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Superoxide and nitric oxide in senescence and aging

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1. ABSTRACT

In this review some aspects of free radical theory of aging are discussed. Many new and interesting findings concerning the role of physiological free radicals superoxide and nitric oxide in senescence and aging development are considered and the mechanisms of processes mediated by these radicals are discussed. It has been known for a long time that being themselves mostly harmless species, superoxide and NO are precursors of really reactive species hydroxyl radicals and peroxynitrite, the initiators of aging and various pathologies. However, contemporary studies demonstrate the other maybe more important ways of damaging activity of physiological free radicals. Numerous studies show that lessening of NO production and its bioavailability could be a starting point of aging development. It results in a decrease in NO inhibition of mitochondrial cytochrome *c* oxidase and an increase in dioxygen consumption. That in its turn leads to an increase in the production of superoxide and the other reactive oxygen and nitrogen species and initiation of apoptosis, In conclusion the possibilities of pharmacological intervention with antioxidants and other antiradical procedures to suppress aging and senescence or even to expand the life span of animals are considered.

2. INTRODUCTION

In 1956 Dr. Harman proposed free radical theory of senescence and aging (1). At that time, the structures of free radicals formed were mostly unknown and all free radicals were considered to be toxic species capable of destroying biomolecules and stimulating aging of living organisms. This hypothesis led to important practical conclusions pointing out for the first time at the possibility of age regulation by antioxidants capable of suppressing free radical formation.

Despite numerous subsequent works where the role of free radicals and antioxidants in aging has been studied, there are still different views concerning many important questions. But this is not surprising taking into account numerous factors regulating free radical formation in normal and aged cells and tissues as well as different functions of free radicals in various enzymatic processes. At present there are many reviews discussing free radical theory of aging. The latest ones are cited here (2-11). In this work we try do not discuss important studies performed with yeast, plants, worms, and fruit flies, which were already considered by many highly competent authors.

At the time of Harman's proposal of free radical theory of aging, the structure of free radicals in biological systems was mainly unknown. Discovery in 1968–1980 years of "physiological" free radicals superoxide $O_2^{\cdot-}$ and nitric oxide $(^{\cdot})NO$ completely changed this situation. It is now known that these radicals, being relatively unreactive and harmless by themselves, are the precursors of hydrogen peroxide and peroxynitrite, the compounds capable of forming highly reactive free radicals by the superoxide-driven Fenton reaction (Reactions 1 and 2) and the decomposition of peroxynitrite (Reactions 3 and 4):

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad [1]$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + HO^{-} \quad [2]$$
$$O_2^{\cdot-} + (^{\cdot})NO \rightarrow {}^{-}OONO \quad [3]$$
$${}^{-}OONO + H^{+} \rightarrow HO^{\cdot} + (^{\cdot})NO_2 \quad [4]$$

Thus, the overproduction of superoxide and NO can lead to the formation of reactive $HO^{\cdot}$ and $^{\cdot}NO_2$ radicals, the initiators of pathological disorders and aging. It is quite possible that an important factor of aging development is the disturbance of $O_2^{\cdot-}$/NO balance in cells, which is responsible for mitochondria dysfunction, the stimulation of apoptosis, and the other pathological changes typical for senescence and aging. All these effects of superoxide, nitric oxide, and $O_2^{\cdot-}$/NO unbalance will be considered below.

3. SUPEROXIDE IN AGING AND SENESCENCE

3.1. Enhanced production of superoxide by mitochondria in aged cells

In previous works superoxide has been considered as an only precursor of damaging reactive species responsible for the aging development because the discovery of nitric oxide has been made much later. In 1972 Harman proposed that the formed mitochondrially superoxide is an important determinant of aging development (12). A view on superoxide as the important initiator of aging has been discussed in numerous works, and now a more that 1900 works on this subject are cited by Medline.

Overproduction of superoxide in aging cells, tissues, animals, and humans has been widely demonstrated. Elevated superoxide production was showed in mitochondria, plasma membranes prepared from the brain, the heart, the liver, and peritoneal macrophages (13-15). Schreiber, *et al.* has found that superoxide production measured by lucigenin-amplified chemiluminescence (CL) in brain slices of old rats after reoxygenation was sharply increased while it was almost nonexistent in the youngest animals (16). Chung, *et al.* also found an increase in superoxide levels in the rat kidney with age supposedly due to the enhanced conversion of xanthine dehydrogenase into xanthine oxidase (a producer of superoxide) (17). Enhanced superoxide formation was found in aortas of aged rats (18).

Moon, *et al.* showed that primary culture smooth muscle cells (SMC) derived from aged mice decreased proliferative capacity in response to alpha-thrombin stimulation and generated higher levels of reactive oxygen species in comparison with the cells from younger mice (19). These effects were explained by dysregulation of cell cycle-associated proteins such as cyclin D1 and p27Kip1 in SMC from aged mice. Chen, *et al.* demonstrated that Leydig cells (the testicular cells responsible for synthesizing and secreting the essential steroid from old rats produced significantly greater levels of superoxide than those from young rats (20). Detection of superoxide by lucigenin-derived CL indicated that superoxide was produced by mitochondria. Enhanced oxygen radical production was also recently demonstrated in aged male and female mice (21).

