ELSEVIER

Molecular Aspects of Medicine 25 (2004) 37–48

MOLECULAR ASPECTS OF MEDICINE

www.elsevier.com/locate/mam

Review

Mitochondrial enzyme activities as biochemical markers of aging

Ana Navarro *

Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Cádiz, 11003 Cádiz, Spain

Abstract

The decrease of neurological performance in normal aging is directly related to brain oxidative stress and inversely related to lifespan. Male mice lifespan was increased by 8–10% (median and maximal lifespan, respectively) in mice with high spontaneous neurological activity, by 21–15% after moderate exercise; and by 25–20% after supplementation with vitamin E. Oxidative stress markers, TBARS and protein carbonyl content, were found increased on aging; a higher content of oxidation products is considered an effective aging factor, specially in the brain, with a majority of postmitotic cells. Mitochondrial enzyme activities, mitochondrial nitric oxide synthase (mtNOS), NADH dehydrogenase and cytochrome oxidase, behaved as markers of brain aging. The decrease in enzyme activities was directly related to the content of oxidation products and to the loss of neurological function in aged mice, this latter was determined in the tighrope and the T-maze tests. The above mentioned conditions that increased mice lifespan were effective to decrease the level of oxidative stress markers, and to retard the decreases in mitochondrial enzyme activities and neurological function associated to aging. The activities of mtNOS, NADH dehydrogenase and cytochrome oxidase may be used as indicators of the effectiveness of antiaging treatments.

Abbreviations: Arg, arginine; Cit, citrulin; FMN and FMNH, oxidized and semiquinone forms of flavin mononucleotide; GSH, glutathione; $HO^{\cdot}$, hydroxyl radical; H_2O_2, hydrogen peroxide; Mn-SOD, manganese superoxide dismutase; mtNOS, mitochondrial nitric oxide synthase; NO, nitric oxide; $ONOO^-$, peroxynitrite; $NADPH_2$, reduced nicotine adenine dinucleotide phosphate; O_2^-, superoxide anion radical; $^1O_2$, singlet oxygen; $ROO^{\cdot}$, peroxyl radical; TBARS, thiobarbituric acid reactive substances; UQ, $UQH^{\cdot}$, and UQH_2, oxidized, semiquinone and reduced forms of ubiquinone

Keywords: Aging; Oxidative stress; Neurological activity; Moderate exercise; Vitamin E; mtNOS; NADH-dehydrogenase; Cytochrome oxidase

* Tel.: +34-956-015231; fax: +34-956-015230.
E-mail address: ana.navarro@uca.es (A. Navarro).

0098-2997/$ - see front matter
doi:10.1016/j.mam.2004.02.007

Contents

1. Aging and the mitochondrial free-radical hypothesis of aging

Aging is a process with a general decline of physiological functions, with a more marked effect on brain functions, such as neuromuscular coordination, cognitive performances and environmental awareness. The decrease in neurological activities of normal aging has been found directly related to brain oxidative stress (Forster et al., 1996; Navarro et al., 2002), and inversely related to lifespan (Navarro et al., 2002).

The free radical theory of aging emerged from the views of Gerschman et al. (1954) that postulated that oxygen free radicals were the common molecular mechanism of oxygen and radiation toxicity, and of Harman (1956) that considered that free radicals generated as byproducts of biological oxidations produce random and cumulative cellular damage leading to tissue and organ aging. The association between aging and mitochondria is based upon the role of the organelles in furnishing cell energy and the physiological decline of organ energy expenditure on aging (Harman, 1972; Miquel and Fleming, 1986; Sohal, 1993). The mitochondrial hypothesis of aging considers mitochondria as the pacemaker of tissue aging due to continuous mitochondrial production of reactive oxygen and nitrogen species (O_2^-, H_2O_2, NO, $ONOO^-$, $HO^\cdot$, $ROO^\cdot$, and $^1O_2$). It is understood that the reactive species, although kept in low steady state concentrations by antioxidant enzymes, reductants and quenchers, are able to react and damage biomolecules leading to cumulative oxidative damage (Chance et al., 1979).

