Beneficial effects of moderate exercise on mice aging: survival, behavior, oxidative stress, and mitochondrial electron transfer

Ana Navarro, Carmen Gomez, José M. López-Cepero, and Alberto Boveris. 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, 2004. First published November 13, 2003; 10.1152/ajpregu.00208.2003.—Moderate exercise in a treadmill (10, 15, and 20 cm/s, for 5 min each, weekly) from 28 to 78 wk of age extended male and female mice life span by 19 and 9% accompanied by 36 and 13% and 13 and 9% increased performance in behavioral assays (tightrope and T-maze tests) at 52 wk of age. Moderate exercise significantly decreased the aging-associated development of oxidative stress by preventing 1) the increase in protein carbonyls and thiobarbituric acid-reactive substances contents of sub mitochondrial membranes; 2) the decrease in antioxidant enzyme activities (Mn- and Cu,Zn-superoxide dismutase and catalase); and 3) the decrease in mitochondrial NADH-cytochrome-c reductase and cytochrome oxidase activities observed at 52 wk of mice age in brain, heart, liver, and kidney. These effects were no longer significant at 78 wk of age in mice. Moderate exercise, started at young age in mice, increased life span, decreased oxidative stress, and prevented the decline of cytochrome oxidase activity and behavioral performance at middle age but not at old age.

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**Materials and Methods**

**Animals.** Mice of the CD-1 strain, inbred at the Department of Experimental Animals of the University of Cadiz for five generations, were housed in groups of five animals at 24 ± 1°C with 12:12-h light/dark cycles and had full access to water and food (A04 diet, Panlab LS, Barcelona, Spain). Mice were periodically checked to verify their pathogen-free condition. Animal experiments were carried out in accordance with the 86/609/CEE European Community regulations and the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society.

**Exercise and behavioral tests.** Moderate exercise was imposed on a group of mice aged 28 wk as training in a treadmill (10, 15, and 20 cm/s, for 5 min each, every 7 days) up to 78 wk of age. Individual mice were subjected to two behavioral assays, the tightrope test and the T-shaped maze test, every 2 wk (29). In the tightrope test for evaluation of neuromuscular coordination (13, 28, 29), mice were placed in the middle of a 60-cm tightrope hanging from their anterior legs and the test was considered successful when mice reached the column at the end of the rope in <60 s. In the maze test for evaluation of spontaneous exploratory and cognitive activity (13, 29), mice were challenged in a T-shaped maze with 50-cm arms and the test was considered successful when mice moved toward the T-intersection in <60 s.

**Isolation of mitochondria.** Brain, heart, liver, and kidney mitochondria were isolated from the whole organs and kidney cortex homogenized in 0.23 M mannitol, 0.07 M sucrose, 15 mM MOPS-KOH (pH 7.2) at a ratio of 9 ml of homogenization medium/1 g of tissue in a Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 700 g for 10 min, the supernatant was centrifuged at 8,000 g for 10 min to precipitate mitochondria that were washed in the same conditions (35). The mitochondrial suspensions, ~20 mg protein/ml, present antioxidant enzyme activities, and mitochondrial electron transfer activities, in four organs, brain, heart, liver, and kidney. These effects were more pronounced at 52 wk of age, while the effects were less significant at 78 wk of age. Moderate exercise, started at young age in mice, increased life span, decreased oxidative stress, and prevented the decline of cytochrome oxidase activity and behavioral performance at middle age but not at old age.

AGING AND AGE-ASSOCIATED NEURODEGENERATION in mice are related to marked decreases in neuromuscular and exploratory functions with inverse statistical relationships between mice performance in behavioral tests and indicators of cellular brain oxidative stress (17, 29). The life span of different rodent strains was reported inversely related to the quality of their behavioral responses to stress, linking individual performance to aging and suggesting an environment-triggered regulatory response that modulates age-dependent neurodegeneration (14, 15, 19). Moreover, the performance in behavioral tests negatively correlated with molecular indicators such as whole brain thiobarbituric acid-reactive substances (TBARS) contents and with brain mitochondrial electron transfer activities (29), indicating an association between oxidative stress, mitochondrial dysfunction, and loss of behavioral capabilities (29).

