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REVIEW

Lability of the Nrf2/Keap/ARE Cell Defense System in Different Models of Cell Aging and Age-Related Pathologies

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Abstract—The level of oxidative stress in an organism increases with age. Accumulation of damages resulting in the disruption of genome integrity can be the cause of many age-related diseases and appearance of phenotypic and physiological signs of aging. In this regard, the Nrf2 system, which regulates expression of numerous enzymes responsible for the antioxidant defense and detoxification, is of great interest. This review summarizes and analyzes the data on the age-related changes in the Nrf2 system *in vivo* and *in vitro* in various organs and tissues. Analysis of published data suggests that the capacity for Nrf2 activation (triggered by the increased level of oxidative stress) steadily declines with age. At the same time, changes in the Nrf2 activity under the stress-free conditions do not have such unambiguous directionality; in many studies, these changes were statistically insignificant, although it is commonly accepted that the level of oxidative stress steadily increases with aging. This review examines the role of cell regulatory systems limiting the ability of Nrf2 to respond to oxidative stress. Senescent cells are extremely susceptible to the oxidative damage due to the impaired Nrf2 signaling. Activation of the Nrf2 pathway is a promising target for new pharmacological or genetic therapeutic strategies. Suppressors of the Nrf2 expression, such as Keap1, GSK3, c-Myc, and Bach1, may contribute to the age-related impairments in the induction of Nrf2-regulated antioxidant genes. Understanding the mechanisms of regulatory cascades linking the programs responsible for the maintenance of homeostasis and cell response to the oxidative stress will contribute to the elucidation of molecular mechanisms underlying aging and longevity.

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Keywords: Nrf2, Keap1, aging, lifespan, oxidative stress, age-related disorders, antioxidants

INTRODUCTION

Lifespan is a stable species-specific parameter similar to body size or fecundity. Its duration, as well as underlying mechanisms, should be at least partially programmed in the genome [1-3], despite that from the evolutionary point of view, the primary objective of living creatures is adaptation to the environment, including survival, food availability, and propagation, rather than longevity *per se*. Aging and anti-aging programs, currently envisioned as a set of gene-related signaling cascades, belong to the internal cues that determine the lifespan, as well as the pace and shape of aging [4]. Regulation of homeostasis and repair processes involves a multilevel network of interconnected reactions, the efficiency of which decreases with age [1-6]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor participating in the maintenance of cell redox balance and signal transduction. Nrf2 plays a central role in the reduction of the intracellular oxidative stress, slowing of cell aging, and prevention of age-related diseases [4-9].

Among other things, Nrf2 is a major regulator of cell homeostasis that controls the maintenance of genome stability. It regulates more than 1% human genes participating in biotransformation, redox homeostasis, energy metabolism, DNA repair, and proteostasis, as well as

Abbreviations: ARE, antioxidant response element; Bach1, BTB domain and CNC homolog 1; Gcl, glutamate-cysteine ligase; Gclc, glutamate-cysteine ligase catalytic subunit; Gclm, glutamate-cysteine ligase modifier subunit; GSK3, glycogen synthase kinase 3; Ho-1, heme oxygenase 1; Keap1, Kelch-like ECH-associated protein 1; Nqo1, NAD(P)H:quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; NSPC, neural stem progenitor cell; PDL, population doubling level; ROS, reactive oxygen species; RPE, retinal pigment epithelium; VSMC, vascular smooth muscle cell.

transcription of antioxidant and detoxifying enzymes that collectively comprise a powerful cell defense system [4, 5]. A common feature of such genes is the presence of antioxidant response elements (AREs) in their promoters. Nrf2 strongly affects multiple physiological and pathological events; therefore, its activity is tightly regulated, mainly via control of the protein stability. Nrf2 is activated by oxidative stressors and electrophilic agents. The products of Nrf2-targeted genes regulate multiple defense functions, including drug detoxification, pentose phosphate pathway, and autophagy [7]. In addition, Nrf2 regulates production of glutathione and enzymes of glutathione homeostasis, in particular, glutamate-cysteine ligase catalytic (Gclc) and modifier (Gclm) subunits that together form the glutamate-cysteine ligase (Gcl) heterodimer. Nrf2 also mediates induction of some other antioxidant proteins [e.g., thioredoxin, peroxiredoxin, sulfiredoxin, ferritin, metallothionein, and heme oxygenase-1 (Ho-1)] and phase I and phase II drug-metabolizing enzymes [e.g., aldo-keto reductase, glutathione S-transferase and NAD(P)H:quinone oxidoreductase 1 (Nqo1)] [10]. Moreover, Nrf2 directly inhibits expression of proinflammatory cytokine genes by binding to their proximal regulatory regions [11]. Altogether, it makes Nrf2 an element of the unique anti-aging program and a guardian of healthspan and longevity [5]. According to Skulachev et al. [4], Nrf2 is a component of one of the most robust cell anti-aging programs (Fig. 1).

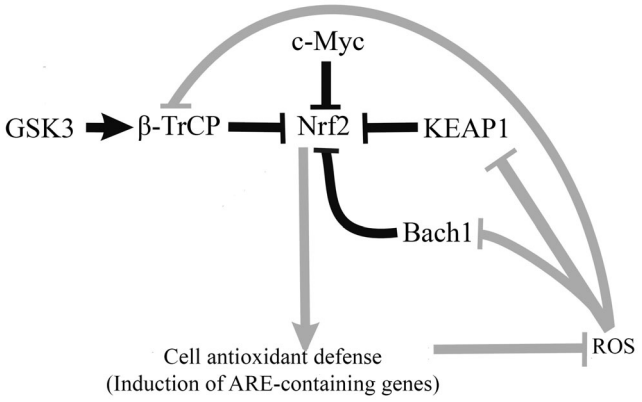

Fig. 1. Regulation of Nrf2 transcription factor that controls expression of more than 200 cytoprotective enzymes responsible for detoxification and antioxidant defense. Nrf2 activation is caused by reactive oxygen species (ROS). Glycogen synthase kinase 3 (GSK3) activated via multiple signaling cascades, acts a suppressor by inhibiting Nrf2. Gray lines, Nrf2 stimulation (and subsequent expression of antioxidant enzymes); black lines, Nrf2 inhibition; arrows, direct stimulation, including catalysis; lines with blunt ends, inhibition. Designations: ROS, reactive oxygen species; Bach1, BTB domain and CNC homolog 1; GSK3, glycogen synthase kinase 3; Keap1, Kelch-like ECH-associated protein 1; Nrf2, NFE2-related factor 2 (NFE family transcription factor 2); β-TrCP, β-transducin repeat containing protein.

The involvement of the Nrf2/Keap1/ARE axis in the cell defense and its regulation by circadian biorhythms was discussed in our previous review [9]. Here, we analyzed the age-related changes in the Nrf2/Keap1/ARE pathways in the context of aging and anti-aging programs.

The functioning of cell antioxidant system, including the Nrf2/ARE pathway, diminishes with age, resulting in the downregulated expression of the Nrf2-targeted genes [5, 8, 12-17]. This leads to the elevation of the reactive oxygen species (ROS) level in tissue, glutathione depletion, and increased oxidative damage to proteins, DNA, and lipids [12, 13]. Another consequence is upregulated expression of NF-κB-targeted genes, such as intercellular adhesion molecule 1 (ICAM-1) and interleukin-6 (IL-6) [14, 18] genes.