Mitochondria are the main intracellular source of oxidizing free radicals; two free radicals, O_2^- and NO, are primarily and continuously produced in these organelles. Other reactive species such as H_2O_2, $ONOO^-$, $HO^\cdot$, $ROO^\cdot$, and $^1O_2$ derive from the primary production of O_2^- and NO. The mitochondrial electron transfer chain is the main source of O_2^- in aerobic mammalian cells (Chance et al., 1979). The two main

reactions producing O_2^- are: (a) the autooxidation of ubisemiquinone, known as the Boveris–Cadenas reaction (reaction (1)) (Boveris and Cadenas, 1975; Boveris et al., 1976; Cadenas and Boveris, 1980), that accounts for about 75% of mitochondrial O_2^- production and (b) the autooxidation of the flavin semiquinone of NADH dehydrogenase, that accounts for about 25% of the total mitochondrial O_2^- production (reaction (2)) (Turrens and Boveris, 1980).

$$UQH^{\cdot} + O_2 \rightarrow UQ + H^+ + O_2^- \quad (k = 8 \times 10^3\ M^{-1}\,s^{-1}) \tag{1}$$

$$FMNH^{\cdot} + O_2 \rightarrow FMN + H^+ + O_2^- \tag{2}$$

Reaction (1) releases O_2^- towards both sides of the inner mitochondrial membrane: to the matrix side (N side) where it encounters matrix Mn-SOD (Boveris and Cadenas, 2000) and to the intermembrane space from where it can be released to the cytosol through a voltage-dependent anion channel (Han et al., 2003).

A few years ago, Ghafourifar and Richter (1997) and Giulivi et al. (1998) originally described the mitochondrial production of NO. A specialized mitochondrial nitric oxide synthase (mtNOS) (Elfering et al., 2002) carries out a classic NOS reaction (reaction (3)) that requires $NADPH_2$ ($K_M = 15$ μM), arginine ($K_M = 6$–12 μM), O_2 ($K_M = 37$–73 μM), and Ca^{2+}/calmodulin for enzyme activity (Tatoyan and Giulivi, 1998; Boveris et al., 2002; Alvarez et al., 2003).

$$Arg + NADPH_2 + O_2 \rightarrow Cit + H_2O + NO \tag{3}$$

Both free radicals, O_2^- and NO, are metabolized into the mitochondrial matrix through the formation of relatively stable and non-radical species, H_2O_2 and $ONOO^-$ (Poderoso et al., 1999). However, these species are also potentially harmful; they yield, after homolytic splitting, $HO^{\cdot}$ (Radi, 1998), a strong oxidant which abstracts hydrogen atoms and initiates the free radical reactions of lipoperoxidation. This free radical process, with initiation, propagation, inhibition and termination reactions, generates $ROO^{\cdot}$ and $^1O_2$. The species H_2O_2 and $ONOO^-$ are detoxified by specific enzymes and reductants in the mitochondrial matrix. Hydrogen peroxide is decomposed by glutathione peroxidase in the mitochondrial matrix (catalase has also been reported as an intramitochondrial enzyme; Radi et al., 1991) and by cytosolic catalase and glutathione peroxidase after diffusion to the cytosol. Peroxynitrite is effectively reduced by $NADH_2$, UQH_2 and GSH in the mitochondrial matrix (Valdez et al., 2000).

The increased steady state concentrations of any of the reactive oxygen or nitrogen species constitutes the chemical basis of the biological situation of oxidative stress. The persistence of an oxidative stress situation is considered an effective aging factor.

2. Mice survival and the effect of selected conditions

There is a current and active interest in the changes in mitochondrial properties that occur during aging (Trounce et al., 1989; Benzi et al., 1992; Hayakawa et al.,

1993; Martinez et al., 1994; Sohal et al., 1994; Nakahara et al., 1998; Sastre et al., 1998; Beckman and Ames, 1998a,b; Vina et al., 2003; Chomyn and Attardi, 2003), an interest that extends to antioxidant treatments, to conditions that retard the decline of mitochondrial functions associated to aging, and to the development of animal models for human aging. Mice and rats are commonly used for the convenience of their relative short lifespan, which are, depending on the strains, in the range of 65 to 150 weeks (Finch and Pike, 1996; Finch and Tanzi, 1997). Rhesus monkeys are also used; the model is closer to humans but it is slower and more expensive (Ramsey et al., 2000).

The kinetics of aging are described by survival curves (Finch and Pike, 1996). Fig. 1 shows that male mice, either with high spontaneous neurological activity, or subjected to moderate exercise, or supplemented with vitamin E, show increased median and maximal lifespan. Male mice show 10–15% more effect than females for the same treatments and conditions (Navarro et al., 2002; Navarro et al., 2004).

