

## Regular exercise improves cognitive function and decreases oxidative damage in rat brain

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Received 6 December 1999; accepted 31 March 2000

### Abstract

The biochemical mechanisms by which regular exercise significantly benefits health and well being, including improved cognitive function, are not well understood. Four-week-old (young) and 14-month-old (middle aged) Wistar rats were randomly assigned to young control and young exercised, middle-aged control and middle-aged exercised groups. Exercise groups were exposed to a swimming regime of 1 h a day, 5 days a week for 9 weeks. The passive avoidance test showed that middle-aged exercised rats had significantly ( $P < 0.05$ ) better short- (24 h) and long-term (72 h) memory than aged-matched control rats. Conditioned pole-jumping avoidance learning was improved markedly in both age groups by exercise. Brain thiobarbituric acid-reactive substances and 8-hydroxy-2'-deoxyguanosine content in the DNA did not change significantly, while the protein carbonyl levels decreased significantly ( $P < 0.05$ ) in both exercised groups. This decrease was accompanied by an increase in the chymotrypsin-like activity of proteasome complex in the exercised groups, whereas trypsin-like activity did not differ significantly between all groups. The DT-diaphorase activity increased significantly ( $P < 0.05$ ) in the brain of young exercised animals. These data show that swimming training improves some cognitive functions in rats, with parallel attenuation of the accumulation of oxidatively damaged proteins. © 2000 Elsevier Science Ltd. All rights reserved.

### 1. Introduction

There is a paradox regarding the effect of exercise on health and well being because while it can induce free radical formation which may be detrimental for cellular functions, it also reduces a variety of age-related diseases. The biochemical mechanisms by which regular exercise significantly benefits health and well being, including depression of the incidence of certain diseases are not well understood (Holloosy, 1993;

Sarna et al., 1993; Blair et al., 1995). The favorable effects of exercise on cardiovascular function are well documented and the close connection between cardiovascular health and cognitive function suggests also that exercise could promote brain function (Hicks and Birren, 1970). Indeed evidence suggests that in humans there is a link between physical fitness and cognitive performance (Chodzko-Zajko and Moore, 1994). Despite a wealth of studies very little is known about how exercise affects cognitive function. However, it has been suggested that exercise maintains cerebrovascular integrity (impaired cerebral circulation has adverse effects) (McFarland, 1963), increases capillary growth (Black et al., 1987), increases dendritic connections (Pysh and Weiss, 1979), and enhances the effi-

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ciency of the processing functions of the central nervous system (Dustman et al., 1990).

Reactive oxygen species have been implicated in neurological diseases and age-related decline in cognitive processes which suggest that these species play a role in brain function (Halliwell, 1992; Cadet and Brannock, 1998; Loeffler et al., 1998; Varadarajan et al., 1999; Ahlemeyer and Kriegstein, 2000). Direct evidence has emerged from the work of Carney et al. (1991) in which 2-week administration of *N*-tert-butyl-alpha-phenylnitron decreased age-associated increases in the accumulation of reactive carbonyl derivatives (RCD) in the brain of gerbils and improved short-term memory. Similar data have also been obtained in the rat model (Socci et al., 1995). However, it is not known whether this causative link between RCD accumulation and cognitive function is restricted to aged-associated changes. We tested the validity of this relationship in young and middle-aged rats using an exercise protocol. There are a few studies on the effects of exercise on oxidative damage or antioxidant status of brain (Suzuki et al., 1983; Somani, 1994; Radák et al., 1995b) and the findings are conflicting. Suzuki et al. (1983) reported that voluntary exercise increased the thiobarbituric acid reactive substance (TBARS) in the brain of rats, whereas we were not able to detect any increase after exhaustive exercise which increased the TBARS level in the muscle, liver and kidney (Radák et al., 1995a, 1995b, 1996).