Regular physical exercise seems to retard the accumulation of cell damage and physiological dysfunction that is characteristic of the aging process (23, 31). There is ample evidence of the reduction of skeletal muscle mass associated with aging and also of the beneficial effects of regular exercise in increasing muscle mass and strength in elderly individuals. The available evidence extends from experimental animals to humans and from biochemical markers to physiological parameters and behavioral performances (7, 16, 27, 34, 38). A series of reports documented that the beneficial effects of exercise are extended to other organs, such as mouse (6, 37) and human heart (5, 32) and human brain (10).

Exercise has long been considered associated with oxidative stress based on experimental data showing an increase of oxidative stress markers after high-intensity exercise and on the unsupported assumption that the production of oxygen free radicals is linearly related to the rate of mitochondrial oxygen uptake (2, 11, 12, 22).

In the present work we studied the effects of moderate treadmill exercise in aging mice by determining macroscopic indicators, such as survival and behavioral performances, as well as biochemical parameters, such as oxidative stress markers, antioxidant enzyme activities, and mitochondrial electron transfer activities, in four organs, brain, heart, liver, and kidney.
were immediately frozen in liquid N₂ and kept at −80°C. Mitochondrial samples were thawed and frozen twice and homogenized by passage through 15/100000 pipet tip and called submitochondrial membranes (29). Proteins were determined by using the Folin reagent.

Biochemical markers of oxidative stress. The mitochondrial content of the oxidation products, TBARS and protein carbonyls, was determined after thawing and homogenization of submitochondrial membrane samples. For TBARS determination, 1 ml of submitochondrial membranes was added to 2 ml of 0.1 N HCl, 0.3 ml of phosphotungstic acid, and 1 ml of 0.67% 2-thiobarbituric acid, heated 30 min in boiling water, and extracted with 5 ml 1-butanol. The absorption of the butanol phase, separated by a brief centrifugation, was determined at 353 nm (ε = 153 mM/cm) and expressed as picomoles TBARS per milligram of mitochondrial protein (18). For protein carbonyl determination, 0.05 ml of submitochondrial membranes was added to 0.05 ml of 10% TCA; the precipitated proteins were resuspended in 0.05 ml of 0.2% (wt/vol) 2,4-dinitrophenyl hydrazine, incubated 1 h at 37°C, precipitated again with TCA, centrifuged, washed with ethanol: ethyl acetate, and dissolved in 6 mM guanidine hydrochloride in phosphate buffer (pH 6.5), and the absorbance was determined at 370 nm (ε = 21 mM/cm). Protein carbonyls are expressed in picomoles per milligram of mitochondrial protein (30).

Mitochondrial electron transfer activities. The membrane-bound activities of Complexes I-III, II-III, and IV were determined spectrophotometrically at 30°C with submitochondrial membranes suspended in 100 mM phosphate buffer (pH 7.4) (1, 29). For NADH-cytochrome c reductase (Complexes I-III) and succinate-cytochrome c reductase (Complexes II-III) activities, submitochondrial membranes were added to 0.2 mM NADH or 20 mM succinate as substrates, 0.1 mM cytochrome c³⁻, and 1 mM KCN, and the enzymatic activity was determined at 550 nm (ε = 19 mM/cm) and expressed as nanomoles cytochrome c oxidized at 10 M/s for pure tetramer catalase (39). Enzyme activities were increased by 19 and by 9% in males and females, and maximal life span was increased by 15–21% and by 8–17% in males and females. Exercised males exhibited a 29% increase in the fraction of animals showing longevity (Fig. 1).