Activation of the Nrf2 pathway includes several stages, such as biosynthesis of the Nrf2 protein, its translocation to the nucleus, binding to the AREs in the target genes (including the Nrf2 gene itself), expression of the target genes, and production of the encoded proteins. Here, we propose a method for quantitative assessment of the age-related dynamics in the activity of the Nrf2 pathway components in various organs in order to reveal those most sensitive to the age-related changes, as well as to identify the tissues most resistant to the oxidative stress due to the upregulated expression of Nrf2-targeted genes and experimental settings for their induction. In addition, this analysis might be used for evaluating the extent of age-related changes in the Nrf2 pathway caused by Nrf2 activators.

Numerous studies have been aimed at comparing the Nrf2 mRNA and protein levels, as well Nrf2 ability for activation in response to oxidative (electrophilic) stress in different tissues of rats (Fischer and Sprague–Dawley) and mice (C57BL/6J) of various age [5, 8, 12-15, 17]. Essentially, the Nfr2 expression level determines the content of active antioxidant enzymes, the activity of which increases in aging cells both *in vitro* and *in vivo*. Usually, the activation of Nrf2 is evaluated based on the activity of the following antioxidant enzymes: Nqo1, Ho-1, and two Gcl subunits (Gclc and Gclm). An increase in the nuclear content of Nrf2 after its translocation also serves as an indicator of Nrf2 functional activation.

Here, we will review the studies on the age-related expression of Nrf2 mRNA and protein, as well as Nrf2-targeted genes in various organs and tissues. The data from the experimental rodent models discussed below cover diverse life stages usually lasting for 28-32 months. It is important to note that in different studies, the definition "old" may be applied to animals that substantially differ in age. In particular, the life stages of mice include "maturation" (2-5 months of age) and "mid-age" (12-24 months) characterized by a low mortality rate and prevalence of cancer, glomerulonephritis, and other degenerative diseases. At the age of 24-26 months, the incidence of chronic degenerative diseases and mortality rate increase significantly [16]. Hence, older mice are rarely studied to minimize the potential effects of acute/chronic patholo-

gies in various organs on the overall animal status. It is believed that in the case of healthy aging, a six-month-old mouse matches a 30-year-old human, whereas a 21-month-old mouse is equivalent to a 60-year-old human [16]. Moreover, it should be noted that virtually all available data on the altered Nrf2 activity and expression have been produced in the cross-sectional rather than longitudinal studies. Hence, although the term "age-related changes of Nrf2 activity and expression" will be used hereinafter, it should be emphasized that this parameter has been compared in animals of different age.

EXPRESSION OF THE Nrf2 PROTEIN AND mRNA AND Nrf2-TARGETED GENES IN VARIOUS ORGANS AND TISSUES

Liver. Shih & Yen [19] investigated Nrf2 expression (by Western blotting), the level of oxidative stress, and Nrf2 activity in liver extracts from 2-, 12-, and 18- to 24-month-old Sprague–Dawley rats. Such studies are of particular interest because liver is characterized by the highest levels of antioxidant and detoxification enzymes, whose expression is regulated by Nrf2. The extent of oxidative stress increased with age. Thus, compared to the 2-month-old rats, the level of protein carbonylation in 12-month-old and 18- to 24-month-old rats increased 1.5- and 3-fold, respectively, whereas the lipid peroxidation [according to the TBARS (thiobarbituric acid reactive substances) assay) increased 1.5-fold in 18- to 24-month-old. The Nqo1 activity gradually declined with age and comprised ~5000, ~3000, and ~1000 nmol/min·mg protein in 2-, 12- and 18- to 24-month-old rats, respectively ($p < 0.05$). However, the activity of catalase was the same in 2-month-old and 12-month-old rats (~20 nmol/min·mg protein), whereas at the age of 18 to 24 months, it was ~15 nmol/min·mg protein ($p < 0.05$). Although the expression of antioxidant enzyme did not change significantly in the 2-month-old and 12-month-old rats, it decreased to 53% for glutathione peroxidase, 60% for glutathione reductase; 62% for Nqo1, and 69% for catalase, respectively) in 18- to 24-month-old rats vs. 2-month-old animals ($p < 0.05$). The expression level of Nrf2 in the liver was the same in the 2-month-old and 12-month-old rats, but ***decreased*** more than 2-fold in the 18- to 24-month-old animals vs. 2-month-old rats ($p < 0.05$).

Similar data were obtained by Suh et al. [12], who showed (by Western blotting) that aged (24- to 28-month-old) Fischer 344 rats had a decreased content of both total Nrf2 (by 56.2%, $p < 0.001$) and its nuclear (i.e., transcriptionally active) fraction (by 51.7%, $p < 0.0001$) vs. young (2- to 5-month-old) animals. The binding of Nrf2 to the AREs in the liver nuclear extracts isolated from aged rats was also ***lower*** (by ~40%) than in young animals (according to the gel-shift assay). The downregulation of the Nrf2 transcriptional activity resulted in the decreased glutathione production [12].

One of the biggest large-scale-studies conducted by Xu et al. [17] investigated changes in the Nrf2 pathway in liver samples collected from Sprague–Dawley rats at 11 time points [prenatally (–2) and on postnatal days 1, 7, 14, 21, 28, 35, 60, 180, 540, and 800]. Similar to the expression of Nrf2 and Kelch-like ECH-associated protein-1 (Keap1), expression of mRNAs for the Nrf2 pathway genes (*Gclm*, *Nqo1*, *Ho-1*) was high in the fetal liver, decreased at birth, and reached its first peak of expression on postnatal day 7, followed by gradual decrease by the age of six months ($p < 0.001$). Expression of all studied genes remained elevated until the age of 18 months and then declined by the age of 26 months. Expression of Nrf2, Nqo1, Ho-1, Gclc, and Gclm proteins changed in a similar manner.

Smith et al. [20] demonstrated that the Nrf2 mRNA expression level did not differ in the hepatocyte cultures derived from young (4 to 6 months) and aged (24-28 months) Fischer 344 rats ($p > 0.05$). In contrast, both baseline and anethole trithione (A3T)-induced expression of Nrf2-dependent genes ***decreased*** in hepatocytes from the aged animals. And *vice versa*, upregulated Nrf2 expression in aged rats was able to restore gene induction. Additional experiments identified six miRNAs, the content of which increased more than 2-fold with age ($p < 0.05$). It was suggested that one them, miRNA-146a, binds the Nrf2 mRNA. Transfection of hepatocytes derived from young rats with the miRNA-146a mimetic attenuated Nrf2 translation by 55%, which corresponded to the age-related Nrf2 loss [20].

Spinal cord. Duan et al. [8] examined the age-related changes in the expression of Nrf2, Ho-1, and Nqo1 proteins both in *in vitro* and *in vivo*. It was found that the content of Nrf2 and Ho-1 proteins in the spinal cord astrocytes in 13-month-old vs. 1-month-old C57Bl6/J mice ***decreased*** 2-fold ($p < 0.05$), while no difference in this parameter was observed between the 1-month-old and 5-month-old animals. The level of Nqo1 expression was similar in all examined age groups. Moreover, comparing expression of these proteins in a stationary culture of spinal cord astrocytes on days 14, 30, and 60 of culturing (culture medium was changed twice a week) revealed that the levels of Nrf2 and Nqo1 proteins on day 60 ***decreased*** 2-fold vs. day 14 ($p < 0.05$), while no significant differences were observed for the cell culture on days 14 and 30. Unlike Nqo1, the level of Ho-1 on days 30 and 60 was 2.3- and 4-fold ***lower***, respectively, than on day 14 ($p < 0.05$).

Bronchi. When using (presumably for the first time) human primary bronchial epithelial cells derived from volunteers of various age, Zhou et al. [21] confirmed the results of earlier studies conducted in animals and showed that the baseline Nrf2 expression ***decreased*** in elderly (60 to 69-year-old) vs. young (25 to 29-year-old) subjects. At

the same time, the baseline expression of the three Nrf2-driven genes (*GCLC*, *GCLM*, and *NQO1*) was ***higher*** in elderly vs. young subjects [21].