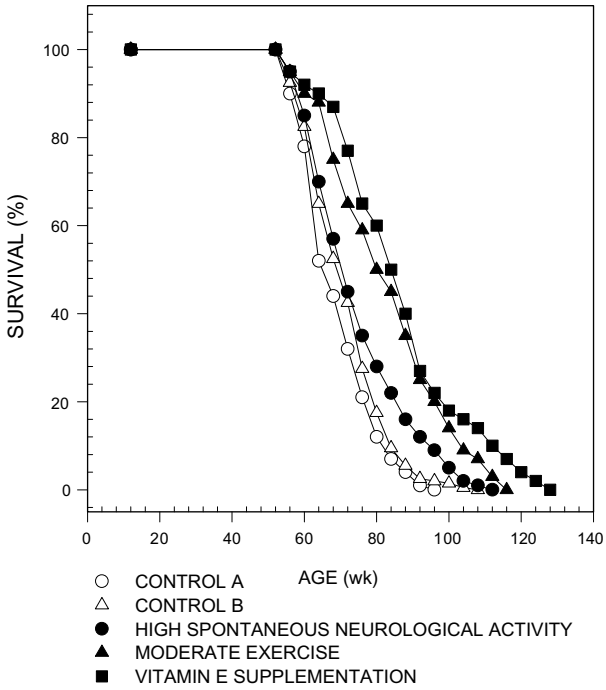

Fig. 1. Male mice survival curves (40 mice in each group). Median lifespan: control A (for high spontaneous neurological activity), 65 ± 2 weeks; control B (for moderate exercise and vitamin E supplementation), 67 ± 2 weeks; high spontaneous neurological activity, 71 ± 2 weeks ($p < 0.05$); moderate exercise, 81 ± 2 weeks ($p < 0.05$); vitamin E supplemented, 84 ± 2 weeks ($p < 0.05$). Maximal lifespan (intersection of the main slope with abscissa): control A, 80 ± 2 weeks; control B, 85 ± 2 weeks; high spontaneous high neurological activity 88 ± 2weeks ($p < 0.05$); moderate exercise, 98 ± 2 weeks ($p < 0.01$); vitamin E supplemented, 102 ± 3 weeks ($p < 0.05$).

Mice with high spontaneous neurological activity, selected according to their performance in the tightrope (neuromuscular coordination) and the T-maze (exploratory activity) tests, showed 8% and 10% increased median and maximal lifespan as compared with their controls (Fig. 1; Navarro et al., 2002). Moderate exercise, imposed on mice aged 28 weeks as weakly training in a treadmill (10, 15 and 20 cm/sec for 5 min each) for the entire mice lifespan, increased 21% and 15% the median and maximal lifespan (Fig. 1; Navarro et al., 2004). Vitamin E supplementation (4.3 g *d*, *l*-α-tocopherol acetate/kg of mice food) from 28 weeks of age and for the entire mice life, increased 25% and 20% the median and maximal lifespan (Fig. 1). The used level of vitamin E in the diet corresponds to a daily intake of about 10 mg vitamin E/mouse. The normal vitamin E content in commercial laboratory food for rodents is about 30–50 mg/kg; in vitamin E supplementation these values are increased to the range of 400–5000 mg/kg. Morley and Trainor (2001) reported that vitamin E (400 mg/kg) has no effect on mice median lifespan (about 116 weeks). On the other hand, Reckelhoff et al. (1998) reported that vitamin E at 5000 mg/kg given from 52 to 88 weeks of rat age prevented the decline of renal function and glomerular filtration and the increases in 8-isoprostanes and in heme oxygenase activity occurring in aging rats. Meydani et al. (1998) reported that vitamin E (1.3 nmol/g mouse, equivalent to 1.2 mg vitamin E/day/mouse) given to mice aged 72 weeks had no effect on median and maximal lifespan, but reduced lipoperoxidation and increased immune function (Meydani et al., 1998).

3. Mitochondrial alterations upon aging

The study of the effect of aging on mitochondrial properties is usually conducted on mitochondria isolated from organs of aging mice and rats. The liver and the heart are used as models of organs that retain a capacity of regeneration during aging, the kidney cortex is used as an organ with a restricted regeneration capacity, and the brain as an organ, with mostly postmitotic cells, that has almost no regeneration capacity. In the four cases, the ability to undergo apoptosis is directly related to the regeneration capacity. Studies using brain regions or areas, such as cortex, cerebellum, and hippocampus are being developed.

The mitochondrial alterations observed upon aging include an increased content of oxidation products and a diminished functional activity; mitochondria in the described condition are called dysfunctional mitochondria.

An increase of the mitochondrial content of oxidation products, a condition that is the definition of oxidative stress, has been reported in aged rodents. The markers that were found increased are: protein carbonyls (Forster et al., 1996; Stadtman, 2002; Navarro et al., 2004), TBARS (Navarro et al., 2004), ROOH, and 8-HO-dG (Ames, 1988; Shigenaga et al., 1994; Sastre et al., 1998). The oxidants produced in the biochemical free radical reactions exhibit selectivity in damaging mitochondrial macromolecules and membrane functions (Ames, 1988; Sohal and Orr, 1998; Ames et al., 2002).

