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Original Research Communication

Dietary Thioproline Decreases Spontaneous Food Intake and Increases Survival and Neurological Function in Mice

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ABSTRACT

Male mice on a diet supplemented with thioproline (*l*-thiazolidine-4-carboxylic acid), a physiological metabolite of 5-hydroxytryptamine, at 2.0 g/kg of food from 28 weeks of age and for their entire life, showed a 23–29% increased median and maximal life span. These survival increases were associated with improved neurological functions. Compared to control mice, thioproline-supplemented mice had a 20% lower integral spontaneous food intake, and 10% lower body weight at 100 weeks of age. Body weight showed a statistically significant inverse relationship with survival and neurological performances. Thioproline-supplemented mice exhibited a 58–70% decrease of the age-dependent oxidative damage in brain and liver mitochondria at 52 weeks (old mice) and 78 weeks (senescent mice) of age, respectively. The age-associated decrease of brain mitochondrial enzyme activities, NADH-dehydrogenase, cytochrome *c* oxidase, and mitochondrial nitric oxide synthase (mtNOS), in old and senescent mice were markedly prevented (51–74%) by thioproline. *In vitro,* thioproline neither exhibited direct antioxidant activity nor had any effect on the electron transfer or mtNOS functional activities of brain and liver mitochondria. It is surmised that thioproline induces an anorexic effect associated with improved survival and neurological function through a decreased oxidative damage and regulation that may involve hypothalamic appetite centers. *Antioxid. Redox Signal.* 9, 131–141.

INTRODUCTION

There is a current interest in defining conditions that increase mammalian life span. Previous reports had shown that moderate physical exercise (32), higher spontaneous neurological activity (34), and diet supplementation with high doses of vitamin E (33) or acetylcarnitine and lipoic acid (20, 26) extend mouse life span, improve neurological function, and ameliorate the mitochondrial dysfunction associated with aging.

Thioproline (*l*-4-thioproline, *l*-thiazolidine-4-carboxylic acid) is a normal brain metabolite produced in the catabolism of 5-hydroxytryptamine (41, 50); thioproline nitroso-derivatives, *N*-nitroso-*l*-4-thioproline and *N*-nitroso-*l*-4-methylthioproline, have been identified in human urine (43). Thioproline has been reported to have *in vivo* antioxidant effects and as such to improve the immune response in aged mice (12) and to increase median life span associated with reduced oxygen consumption in *Drosophila melanogaster* (28, 29). Moreover, thioproline inhibited chemical carcinogenesis (19, 37) an action interpreted as inhibition of the *in vivo* nitrosation of *N*-benzylmethylamine (42) and has been used in the treatment of squamous cancer in humans as the drug Norgamem (2, 21).

Aging has been associated with mitochondrial dysfunction, a concept supported by the understanding of the function of these organelles as the main source of cell energy and by the physiological decline of organ metabolism and energy expenditure upon aging (24, 39). There is also an active research on the mechanisms of the decline in mitochondrial function inher-

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ent in aging (5, 30, 47) that may support strategies for preservation of mitochondrial function (32, 33). The mitochondrial alterations observed upon aging are: an increased content of the oxidation products of phospholipids, proteins, and DNA; diminished rates of electron transfer by mitochondrial complexes I and IV; decreased active state 3 respiration without uncoupling or decreased F_1-ATP synthase activity (31); and decreased activities of translocating enzymes, such as carnitine acetyltransferase (31) and adenine nucleotide translocase (51). Brain mitochondria isolated from aged animals exhibit a decrease of complexes I and IV electron transfer and levels of oxidation products that are linearly related to the age-dependent impairment of neurological function, as determined by tests of neuromuscular coordination and of exploratory function (31).

This study examines the effects of chronic dietary supplementation with thioproline on the physiological and biochemical indicators of mice aging. Survival and neurological performance were taken as physiological indicators of aging (31) and the mitochondrial content of oxidative damage products, the electron transfer activities of complex I and IV, and the activity of mitochondrial nitric oxide synthase (mtNOS) (31) were considered as biochemical indicators of aging.