Moderate treadmill exercise also improved the performance of trained mice in the tightrope and T-maze tests over the period from 28 to 78 wk of age (Figs. 2 and 3). The significant improvement at 52 wk of age, when biochemical markers were determined and body weight was 50.8 ± 1.3 g for males and 41.3 ± 0.8 g for females, was 36–37% in the tightrope test and 9–13% in the T-maze test, similar for male and female mice. At 78 wk of age the exercise effect was in the 6–10% range, without statistical significance.

Exercise, aging, and oxidative stress markers. Two markers of mitochondrial oxidative stress increased significantly in mice from 28 to 78 wk of age. TBARS (indicated as males-females) increased by 39–20% at 52 wk and by 55–46% at 78 wk of age in brain, heart, liver, and kidney. Protein carbonyls (similarly indicated) increased by 43–26% at 52 wk and by 63–50% at 78 wk in the same organs (Table 1). Protein carbonyls resulted in a more sensitive marker of the oxidative stress associated to aging. Protein carbonyls and TBARS were ≈12% higher in males than in females, considering the two markers, the three time points, and the four organs. Moderate exercise from 28 to 52 wk of age prevented by 43 ± 14% in males and females taken together (P < 0.05), the increase in the level of oxidative stress markers in the same time period. However, the effect was lost at 78 wk of age (Table 1). Table

![Fig. 1. Effect of moderate exercise on mice survival. Forty mice were in each experimental group. Median life span: control males, 69 ± 2 wk; exercised males, 81 ± 2 wk (P < 0.05); control females, 81 ± 2 wk; exercised females, 89 ± 3 wk (P < 0.05). Maximal life span (intersection of the main slope with abscissa): control males, 85 ± 2 wk; exercised males, 98 ± 2 wk (P < 0.05); control females, 106 ± 2 wk; exercised females 114 ± 3 wk (P = 0.05). Maximal life span (last survivor): control males, 108 ± 2 wk; exercised males, 114 ± 3 wk; control females, 116 ± 3 wk; exercised females 136 ± 3 wk (P < 0.05). Fraction of the population showing longevity (29): control males, 7%; exercised males, 9%; control females 16%; exercised females, 16%.

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considered organs, including males and females and control and exercised mice. The first correlation seems to indicate that protein oxidation accounts for the loss of mitochondrial enzyme activity associated with age and partially prevented by exercise in the mitochondria membranes of brain, heart, liver, and kidney. The second correlation, more provocative, points to the cytochrome oxidase activity of the four organs as an indicator of whole behavioral and physiological functions, referred in this specific case to mice neuromuscular coordination.

NADH-cytochrome-c reductase activity, considered in groups and organs as a whole, decreased by 14 and 27% at 52 wk and 78 wk of mice age. Moderate exercise partially (31%) prevented the loss of activity at 52 wk of age.

Pairing the enzymatic activities of the different age and exercise groups, females exhibited higher mitochondrial electron transfer activities than males: 7–8% in brain and heart and 16–18% in liver and kidney.

Exercise, aging, and antioxidant enzymes. The activities of the antioxidant enzymes Mn-SOD, Cu,Zn-SOD, and catalase were found decreased on aging in the four organs of both male and female mice. Compared with the reference young adults of 28 wk of age, there were losses of 18 and 14% in the activity of the three enzymes considered together at 52 wk, and of 38 and 31% at 78 wk of age, in males and females, respectively. Moderate exercise from 28 to 52 wk of age was able to significantly decrease the loss of enzymatic activities at 52 wk by 67 ± 23% and 98 ± 21% in male and female mice, respectively (P < 0.05 in both cases). However, the beneficial effect of exercise was reduced to 7–9%, without statistical

1 indicates the biological significance of differences between experimental groups in terms of aging, exercise, and animal sex.