Dysfunctional mitochondria show impairment of oxidative phosphorylation, decreased rates of electron transfer and decreased enzymatic activities. Uncoupling of oxidative phosphorylation with an increase in state 4 respiration, a process that indicates increased passive H^+ permeability, and decreases in respiratory control and membrane potential have been reported (Nakahara et al., 1998; Sastre et al., 1998; Hagen et al., 1998).

Concerning the electron transfer activities of the mitochondrial respiratory chain, reduced activities of Complexes I and IV (NADH-ubiquinol reductase and cytochrome oxidase) have been found on aging rat and mice, whereas Complexes II and III were unaffected (Lenaz et al., 2000; Navarro et al., 2002; Navarro et al., 2004). Cytochrome oxidase was reported decreased in ged rodents, as detected by histochemistry and by determination of enzymatic activity (Trounce et al., 1989; Benzi et al., 1992; Martinez et al., 1994; Navarro et al., 2004). Decreased state 3 respiration rates with NADH-dependent substrates, a process in which Complexes I, III and IV are involved, and with succinate as substrate, a process in which Complexes II–IV are involved, have been observed (Junqueira and Boveris, personal communication).

Two enzymatic activities that are essential for mitochondrial function have been reported selectively decreased on aging: adenine nucleotide translocase, that catalyzes the fast ADP/ATP exchange between cytosol and mitochondria (Yan and Sohal, 1998); and acyl carnitine transferase, that catalyzes fatty acid transport to the mitochondrial matrix (Liu et al., 2002).

Table 1 illustrates about the occurrence of mitochondrial oxidative stress and dysfunction in the brain mitochondria of aging male mice. The oxidative stress markers, TBARS and protein carbonyls, increased with aging by about 45–70% at 52–78 weeks respectively; moderate exercise and vitamin E supplementation, started at 28 weeks of age, were effective in preventing by 48–82%, the described increase in the two oxidative stress markers. The inner membrane mitochondrial enzyme activities: (i) NADH-cytochrome c reductase, (ii) cytochrome oxidase and (iii) mtNOS were markedly decreased upon aging (Table 1). The 15–34% decrease of NADH-cytochrome c reductase activity (Complexes I + III) at 52–78 weeks of age, is attributed to Complex I decreased activity, since succinate cytochrome c reductase activity (Complex I + II) was not affected by aging (Navarro et al., 2002; Navarro et al., 2004). Cytochrome oxidase activity decreased by 22–35% at 52–78 weeks of age. NADH-dehydrogenase activity was 12–18% protected at 52–78 weeks of age by the conditions of high spontaneous neurological activity, moderate exercise, and supplementation with vitamin E. Cytochrome oxidase activity was 8–17% protected at 52–78 weeks by the three experimental conditions. The activity of mtNOS decreased by 40–73% at 52–78 weeks of age, and this decrease was prevented 15–43% by the three experimental conditions.

Oxidative damage negatively correlated with mitochondrial enzyme activities (Fig. 2), which supports the view that intermediates and oxidation products of the lipoperoxidation process constitute the molecular mechanism of the decreased enzymatic activity.

Table 1
Biochemical markers of brain mitochondrial aging in male mice

Age	28 weeks	52 weeks	78 weeks
Control mice			
TBARS	5.2 ± 0.4	8.1 ± 0.4[a]	9.1 ± 0.4[a]
Protein carbonyls	55 ± 4	74 ± 5[a]	91 ± 5[a]
MtNOS	0.59 ± 0.06	0.33 ± 0.04[a]	0.16 ± 0.02[a]
NADH-cytochrome c reductasa	319 ± 10	270 ± 10[a]	210 ± 10[a]
Cytochrome oxidase	116 ± 8	90 ± 7[a]	75 ± 8[a]
High spontaneous neurological activity			
TBARS	5.3 ± 0.4	7.9 ± 0.5[a]	8.2 ± 0.4[a]
Protein carbonyls	52 ± 3	70 ± 5[a]	82 ± 5[a]
MtNOS	0.64 ± 0.05	0.51 ± 0.05	0.32 ± 0.03[a]
NADH-cytochrome c reductasa	335 ± 11	282 ± 10[a]	234 ± 10[a]
Cytochrome oxidase	121 ± 8	106 ± 8	95 ± 9[a]
Moderate exercise			
TBARS	5.2 ± 0.4	5.6 ± 0.5	8 ± 0.4[a]
Protein carbonyls	55 ± 5	59 ± 4	75 ± 4[a]
MtNOS	0.60 ± 0.06	0.53 ± 0.04	0.31 ± 0.04[a]
NADH-cytochrome c reductasa	321 ± 11	285 ± 10[a]	279 ± 10[a]
Cytochrome oxidase	118 ± 8	111 ± 7	100 ± 8[a]
Vitamin E supplementation			
TBARS	5.9 ± 0.3	5.5 ± 0.4	5.9 ± 0.4
Protein carbonyls	50 ± 5	59 ± 4	63 ± 5[a]
mtNOS	0.62 ± 0.05	0.54 ± 0.05	0.43 ± 0.06[a]
NADH-cytochrome c reductasa	322 ± 11	290 ± 10[a]	283 ± 11[a]
Cytochrome oxidase	120 ± 8	112 ± 8	102 ± 8[a]