Muscles. It was found that with age, Fischer 344 rats accumulate defective mitochondria characterized by the damaged cristae, swelling, and formation of intramitochondrial vacuoles [22]. Moreover, whereas the ROS level in the tongue tissue increased 1.36-fold ($p = 0.001$), the content of Nrf2 protein (according to Western blotting) ***decreased*** 1.75-fold ($p = 0.021$) in the aged (22-month-old) vs. young (7-month-old) rats [22].

Cardiovascular system. The activity of the Nrf2/ARE axis in the heart declines with age [23]. In particular, it was demonstrated that the Nrf2 level reduced almost 3-fold in the myocardium nuclear extracts from the aged (over 23-month-old) vs. young (2-month-old) C57/Bl6/SJ mice (0.35 ± 0.12 vs. 1.0 ± 0.18, respectively). Moreover, expression of the antioxidant target genes in the heart was lowered by 40% [23].

Age-related dysfunction of Nrf2 in the endothelial cells altered cell signaling and contributed to the development of vascular disorders [24]. Induction of aging program in cerebral arteries is related to the upregulation of a wide range of pro-inflammatory cytokines and chemokines that contribute to the emergence of the senescence-associated secretory phenotype (SASP). The expression level of molecular markers of aging (p16INK4a, p21) in cerebral arteries was found to be higher in aged (24-month-old) vs. young (3-month-old) C57BL/6J mice [25].

Ungvari et al. [14] compared the expression of Nrf2 mRNA and protein and of Nrf2 target genes in the aorta from 3-, 12-, 18-, 24-, and 28-month-old Fischer 344x Brown Norway (F344xBN) rats. Analysis of the intensity of nuclear fluorescence using redox-sensitive dye dihydroethidium (DHE) uncovered a marked age-related increase in the O_2^- production in the aorta. Blood vessels from the aged rats demonstrated mainly cytosolic Nrf2 staining, whereas the signal from the nuclear Nrf2 was much less pronounced and similar in both young (3-month-old) and aged (24-28-month-old) animals. Therefore, the age-related decrease was observed for the Nrf2 nuclear fraction responsible for the ARE binding and upregulation of transcription of antioxidant and phase II detoxifying enzymes. The extent of Nrf2 binding to the ARE (according to ELISA) in arterial nuclear extracts in 12-month-old rats was ***lower*** than in the 3-month-old animals and remained at this level in all examined age groups ($p < 0.05$). The content of nuclear Nrf2 in the aorta declined with age together with the total Nrf2 content (cytosolic + nuclear), but the significant ($p < 0.05$) decrease in the Nrf2 mRNA (70% of expression in 3-month-old rats) and protein (30% of protein content in 3-month-old rats) was observed starting only from the age of 24 months. Similar age-related dynamics in the mRNA (RT-PCR) and protein (Western blotting) content was also found for the Nrf2-targeted genes. The level of Gclc mRNA in 24-month-old rats decreased down to 60% vs. 3-month-old animals ($p < 0.05$). The content of Nqo1 and Ho-1 mRNAs decreased to 70 and 60%, respectively, and was significant already in 12-month-old animals ($p < 0.05$, compared with 3-month-old rats). The levels of Gclc and Nqo1 proteins decreased to ~70 and 25%, respectively, in 24-month-old rats vs. 3-month-old rats [14]. Ungvari et al. showed that that the baseline Gclc mRNA level in the aorta remained the same in the 3 to 18-month-old animals and then declined starting from the age of 24 months. Such decrease could be also induced in response to H_2O_2 in young (3-month-old), but not in aged (24-month-old) rats.

Another study published by the same group reveled that aging is accompanied by the elevated oxidative stress [15]. Thus, the content of 8-iso-prostaglandin F2a (8-iso-PGF2α, a biomarker of lipid peroxidation) in the carotid arteries was 6 times higher in aged (22.2 ± 1.7-year-old) vs. young (10.5 ± 0.9-year-old) rhesus macaques (*Macaca mulatta*). Aged macaques also demonstrated a 1.8-fold increase in the content of 4-hydroxy-2-nonenal (4-HNE, secondary oxidation product that forms stable adducts with proteins) and augmented inflammation (upregulated NF-κB activity). The levels of the antioxidants glutathione and ascorbate were decreased 4- and 8-fold, respectively, in aged vs. young animals. However, the age-related promotion of the oxidative stress caused no Nrf2 activation (no significant difference in the levels of the Nrf2 mRNA and protein, as well as in the expression of the Nrf2-targeted genes for Nqo1, Gclc, and Ho-1 was observed). Perhaps, this can be explained by the age of animals compared in the studies, as the age of the "old" animals used in the experiments was only a half of the lifespan typical for this species [15].

The data on the age-related alterations in the Nrf2 activity are shown in table. Notwithstanding the data from the mid-age groups (age-related dynamics) and differences in the age of the "youngest" and "oldest" age groups, there is a 2 to 4-fold age-related decrease in the expression of Nrf2 (both protein and mRNA) and targeted genes in almost all tissues. The most prominent decline is observed in the muscles, which matches their status as the most metabolically active tissue. Vascular endothelial cells are the most resistant to the Nrf2 downregulation (table). It should be mentioned that the data obtained for the primary cell cultures prepared from tissues isolated from animals of different age (i.e., different environment) were the opposite [13, 21, 26-28]. Tissue cells derived from old vs. young animals had a ***higher level*** of the Nrf2 mRNA and protein, which is in agreement with the notion that Nrf2 is activated by the oxidative stress (whereas the majority of pathways regulating Nrf2 are inactivated) (Fig. 1). Likewise, a more pronounced oxidative stress should be matched by a higher Nrf2 content, as well as activated transcription of its target genes