Exercise, aging, and mitochondrial electron transfer. Aging decreased NADH-cytochrome-c reductase activity (indicated as male-female) to 87–87% at 52 wk and to 70–77% at 78 wk in brain, heart, liver, and kidney mitochondria. Moderate exercise partially prevented, by ~40% in average, the decline in enzyme activity observed in the four organs at 52 wk; however, this beneficial effect of exercise was not observed at 78 wk of age (Table 2). Succinate-cytochrome-c reductase activity was neither affected by aging nor by exercise, a fact that indicates the selectivity of the effect. Table 2 indicates the biological significance of the differences of electron transfer activities between experimental groups in terms of aging, exercise, and animal sex.

On the other hand, cytochrome oxidase activity resulted a useful marker of the effects of aging and exercise in mitochondrial electron transfer. The cytochrome oxidase activity of the young adult mice of 28 wk of age, taken as reference, was decreased by aging to 77 ± 11% at 52 wk and to 61 ± 15% at 78 wk of age (P < 0.05 in both cases), considering the four organs and males and females together. Moderate exercise prevented the loss of cytochrome oxidase activity, as considered, by 82 ± 21% at 52 wk of age (P < 0.05) and by 27 ± 20% at 78 wk of age (P = 0.25). Moreover, two interesting correlations were observed with cytochrome oxidase activity: 1) a negative correlation with protein carbonyl content (Fig. 4) and 2) a positive correlation with mice neuromuscular coordination (Fig. 5). The correlations were made for each of the
significance, at 78 wk of age in mice. Table 3 indicates the biological significance of the differences in antioxidant enzyme activities between experimental groups in terms of aging, exercise, and animal sex.

**DISCUSSION**

Aging is associated with a general decline of physiological functions with a more marked effect on those that depend on central nervous system, such as behavior and cognitive performances. The decrease in mice behavioral performances occurring on normal aging has been found correlated to brain oxidative stress, determined as protein carbonyls and TBARS content (17, 29).

The mitochondrial hypothesis of aging considers that the primary production of free radicals, superoxide radicals (O₂⁻), and nitric oxide (NO) by mitochondria sustains free radical reactions that selectively damage critical macromolecules in the organelle (8, 17, 21). In such a way mitochondria are the main source and the main target of cellular free radicals. An increase in the rate of free radical production or a decrease in the content of antioxidant enzymes equally leads to oxidative stress, enzyme inactivation, and mitochondrial and cellular dysfunction. The activities of NADH-dehydrogenase and cytochrome oxidase, constitutive proteins of the mitochondrial inner membrane, are recognized markers of aging (16, 25, 29). Other mitochondrial proteins, such as adenine nucleotide translocase (33), acyl carnitine trasferase (21), cytochrome oxidase (3, 26, 35), and citrate synthase (29), are also considered as selective targets in the free radical-mediated damage associated with oxidative stress and aging.

Moderate exercise produced a series of beneficial effects in aging mice: 1) increased survival, with increased median and maximal life span; 2) improved the performance in behavioral tests of neuromuscular function and exploratory activities; 3) decreased the level of oxidative stress markers in the mitochondrial membranes of brain, heart, liver, and kidney; 4) prevented the age-associated decrease of antioxidant enzyme activities in the same organs; and 5) prevented the age-associated decline of mitochondrial cytochrome oxidase and NADH-dehydrogenase activities in the same organs.

The three effects of moderate exercise observed at the biochemical level, i.e., upregulation of antioxidant enzyme activity, decrease in oxidative stress markers, and increased activity of mitochondrial electron transfer enzymes, are logically related. An upregulated level of antioxidant enzymes will decrease the rate of mitochondrial and cellular free radical reactions, which in turn will decrease the levels of by-products or markers of free radical reactions. Increased TBARS and protein carbonyls in the mitochondrial membranes impair membrane-bound enzyme activities leading to mitochondrial and cellular dysfunction.