The present study was designed to assess whether regular swimming in rats induces alterations of the accumulation of oxidative stress markers in the brain and cognitive function. Previous findings suggest that the accumulation of RCD might influence cognitive function, and we, therefore, measured two kinds of activity of the proteasome enzyme complex which could provide information on the mechanism of the accumulation of oxidatively modified proteins. Epidemiological studies have shown that exercise decreases the incidence of certain forms of cancer (Sarna et al., 1993; Blair et al., 1995). Because DT-diaphorase appears to play an important role in anti-tumor action we were interested in whether exercise alters the activity of this enzyme.

## 2. Materials and methods

### 2.1. Animals

Twelve young (4-week old) and 12 middle-aged male Wistar rats (14-month old) were used for the study and cared for according to the "Guiding Principles for the Care and Use of Animals". The study was approved by the local Animal Welfare Committee. The rats were randomly assigned to control and exercised

groups: young control (C1); young exercised (E1); middle-aged control (C2); and middle-aged exercised (E2).

### 2.2. Training protocol and behavioral tests

All exercised rats were subjected to swimming exercise for 9 weeks. Water temperature was maintained at 32°C and swimming duration was 60 min per day, 5 days a week for 6 weeks; then for the remaining 3 weeks it was increased to 90 min a day for 5 days a week. Swimming was selected because no electric shock was required to promote this exercise protocol, and therefore, the stimuli of exercise would not interfere with the stimuli used during passive avoidance and conditioned avoidance tests. The control rats were transported to the experimental room and handled the same as the experimental animals without placing them in the swimming pool. One day after the last training session cognitive function was assessed with the passive avoidance learning test and conditioned pole-jumping avoidance test. An interval of 1 or 2 days was inserted into the schedule between the behavioral tests.

### 2.3. Retention of passive avoidance response

The passive avoidance behavior was investigated in a one-trial step-through paradigm (Ader et al., 1972). The apparatus consisted of a dark compartment (40 × 40 × 40 cm) and a well-lit platform attached to the front side of the dark chamber. A small sliding door separated the two compartments. On the first day of testing an 1 min adaptation was allowed in the dark compartment which was then followed by a single trial by placing the animal on the illuminated platform and allowing it to enter the dark chamber. On day 2, after entering the dark chamber an electric footshock (0.6 mA, 3 s) was delivered. The measurement of the passive avoidance response was done by the registration of latency in entering the dark compartment 1 day (short-term) and 3 days (long-term) following the electric footshock. Avoidance to enter the dark compartment during a 3-min time period was set to 100% and a time-related percentage was given when the animals entered (50% was given to the animal if they entered the chamber after 1.5 min of the start).

### 2.4. Pole-jumping conditioned avoidance behavior test

The active avoidance pole-jumping response was conditioned for a visual stimulus by the procedure described by Nyakas et al. (1991). The conditioning stimulus was delivered by a lamp positioned on the top of a vertical pole, which was centered in a glass-wall box. The unconditioned stimulus was an electric

footshock of 0.45 mA intensity delivered through the metal grid floor; this lasted for a maximum of 10 s. The unconditioned stimulus automatically followed 5 s after the conditioning stimulus. The inter-trial interval varied between 50 and 70 s. Before conditioning, three trials were given, in which a 15 s continuous electric footshock was delivered to enforce the escape of the rat from the floor onto the pole. The test to acquire the conditioned avoidance response consisted of 3 days and 10 trials were done a day. The number of conditioned avoidance responses (CAR) was measured and served to evaluate the learning capability of the animals and was expressed in points. Three points were given if the animal jumped to the pole in the time period between the light and electric stimuli. One point was given if the animal jumped to the pole during the time of electric stimuli.

### 2.5. Assays

For the estimation of malondialdehyde, a peroxidation marker, the TBARS method, was used (Ohkawa et al., 1979). To get more reliable data on lipid peroxidation and related oxidative damage we measured the 4-hydroxynonenal by commercially available monoclonal antibody as described by the supplier (Japan Institute for the Gerontology, Saitama, Japan). The measurement of RCD was done from the same sample preparation as described earlier (Radák et al., 1997) by both a spectrophotometric method and by Western blot using anti-2,4-dinitrophenylhydrazine (DNPH) antibodies.