Age-related changes in expression of Nrf2 and its target genes

Organ/(species)	Young, months	Old, months	Nrf2 mRNA and protein levels (old vs. young animals)	Expression level of the Nrf2 target genes (old vs. young animals)	References
Liver (Fischer 344 rats)	4-6	24-28	Nrf2 protein level ***decreased*** by 42%; mRNA level was unaffected	mRNA levels for Gclc, Gclm, and Gst2a were ***decreased*** by 19.6, 31.4, and 40%, respectively	[20]
Liver (Fischer 344 rats)	2-5	24-28	Nrf2 level ***decreased*** 1.79-fold for total Nrf2 protein and 2-fold for its nuclear fraction	Nrf2 binding to ARE ***decreased*** by 40%	[12]
Liver (Sprague–Dawley rats)	2	18-24	Nrf2 protein level ***decreased*** 2-fold	expression level comprised 60, 62, and 69% for glutathione reductase, Nqo1, and catalase, respectively	[19]
Liver (Sprague–Dawley rats	2-5	24-28		Nrf2 binding to the Gclc ARE ***decreased*** (50 ± 17% of that in young rats)	[68]
Liver (Sprague–Dawley rats)	6	26	Expression level of the Nrf2 gene *was the same* in 6-month-old vs. 26-month-old rats	expression of the Gclm, Nqo1, Gclc, and Ho-1 genes *did not differ* in 6-month-old vs. 26-month-old rats	[17]
Skeletal muscles (Fischer 344 rats)	7	22	Nrf2 protein level ***decreased*** 1.75-fold		[22]
Skeletal muscles and heart (C57BL/6 mice)	2	22	Nrf2 mRNA level ***decreased*** 3.5- and 2-fold in muscles and heart, respectively	Nrf2/ARE binding ***decreased*** 2.1- and 1.9-fold in the heart and skeletal muscles, respectively; Gclc and Gclm mRNA levels in the heart decreased 2- and 1.5-fold, respectively; in skeletal muscles, the Gclc mRNA level remained *unchanged*, while the Gclm mRNA level ***decreased*** 1.5-fold	[31]
Heart (C57BL/6/SJ mice)	2	23	Nrf2 protein level ***decreased*** 3-fold	Nrf2/ARE binding ***decreased*** 1.65-fold	[23]
Heart (Wistar rats)	9	24	Nrf2 protein level was *unchanged*		[51]
Blood vessels (rhesus macaques)	126	264	*no age-related changes* in the Nrf2 mRNA and protein levels	*no age-related changes* in mRNA and protein level for the Nrf2 target genes (Nqo1, Gclc, and Ho-1)	[15]
Blood vessels (F344xBN rats)	3	24	Nrf2 mRNA level ***decreased*** 1.4-fold; Nrf2 protein level ***decreased*** 3.3-fold	Nrf2 binding to ARE ***decreased*** 2-fold in old rats	[14]
Blood vessels (Fischer 344 rats)	6	24	Nrf2 protein level ***increased*** 1.5-fold in the nucleus (but not in the cytosol) of the primary endothelial cell culture from old rats	Nrf2 binding to ARE in the primary endothelial cell culture from old rats ***increased*** by 33.5 ± 17%	[27]
Spinal cord (ICR mice)	5	13	Nrf2 protein level ***decreased*** 2-fold	Ho-1 protein level in the spinal cord ***increased*** 2-fold, the content of Nqo1 remained unchanged	[8]
Retinal pigment epithelium (C57Bl6/J mice)	2	15		expression of Nqo1 and Ho-1 in the culture of primary retinal pigment epithelial cells ***increased*** 11- and 10-fold, respectively	[28]
Bronchi (humans)	21-29*	60-69*	Nrf2 protein level in primary bronchial epithelial cell culture ***decreased*** 1.34-fold	levels of GCLC, GCLC and NQO1 proteins ***increased*** 1.8-, 2.2-, and 2.5-fold, respectively	[21]

Note. * Years.

encoding enzymes of the antioxidant defense system. Physical exercises represent another interesting example of environment impact on the Nrf2 pathway. Safdar et al. [13] showed that in elderly (~70-year-old) individuals leading an active lifestyle, the content of the Nrf2 protein increases, rather than decreases, compared to the adult subjects. Similar data were obtained in exercised rats [26].

AGE-RELATED CHANGES IN THE ABILITY OF THE Nrf2/ARE AXIS TO BE ACTIVATED BY THE EXOGENOUS AND ENDOGENOUS OXIDATIVE STRESS

Cell culturing in glucose-supplemented medium. The potential of cells to respond to oxidative stress and their ability to activate Nrf2 (as a major regulator of highly coordinated antioxidant response) change with age. Ungvari et al. [15] showed that the treatment with high glucose concentrations (30 mM) or H_2O_2 (14-100 μM) markedly promoted the transcriptional activity of Nrf2 and upregulated expression of the Nrf2-targeted genes in the vascular smooth muscle cells (VSMCs) derived from young (10.5 ± 0.9-year-old) *Macaca mulatta* animals at the early passages (passages 4-5). In contrast, cultured VSMCs derived from old macaques (22.2 ± 1.7-year-old) displayed no evident rise of Nrf2 activity and expression of Nrf2 target genes after expose to H_2O_2 or high-level glucose, which evidenced a decreased ability of cells derived from old animals to respond to oxidative stress. Ungvari et al. [14] also examined Nqo1 induction in the aorta after exposure to H_2O_2 and glucose and found that it was downregulated in old rats (24-month-old vs. young (3-month-old) animals. Moreover, the authors demonstrated that albeit no changes in the Nqo1 expression was observed, its induction by H_2O_2 was moderate in the carotid arteries and VSMCs derived from the aged (20-year-old) vs. young (10-year-old) macaques.

VSMCs derived from old (24-month-old) Fischer 344 rats and grown in either standard (supplemented with 5 mM glucose; $p = 0.02$) or glucose-enriched medium (12.5 mM, $p = 0.01$) had a ***higher*** nuclear (but not cytosolic) Nrf2 protein content [27] vs. cells from the young (6-month-old) animals. The binding of Nrf2 to the ARE motif in VSMCs derived from the old mice and exposed to the high-glucose medium (25 mM, 24 h) or grown in the standard medium (5 mM glucose) ***increased*** by 54.5 ± 14.1 and 33.5 ± 17.4%, respectively, compared to the cells from younger animals, resulting in the upregulated *Gclc* expression [27]. Therefore, old animals had both decreased baseline as well as H_2O_2- and glucose-induced nuclear Nrf2 levels.

Oxidative stress in the retinal pigment epithelium (RPE). Modeling age-related changes in the retina is important for understanding the role of oxidative stress in pathogenesis of age-related macular degeneration, which is one of the most common age-related vision disorders [28]. Sachdeva et al. [28] compared the RPE in young (2-month-old) and aged (15-month-old) C57Bl6/J mice under stress-free and stressful conditions (sodium iodate, 5 mg/kg). In the absence of stress, the expression of the Nrf2 target genes (*Nqo1* and *Ho-1*) in the RPE of aged mice was 11- and 10-fold, respectively, ***higher*** than in the young animals ($p < 0.05$) (according to RT-qPCR), thus indicating an age-related increase in the baseline oxidative stress in old mice and higher RPE susceptibility to the oxidative damage due to the impaired Nrf2 signaling. In the RPE of young mice, expose to $NaIO_3$ caused a 2.2-fold increase in the Nrf2 mRNA content and relevant increase in the amount of transcripts for Gclm, Ho-1, and Nqo1 (2-, 9-, and 10-fold, respectively). However, no stimulation of the Nrf2 expression by $NaIO_3$ was observed in the RPE from old mice ($p < 0.05$) [28]. Moreover, the RPE from old vs. young animals exposed to sodium iodate had a higher level of superoxide anion and malonic dialdehyde, which evidences an insufficient defense against oxidative damage. Conversely, Nrf2 overexpression in the RPE preserved cell morphology and improved mouse survival in the retinal degeneration models, suggesting that it can be used as a potential therapeutic approach for treating disorders related to the RPE degeneration [29].

Compensation of age-related changes by Nrf2 activators. The use of Nrf2 activators is one of the commonly accepted ways to compensate for the impairments in the Nrf2 action. Usually, such activation is achieved by the administration of agents capable of interacting with cysteine thiol groups in Keap1, which is a major Nrf2 inhibitor [7, 10, 30].

Lipoic acid. (R)-alpha-lipoic acid induces Nrf2 activation *in vitro* and elevates glutathione level *in vivo*, presumably, due to the formation of lipoyl-cysteinyl mixed disulfides on Keap1 (Nrf2 inhibitor), thus hindering its binding to Nrf2. Another putative mechanism is protein kinase C-mediated Nrf2 phosphorylation [10]. In aged Fischer 344 rats (24- to 28-month-old), injection of lipoic acid (40 mg/kg, intraperitoneally) increased the content of nuclear Nrf2, its binding to the ARE motif, and Gclc level (1.7-fold) (but caused no induction of Gclm), as well as upregulated the Gcl activity (24 h after injection) compared to the values observed in young (2- to 5-month-old) animals [12].