3.2. Enhanced production of superoxide by NADPH oxidase and xanthine oxidase with aging

Besides of mitochondria there are two other major producers of superoxide, which can be responsible for superoxide enhancement in the age: NADPH oxidase and xanthine oxidase. It is still unclear, which one prevails in aging cells. Oudot, *et al.* found that the activity and expression of NADPH oxidase increased in rat aortas with the age that was associated with an increased release of reactive oxygen species in endothelial cells (22). On the other hand, Newaz, *et al.* concluded that the main producer of free radicals in aged rats was xanthine oxidase and not NADPH oxidase (23). Chaves, *et al.* suggested that there is a correlation between NADPH oxidase and protein kinase C during oxygen radical production by granulocytes from old humans (24). It is of interest that intracellular superoxide production by macrophages decreased but extracellular superoxide increased in old mice (25). Recently, Kozlov, *et al.* demonstrated that skeletal muscles, heart, and lung were main sources of oxygen radicals in old rats (26).

3.3. A decrease in superoxide dismutase activity with age

Another cause of the increased production of reactive oxygen species with age is a decrease in the activity of superoxide dismutases CuZnSOD and MnSOD (see, below). Thus Didion *et al.* demonstrated that loss of a single copy of the gene for (CuZnSOD) increased vascular superoxide levels and produced vascular dysfunction with aging (27). They found that vascular superoxide levels increased in aorta in wild-type (CuZnSOD (+/+) old mice and even more strongly in heterozygous CuZnSOD-deficient (CuZnSOD (+/-) old mice with aging. Aging significantly increased brain superoxide levels in normotensive female rats and even more in spontaneously hypertensive stroke prone female rats (28). In the last case an increase in the superoxide level might be associated with the down-regulation of Cu/ZnSOD. Weir and Robaire have showed that hydrogen peroxide and superoxide production increased significantly when SOD activity decreased in the rat aging spermatozoa (29).

Role of superoxide dismutase (SOD) in aging is undoubtedly a very important one. As early as in 1976, Kellogg and Fridovich showed that the activity of SOD, a major enzyme responsible for the suppression of superoxide levels by its dismutation to hydrogen peroxide and dioxygen, in brain and lung of long-living rats are

higher than in short-living rats (30). Later on, a correlation between SOD activity and a life-span has been shown for many animal species (31,32). Therefore, it might be suggested that a higher SOD activity is favorable for increased life-spans of living species.

In accord with this proposal, SOD activity, as a rule, decreases with aging. Reiss and Gershon found a decrease in SOD activity in liver, heart, and brain of aged rats and mice (33). A decrease in CuZnSOD was observed in the liver of rats between 6 and 29 months of age (34). The decrease was paralleled by the reduction of the levels of mRNA species coding and nuclear transcription of SOD genes. Life-long dietary restriction increased SOD expression in liver tissue from 18-month-old rats. It has also been demonstrated that SOD activity decreased in the brain of old rats (35).

Some studies demonstrate the different roles of SOD enzymes in aging. Park, *et al.*) showed that the activity of mitochondrial superoxide dismutase decreased in senescence-accelerated mice (36). Significant down regulation of MnSOD mRNA expression was observed in the hippocampus of aged rats (37). Similarly, Sun, *et al.* found the decreased activity of extracellular SOD in vessels of aged rats (38). At the same time, Van der Loo, *et al.* showed that the inhibition of CuZnSOD had no effect on superoxide production in aged rats although its expression decreased in an age-dependent manner (39). Furthermore, CuZnSOD lost its membrane association with increasing age and was relocated to the mitochondria, possibly to counter-balance age-associated oxidative stress. These findings can be important for understanding of redox mechanisms regulating age development.

3.4. A decrease in superoxide dismutase activities with the age in humans

The reduction of SOD activity with age has been also shown in humans. Thus, Pansarasa, *et al.* has shown that total SOD activity decreased significantly in human muscle samples obtained from hospitalized 66-75-year-old patients (40). It is interesting that the activities of two H_2O_2 detoxifying enzymes (glutathione peroxidase and catalase) were not changed with the age pointing out at the exclusive role of superoxide and not hydrogen peroxide in age development. Inal, *et al.* found that erythrocyte SOD was significantly lower in healthy 41-69 years old men comparing to younger men (41). Di Massimo, *et al.* determined a decrease in extracellular SOD activity in plasma of healthy men of 30-90 years (42,43). In another work it was also shown that SOD levels decreased in old men (74 – 98 years old) comparing to young subjects of 20 – 40 years old (44).