In brief, proteins precipitated with trichloroacetic acid were suspended and incubated in a solution containing 10 mM DNPH and 2 N HCl for 1 h at 15°C. The resulting protein hydrazones were pelleted in a centrifuge at 11,000  $\times g$  for 5 min. The pellets were washed three times with ethanol–ethyl acetate (1:1) and then once with acetone. The final precipitates (1 mg protein) were dissolved in 1 ml buffer containing 8 M urea and 5% 2-mercaptoethanol using a sonicator for 10 min. Duplicate polyacrylamide gel electrophoresis of derivatized proteins was carried out in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. After the electrophoresis the proteins were transferred to nitrocellulose membranes. Then the membranes were soaked in PBS containing 3% skim milk, 0.05% Tween and 0.05% sodium azide and then treated with anti-DNPH antibody (Radák et al., 1998). After washing the buffer without antibodies, the membranes were treated with  $^{125}\text{I}$ -Protein A (0.02  $\mu\text{Ci}/\text{ml}$ ). Finally, the radioactive signals were quantified by BAS 2000 Bioimaging Analyzer (Fuji Film, Japan).

The isolation of nuclear DNA and the measurement of 8-hydroxy-2'-deoxyguanosine (8-OHdG) were performed as described by Kaneko et al. (1997). In

brief, after the isolation of DNA, the aqueous solution containing 50  $\mu\text{g}$  DNA was adjusted to 45  $\mu\text{l}$ , and 5  $\mu\text{l}$  of 200 mM sodium acetate buffer (pH 4.8) and 5  $\mu\text{g}$  of nuclease P1 were added. After purging with a nitrogen stream, the mixtures were incubated at 37°C for 1 h to digest the DNA to nucleotides. Then, 5  $\mu\text{l}$  of 1 M Tris–HCl (pH 7.4) and 0.65 units of alkaline phosphatase were added and the mixture was incubated at 37°C for 1 h to hydrolyze the nucleotides to nucleosides. Nucleosides were analyzed by the HPLC/ECD system, which consisted of a Pegasil ODS column connected to a Shimadzu LC-10 pump (Tokyo, Japan) coupled to an ECD (ESA Coulechem II 5200; Bedford, MA tor 1:150, 2:350 mV). The solvent system used was a mixture (pH 5.1) of 6% methanol, 12.5 mM citric acid, 30 mM sodium hydroxide, 25 mM sodium acetate, and 10 mM acetic acid. The flow rate was 1.4 ml/min. The amount of 8-OHdG in samples was expressed relative to the amount of dG as calculated from the absorbance at 260 nm with an UV detector (Shimadzu UVD-10), while the amounts of 8-OHdG were simultaneously measured by ECD.

Proteasomes have at least five distinct peptidase activities (Cardozo and Michaud, 1993) and among these two types of peptidase activities were measured for each fraction of the gradient as described previously (Hough et al., 1987; Hayashi and Goto, 1998). These peptidase activities were determined fluorometrically by measuring the release of 7-amino-4-methyl-coumarin (AMC) from the peptides succinyl-Leu-Leu-Val-Tyr-MCA (SUC-LLVY-MCA) and butyloxycarbonyl-Leu-Arg-Arg-MCA (BOC-LRR-MCA) for chymotrypsin-like and trypsin-like activities, respectively.

For the determination of DT-diaphorase activity the Erstner (1967) method was used. The assay mixture contained 25 mM Tris–HCl (pH 7.4), 0.3 mM NADH, 0.04 mM 2,6-dichloroindophenol and 0.2% Tween-20. The reaction was started by the addition of the enzyme, and the reduction was followed on a spectrophotometer at 600 nm.

To appraise the effect of physical exercise citrate synthase (CS) was measured as described by Shepherd and Garland (1969).

### 2.6. Statistical analysis

The open-field, passive avoidance and pole-jumping test data were evaluated by Whitney-U test. The biochemical data were assessed by ANOVA, followed by Scheffé's post-hoc test. When applicable, an unpaired Student's t-test was used. The significance was set at  $P < 0.05$ .