Sulforaphane. Isothiocyanate sulforaphane from cruciferous plants is another Nrf2 activator that upregulated Nrf2 mRNA (RT-qPCR) 4-fold in the cardiac tissue of old C57BL/6 (21-22 months) mice (0.17 ± 0.06 vs. 0.67 ± 0.13; before and after, respectively; $p < 0.001$) and 6-fold in young mice (0.35 ± 0.04 and 2.12 ± 0.50, before and after, respectively; $p < 0.0001$) when taken with food for 12 weeks. In skeletal muscles, it increased the Nrf2 content 2-fold in young mice (0.90 ± 0.37 and

1.77 ± 0.82 before and after, respectively; $p < 0.01$) and almost 3.5-fold in old mice (0.26 ± 0.07 and 0.90 ± 0.40 before and after, respectively; $p < 0.05$). Interestingly, the expression of Nrf2 in old mice receiving dietary sulforaphane was 2 times higher (heart) or remained the same (skeletal muscles) as in healthy young animals untreated with sulforaphane. The Nrf2/ARE binding (measured with a Nrf2-DNA-binding ELISA kit) was ***decreased*** 2.1- and 1.9-fold in the heart and skeletal muscles, respectively, in old vs. young mice ($p < 0.001$). Sulforaphane also improved the Nrf2/ARE binding 1.225- and 1.17-fold ($p < 0.05$) in the heart and skeletal muscles, respectively, of young mice. Interestingly, although the level of Nrf2 binding in old mice receiving sulforaphane with food was increased 1.67-fold ($p < 0.01$) and 2.08-fold ($p < 0.001$) in the heart and skeletal muscles, respectively, it did not reach the level typical of young animals untreated with sulforaphane. However, sulforaphane improved the overall physical body state, mitochondrial function, glucose tolerance, and activation/differentiation of skeletal muscle satellite cells to the level found in young (2-month-old) mice [31].

Tert-butylhydroquinone. The efficacy of Nrf2 activation also depends on age. The period within which Nrf2 stays in the activated form depends on the stimulus generated by the inducer, as well as the animal's age. For instance, *tert*-butylhydroquinone (tBHQ) promoted Nrf2 binding to the ARE in primary astrocytes derived from aged (24-month-old) Wistar rats only within 30 min. At the same time, its activity in the astrocytes isolated from the young (9-month-old) animals was sustained for up to 180 min [32].

Nrf2 inactivation in the models of age-related pathologies. *Muscles.* Miller et al. [33] revealed that the levels of Nqo1 mRNA and protein were sharply ($p < 0.001$) decreased in the skeletal muscles of 2-month-old *Nfe2l2*$^{-/-}$ mice (hereinafter denoted as *Nrf2*$^{-/-}$) compared with the wild-type C57/BL6x animals. Aged mice (over 24-month-old) demonstrated elevated ROS levels, and lower glutathione content in skeletal muscles vs. the young animals. Moreover, the signs of oxidative stress (ROS levels, HNE-positive proteins, ubiquitination, proapoptotic signals) suggested that skeletal muscles in old mice were much more susceptible to the impairments in the Nrf2 pathway regulation. Kitaoka et al. [34] showed that mitochondrial markers of oxidative stress (hydroxynonenal and protein carbonyl levels) in old *Nrf2*$^{-/-}$ mice were markedly elevated due to the decreased expression of the Nrf2-targeted antioxidant genes. Although mitochondrial respiration decreased with age, no differences were observed between the *Nrf2*$^{-/-}$ and age-matched wild-type mice. The activity of cytochrome oxidase *c* was lower in the wild-type old and *Nrf2*$^{-/-}$ mice vs. young wild-type animals. Mitochondrial ROS production in the old *Nrf2*$^{-/-}$ mice (calculated per oxygen consumption) was increased. However, the *Nrf2* knockout did not affect the age-related decline in the muscle mass-to-body weight ratio, which assumes that the Nrf2 deficiency exacerbates the age-related mitochondrial oxidative stress without affecting the decrease in the respiratory function of skeletal muscles [34].

Age-related hearing impairment. In the human auditory system, Nrf2 was detected (by immunostaining) mainly in the apical, medial, and basal regions of the organ of Corti, inner and outer hair cells, and supporting cells in the cochlea [35, 36]. To examine the age-related differences in the Nrf2 immunoreactivity in the organ of Corti, the studied subjects (18-92 years of age) were divided into the adult (<70-year-old) and elderly (70 to 92-year-old) groups. The data on the mean Nrf2-positive area were averaged for each sample and subjected to the repeated one-way analysis of variance (ANOVA). Although the Nrf2 immunoreactivity was detected even in the organ of Corti from the oldest person (92 years of age), it was found to be decreased by 20% in the elderly group ($p \leq 0.0372$) [36]. Noise-induced hearing loss (NIHL) is one of the most common sensorineural hearing impairments. The pathogenesis of NIHL is closely related to the ischemia–reperfusion-induced cochlear damage caused by the reduced blood flow and free radical production due to the excessive noise [35]. The damage and loss of hair cell caused by oxidative stress, which are considered the cause for hearing loss, are closely associated with the cell antioxidant status and Nrf2 function [35, 36]. The knockout of the *Nrf2* genes makes the auditory organs (and hair cells, in particular) more susceptible to noise [37]. Thus, *Nrf2*$^{-/-}$ mice demonstrated more pronounced hearing impairments 7 days after noise exposure compared to the wild-type mice. When used before (but not after) the noise exposure, Nrf2 activator CDDO-Im (2-cyano-3,12 dioxooleana-1,9 dien-28-imidazolide) protected the integrity of hair cells and improved hearing in wild-type mice, but had no effect in the *Nrf2*$^{-/-}$ animals. This confirms the efficacy of Nrf2 activation for preventing NIHL and protecting the cochlea from the noise-induced damage [38].

Cardiovascular system. Valcarcel-Ares et al. [39] found that suppression of the Nrf2 signaling (with siRNA for the Nrf2 knockdown or overexpression of the Nrf2 inhibitor Keap1) impaired angiogenesis in the cultured endothelial cells from the human coronary artery and impaired their proliferation and adhesion to the glycoproteins vitronectin and collagen. Disturbed Nrf2 signaling also resulted in the suppression of cell migration and affected the ability of these cells to form capillary-like structures [39].

Cell aging and expression of molecular markers of senescence. Kapeta et al. [40] investigated the role of Nrf2 in the replicative senescence of human lung fibroblasts (HFL-1). In brief, cultured fibroblasts were replated (1 : 2) after forming a confluent monolayer. After the cells reached a certain population doubling level (PDL), deter-

mined by the formula $\ln(N_f/N_i)/\ln 2$, where N_f is the number of cells after subcultivation and N_i is the initial number of live cells in the culture, they were considered as replicatively senescent (unable to double within a certain period of time). In the replicatively senescent HFL-1 fibroblasts (~42 PDL), the levels of oxidative stress and aging-related markers (percentage of cells positively stained for senescence-associated β-galactosidase and expression of senescence markers p16INK4a and p21) were higher [40]. In addition, cultured HFL-1 cells demonstrated a lower content of the Nrf2 protein (~65%) and mRNA (45%) and downregulated expression of *Nqo1* (Nrf2 target gene) vs. young cells. Additional experiments have shown that young HFL-1 cells treated with the triterpenoid 18-glycyrrhetinic acid (Nrf2 inducer) avoided senescence (unlike the control cells). The action of this compound is mediated via the Nrf2/Keap1 pathway, as 18-glycyrrhetinic acid increased the content of Nrf2 3 times, while decreasing the content of Keap1 3 times [40].

Nrf2$^{-/-}$ fibroblasts have a short lifespan [41]. Moreover, Jódar et al. [41] showed that *Nrf2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) are prone to immortalization after PDL 15. In contrast, selective Nrf2 activators, such as rapamycin [42], prevented cell aging and extend the lifespan of normal MEFs [43]. Nrf2-regulated enzymes, e.g., superoxide dismutase 1 (Sod1), can also prevent aging and inflammation in some cases [44]. Senescent cells, in their turn, contribute to the development of age-related pathologies and loss of function, whereas their targeted depletion improves physiological functions and prolongs lifespan [42]. Thus, the levels of p16INK4a and p21 markers in the cerebral arteries are upregulated in 24-month-old mice compared to the young control animals [25].