3.5. Controversy in the effects of aging on superoxide dismutase activity

Notwithstanding the above data, the changes of SOD activity with aging can be different in different cells and tissues. Although many works demonstrate the deterioration of SOD enzymes and decreasing their activities with the age, some authors found an increase in SOD activities with the age, probably as a response to the augmentation of oxidative stress. Thus Scarpa *et al.* (45) showed that the concentrations of CuZnSOD and MnSOD increased with the age in the brain of rats from 3 days before birth to the 30 months age (45). Increase in mitochondrial MnSOD has been also found in both subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) in the myocardium of old rats (46).

Barreiro, *et al. (*47) showed that the levels of reactive carbonyls, malondialdehyde-protein adducts, 3-nitrotyrosine, catalase, and MnSOD were significantly greater in the external intercostals of elderly subjects comparing to the young controls (47). It has been also shown that the activity of erythrocyte SOD in nonagenarians (persons 90 years old or between 90 and 100 years old) was higher than in the 80 year old subjects (48).

4. INTERPLAY BETWEEN SUPEROXIDE AND NITRIC OXIDE IN AGING AND SENESCENCE

Deteriorating effects of superoxide in the age (see, above) demonstrate an importance of superoxide regulation in aging and senescence. One of the major factors affecting superoxide levels in aging is a decrease in the activities of SODs. Another important factor is the deregulation of the $O_2^{\cdot-}$/NO balance. Although some findings suggest that nitric oxide itself can promote aging development, its principal effects undoubtedly depend on the interplay between NO and superoxide. The formation of peroxynitrite in the reaction of nitric oxide with superoxide (Reaction 3) is an important route to the formation of reactive oxygen and nitrogen species. However, it is not an only way to free radical-mediated damage in aging. In 1994 several groups of authors showed that nitric oxide is able to inhibit reversibly mitochondrial cytochrome *c* oxidase (49-52). These findings suggest that there is competition between dioxygen and nitric oxide, which regulates dioxygen consumption and the formation of reactive oxygen species. The subsequent studies showed, how interplay between superoxide and nitric oxide stimulated the aging development.

In 1995 Mollace, *et al.* showed that NO synthase activity, as expressed by citrulline and nitrite formation in brain homogenates, decreased in 24-month old rats comparing to young rats (53). Similar results have been obtained in the other works. Amrani *et al.* concluded that in rats, basal and stimulated release of nitric oxide by the coronary endothelium deteriorated with the age (54). Gerhard, *et al.* showed that endothelium-dependent vasodilation declined steadily with the increasing age in healthy human subjects supposedly due to a decrease in NO bioavailability (55).

The effects of age on NO production might be cell- and tissue-dependent. Tschudi, *et al.* found that the age differently altered endothelial NO release in rat aortas and pulmonary arteries: the initial rate of the NO release and the peak of NO concentrations significantly declined with the age only in aortas, while they increased in pulmonary arteries (56). Der Loo, *et al.* showed that levels of NO decreased in old rats, due to a 3-fold increase in

endothelial superoxide production despite a 7-fold increase in the expression of NO-produced enzymes (57). Chou, *et al.* found that aging reduced the activity of endothelial nitric oxide synthase (eNOS) in old rats but not in old spontaneously hypertensive (SHR) rats (58). Smith and Hagen suggested that the reduction of endothelial-derived nitric oxide led to a loss of vasomotor function of the major conduit arteries due to the age-related alterations in eNOS (59). They found that the levels of eNOS phosphorylation in aortas from aged rats were almost 50% lower, than those in aortas from young animals. Lower eNOS phosphorylation apparently depended on a loss of constitutive Akt/protein kinase B activity. Although neuronal NO release increased in mesenteric arteries from old spontaneously hypertensive rats, Ferrer, *et al.* has shown that NO bioavailability decreased due to the simultaneously enhanced superoxide production and peroxynitrite formation with the age (60).

Csiszar, *et al.* found that flow-induced NO-mediated dilation of coronary arterioles was significantly diminished in aged rats due to increased superoxide production (61). It was suggested that the decreased expression of eNOS, the increased activity of NADPH oxidase, and the increased expression of inducible NO synthase (iNOS) resulted in the enhancement of peroxynitrite formation. Di Massimo, *et al.* concluded that progressive reduction of plasma NO availability in healthy old men depended on a decrease in extracellular SOD activity (42,43). It has been shown that in mouse carotid arteries the aging-induced impairment of NO reactivity is due to increased superoxide formation (62). Sun, *et al.* concluded that an increased production of superoxide, the reduced activity of SOD, and an impaired shear stress-induced activation of eNOS are the causes of decreased shear stress-induced release of NO in vessels from aged rats (38). Woodman, *et al.* showed that aging impaired vasodilator responses in soleus muscle feed arteries by attenuating NO- and prostacyclin-mediated, endothelium-dependent dilation in old rats (63).