The quantitative differences observed in the effect of moderate exercise on the activities of cytochrome oxidase and NADH dehydrogenase suggest a complex genomic regulation for the expression of these two enzymes, both with structural polypeptides coded in mtDNA. It is worth noting the convenience of determining the biological activity of NADH-dehydrogenase (Complex I) as the difference between NADH-cytochrome-c reductase (Complexes I and III) activity and succinate cytochrome-c reductase (Complexes II and III). In some cases the use of externally added electron acceptors, such as CoQ₉ or CoQ₆, does not reveal the decline in enzyme activity associated with aging (24, 25).

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**Table 1. Effect of moderate exercise on oxidative stress markers of submitochondrial membranes of brain, heart, liver, and kidney in aging mice**

<table>
<thead>
<tr>
<th>Organ/Marker</th>
<th>Control 28 wk</th>
<th>Control 52 wk</th>
<th>Control 78 wk</th>
<th>Exercised 52 wk</th>
<th>Exercised 78 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain/TBARS</td>
<td>5.5±0.3</td>
<td>8.5±0.4*</td>
<td>8.9±0.4*</td>
<td>5.7±0.5†</td>
<td>9.1±0.4*</td>
</tr>
<tr>
<td>Brain/protein carbonyls</td>
<td>56±3</td>
<td>79±5*</td>
<td>93±5*</td>
<td>61±3†</td>
<td>81±3*</td>
</tr>
<tr>
<td>Heart/TBARS</td>
<td>8.7±0.4</td>
<td>11.0±0.4*</td>
<td>11.8±0.5*</td>
<td>9.6±0.4†</td>
<td>12.1±0.2*</td>
</tr>
<tr>
<td>Heart/protein carbonyls</td>
<td>30±2</td>
<td>55±3*</td>
<td>69±5*</td>
<td>47±3</td>
<td>72±5*</td>
</tr>
<tr>
<td>Liver/TBARS</td>
<td>3.7±0.2</td>
<td>4.5±0.2*</td>
<td>4.8±0.2*</td>
<td>4.0±0.2</td>
<td>4.6±0.3*</td>
</tr>
<tr>
<td>Liver/protein carbonyls</td>
<td>129±5</td>
<td>169±7*</td>
<td>187±7*</td>
<td>140±7†</td>
<td>184±8*</td>
</tr>
<tr>
<td>Kidney/TBARS</td>
<td>9.6±0.5</td>
<td>14.9±0.5*</td>
<td>18.5±0.4*</td>
<td>12.1±0.4†</td>
<td>17.4±0.5*</td>
</tr>
<tr>
<td>Kidney/protein carbonyls</td>
<td>174±5</td>
<td>206±5*</td>
<td>253±6*</td>
<td>183±6†</td>
<td>260±7*</td>
</tr>
</tbody>
</table>

Values are means ± SE in pmol/mg mitochondrial protein. Eight mice in each experimental group. P < 0.05: *, for aging compared with younger mice; †, for moderate exercise; ‡, for females different from males.
The effects of moderate exercise in females was weaker than in males. This applies to life span, behavioral performances, oxidative stress markers, and enzyme activities.