Corenblum et al. [45] showed that even that the activity of neural stem progenitor cells (NSPCs) in Fisher 344 rats continuously declines with age, there is a critical period during the mid-age (13-15 months of age), when NSPC survival and regeneration sharply decrease. This specific temporal pattern for the compromised NSPC potential is accompanied by the decrease in the Nrf2 expression ($p < 0.05$), after which (as well as before), no prominent changes in this parameter are observed until the age of 26 months. Suppression of the Nrf2 expression in young NSPCs with short interfering RNA significantly decreased NSPC survival and regeneration. On the contrary, Nrf2 overexpression in old NSPCs makes them similar to young cells and promotes their survival and regeneration. Moreover, NSPCs from *Nrf2*$^{-/-}$ mice also demonstrated impaired proliferative and neurogenic potential [45].

In another model of the oxidative stress-induced aging called the stress-induced premature senescence (SIPS), cells with the suppressed activity of the Nrf2 inhibitor caveolin-1, which interferes with the Nrf2 nuclear translocation, exhibited less prominent signs of senescence (with the 2-fold increase in the of Nrf2 expression). Interestingly, the inhibitory effect of caveolin-1 was not mediated by Keap1 [46].

A relation between Nrf2 activity, lifespan, and longevity. Nrf2 is a crucial regulator of lifespan [47-50]. It is assumed that long-living species have an active cytoprotective Nrf2-dependent pathway. Overexpression of the downstream components in the Nrf2 pathway prolongs the lifespan of *Drosophila* [48]. Long-living *Caenorhabditis elegans* models demonstrate an upregulated expression of Skn-1 (Nrf2 homolog in this organism) [49]. The activity of Nrf2 is also a crucial modifier of lifespan in vertebrates [50]. However, the total Nrf2 content does not necessarily decline with age. For instance, the levels of Nrf2 protein (and its nuclear translocation) were similar in the hearts of adult and young rat in the absence of oxidative stress (however, the efficiency of the Nrf2–ARE binding was impaired, thus reducing expression of the major antioxidant enzymes Ho-1, GST, and Gcl) [51].

In addition, the content of Nrf2 in the long-living naked mole-rat *Heterocephalus glaber* is comparable with that found in other rodent species with a shorter species-specific lifespan [5]. Lewis et al. [5] suggested that the defense function of Nrf2 is related to its activity rather than its content. In this case, a reduced Keap1 level was essential for allowing Nrf2 to avoid ubiquitination and subsequent proteasomal degradation. Moreover, the authors also assumed a particularly important role of the two Nrf2 modifiers – p62/SQSTM1 (autophagosome-related protein that counteracts Nrf2 proteasomal degradation) and β-TrCP (E3 ubiquitin ligase that promotes Nrf2 proteasomal degradation) [5] (see below). Other physiological Nrf2-related adaptations include constitutively elevated expression of Nrf2 (1.5-fold) and Nrf2 pathway downstream components in the thirteen-lined ground squirrel *Spermophilus tridecemlineatus* in the preparation for hibernation. These parameters return back to the normal values upon animal arousal and return to the active life [52].

The major data on the age-related changes in the levels of Nrf2 protein are shown in Fig. 2. Notwithstanding the data for the mid-age animals (age dynamics) and differences for some age groups of young(est) and old(est) animals, the age-related decline in the Nrf2 protein level is less pronounced in the liver (major site for the antioxidant defense and detoxification), as the Nrf2 content did not decrease ***below*** 50% of the baseline level. Continuous decline in the Nrf2 protein content is typical for the cardiovascular system, while the nervous system holds an intermediate position.

Next, these results were compared with the data for other stages of Nrf2 activation (including expression of the Nrf2-encoding *Nfe2l2* gene and Nrf2-targeted genes, as well as Nrf2-ARE binding). The data are summarized in the table.

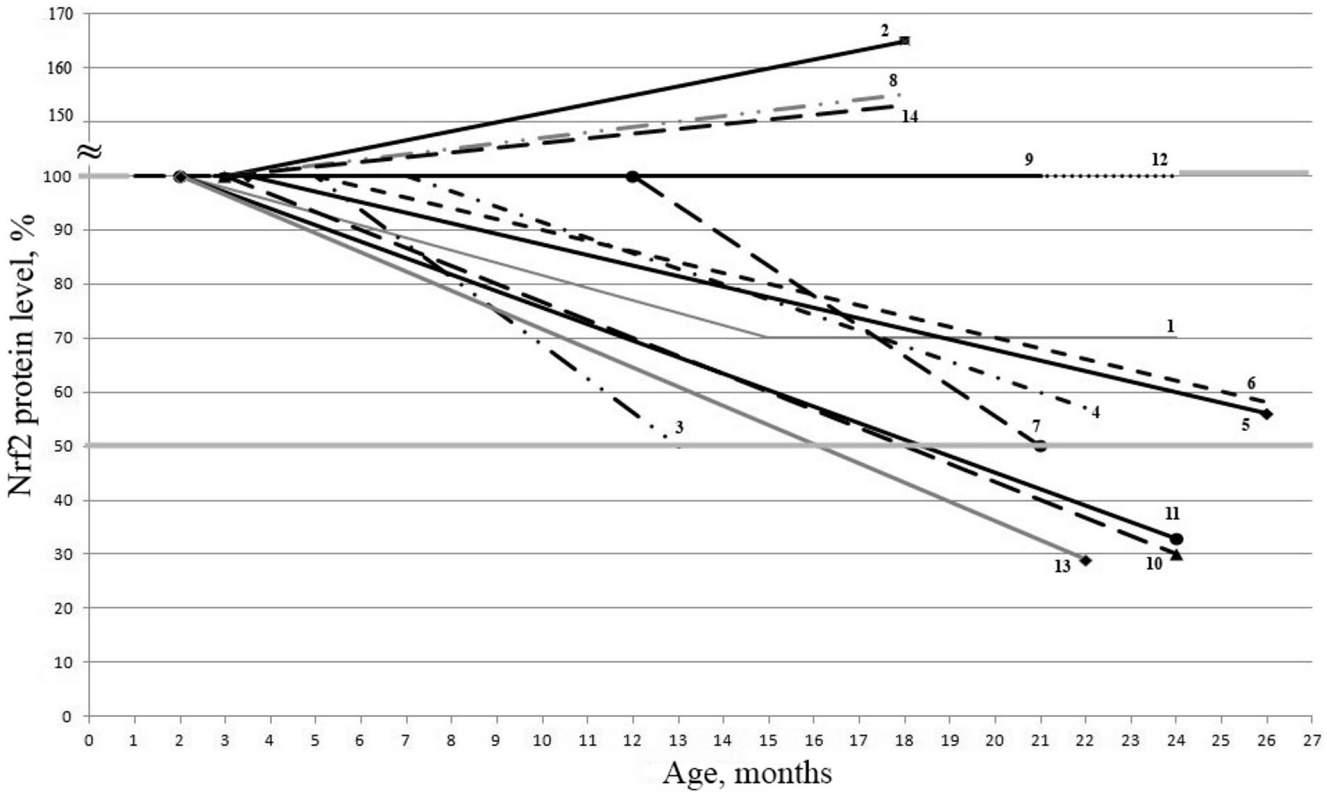

Fig. 2. Age-related changes in the Nrf2 protein content in laboratory animals (rats and mice). Nrf2 protein level in young animals is taken as 100%. The data from the following studies are shown: 1, Corenblum et al. [45], rats, brain; 2, Zhang et al. [67], mice, brain (cerebellum); 3, Duan et al. [8], mice, spinal cord; 4, Baek et al. [22], rats, tongue; 5, Suh et al. [12], rats, liver; 6, Smith et al. [20], rats, liver; 7, Shih and Yen [19], rats, liver; 8, Zhang et al. [67], mice, liver; 9, George et al. [26], rats, kidneys; 10, Ungvari et al. [14], rats, blood vessels; 11, Gounder et al. [23], mice, heart; 12, Silva-Palacios et al. [51], rats, heart; 13, Bose et al. [31], mice, heart; 14, Zhang et al. [67], mice, lungs.