As it was noted above, NO is able to inhibit reversibly mitochondrial cytochrome *c* oxidase and by this to regulate dioxygen consumption and the formation reactive oxygen species. Regulation of the activity of this enzyme by nitric oxide could be of utmost importance in aging. In 2003 Adler, *et al.* demonstrated that the NO-mediated control of cardiac dioxygen consumption by bradykinin or enalaprilat was markedly reduced in 23-mo-old Fischer rats (64). In subsequent work these authors showed that NO availability decreased in the aging kidney due to scavenging of NO by superoxide produced by NADPH oxidase (65).

5. CYCLOOXYGENASE-CATALYZED FREE RADICAL OVERPRODUCTION IN THE AGE

As is seen from the above, several major enzymes are responsible for superoxide and nitric oxide production in the age: mitochondrial respiratory chain, xanthine oxidase, NADPH oxidases, and NO synthases. However, the other prooxidant enzymes might also contribute to aging development through the production of reactive oxygen and nitrogen species. Among them are the enzymes cyclooxygenase-1 and cyclooxygenase-2, which can produce free radicals as intermediates during their catalytic cycles. Some works described an increase in the formation of cyclooxygenase products prostaglandins and prostanoids in old animals. Roberts and Reckelhoff demonstrated a sharp increase in isoprostane levels in plasma and plasma lipids in aged rats (66). Mukai, *et al.* showed that the inhibitors of cyclooxygenase-2 (COX-2) restored the enhanced endothelium-dependent relaxation in aortas from aged rats suggesting, an involvement of COX-2-derived vasoconstricting eicosanoids in aging development (67). Kim, *et al.* showed that the levels of prostaglandins E(2) (PGE (2)) and PGI (2) and thromboxane A(2) (TXA (2)) were elevated in old rats (68). It should be mentioned that an earlier work by Yamamoto, *et al.* is of a special interest because it demonstrated a correlation between the life spans of humans and rats and the formation of nonenzymatic peroxidation products (69). The content of these products increased in the range: humans < Sprangue-Dawley rats < Nagase analbuminetic rats and correlated well with the life spans of humans and rats.

6. GENE REGULATION OF FREE RADICALS IN THE AGE

In 1999 Migliaccio, *et al* concluded that no genes are known to increase individual life span (70). However they demonstrated that targeted mutation of the mouse *p66shc* gene, a cytoplasmic signal transducer involved in the transmission of mitogenic signals, induced stress resistance and prolonged life span. Thus, p66shc could be a part of the signal transduction pathway that regulates stress apoptotic responses and life span in mammals. Later on, it has been shown that there is a decrease in superoxide production and an increase in life span (about 30%) in old mice lacking *p66shc* (p66shc-/-) in comparison with control animals (71). Another gene regulating the aging development is the pro-apoptotic gene *gadd153/chop*, which is elevated in liver of old rats (72).

At present, significant attention has been drawn to the discovery of the aging-suppressor gene *klotho*. Because high expression of *klotho* gene in the brain, it was proposed that *klotho* gene is involved in the regulation of brain aging. Furthermore, it has been shown that *klotho* gene presents in the blood and that its serum level decreased with age in humans from 0 to 91 years. Thus, these findings suggest that *klotho* gene is a serum factor related to human aging (73).

Experimental findings showed that klotho protein might be involved in the regulation of antioxidative defense. Saito, *et al*. showed that the free radical scavenger (T-0970), which effectively decreased plasma levels of 8-epi-prostaglandin, suppressed angiotensin II-induced downregulation of *klotho* (74). Nagai, *et al.* suggested that oxidative stress had a crucial role in the aging-associated cognition impairment in *klotho* mutant mice (75). Mitobe, *et al.* demonstrated that oxidant stress injury by hydrogen

peroxide dose-dependently reduced *klotho* expression in mice (76). In 2005 it has been shown (77,78) that *klotho* is indeed an aging suppressor gene, which is able to extend lifespan when overexpressed in mice. It was also found that klotho protein increased resistance to oxidative stress at the cellular and whole organism levels in mammals. Klotho protein activated the FoxO forkhead transcription factors that are negatively regulated by insulin/IGF-1 signaling, thereby inducing expression of MnSOD. This in turn facilitated removal of reactive oxygen species and increased oxidative stress resistance. Ikushima, *et al.* showed that *klotho* overexpression in mice decreased hydrogen peroxide-induced apoptosis in endothelial cells (79). Caspase-3 and caspase-9 activities were lower in klotho-treated HUVEC than in control cells. It was also found that klotho protein interfered with H_2O_2-induced premature cellular senescence. Authors concluded that *klotho* acts as a humoral factor capable of reducing apoptosis and cellular senescence in vascular cells.