### 3. Results

Nine weeks of swimming did not result in significant differences in body mass between exercised and control groups. An age-associated decline in performance of the passive avoidance test ( $P < 0.05$ ), representing both short-term (24 h) and long-term (72 h) memory (Fig. 1), was found by comparing the achievement of C1 and C2 rats. This decline was prevented by exercise as shown in the data for E2 ( $P < 0.05$ ). In other words, passive avoidance response revealed that exercise improved short- and long-term retention of a learned response. The pole-jumping active avoidance test data showed that exercised rats were able to achieve significantly greater success ( $P < 0.05$ ) with the learning process as measured by this test (Fig. 2).

Interestingly, 9 weeks of swimming did not increase the activity of CS in the brain. The oxidative damage markers of lipids and DNA did not show changes in any group, nor were the 4-hydroxynonenal signals detectable, suggesting 4-hydroxynonenal modified proteins are negligible (Table 1). The RCD appeared to increase in C2 compared with C1 and this increase was attenuated by exercise ( $P < 0.05$  (Fig. 3)). The chymo-

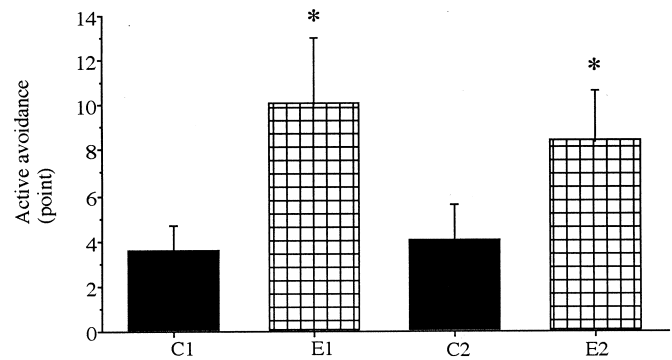


Fig. 2. The active avoidance test was done to measure the ability of rats to respond to unconditioned visual stimuli followed 5 s later by a conditioned stimuli. The graph shows the collected points. The performance of exercised rats was significantly better than control in both age groups. Values are means  $\pm$  SD for six animals per group. \* $P < 0.05$  the effect of exercise.

trypsin-like activity of proteasome complex showed a significant increase as a result of exercise, while trypsin-like activity (breakdown of BOC-LRR-MCA) did not change significantly (Fig. 4). The activity of DT-diaphorase increased in E1 compared with C1