Nrf2 pathway regulators. Systems involved in the Nrf2 suppression are components of the aging programs [4]. The most studied of them are the redox-dependent Keap1 and redox-independent system of β-transducin repeat-containing protein (β-TrCP) and glycogen synthase kinase 3 (GSK3) (Fig. 1). c-Myc is another inhibitor of Nrf2 action [53] (Fig. 1). Although Bach1 protein is not a direct Nrf2 inhibitor, it competes with Nrf2 for the binding to the ARE motif [10]. Beside GSK3, the activity of other kinases (e.g., Fyn kinase) may result in the Nrf2 inactivation followed by its export from the nucleus [54].

Keap1. Keap1 is the major Nrf2 regulator [54]. Although it was reported that *Keap1*$^{-/-}$ mice are nonviable and die within the first three postnatal weeks [55], downregulation of Keap1 promotes toxin resistance and in some cases, may extend the lifespan of species such as *Drosophila melanogaster* [56]. Interestingly, in more evolutionarily advanced birds (taxon Neoaves), Keap1 has underwent a profound remodeling and has virtually lost its potential to inhibit Nrf2 [57]. Constitutively active Nrf2 and, hence, induction of antioxidant enzymes are of critical importance for animals with rapid metabolism [57, 58]. SAMP8 mice characterized by accelerated aging have the same level of Keap1 protein as senescence-resistant SAMR1 mice [18]. No age-related changes in the Keap1 mRNA content were observed in 10 to 50-day-old *Drosophila* flies [58]. It was reported that excessive Keap1 amounts impair cell homeostasis and are observed in the models of premature aging [59-61]. Palsamy et al. [61] demonstrated that the unfolded protein response caused by stressors and endoplasmic reticulum oxidants leads to the decrease in the Keap1 promoter methylation followed by the upregulated Keap1 expression, resulting in the suppressed expression of the Nrf2 target genes and Nrf2 itself in human lens epithelium. Aging is accompanied by the decrease in the extent of Keap1 promoter methylation in the lens, which reaches its minimum at the age of ~75 years, thus creating prerequisites for the age-related rise in the Keap1 expression [61]. However, Safdar et al. [13] and Xu et al. [17] found no age-related increase in the Keap1 content.

Keap1 can act as a cell cycle regulator independently of Nrf2. Nrf2-depleted cells demonstrate a profoundly altered regulation of mitochondrial homeostasis due to the Keap1-driven degradation of Miro2 protein [62]. Moreover, Keap1 negatively regulates proliferation of endothelial cells and causes their aging [59, 60].

Sachdeva et al. [28] examined the effects of genetic ablation of Keap1 in the RPE of young (2-month-old) and mid-age (15-month-old) C57Bl6/J mice in the stress-free and stress-induced (sodium iodate, 5 mg/kg) settings [28]. They compared the oxidative stress-induced expression of Nrf2 and its target genes in mice with the tamoxifen-driven Cre recombinase transgene (Tam-Cre; Keap1loxP) and age-matched Keap1loxP animals expressing Keap1 at the wild-type level [28]. Exposure to tamoxifen downregulated Keap1 expression by 50% (assessed by RT-qPCR in Tam-Cre Keap1loxP vs. Keap1loxP mice lacking tamoxifen-driven Cre recombinase transgene). The treatment with $NaIO_3$ upregulated expression of the Nrf2-targeted *Nqo1* gene 2.5-fold in old vs. young mice ($p < 0.05$), thus evidencing partial recovery of the cell response to oxidative stress altered by Nrf2 in the RPE. The amount of transcripts of the *Gclm* and *Ho-1* genes in 15-month-old mice was not changed by the Keap1 knockdown. Exposure to $NaIO_3$ of the old mice with the wild-type Keap1 level (Keap1loxP) caused no activation of Nqo1 expression, which agrees with the above data. Induction of the Nrf2 pathway by $NaIO_3$ was partially restored in the RPE of aged (15-month-olds) C57Bl6/J mice after knockdown of the Nrf2 negative regulator Keap1 (Tam-Cre; Keap1loxP) compared to the Keap1loxP mice. These data demonstrate that the RPE of old mice is more susceptible to the oxidative damage due to the impaired Nrf2 signaling pathway. Activation of this pathway may be a highly promising approach for developing novel pharmacological or genetic therapeutic strategies [28]. The clinical importance of the Nrf2/Keap1 imbalance may be related to the age-related disorders [60].

THE ROLE OF INDIVIDUAL COMPONENTS OF THE Nrf2/ARE AXIS IN THE AGE-RELATED CHANGES, AGING, AND LONGEVITY

When speaking about the role of a particular protein in aging or anti-aging programs, the most commonly referred to features are age-related increase in its content (or activity), involvement in the developing of age-related pathologies, and counteraction of processes of cell repair and antioxidant defense (for Keap1, it implies the binding to Nrf2 followed by the proteasomal degradation of the latter). The (over)activation of Keap1 impairs the functioning of the cell antioxidant system. Although Keap1 was shown to be a subject of the nuclear import/export [62], the vast majority of its activity is detected in the cytosol. At the same time, it was found that it is the nuclear Nrf2 fraction (rather than the cytosolic one) that decreases with age.

Thus, it can be concluded that despite that fact that Keap1 is the most powerful Nrf2 inhibitor and, consequently, the major target for pharmacological intervention [30], it cannot be fully considered as a protein involved in the aging program. Instead, this role belongs to proteins responsible for nuclear Nrf2 shuttling, as well as for the Nrf2 activity as a transcription factor.

Bach1 and c-Myc. It is believed that the ARE-governed gene expression depends on the balance between Nrf2 and Bach1 in the nucleus [63]. Bach1 downregulates gene expression by competing with Nrf2 for binding to the ARE in the gene promoters, which results in the loss of inducibility for some of the Nrf2-regulated genes [64]. The heme level affects the intracellular distribution of Bach1, its nuclear export, and activation of Ho-1 expression [65]. Participation of the Bach1 residues C557 and C574 in the ARE/Ho-1 dissociation suggests that the Bach1 activity depends on the cell redox status [66]. The content of Bach1 increases with age; thus, it was found to be higher in aged (21-month-old) C57BL/6J mice vs. young (6-month-old) animals [67]. Likewise, bronchial epithelial cells of elderly (67-69 years of age) vs. young (28-29 years of age) individuals also contained ***higher*** amounts of the BACH1 protein [21]. Shenvi et al. [68] found that Nrf2 binding to the Gclc gene ARE was markedly ***lower*** ($50 \pm 17\%$, $p \leq 0.05$) in aged (24 to 28-month-old) vs. young (2 to 5-month-old) Fischer 344 rats and that the activity of the ARE-driven transcriptional response decreased with aging in the presence of Bach1. Moreover, the baseline level of the c-Myc and Bach1 proteins also increased with age in all tissues: the content of c-Myc was elevated by 170, 87, and 90% and the content of Bach1 increased by 80, 50, and 150% in the cerebellum, liver, and lungs, respectively. Chronic exposure to the airborne nano-sized particulate matter (nPM) increase the tissue levels of c-Myc in 6-month-old mice, but not in older animals. In contrast, the content of Bach1 was not affected by nPM in the 6-month-old mice, but was elevated in the older animals [67].

p62/sequestosome1 (SQSTM1) is an adapter protein that binds and delivers ubiquitinated proteins to autophagosomes during autophagy. It contains the STGE motif similar to that of the Keap1-interacting ETGE sequence in the Neh2 domain of Nrf2. This motif accounts for the direct interaction between p62 and Keap1, so that p62 sequesters Keap1 in autophagosomes for its subsequent degradation [69]. Autophagy may be another important process for the Nrf2 regulation during senescence. Expression of p62 declines with age [70] and its loss and/or decline reduce the lifespan [71]. It was shown that $p62^{-/-}$ mice develop the senile phenotype faster and their tissues are characterized by the prooxidant environment due to the impaired electron transport in the mitochondrial respiratory chain.