7. FREE RADICAL-MEDIATED DAMAGE IN THE AGE THROUGH SIGNALING BY PROTEIN KINASES

Involvement of enzymatic signaling by protein kinases in the age development might be a one of the important mechanisms of free radical-mediated damage. Mechanisms of free radicals formation by protein kinases, which mostly catalyze heterolytic processes, will be considered below; here we will look at published experimental findings. It has been suggested that two protein kinases, the extracellular signal-regulated kinase (ERK) and Akt/protein B kinase are important signaling molecules, which protect against free radical damage in the age. Thus Ikeyama, *et al.* showed that lower survival of rat old hepatocytes was associated with the reduced activation ERK and Akt kinase (80). This conclusion was supported by findings that the inhibition of ERK and Akt activities in young cells markedly increased their sensitivity to hydrogen peroxide; at the same time caloric restriction increased the life span of rats. Smith and Hagen showed that a loss of Akt/protein kinase B activity caused a decrease in eNOS phosphorylation in aortas from old rats (59). Jin, *et al.* also demonstrated that PI3-kinase activity and Akt phosphorylation (PI3-kinase/Akt pathway) were significantly reduced in the kidney of old rats after treatment with the superoxide producer menadione (81). In contrast, the activities of the other mitogen-activated protein kinases (MAPK) JNK1 and AMPK were higher in old than in young animals.

Rice, *et al.* found that signaling by mitogen activated protein kinases (MAPKs) was simultaneously enhanced with superoxide overproduction in aortas from old rats (82). GM-CSF-dependent ERK signalling pathway turned out to be defective in neutrophils in elderly humans. A decline in ERK1/2 activation could be a source of the GM-CSF-dependent impairment of neutrophil respiratory burst with the age (83).

As is seen from the above data, the activity of Akt/protein kinase B could be considered a positive factor in fighting the age development and senescence. However, the situation is not so simple because some findings contradict this conclusion. Thus Miyauchi, *et al.* showed that in their experiments the inhibition of Akt activity extended a lifespan of primary cultured human endothelial cells in contrast to the other data, which demonstrated that the activation of Akt promoted proliferation and survival of mammalian cells (84), These authors suggested that constitutive activation of Akt promoted senescence-like arrest of cell growth via a p53/p21-dependent pathway and inhibition of forkhead transcription factor FOXO3a through the regulation of reactive oxygen species. Negative effects of Akt/Foxo1 pathway in the regulation of longevity were also showed in long-lived growth hormone receptor knockout mice (85). Li, *et al.* found that Akt activity increased in vascular smooth muscular cells from old rats that might cause the phosphorylation and inactivation of FOXO3a and down-regulation of MnSOD transcription (86).

8. FREE RADICAL-MEDIATED APOPTOSIS IN THE AGE

Most scientists are agreeing that apoptosis, or programmed cell death, increases during the age, although there are contradictory data showing that long-living mice are characterized by the enhanced level of apoptosis (87,88). Role of free radicals in apoptotic processes is also well documented. Thus Kokoszka, *et al.* showed that mitochondrial production of reactive oxygen species, oxidative stress, functional decline, and the initiation of apoptosis appear to be central components of aging in normal mice and in the mice with partial or complete deficiencies in the mitochondrial antioxidant enzyme manganese superoxide dismutase (MnSOD) (89). Phaneuf and Leeuwenburgh suggested that oxidative stress is an apoptosis-inducing signal, which could increase in the aging rat heart (90). They found that cytosolic cytochrome *c* content was significantly elevated in old animals. Furthermore, Bcl-2, an antiapoptotic protein, strongly decreased with age, whereas Bax, a proapoptotic protein, remained unchanged. It was found that aged rats exhibited an increase in the ratio Bax mRNA/Bcl-2 mRNA and cardiomyocyte apoptosis in a rat model of myocardial ischemia-reperfusion (MI/R) (91). Hoffmann, *et al.* showed that aging of endothelial cells is associated with decreased NO synthesis and increased sensitivity to apoptosis (92).

9. MECHANISMS OF FREE RADICAL-MEDIATED DAMAGE IN AGING AND SENESCENCE

9.1. Mitochondria as a source of aging development

At present, the most popular free radical theory of aging is based on the Harmon mitochondrial hypothesis. He supposed that mtDNA mutations, which are accumulated progressively during life are directly responsible for a deficiency in cellular oxidative phosphorylation activity, leading to an enhanced oxygen radical production. In its turn, increased ROS production leads to the increased rate of mtDNA damage causing a "vicious cycle" of exponentially increasing oxidative damage and mitochondrial dysfunction (93). Thus, this hypothesis highlights an importance of leak of superoxide,

a product of dioxygen one-electron reduction, from the normal two-electron reduction of dioxygen by mitochondrial respiratory chain. Until now, this hypothesis remains an important one, although it might overestimate the role of superoxide production by mitochondria because its real levels under both physiological and pathophysiological conditions are still uncertain. In addition, the last data seem to cast doubts at direct relationship between mtDNA damage and aging (95).

Despite possible effects of mtDNA mutations on aging development, there is no reason to believe that it is an only cause of free radical-mediated damage in mitochondria. Thus Miro, *et al.* found a progressive, significant increase of heart membrane lipid peroxidation with aging in the hearts from human donors (95). Conversely, neither absolute nor relative enzyme activities of complex I, II, III and IV of mitochondrial respiratory chain decreased with age.