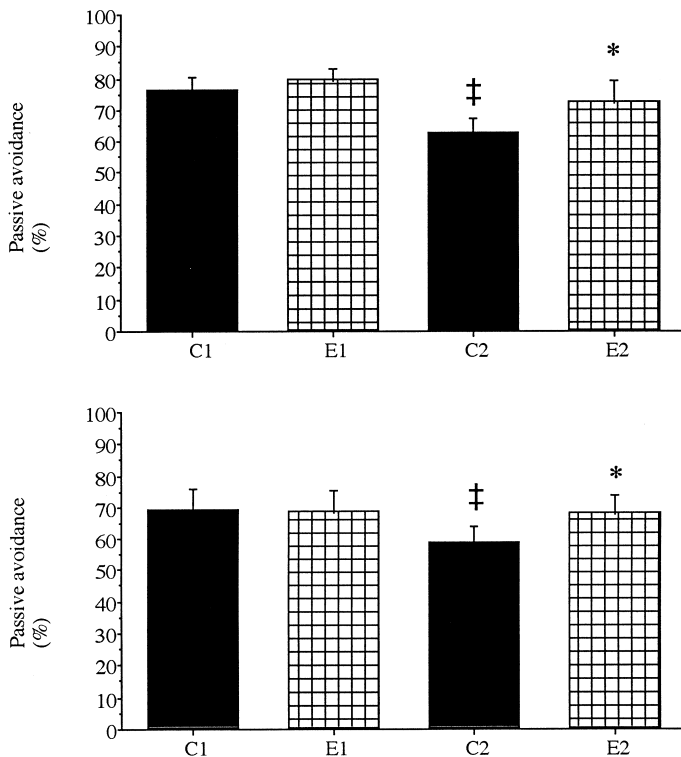


Fig. 1. On the second day of the passive avoidance test electric shock was delivered to the rats when they entered the dark chamber. Panel A shows the degree of memory 24 h and panel B 72 h after the shock. Values are means  $\pm$  SD for six animals per group. \* $P < 0.05$  the effect of exercise, † $P < 0.05$  the effect of aging. C1: young control; E1: young exercised; C2: middle-aged control; E2: middle-aged exercised.

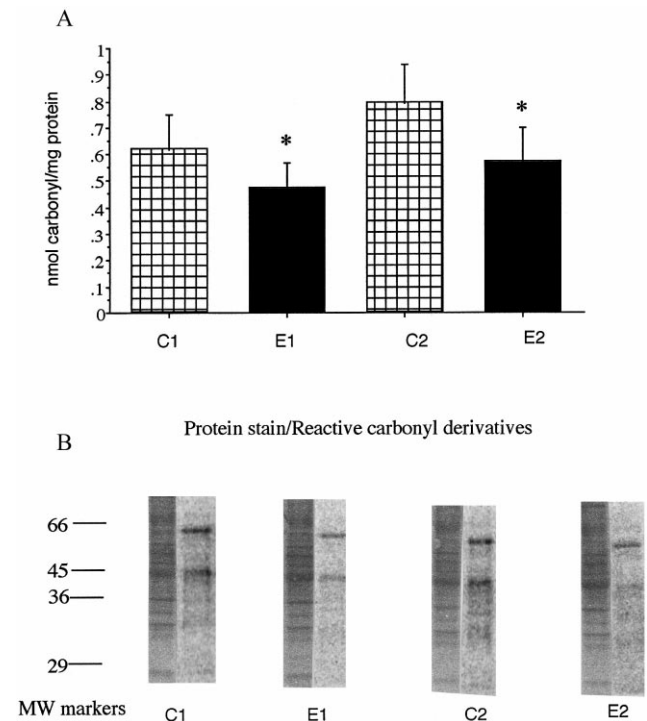


Fig. 3. Accumulation of reactive carbonyl derivatives (RCD) in the brain of rats was reduced by exercise training in both age groups. Panel A shows the data of spectrophotometric measurements. Values are means  $\pm$  SD for six animals per group. \* $P < 0.05$  the effect of exercise, † $P < 0.05$  the effect of aging. Panel B displays the immunoblot data. The RCD signals are to the right of Coomassie Blue panels, and the molecular weight markers are at left.

Table 1

The activity of citrate synthetase and content of 8-OHdG and TBARS in rat brain<sup>a</sup>

|  | C1 <sup>b</sup> | E1 <sup>b</sup> | C2 <sup>b</sup> | E2 <sup>b</sup> |
|--|-----------------|-----------------|-----------------|-----------------|
| CS (nmol/mg protein)                   | 23.7 ± 1.4      | 24.0 ± 1.3      | 19.5 ± 0.5      | 19.8 ± 0.8      |
| DNA damage (8-OHdG/10 <sup>6</sup> dG) | 0.477 ± 0.07    | 0.489 ± 0.05    | 0.634 ± 0.08    | 0.589 ± 0.03    |
| TBARS (nmol/mg protein)                | 0.295 ± 0.01    | 0.248 ± 0.06    | 0.331 ± 0.02    | 0.303 ± 0.02    |

<sup>a</sup> Means ± SD of six animals.<sup>b</sup> C1: young control; E1: young exercised; C2: middle-aged control; E2: middle-aged exercised.

( $P < 0.05$ ), but no difference was observed for the middle-aged groups (Fig. 5).