GSK3β. Proteins may be phosphorylated by GSK3β kinase, which often results in their subsequent ubiquitination and proteasomal degradation mediated by the relevant adaptor proteins (e.g., F-box proteins). The GSK3β activity is elevated with age both *in vivo* and *in vitro*. In particular, the baseline level for the inactive GSK3 protein

in aged (18-month-old) Syrian hamsters (*Meso-cricetus auratus*) was significantly ***lower*** than in the young (1 to 3-month-old) animals [72]. Moreover, the fraction of the inactive protein was also twice ***lower*** in the 1.5-year-old vs. 3-week-old female animals [73]. At the age of 10 months, the activity of hepatic Akt and GSK3β was substantially ***higher*** in SAMP8 vs. SAMR mice. It is believed that the decrease in the Nrf2 nuclear translocation due to the elevated GSK3β activity may promote oxidative stress in SAMP8 mice [18]. It was found that despite virtually unchanged total GSK3β content in the stationary culture of mouse primary cortical neurons, the fraction of GSK3β phosphorylated by the S9 residue (i.e., inactive enzyme) decreased from >30% at 3 days-*in vitro* (DIV) to 15% at 12 DIV [73]. Compared to mid-age (PDL, 38-41) and young (PDL, 26-30) human WI-38 fibroblasts, old cells (PDL, 58-64) displayed typical signs of senescence, including enlarged size, flattened shape, and elevated senescence-associated β-galactosidase activity, which lacked in the young and mid-age cells. The nuclei in the old cells demonstrated an increased content of both GSK3 isoforms – GSK3α and GSK3β [74]. Another evidence in favor of the GSK3β involvement in the aging program may be changes in its activity observed in the age-related diseases. In particular, GSK3β was shown to selectively phosphorylate tau-protein at the residues sites typically hyperphosphorylated in the protein found in the brain neurons of patients with the Alzheimer's disease (AD) [75]. Moreover, the brain level of GSK3β in AD patients is increased by 50% [75]. Inhibition of GSK3β attenuated the cognitive deficits associated with AD and other disorders. The GSK3β activity was found to be elevated in the cellular (growth factor deprivation) and animal (cerebral ischemia) models of neurodegeneration [76]. The pro-inflammatory GSK3β potential is accounted for by the induction of IL-1β, IFN-γ, IL-6, and IL-12 and suppression of IL-10 synthesis [77]. Our interest in this kinase within the framework of this article is due to its regulatory effects on Nrf2. These effects are mediated via at least three pathways: (i) GSK3 and βTrCP are directly involved in the Nrf2 degradation by promoting its ubiquitination and proteasomal cleavage (and not just its inactivation as in the case of other kinases); (ii) GSK3 drives the ultradian rhythms of Nrf2; it phosphorylates Fyn kinase, which is translocated to the nucleus, where it modifies Nrf2 for the following export to the cytosol; and (iii) GSK3 phosphorylates proteins involved in the positive feedback loop regulating circadian rhythms (Bmal1 and Clock), leading to their proteasomal degradation and downregulation of Nrf2 expression (it should be noted that the promoter regions of the genes regulating biorhythms, such as *Cry1*, *Cry2*, and *Rev-erbα*, as well as the Nrf2-encoding *Nfe2l2*, contain the E-box, so that their transcription is positively regulated by the Clock/Bmal1 complex [9, 78]). Altogether, the above data indirectly confirm GSK3β as an important component of the aging program.

CONCLUSIONS

Senescence is related to the elevated ROS production and increased oxidative stress paralleled by the decrease in the activity of major antioxidant enzymes and compounds (superoxide dismutases, catalase, glutathione), which contribute to the development of various pathologies [24]. The evolutionarily conserved pathway of the Nrf2-mediated antioxidant response maintains the cellular redox homeostasis and sustains it at the level typical for young cells by regulating transcription of cytoprotective genes. The level of Nrf2 expression declines with age both in *in vivo* and *in vitro* settings, which results in more pronounced oxidative stress. Age-related changes may result in the impaired expression of the Nrf2 target genes (*Nqo1*, *Ho-1*, *Gcl*) due to the decrease in the total content of Nrf2 mRNA and protein, decline in the amount of the Nrf2 nuclear fraction, impaired Nrf2 binding to the AREs, and suppression of the Nrf2-driven expression. Thus, impairments in the cell antioxidant defense in old animals due to the Nrf2 deficiency may result in the accumulation of harmful amounts of ROS [22]. Genetic ablation of Nrf2 aggravates the age-related induction of senescence markers and inflammatory SASP cues [25], as well as accelerates cell aging by causing chronic inflammation [25, 79, 80]. Nrf2-deficient mice also display an elevated sensitivity to some pharmacological and ecological toxins [81]. An insufficient activity of the Nrf2 pathway plays a crucial role in the emergence and development of certain toxic and chronic diseases related to the oxidative stress. Nrf2-deficient animals are more susceptible to the development of age-related disorders, such as cardiovascular diseases [82], skeletal muscle atrophy [83], cancer [46], and retinal diseases [84], and are characterized by a shorter lifespan and premature aging [46]. In particular, the age-related decrease in the major cellular antioxidant glutathione may be related to the impaired regulation of the ARE-driven gene expression. Chemoprotective agents targeting Keap1, such as lipoic acid, sulforaphane, and other Nrf2 activators, can only partially compensate for this loss [30, 85-92] by suppressing the Keap1-mediated degradation pathway, but not fully abrogating it. Moreover, their effect progressively decreased with age. The most probable candidates for the role of a "grey eminence" implicitly accounting for the age-related decline in the Nrf2 activity are c-Myc, GSK3β, and Bach1.

Therefore, along with comparison of the defense system functioning in short- and long-living species [2, 5, 6, 57, 93-98] and search for the novel antioxidants [99-101] and Nrf2 activators [30, 85-89], the promising approach in biogerontology studies may be the design and synthesis of substances with multiple effects, such as a hybrid compound derived from sulforaphane (Nrf2 activator) and melatonin (regulator of biorhythms) [102], as well as molecules simultaneously acting as GSK3β inhibitors

and Nrf2 activators (2,4-dihydropyrano[2,3-c]pyrazoles [103]) or capable of regulating the Nrf2 inactivation pathways (GSK3β, FYN, and DYRK1A) [104]. Hence, the Nrf2 signaling pathway as the major regulator of highly coordinated antioxidant response, may be as a therapeutic target for protecting the cells from the age-related ROS accumulation and preventing age-related pathologies via the redox homeostasis restoration. Moreover, future studies of the Nrf2 signaling in humans and on the ability of different substances to activate Nrf2 in order to prevent chronic age-related disorders caused by the cell senescence will provide a deeper insight into the role of Nrf2 activation as a potential approach to promoting longevity.

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