Concerning the effect of exercise on the organ rate of free radical reactions, it was thought for years that the increased rate of oxygen consumption derived from exercise would be associated with a higher rate of mitochondrial free radical production. Because beneficial effects of exercise were often observed, the question was addressed as the “exercise-free radical paradox” (11, 31). However, moderate exercise sets an increased ATP demand and shifts the mitochondrial metabolic state in cells from mainly state 4 (the resting state with low ADP levels and slow respiration), to mainly state 3 (the active state, with high levels of ADP and fast respiration and ATP synthesis). In this transition, the state 4 relatively high rate of superoxide radical and hydrogen peroxide production is shifted to the state 3 lower rate of superoxide radical and hydrogen peroxide production (4, 8). In this way the beneficial effects of moderate exercise are better explained by a lower generation rate of mitochondrial superoxide radical and by the increased levels of mitochondrial Mn-SOD and the antioxidant enzymes. The observed increase in oxidation products after high-intensity or exhaustive exercise is more recently explained by the release of xanthine oxidase from the liver (36) and by the translocation of hemoglobin and myoglobin from their biological compartments (11).
It seems that moderate exercise triggers regulatory responses that retard some age-dependent processes such as the impairment of behavioral performances, the development of cellular oxidative stress, and the decrease of mitochondrial activities. The effect, observed in four organs, brain, heart, liver, and kidney, seems to support the concept advanced by Welle and Glueck (37) and by Churchill et al. (9) that exercise beneficial effects involve genomic modulation. In the same line of thought, Bronikowski et al. (6) reported that voluntary exercise partially prevented the aging-dependent decline in heart gene expression and suggested that adaptive physiological mechanisms are induced to retard the aging effects at the transcriptional level. Moreover, after an analysis of age-associated increases in oxidation products, Radak et al. (31) suggested that moderate exercise induced an increase in the activity of DNA repair systems and in the resistance against oxidative stress in rat skeletal muscle. It is important to note that the exercise effect seems to extend to humans; Colcombe et al. (10) recently reported that aerobic exercise reduces brain tissue loss in aging humans.

Most interestingly, moderate exercise extended mice survival. The effect was moderate but was observed both as an increased median life span and as an increased maximal life span. Moderate exercise not only extended survival, but improved the quality of neuromuscular and exploratory performances as determined by the tightrope and T-maze tests. It is apparent that moderate exercise at middle age in mammals may

Table 3. Effect of moderate exercise on antioxidant enzymes of brain, heart, liver, and kidney in aging mice

<table>
<thead>
<tr>
<th>Organ/Activity</th>
<th>Control</th>
<th>Exercised</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28 wk</td>
<td>52 wk</td>
</tr>
<tr>
<td>Brain/Mn-SOD</td>
<td>0.51±0.04</td>
<td>0.42±0.02*</td>
</tr>
<tr>
<td>Brain/Cu,Zn-SOD</td>
<td>13.2±0.8</td>
<td>10.3±0.7*</td>
</tr>
<tr>
<td>Brain/Catalase</td>
<td>0.126±0.010</td>
<td>0.112±0.009</td>
</tr>
<tr>
<td>Heart/Mn-SOD</td>
<td>1.24±0.09</td>
<td>1.09±0.07*</td>
</tr>
<tr>
<td>Heart/Cu,Zn-SOD</td>
<td>9.9±0.9</td>
<td>8.1±0.7</td>
</tr>
<tr>
<td>Heart/Catalase</td>
<td>0.113±0.010</td>
<td>0.095±0.008*</td>
</tr>
<tr>
<td>Liver/Mn-SOD</td>
<td>0.63±0.03</td>
<td>0.54±0.03*</td>
</tr>
<tr>
<td>Liver/Cu,Zn-SOD</td>
<td>12.3±0.8</td>
<td>9.7±0.8*</td>
</tr>
<tr>
<td>Liver/Catalase</td>
<td>1.19±0.09</td>
<td>0.92±0.06*</td>
</tr>
<tr>
<td>Kidney/Mn-SOD</td>
<td>0.38±0.03</td>
<td>0.29±0.02*</td>
</tr>
<tr>
<td>Kidney/Cu,Zn-SOD</td>
<td>6.8±0.5</td>
<td>5.5±0.4</td>
</tr>
<tr>
<td>Kidney/Catalase</td>
<td>0.93±0.07</td>
<td>0.72±0.05*</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol of enzyme/g of tissue for the 3 enzymes. Eight mice in each experimental group. P < 0.05: *, for aging compared with 28-wk-old mice; †, for moderate exercise; ‡, for females different from males. SOD, superoxide dismutase.
increase life span, likely by a decrease in cellular oxidative stress and by preventing the decreased mitochondrial functions that accompany the age-associated decline of physiological functions.

GRANTS

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REFERENCES