#### 4. Discussion

Exercise in humans is reported to ameliorate and/or retard the age-associated decline in cognitive function (Chodzko-Zajko and Moore, 1994). In the present work, we showed that moderate regular exercise decreased the accumulation of RCD in rat brain. This decrease might be involved in the mechanism by which exercise improves the learning and memorizing capability of animals. The improved memory function, as observed by passive avoidance learning and a mark-

edly facilitated active avoidance learning performance, was observed after 9 weeks of exercise. In the pole-jumping active avoidance test the cognitive performance was probably facilitated by the enhanced physical condition of the animals, that resulted from regular swimming. However, it seems very unlikely that muscle action alone, without cognitive drive, could make such a significant difference. The present data indicate that there is a relationship between the oxidative modification of proteins and brain function, which is in accordance with previous observations (Carney et al., 1991; Socci et al., 1995). Data reported by Forster et al. (1996) are in accordance with our findings in that they found a strong negative correlation between the accumulation of RCD in brain proteins and cognitive performance in aging mice. Moreover, they observed that the age-associated loss in motor coordination is correlated with oxidative damage of proteins in brain, as compared to a proposal that a functional relationship exists between the decline in cognitive function and motor coordination and oxidative modifications of proteins (Forster et al., 1996). In most of the related studies on aging, accumulation of oxidative modification in proteins is reported to be more enhanced in older animals than in younger ones (Stadtman, 1992; Sohal and Weindruch, 1996; Forster et al., 1996; Goto and Nakamura, 1997). In the current study we used young, still growing, and middle-aged rats and the

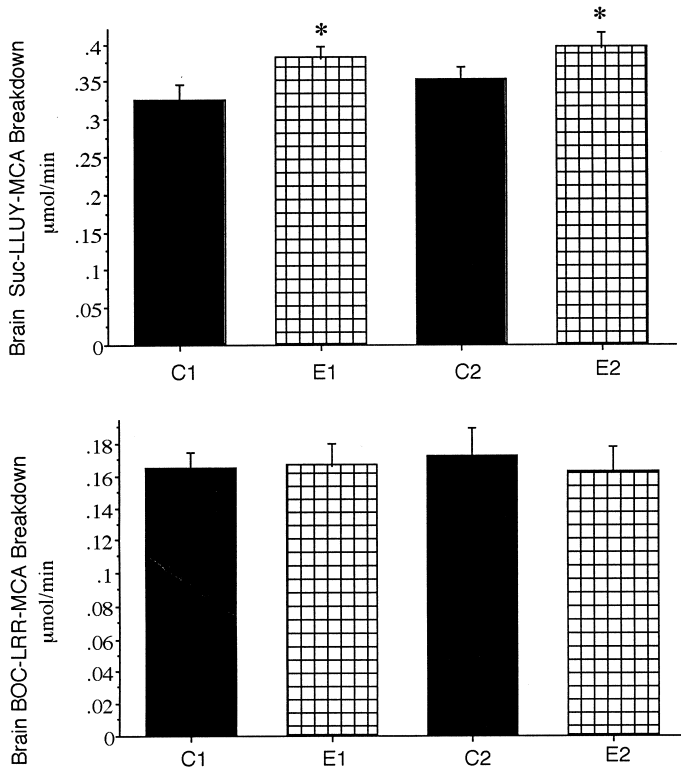


Fig. 4. The peptidase-like activity (breakdown of SUC-LLVY-MCA) of proteasome complex is shown in panel A, while the chymotrypsin-like and trypsin-like activity (breakdown of BOC-LRR-MCA) are found in panel B. Values are means ± SD for six animals per group. \* $P < 0.05$  the effect of exercise.