Values are means ± SE; 8 mice in each experimental group. TBARS and protein carbonyls are expressed in pmol/mg mitochondrial protein, and the enzymatic activities in nmol of substrate or product/min mg mitochondrial protein.

[a] $p < 0.05$ for aging (52 and 78 weeks of age) as compared with younger mice.

4. Brain mitochondrial enzyme activities, behavior and survival

The retard in the decline of brain mitochondrial enzyme activities observed in the high spontaneous neurological activity mice, in the moderate exercise group, and in the mice supplemented with vitamin E correlated with an increased mice survival (Fig. 3). These animals not only show extended lifespan, but also exhibit an improved quality of neuromuscular and exploratory performance, as determined by the tightrope and T-maze tests (Fig. 3). It is apparent that spontaneous high neurological activity, moderate exercise, and vitamin E supplementation may increase lifespan, likely by a decrease in cellular oxidative stress and by preventing the

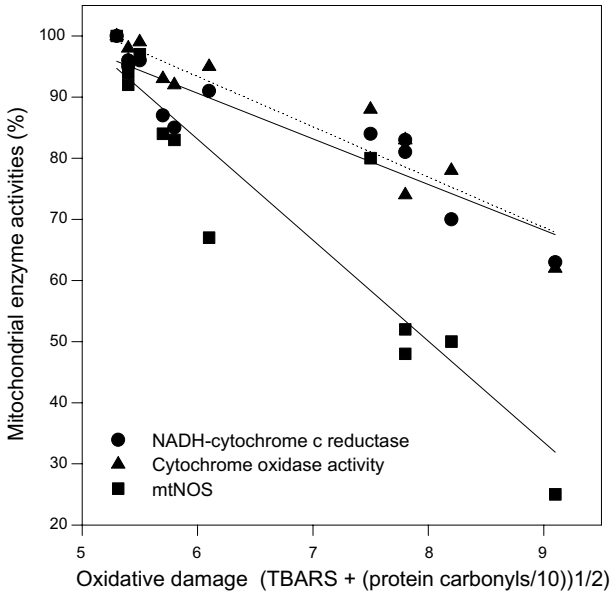

Fig. 2. Correlation between oxidative damage ($0.5 \times$[TBARS + (protein carbonyls/10)]), and the mitochondrial enzyme activities: NADH-cytochrome c reductase, cytochrome oxidase and mitochondrial nitric oxide synthase (Activities expressed as the percentage of activity at 28 weeks). Oxidative damage, vs. NADH cytochrome *c* reductase activity $r^2 = 0.84$, $p < 0.05$; id. vs. cytochrome oxidase activity, $r^2 = 0.89$, $p < 0.05$; id. vs. mtNOS, $r^2 = 0.86$, $p < 0.05$.

decreased mitochondrial functions that accompany the age-associated decline of physiological functions. It seems that these three condition are able to trigger regulatory responses that retard age-dependent processes, such as the impairment of behavioral performances, the development of cellular oxidative stress, and the decrease of mitochondrial activities. This effect supports the concept that both high neurological activity (spontaneous or by imposed exercise) and antioxidant supplementation exert a beneficial effect involving genomic regulation (Churchill et al., 2002; Radak et al., 2002; Welle and Glueck, 2003; Bronikowski et al., 2003; Lores-Arnaiz et al., 2004). The effect seem to extend to humans, since aerobic exercise reduces brain tissue loss in aging humans (Colcombe et al., 2003).