Recent studies demonstrate significance of competition between superoxide and nitric oxide in the reaction with cytochrome *c* oxidase leading to superoxide overproduction, mitochondrial damage, and cell death. It is reasonable to suggest that a decrease in NO levels with the age will shift the equilibrium of Reaction 5 to the right and correspondingly increase dioxygen consumption (96-98):

cytochrome oxidase (NO) + O_2 ⇔ cytochrome oxidase (O_2) + NO [5]
NO ↓⇒ O_2 consumption ↑

An increase in dioxygen consumption can lead to the enhanced electron leak from mitochondrial carriers and the overproduction of superoxide:

O_2 consumption ↑ ⇒$O_2^{\cdot-}$ ↑

On the whole, the reduction of nitric oxide levels can initiate a new "vicious" cycle of the formation of reactive oxygen and nitrogen species and a decrease in nitric oxide availability in the age:

NO ↓ ⇒ O_2 consumption ↑ ⇒ $O_2^{\cdot-}$ ↑ ⇒ NO ↓

The well known ways of superoxide-mediated damage are the superoxide-dependent Fenton reaction (Reactions 1 and 2) and the interaction with NO (Reactions 3 and 4). However, we recently suggested a new mechanism of superoxide-initiated damage in the age through the activation uncoupling protein 2 (UCP2) and the stimulation of apoptosis (96,99,100). It has been shown that superoxide is able to activate UCP2 after the stimulation of proton leak under hyperglycemic conditions in cells (101). Echtay, *et al.* showed that UCP2 activation depended on ubiquinone (coenzyme CoQ), an obligatory cofactor of proton transport (102). We suggested that superoxide reduces the ubiquinone molecule bound to protein into a negatively charged semiquinone radical anion, which scavenged a proton and stimulated proton leak (96):

$O_2^{\cdot-}$ + Q(UCP2)⇒O_2 +$Q^{\cdot-}$(UCP2) [6]

$Q^{\cdot-}$ (UCP2)+H^+ ⇒HQ($^{\cdot}$)(UCP2) [7]

Thus the activation of UCP2 protein by superoxide leads to the enhanced proton leak and the inhibition of oxidative phosphorylation in mitochondria.

Another way to damaging effects in age development due to superoxide overproduction is the initiation of apoptosis. The ability of superoxide to initiate apoptosis without hydrogen peroxide or peroxynitrite points out at the existence of a unique superoxide-mediated proapoptotic pathway (103,104). This pathway cannot include the oxidation of phospholipids as it sometimes was proposed because superoxide is not an oxidant. But superoxide is able to reduce biomolecules having one-electron reduction potentials more than -0.16 V (the reduction potential of dioxygen) such as cytochrome *c*. It has been shown that superoxide and not hydrogen peroxide or peroxynitrite induced rapid cytochrome *c* release from mitochondria (105). There are two membrane complexes of cytochrome *c* in mitochondria existing as an electrostatically loose-bound conformation and a tight complex with partial embedding of cytochrome into the membrane. We suggested that the initiation of apoptosis by superoxide begins by the reduction of electrostatically bound cytochrome *c via* Reaction 8 (99):

$O_2^{\cdot-}$ + cyt.c $^{3+}$ ⇒ O_2 +cyt.c $^{2+}$ [8]

Correspondingly, the following mechanism of superoxide-induced cytochrome *c* release can be proposed:

$O_2^{\cdot-}$ +(bound)cyt.c^{3+}⇒O_2+ (bound)cyt.c^{2+}[9]
$O_2^{\cdot-}$ +(bound)cyt.c^{2+}⇒(bound)cyt.c^{2+}($O_2^{\cdot-}$)[10]
Release to cytosol
(bound)cyt.c^{2+}($O_2^{\cdot-}$) ⇒ cyt.c^{2+}($O_2^{\cdot-}$) [11]
cyt.c^{2+}($O_2^{\cdot-}$)⇔ cyt c^{2+} + $O_2^{\cdot-}$ [12]

9.2. Superoxide-mediated enzymatic catalysis

There are various enzymatic producers of superoxide and nitric oxide in cells such as xanthine oxidase, NADPH oxidases, peroxidases, and NO synthases in addition to mitochondria (see, above), therefore mitochondria cannot be a only origin of free radical damage in the age. We already discussed experimental findings concerning the participation of superoxide in aging through the catalysis by protein kinases and phosphatases. Now we will consider the possible mechanisms of these reactions.