MATERIALS AND METHODS

Animals and thioproline supplementation

Mice of the Swiss CD-1/UCadiz strain inbred at the Department of Experimental Animals of the University of Cadiz (32–34) were housed in groups of five animals at 22 ± 2°C with 12 h/12 h light/dark cycles. Mice used in this study (CD-1/UCadiz) belong to a senescence accelerated strain similar to AKR, SAM, NZB/Lac, and SJL/J that exhibit a median life span of 36–57 weeks and a maximal life span of 52–83 weeks (16). The control group received standard laboratory animal food (A04 diet, Panlab LS, Barcelona, Spain) with *ad libitum* access to food and water, whereas thioproline-supplemented mice received the same food added with 2.0 g *l*-4-thioproline (*l*-4-thiazolidin carboxylic acid)/kg of food, from 28 weeks of age and for their entire lives. Thioproline withdrawal was imposed to a group of eight thioproline-supplemented mice at week 52. Mice were weekly weighed and checked to verify their pathogen-free condition. The food provided in the cages was daily weighed, before and after being exposed to mice. 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.

Survival curves

Mouse survival was controlled daily. Male mice (n = 50); thioproline-supplemented male mice (n = 78), female mice (n = 40); and thioproline-supplemented female mice (n = 40) provided the data for the survival curves and the statistics for equality of survival distributions.

Behavioral tests

Individual mice were subjected every 2 weeks to two behavioral assays: the tightrope and the T-shaped maze tests. In the tightrope test for evaluation of neuromuscular coordination (34), mice were placed hanging from their anterior legs in the middle of a 60 cm tightrope and the test was considered successful when mice reached the column at the end of the rope in less of 60 s. In the maze test for evaluation of spontaneous exploratory and cognitive activity (34), mice were challenged in a T-shaped maze of 50 cm arms; and the test was considered successful when mice moved towards the T-intersection in <60 s. Mice did not receive any food reward that may encourage movement towards the T-junction. There was no familiarity or improved response developed with the use of the T-maze, since successes decreased with time.

Isolation of mitochondria

Brain and liver mitochondria were isolated from whole organs homogenized in 0.23 *M* mannitol, 0.07 *M* sucrose, EDTA 1 m*M*, and Tris-HCl 10 m*M*, pH 7.4, at a ratio of 9 ml of homogenization medium/g of tissue in a Potter homogenizer with a Teflon pestle. The homogenate was centrifuged at 700 *g* for 10 min and the supernatant at 8000 *g* for 10 min to precipitate mitochondria that were washed in the same conditions (31, 32, 34). Mitochondrial suspensions, at about 20 mg protein/ml, were used immediately after isolation for O_2 uptake determination or frozen in liquid N_2 and kept at −80°C. Mitochondrial samples were twice frozen and thawed and homogenized each time by passage through a tuberculin needle. The procedure yielded a preparation with disrupted mitochondrial membranes that had 0.19–0.25 nmol cytochrome aa_3/mg protein and that was used for determination of enzyme activities and oxidative stress markers. Protein content of the samples was determined using the Folin reagent, with bovine serum albumin as standard.

Biochemical markers of oxidative damage

Protein carbonyls and thiobarbituric acid-reactive substances (TBARS) were determined in mitochondrial membranes by the assays of Fraga *et al.* (18) and of Oliver *et al.* (36), modified as described (31) and expressed in pmol/mg of mitochondrial protein.

Mitochondrial electron transfer activities

The membrane-bound activities of complexes I-III, II-III, and IV were determined spectrophotometrically at 30°C with the mitochondrial membranes suspended in 100 m*M* phosphate buffer, pH 7.4. For NADH-cytochrome *c* reductase (the activity of complexes I and III) and succinate-cytochrome *c* reductase (the activity of complexes II and III), mitochondrial membranes were added with 0.2 m*M* NADH or with 20 m*M* succinate as substrates, 0.1 m*M* cytochrome c^{3+}, and 1 m*M* KCN, and the enzymatic activity was determined at 550 nm (ϵ = 19 $mM^{-1}cm^{-1}$) and expressed as nmol cytochrome *c* reduced/min × mg protein. Cytochrome oxidase (complex IV) was determined in the same buffer added with 0.1 m*M* cytochrome c^{2+}, which was prepared by reduction with $NaBH_4$ and HCl. The rate of cytochrome *c* oxidation was calculated as the first order reaction constant (k')/mg protein and expressed as nmol cytochrome c oxidized at 10 μ*M* cytochrome *c*/ min × mg protein, which gives rates of electron transfer of the order of physiological O_2 uptake (32, 34).