5. Concluding remarks

Mitochondrial enzymatic activities, mtNOS, NADH dehydrogenase and cytochrome oxidase, can be used as markers of brain aging; the decreases in enzymatic activity are directly related to the loss of neurological function in aged mice. High spontaneous neurological activity, moderate exercise and supplementation with vitamin E were three experimental conditions that partially prevented the losses of

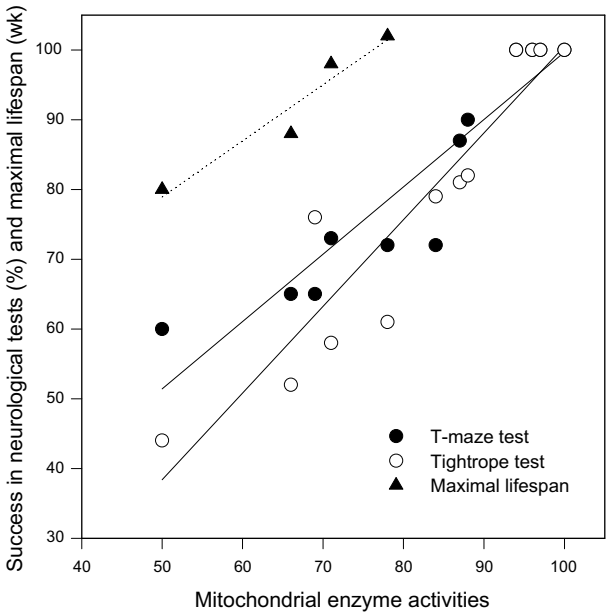

Fig. 3. Correlation between mitochondrial inner membrane enzymatic activities (mean value of NADH-cytochrome c reductase, cytochrome oxidase and mtNOS activities in percentage of the activity at 28 weeks) at 28, 52 and 78 weeks of age with (a) success in the T-maze and the tightrope tests at the same time points; (b) maximal lifespan (in this case with the enzyme activities at 78 weeks of age). Correlations of mitochondrial enzyme activities: to T-maze success, $r^2 = 0.87$, $p < 0.05$; to tightrope success, $r^2 = 0.87$, $p < 0.05$; and to maximal lifespan, $r^2 = 0.93$, $p < 0.05$.

brain mitochondrial enzyme activities and neurological function which are characteristics of aging, and, at the same time, extended mice lifespan.

Acknowledgements

Supported by grants FIS 99-1033 and 02-1354 from Ministerio de Sanidad y Consumo de España, and by Plan Andaluz de Investigación 2000-03 (CTS-194).

References

Alvarez, S., Valdez, L.B., Zaobornyj, T., Boveris, A., 2003. Oxygen dependence of mitochondrial nitric oxide synthase activity. Biochem. Biophys. Res. Commun. 305, 771–775.

Ames, B.N., 1988. Measuring oxidative damage in humans: relation to cancer and ageing. IARC Sci. Publ., 407–416.

Ames, B.N., Elson-Schwab, I., Silver, E.A., 2002. High-dose vitamin therapy stimulates variant enzymes with decreased coenzyme binding affinity (increased K(m)): relevance to genetic disease and polymorphisms. Am. J. Clin. Nutr. 75, 616–658.

Beckman, K.B., Ames, B.N., 1998a. Mitochondrial aging: open questions. Ann. N.Y. Acad. Sci. 854, 118–127.

Beckman, K.B., Ames, B.N., 1998b. The free radical theory of aging matures. Physiol. Rev. 78, 547–581.

Benzi, G., Pastoris, O., Marzatico, F., Villa, R.F., Dagani, F., Curti, D., 1992. The mitochondrial electron transfer alteration as a factor involved in the brain aging. Neurobiol. Aging 13, 361–368.

Boveris, A., Arnaiz, S.L., Bustamante, J., Alvarez, S., Valdez, L., Boveris, A.D., Navarro, A., 2002. Pharmacological regulation of mitochondrial nitric oxide synthase. Methods Enzymol. 359, 328–339.

Boveris, A., Cadenas, E., 1975. Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. FEBS Lett. 54, 311–314.

Boveris, A., Cadenas, E., 2000. Mitochondrial production of hydrogen peroxide regulation by nitric oxide and the role of ubisemiquinone. IUBMB. Life 50, 245–250.

Boveris, A., Cadenas, E., Stoppani, A.O., 1976. Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. Biochem. J. 156, 435–444.

Bronikowski, A.M., Carter, P.A., Morgan, T.J., Garland Jr., T., Ung, N., Pugh, T.D., Weindruch, R., Prolla, T.A., 2003. Lifelong voluntary exercise in the mouse prevents age-related alterations in gene expression in the heart. Physiol. Genomics 12, 129–138.

Cadenas, E., Boveris, A., 1980. Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. Biochem. J. 188, 31–37.

Chance, B., Sies, H., Boveris, A., 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59, 527–605.

Chomyn, A., Attardi, G., 2003. mtDNA mutations in aging and apoptosis. Biochem. Biophys. Res. Commun. 304, 519–529.

Churchill, J.D., Galvez, R., Colcombe, S., Swain, R.A., Kramer, A.F., Greenough, W.T., 2002. Exercise, experience and the aging brain. Neurobiol. Aging 23, 941–955.