Catalysis by superoxide of enzymatic heterolytic reactions of etherification and hydrolysis is a consequence of superoxide "super-nucleophile" properties. In accord with the established mechanism protein kinases (PK) catalyze phosphorylation through the interaction of phosphorylated enzyme with the threonine or serine residues of a protein:

PK-O(phos) +HO(Ser)protein⇒ PK-OH + (phos)O(Ser)protein[13]
PK-O (phos) +HO(Thr)protein⇒ PK-OH + (phos)O(Thr)protein[14]

Reactions 13 and 14 are heterolytic nucleophilic processes, in which protons of the serine or threonine residues are substituted by phosphate groups. As the pKa values for aliphatic hydroxyls of serine or threonine are very high, the strong nucleophile is needed to catalyze these reactions. It is quite possible that there are no other intermediates in biological systems with so strong nucleophilic properties as superoxide, which is even a stronger nucleophile than the hydroxyl anion HO^-. Therefore, we suggested that a major role of superoxide in these phosphorylation processes is deprotonation of the serine and threonine residues that should sharply increase the rates of Reactions 13 and 14 (106-108):

$$HO(Ser)X \text{ or } HO(Thr)X + O_2^{\cdot -} \Rightarrow {}^-O(Ser)X \text{ or } {}^-O(Thr)X + HOO^{\cdot} \quad [15]$$

Reaction 15 is always followed by electron transfer reaction between superoxide and perhydroxyl radical $HOO^{\cdot}$.

$$HOO^{\cdot} + O_2^{\cdot -} \Rightarrow HOO^- + O_2 \quad [16]$$

Accordingly, superoxide signaling will sharply accelerate Reactions 13 and 14 because the substitution reactions 17 and 18 with protein anions proceed with much greater rates than with neutral molecules:

$$PK\text{-}O(phos) + {}^-O(Ser)protein \Rightarrow PK\text{-}O^- + (phos)O(Ser)protein \quad [17]$$

$$PK\text{-}O(phos) + {}^-O(Thr)protein \Rightarrow PK\text{-}O^- + (phos)O(Thr)protein \quad [18]$$

Experimental findings considered above suggest that many enzymes can be responsible for superoxide overproduction in the age. As it was showed above, the activation of protein kinases Akt/B and ERK might be protective against free radical damage in aging (59, 80-83). Thus, the overproduction of superoxide in the age could in some cases be a favourable factor due to the enhancement of activation of these enzymes. These data make more complicated already very complex picture of interplay between superoxide and NO in aging. However, it should be mentioned that not all authors agreed with conclusions about protective role of protein kinases Akt/B and ERK in the age (84-86).

10. WHAT COULD BE PRIMARY CAUSES OF AGING AND SENESCENCE DEVELOPMENT?

All free radical theories of aging agree that the starting point of aging development is the overproduction of reactive oxygen and nitrogen species. There are numerous origins of this phenomenon: pathologies associated with free radical overproduction, environmental contamination, irradiation, etc. However, we know that all human beings are mortal and will certainly die even the absence of any external damaging factors. Therefore, we should ask: is it possible to find the origins of free radical-initiated physiological aging in the hypothetical conditions without external damage?

We believe that important causes of physiological aging could be the diet components responsible for the initiation of free radical-mediated damage (97). It is not a question of good or bad diet – any diet contains some damaging compounds. Therefore, it is not by chance that caloric restriction (CR) is a known therapeutic intervention capable of attenuating aging in mammals (see, below). Unsaturated acids are probably the most abundant oxidisable food components. In the past, it was erroneously believed that these unsaturated acids are just the products of lipid peroxidation. Now, it is known that they have a dietary origin and are important components of human diet. They are easily oxidized into prostaglandins and isoprostanes in living organism. These products of enzymatic and nonenzymatic lipid peroxidation are highly toxic compounds, and their formation under physiological conditions may lead to the start of aging. (Recent publication by Aitken, *et al.* points out at the possibility of regulation of MnSOD, the primary defense enzyme against superoxide by nutrient sensing through MnSOD expression mediated by essential amino acid depletion (132)).

11. ANTIOXIDANT TREATMENT AGAINST AGING AND SENESCENCE; POSSIBILITY OF LIFESPAN ENLARGEMENT

Free radical mechanisms of aging and senescence point out at the possibility of antioxidant treatment for suppression of aging development and even the enhancement of lifespan of humans and animals. It is true that experimental findings are not always encouraging, but there are definite results showing principal possibility to affect aging and life span by the use of antioxidants. The application of classic antioxidants and special treatment of aged organisms capable of increasing organism's antioxidant defense have been described (109). Now we will discuss the most important examples of antioxidant treatment of aging processes.

11.1. Calorie restriction

Calorie restriction (CR) feeding most frequently applied as a mean of reducing mammal aging. It has been proposed that CR feeding slows the rate of oxidative damage due to a decrease in mitochondrial superoxide generation (110). Hall, *et al.* showed that CR reduced cellular injury and improved heat tolerance in old rats by lowering radical production and preserving cellular ability to adapt to stress through antioxidant enzyme induction (111). No age-associated increase in mitochondrial protein, lipid peroxidation, or in superoxide generation was detected in calorically-restricted old mice (112). CR enhanced the transcripts of genes involved in reactive oxygen radical scavenging function, tissue development, and energy metabolism such as cytochrome c oxidase III, superoxide dismutases SOD1, and SOD2 in old rats fed with calorie-restricted diet (60% of control diet) for 36 weeks (113). Zou, *et al.* (114) found that CR suppressed the elevated level of superoxide generating xanthine oxidase in serum of old rats (114). Important findings have been recently obtained by Nisoli, *et al.* who found that CR for 3 or 12 months induced endothelial nitric oxide synthase (eNOS)

expression in various tissues of male mice (115). Several other works on antioxidant mechanisms of CR effects on aging development were also cited above.