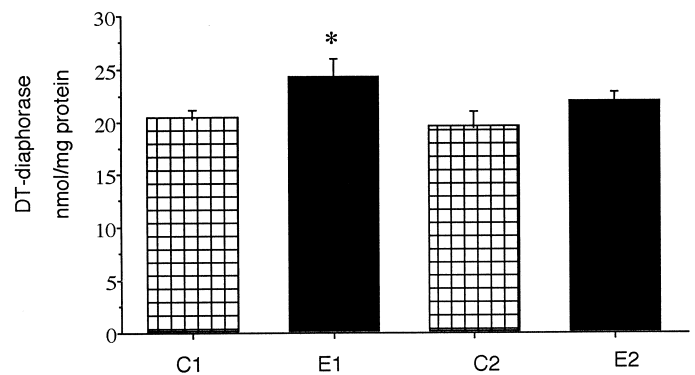


Fig. 5. The activity of DT-diaphorase increased significantly in the brain of E1 rats as a result of regular training. No other changes were observed. Values are means ± SD for six animals per group. \* $P < 0.05$  the effect of exercise.

levels of RCD decreased significantly in the brains of both age groups as a result of regular exercise. This finding permits us to hypothesize, that even normal, steady-state level of oxidative modification of proteins hampers cell function, and the decrease in RCD accumulation is accompanied by better neuronal function.

Physical exercise might act via different pathways including induction of fibroblast growth factor, antioxidant enzyme activities, or concomitant increases in catecholamine secretion and these alterations can lead independently to better cognitive function (Chodzko-Zajko and Moore, 1994; Pagliari and Peyrin, 1995; Sugaya et al., 1996; Ohkuwa et al., 1997). On the other hand, it seems that the steady-state level of lipid peroxidation and 8-OHdG content in the brain are not significantly affected by exercise, and therefore, the measured changes in cognitive function and behavioral stress can not be readily ascribed to the oxidative modification of lipid and DNA of the brain. Obviously more study is needed to confirm that cognitive function is exclusively or mainly dependent upon the modification of protein molecules.

The increases in the activity of antioxidant enzymes in the brain as a response to regular physical exercise is most probably due to the excess formation of free radicals (Radák et al., 1995a, 1995b; Somani et al., 1995). These reactive species might alter the redox state of cells involving pre- and post-translational modifications and cellular homeostasis. It has been shown that accelerated brain aging as in Alzheimer's disease, is associated with increases in post-translational modifications of proteins and decreases in proteolytic removal of altered proteins, resulting in impaired cognitive function (Smith and Perry, 1996). It appears that this impairment, based on neuronal degeneration, is protein mediated and might be due to the accumulation of damaged proteins such as beta-amyloid and paired helical filaments. In the present study, we observed a decline in RCD accumulation and associated with this better memory and learning. This decrease in RCD accumulation and an increase in proteasome activity could mean faster protein turnover, resulting in a smaller amount of physiologically inactive oxidatively modified 'junk'. In this way the oxidatively modified proteins would not hamper cell function, which otherwise might occur because of a massive accumulation of RCD in untrained aging rats.

Muscular activity is dependent upon nervous stimulation. Repeated activation of nerve cells might lead to improved nerve function. Voluntary and/or forced inactivity of the muscular or neural systems might have serious consequences. Eight hours immobilization resulted in an increase in RCD, TBARS and 8-OHdG content in rat brain (Liu et al., 1996). It was suggested that immobilization caused emotional oxidative stress

to the animals. We believe that even if an oxidative stress had occurred due to exercise-induced mild emotional stress in the middle-aged group, it was compensated for and outweighed by the beneficial effects of regular exercise. Epidemiological studies have revealed that regular exercise reduces the incidence of certain type of cancer (Sarna et al., 1993; Blair et al., 1995). DT-diaphorase is regarded as a 'physiological tool' against tumor formation, since it neutralizes quinones and nitrobenzenes and induces antitumor compounds (Erstner and Dallner, 1995). It would be worthwhile to know, whether the exercise-induced increase in DT-diaphorase activity in the brain and other tissues (Radák et al., 1999) is an active contributor to reduced incidence of cancer.

In summary, we suggest regular exercise improves cognitive function in parallel with a decrease in the accumulation of oxidative modification of proteins. Other mechanisms might also be involved in the accumulation of oxidatively damaged macromolecules and the repair process may differ as well. Regular physical exercise seems to be an important means by which the age-associated decline of cognitive function can be efficiently prevented.

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