Colcombe, S.J., Erickson, K.I., Raz, N., Webb, A.G., Cohen, N.J., McAuley, E., Kramer, A.F., 2003. Aerobic fitness reduces brain tissue loss in aging humans. J. Gerontol. A Biol. Sci. Med. Sci. 58, M176–M180.

Elfering, S.L., Sarkela, T.M., Giulivi, C., 2002. Biochemistry of mitochondrial nitric-oxide synthase. J. Biol. Chem. 277, 38079–38086.

Finch, C.E., Pike, M.C., 1996. Maximum life span predictions from the Gompertz mortality model. J. Gerontol. A Biol. Sci. Med. Sci. 51, B183–B194.

Finch, C.E., Tanzi, R.E., 1997. Genetics of aging. Science 278, 407–411.

Forster, M.J., Dubey, A., Dawson, K.M., Stutts, W.A., Lal, H., Sohal, R.S., 1996. Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. Proc. Natl. Acad. Sci. USA 93, 4765–4769.

Gerschman, R., Gilbert, D.L., Nye, S.W., Dwyer, P., Fenn, W.O., 1954. Oxygen poisoning and x-irradiation: a mechanism in common. Science 119, 623–626.

Ghafourifar, P., Richter, C., 1997. Nitric oxide synthase activity in mitochondria. FEBS Lett. 418, 291–296.

Giulivi, C., Poderoso, J.J., Boveris, A., 1998. Production of nitric oxide by mitochondria. J. Biol. Chem. 273, 11038–11043.

Hagen, T.M., Wehr, C.M., Ames, B.N., 1998. Mitochondrial decay in aging reversal through supplementation of acetyl-L-carnitine and *N*-tert-butyl-alpha-phenyl-nitrone. Ann. N.Y. Acad. Sci. 854, 214–223.

Han, D., Antunes, F., Canali, R., Rettori, D., Cadenas, E., 2003. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. J. Biol. Chem. 278, 5557–5563.

Harman, D., 1956. Aging: a theory based on free radical and radiation chemistry. J. Gerontol 11, 298–300.

Harman, D., 1972. The biologic clock: the mitochondria. J. Am. Geriatr. Soc 20, 145–147.

Hayakawa, M., Sugiyama, S., Hattori, K., Takasawa, M., Ozawa, T., 1993. Age-associated damage in mitochondrial DNA in human hearts. Mol. Cell Biochem. 119, 95–103.

Lenaz, G., D'Aurelio, M., Merlo, P.M., Genova, M.L., Ventura, B., Bovina, C., Formiggini, G., Parenti, C.G., 2000. Mitochondrial bioenergetics in aging. Biochim. Biophys. Acta 1459, 397–404.

Liu, J., Killilea, D.W., Ames, B.N., 2002. Age-associated mitochondrial oxidative decay: improvement of carnitine acetyltransferase substrate-binding affinity and activity in brain by feeding old rats acetyl-L-carnitine and/or R-alpha-lipoic acid. Proc Natl. Acad. Sci. USA 99, 1876–1881.

Lores-Arnaiz, S., D'Amico, G., Paglia, N., Arismendi, M., Basso, N., Lores-Arnaiz, M. del R. 2004. Enriched environmnt, nitric oxide production and synaptic plasticity prevent the aging-dependent impairment of spacial cognition. Mol. Aspects Med., this volume.

Martinez, M., Ferrandiz, M.L., De Juan, E., Miquel, J., 1994. Age-related changes in glutathione and lipid peroxide content in mouse synaptic mitochondria: relationship to cytochrome *c* oxidase decline. Neurosci. Lett. 170, 121–124.

Meydani, M., Lipman, R.D., Han, S.N., Wu, D., Beharka, A., Martin, K.R., Bronson, R., Cao, G., Smith, D., Meydani, S.N., 1998. The effect of long-term dietary supplementation with antioxidants. Ann. N.Y. Acad. Sci. 854, 352–360.

Miquel, J., Fleming, J., 1986. Theoretical and experimental support for an "oxygen radical injury" hypothesis of cell aging. In: Johnson, J.E., Waldorf, R., Harman, D., Miquel, J. (Eds.), Free Radicals, Aging and Degenerative Diseases. Alan R.Liss, New York, pp. 51–74.

Morley, A.A., Trainor, K.J., 2001. Lack of an effect of vitamin E on lifespan of mice. Biogerontology 2, 109–112.

Nakahara, H., Kanno, T., Inai, Y., Utsumi, K., Hiramatsu, M., Mori, A., Packer, L., 1998. Mitochondrial dysfunction in the senescence accelerated mouse (SAM). Free Radic. Biol. Med. 24, 85–92.