11.2. Antioxidants

Two classes of antioxidants can be used for the suppression of aging development: free radical scavengers capable of direct reacting with reactive free radicals through H-abstraction (for example vitamins E and C and flavonoids) and compounds capable of oxidizing free radicals by one-electron transfer mechanism (SOD mimics and ubiquinones) (97). There is a great number of works, in which the effects of vitamins E and C on aging and senescence have been studied; obviously, their consideration is out of the limits of this work. We will look only at the most interesting examples.

It has been proposed that vitamin E (α-tocopherol) exhibits favorable action on aging processes and even able to expanse the life span of experimental animals. As early as in 1996, Poulin, *et al.* showed that high vitamin E diet prevented aging-related decline in lymphocytes and brain (116). Reckelhoff, *et al.* found that vitamin E decreased renal lipid peroxidation and the accumulation of F2-isoprostanes in aged rats (117). Probably the most promising results were obtained by Navarro, *et al.* who found that high doses of vitamin E improved neurological performance, brain mitochondrial function, and survival in aged mice (4,118). The median life span was increased by 40% and maximal lifespan by 17% in aged male mice. It should be mentioned that in earlier work the supplementation with vitamin E failed to diminish oxidative damage in old mice (119).

Despite numerous works on vitamin C, its effects on aging development are uncertain and the administration of vitamin C sometime even leads to negative outcome. For example, life-long vitamin C supplementation in combination with cold exposure did not affect oxidative damage or lifespan in mice and even decreased the expression of antioxidant genes (120).

Other antioxidants, for example α-lipoic acid, coenzyme Q10 (ubiquinone 10), and metallothioneins, were also applied in aging studies with various degrees of success (121-123). Special interest was drawn to (-)deprenyl (selegiline) (N-methyl-N- (1-methyl-2-phenyl-ethyl)-prop-2-yn-1-amine), a selective MAO-B inhibitor, which supposedly exhibited exclusively high anti-aging effects in experimental animals. In 1988 Knoll discovered that (-)deprenyl treatment of rats resulted in an increase in the average lifespan up to 197.98±2.36 weeks, i.e. higher than the estimated maximum age of death in the rat (182 weeks) (124). This effect of (-)deprenyl could be due to its antioxidant properties and an increase in SOD activity in the striatum. Kitani, *et al.* found that (-)deprenyl administration sharply increased the activities of CuZnSOD, MnSOD, and catalase in striatum and substantia nigra as well as life expectancy for old male rats (125). Archer and Harrison also observed an increase in the lifespan of old mice after treatment with (-)deprenyl (126). At present the antioxidant properties of (-)deprenyl are considered to be major factors of its anti-aging activity in animals and possibly in humans (127).

Antioxidant enzymes (SOD and catalase) could be, in principle, even more effective inhibitors of aging comparing to the other pharmaceutical agents capable of expanding of lifespan. There are two possibilities of the enhancement of activities of these enzymes: the overexpression of enzymes and transfection into experimental animals or the application of low-molecular enzyme mimics. In 1994 Orr and Sohal demonstrated that the lifespan of Drosophila melanogaster might be increased by the overexpression of superoxide dismutase and catalase (32). Recently Schriner, *at al.* showed that median and maximum lifespans were maximally increased in transgenic mice with overexpressed human catalase localized to the peroxisome, the nucleus, or mitochondria (128). Melov, *et al.* used small synthetic superoxide dismutase/catalase mimetics for the treatment of Caenorhabditis elegans (129). They found that the treatment of wild-type worms with mimetics increased their mean lifespan by a mean of 44 percent. Zhang, *et al.* showed that chronic systemic administration of the SOD)/catalase mimetic (EUK-189) prevented heat stress-induced liver injury by decreasing oxidative damage in aged rats (130). Quick, *et al.* administered a small-molecule synthetic SOD mimetic to wild-type middle age mice (131). They found that chronic treatment with SOD mimetic not only reduced age-associated oxidative stress and mitochondrial radical production, but significantly extended lifespan of mice.

12. CONCLUSIONS

Are there hopes for longer longevity in humans? This question is of course of utmost importance for everybody, and all scientific hypotheses and theories are supposed to answer this question and possibly to give some hope. Free radical theory is no exception. I think that major benefits of this theory before the others possibly equally important are comprehension of detailed mechanisms of pathological events in the age mediated by free radicals. Furthermore, understanding of importance of regulation of the balance between the physiological radicals superoxide and nitric oxide, which are metabolites of normal physiological processes and always presented in living organisms, might encourage the appearance of novel ideas and new treatments.

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