory action [100]. Natural low molecular weight thiols (N-acetyl-L-cysteine and glutathione), ascorbic acid, and also α-tocopherol inhibit dissociation of Nrf2–Keap1 complex and decrease ARE regulated expression [101]. The nuclear oncoprotein prothymosin α competes with Nrf2 for binding to Keap1 and therefore increases dissociation of the complex [94].

Numerous studies have demonstrated the involvement of various protein kinases (JNK1, MEKK1, TAK1, ASK1, ERK2, PERK, p38, PI3K) in regulation of ARE-regulated genes [21, 102-104]. It is suggested that kinases influence the interaction of transcription factor Nrf2 with its cytoplasmic protein inhibitor, Keap1. Protein kinase C is one of the major kinases involved in ARE regulation; the enzyme phosphorylates a serine residue (Ser40) in the Neh2 domain of Nrf2 accompanied by dissociation of its complex with Keap1 [105]. Ser40 phosphorylation did not influence Nrf2 transport into the nucleus and its binding to a DNA regulatory site but did increase stability of Nrf2 and its half-lifetime in cells [99]. Protein kinase C inhibitors (calphostin C and staurosporin) decreased *tert*-butylhydroquinone-induced NQO1 synthesis in HepG2 [105]. Under conditions of oxidative stress, 4-hydroxy-2,3-nonenal and phorone (2,6-dimethyl-2,5-heptadien-4-one) activated atypical protein kinase C; this derepressed Nrf2 and stimulated heme oxygenase-1 synthesis [39]. Induction of heme oxygenase-1, thioredoxin, and peroxiredoxin-1 by hemin in human neuroblastoma (SH-SY5Y) cells depended on PI3K activity [21]. Protein kinase C and PI3K may activate Nrf2 *in vivo* and increase GSTA2 synthesis in rat hepatocytes by regulating peroxynitrite formation [22]. A wide-spread plant phenol, curcumin, increased heme oxygenase-1 in epithelial cells by activating p38 kinase [106]. Inhibition of ERK

and p38 kinases reduced induction of heavy and light chains of γ-GCS in HepG2 cells [51]. However, in human bronchial epithelial cells simulation of synthesis of this enzyme by 4-hydroxy-2,3-nonenal involved activation of AP-1 and JNK-kinases, but it did not depend on ERK and p38 kinases [107]. In human tumor cells (HCT116) expression of AP-1 and induction of apoptosis by curcumin depended on JNK activation rather than ERK and p38 kinases [108].

Protein kinase activation of Nrf2 involves the following pathway. In cytoplasm, Nrf2 rapidly undergoes ubiquitin-dependent degradation, which includes ubiquitination of the Neh6 domain. Phosphorylation of Nrf2 causes simultaneous increase in its free concentration (due to Nrf2 release from the complex with Keap1) and stabilization (due to inhibition of ubiquitination) [99, 102]. This results in Nrf2 accumulation in the nucleus, whereas intracellular localization of Keap1 remains unchanged. It should be noted that although Nrf2 undergoes ubiquitination under conditions of oxidative stress, its half-lifetime is significantly longer. Besides protein kinase activity, derepression of Nrf2 critically depended on the cysteine residue in the BTB domain of Keap1 (Cys151); point mutation of this cysteine residue made Keap1 a constitutive repressor of Nrf2. Under conditions of oxidative stress, this residue covalently binds many high molecular weight compounds, forming Keap1 modifications that are resistant to various reducing agents.

PHYSIOLOGICAL IMPORTANCE OF ARE

The importance of Nrf proteins for living organisms has been convincingly demonstrated in experiments with *nrf*-knockout mice. Homozygous *nrf1* knockout animals died during the initial period (2-3 weeks) of intrauterine development due to impairments of hemopoiesis and development of anemia [109]. Although *nrf2*-knockout mice did not have impairments in development and reproduction compared with wild type animals [110], aged animals were characterized by development of anemia (possibly due to decreased erythrocyte resistance to oxidative damages [111]) and autoimmune glomerulonephritis [112]. In the liver and fibroblasts of *nrf2*$^{-/-}$ mice there was significant reduction of γ-GCS activity and glutathione synthesis [113]. Such animals were also characterized by decreased activity of NQO1 (both basal and stimulated by phenolic compounds or natural isothiocyanates), microsomal epoxide hydrolase, and GST (classes α and μ) and also intracellular levels of mRNAs of these enzymes [13, 80]. Some studies revealed significant decrease of enzyme (GSTP1 and NQO1) induction by xenobiotics with minor change of basal GSTP1 activity [81, 82]. In spleen homogenates of *nrf2*-knockout mice, the decreased expression of genes encoding NQO1, heme oxygenase-1, GSTA4, thioredoxin reductase-1, and γ-GCS was found [111]. Thus, Nrf2 is involved not only in induction but also in maintenance of basal transcription level of some of its target genes. This possibly explains higher sensitivity of *nfr2*$^{-/-}$ mice to hepatotoxic effect of acetaminophen [52, 114] and lung damages induced by bleomycin, butylated hydroxytoluene, and hyperoxia [26, 115].

Myocardial fibroblasts obtained from *nrf2*$^{-/-}$ mice were sensitive to toxic effect of reactive oxygen and nitrogen species [116], whereas T-lymphocytes exhibited higher sensitivity to Fas-receptor mediated apoptosis [9]. Inflammatory reaction in response to carrageenan administration was also more pronounced in these animals; in lungs, it was accompanied by increased infiltration with albumin and neutrophils [23, 117]. Long term treatment with tobacco smoke caused changes typical for lung emphysema, which was more pronounced in knockout mice [57]. In *nrf2*$^{-/-}$ mice antibodies against Fas-receptors and tumor necrosis factor α caused more pronounced hepatocyte damage than in wild type animals [9]. The development of gastrointestinal tract tumors induced by the treatment with benzo[α]pyrene was 50% higher in *nrf2*-knockout than in corresponding control, the dithiolethione analog oltipraz exhibited pronounced anti-carcinogenic effect (causing 2-fold reduction of number of tumors) in wild type but not in the knockout mice [12]. Studies on *nrf2*-knockout animals have clearly demonstrated the importance of ARE in inflammation, carcinogenesis, fibrosis, and also protection against various stress exposures [14, 23, 115].

ARE is now considered as an important regulator of cellular redox balance and inducer of protective mechanisms under conditions of oxidative stress. Regulation of numerous cellular processes requires constant ratio of oxidized and reduced SH group level (redox balance). The universal role of ARE consists in its ability to respond to effects of both ROM and antioxidants including SH-containing compounds, and this is important for maintenance of their balance. Some protein products of ARE-regulated genes play direct protective role, others are involved in replenishment of low molecular weight intermediates or repair functions of vitally important biological macromolecules under conditions of impaired cell redox balance. So the transcription factor Nrf2 and the network of ARE-regulated genes (sensitive to Nrf2 regulation) may be considered as a universal system of cell defense under conditions of oxidative stress. Using ARE as an example, one can see that such defense is rather complex and it is not limited by simple increase in antioxidant level or removal of toxic compounds formed during processes of free radical oxidation. Usually destructive effects of oxidative stress do not have strictly determined targets; these can include membrane, nucleic acids, and enzymes. So, protection against such treatments should involve various organizational levels and structures, which often do not exhibit direct antioxidant effect. This

raises reasonable questions of whether such system is universal and whether various cell types have specific defensive elements [118]. If this defense system changes during cell differentiation, we can suggest that this system also exhibits age-related dependence and determines individual longevity of life.

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