Navarro, A., Gomez, C., Lopez-Cepero, J.M., Boveris, A., 2004. Beneficial effects of moderate exercise on mice aging: survival, behavior, oxidative stress and mitochondrial electron transfer. Am. J. Physiol Regul. Integr. Comp. Physiol. 286, R505–R511.

Navarro, A., Sanchez Del Pino, M.J., Gomez, C., Peralta, J.L., Boveris, A., 2002. Behavioral dysfunction, brain oxidative stress, and impaired mitochondrial electron transfer in aging mice. Am. J. Physiol Regul. Integr. Comp. Physiol. 282, R985–R992.

Poderoso, J.J., Lisdero, C., Schopfer, F., Riobo, N., Carreras, M.C., Cadenas, E., Boveris, A., 1999. The regulation of mitochondrial oxygen uptake by redox reactions involving nitric oxide and ubiquinol. J. Biol. Chem. 274, 37709–37716.

Radak, Z., Naito, H., Kaneko, T., Tahara, S., Nakamoto, H., Takahashi, R., Cardozo-Pelaez, F., Goto, S., 2002. Exercise training decreases DNA damage and increases DNA repair and resistance against oxidative stress of proteins in aged rat skeletal muscle. Pflugers Arch. 445, 273–278.

Radi, R., 1998. Peroxynitrite reactions and diffusion in biology. Chem. Res. Toxicol. 11, 720–721.

Radi, R., Turrens, J.F., Chang, L.Y., Bush, K.M., Crapo, J.D., Freeman, B.A., 1991. Detection of catalase in rat heart mitochondria. J. Biol. Chem. 266, 22028–22034.

Ramsey, J.J., Colman, R.J., Binkley, N.C., Christensen, J.D., Gresl, T.A., Kemnitz, J.W., Weindruch, R., 2000. Dietary restriction and aging in rhesus monkeys: the University of Wisconsin study. Exp. Gerontol. 35, 1131–1149.

Reckelhoff, J.F., Kanji, V., Racusen, L.C., Schmidt, A.M., Yan, S.D., Marrow, J., Roberts, L.J., Salahudeen, A.K., 1998. Vitamin E ameliorates enhanced renal lipid peroxidation and accumulation of F2-isoprostanes in aging kidneys. Am. J. Physiol. 274, R767–R774.

Sastre, J., Millan, A., Garcia, d.l.A., Pla, R., Juan, G., Pallardo, F., O'Connor, E., Martin, J.A., Droy-Lefaix, M.T., Vina, J., 1998. A Ginkgo biloba extract (EGb 761) prevents mitochondrial aging by protecting against oxidative stress. Free Radic. Biol. Med. 24, 298–304.

Shigenaga, M.K., Hagen, T.M., Ames, B.N., 1994. Oxidative damage and mitochondrial decay in aging. Proc Natl. Acad. Sci. USA 91, 10771–10778.

Sohal, R.S., 1993. The free radical hypothesis of aging: an appraisal of the current status. Aging (Milano.) 5, 3–17.

Sohal, R.S., Agarwal, S., Candas, M., Forster, M.J., Lal, H., 1994. Effect of age and caloric restriction on DNA oxidative damage in different tissues of C57BL/6 mice. Mech. Ageing Dev. 76, 215–224.

Sohal, R.S., Orr, W.C., 1998. Role of oxidative stress in senescence. Aging (Milano.) 10, 149–151.

Stadtman, E.R., 2002. Importance of individuality in oxidative stress and aging. Free Radic. Biol. Med. 33, 597–604.

Tatoyan, A., Giulivi, C., 1998. Purification and characterization of a nitric-oxide synthase from rat liver mitochondria. J. Biol. Chem. 273, 11044–11048.

Trounce, I., Byrne, E., Marzuki, S., 1989. Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. Lancet 1, 637–639.

Turrens, J.F., Boveris, A., 1980. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem. J. 191, 421–427.

Valdez, L.B., Alvarez, S., Arnaiz, S.L., Schopfer, F., Carreras, M.C., Poderoso, J.J., Boveris, A., 2000. Reactions of peroxynitrite in the mitochondrial matrix. Free Radic. Biol. Med. 29, 349–356.

Vina, J., Sastre, J., Pallardo, F., Borras, C., 2003. Mitochondrial theory of aging: importance to explain why females live longer than males. Antioxid. Redox. Signal. 5, 549–556.

Welle, S., Glueck, S.B., 2003. In for the long run: focus on ''Lifelong voluntary exercise in the mouse prevents age-related alterations in gene expression in the heart''. Physiol. Genomics 12, 71–72.

Yan, L.J., Sohal, R.S., 1998. Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. Proc Natl. Acad. Sci. USA 95, 12896–12901.