Spectrophotometric determination of mitochondrial nitric oxide synthase (mtNOS) activity

Mitochondrial NO production was determined by the oxyhemoglobin (HbO_2) oxidation assay at 30°C, as described (7). The reaction medium consisted of 0.1 m*M* NADPH, 0.2 m*M* arginine, 1 m*M* $CaCl_2$, 4 μ*M* Cu,Zn-superoxide dismutase (SOD), 0.1 μ*M* catalase, and 25 μ*M* HbO_2 heme, in 50 m*M* phosphate, and 0.5–0.7 mg mitochondrial protein/ml, pH 7.4. A diode array sensitive spectrophotometer (model 8453 Agilent Corporation, Palo Alto, CA) was used to follow the absorbance change at 577 nm with a reference wavelength at the isosbestic point of 591 nm ($\epsilon_{577-591}$ = 11.2 $mM^{-1}cm^{-1}$). Production of NO was calculated from the absorbance change that was inhibited by 2 m*M* N^G-methyl-L-arginine, usually 92–96%, and expressed in nmol NO/min × mg protein.

Mitochondrial oxygen uptake and mtNOS functional activity

Mitochondrial respiration was determined with a Clark electrode in a 1.5 ml chamber at 30ºC, in an air-saturated reaction medium consisting of 0.23 *M* mannitol, 0.07 *M* sucrose, 20 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, 5 m*M* phosphate, 4 m*M* $MgCl_2$, and 0.5–0.7 mg mitochondrial protein/ml, at pH 7.4 (8). Respiratory rates were determined with 10 m*M* succinate or 5 m*M* malate-glutamate as substrates, and state 3 active respiration was established by addition of 0.5 m*M* ADP. Respiration is expressed in ng-at O consumed/min × mg protein.

The mtNOS functional activity was assayed by the determination of the difference between the rates of state 3 respiration of coupled mitochondria at maximal and minimal intramitochondrial NO levels, as described (33, 46) and expressed as percentage of state 3 oxygen uptake. The first condition, high NO levels, was achieved by supplementation with 0.2 m*M* L-arginine and 1 μ*M* superoxide dismutase (SOD), and the second one, low NO levels, by addition of 1 m*M* L-NAME and 20 μM HbO_2.

Total antioxidant activity

The antioxidant capacity of thioproline, proline, methionine, and cysteine were assayed by the ABTS (2,2-azo-bis-di-3-ethyl-benzo-thiazolin-sulfonate) assay (27, 44). The assay is based upon the formation of radical intermediates in the peroxidase reaction of methemoglobin, H_2O_2, and ABTS that yield the radical cation ($ABTS^{\bullet+}$) with intense blue/green color and with absorption maximum at 734 nm. Color formation is suppressed by hydrogen-donating antioxidants linearly related to the antioxidant capacity of the sample. The assay was carried out spectrophotometrically at 734 nm and at 30°C in 50 m*M* phosphate buffer, 0.14 m*M* methemoglobin, 1 m*M* H_2O_2, and 5 m*M* ABTS. The antioxidant capacities of 1 m*M* amino acid solutions were compared with 1 m*M* trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and expressed in trolox units.

Statistics

The survival curves were analyzed by the Kaplan–Meier test. Numbers in tables and figures are mean values ± SEM. The differences between groups were analyzed by the Student–Newman–Keuls as *post hoc* test after significant one-way ANOVA. A p value of < 0.05 was considered statistically significant. Statistical analyses were carried out using a statistical package (SPSS 11.5 for Windows).

RESULTS

Mice supplemented with thioproline (2 g/kg of food) in the diet, starting at 28 weeks of age, exhibited increased survival, an effect that was more marked in males than in females. Male mice receiving thioproline showed a 29% increased median life span (from 62 ± 4 to 80 ± 3 weeks) and a 23% increased maximal life span (from 118 ± 4 to 145 ± 3 weeks). Female mice receiving the same thioproline supplementation showed a more modest effect, a 21% increased median life span and a 10% increased maximal life span (Fig. 1).

Thioproline-supplemented male mice showed a decrease in body weight, clearly noticeable after 12 weeks of thioproline supplementation and with statistically significant differences with the control animals after 50 weeks of age (Fig. 2). At 52, 78, and 100 weeks of age, thioproline-supplemented mice were 4, 10, and 10% lighter than control mice of the same age. The thioproline effect was reversible; drug withdrawal was followed by restoration of body weight gain (Fig. 2, inset).

Control mice had a mean value of *ad libitum* food intake of 6.3 ± 0.1 g/day, whereas the *ad libitum* food intake of thioproline-supplemented mice was 5.1 ± 0.1 g/day, thus implying a spontaneous decrease in food intake of about 19% in thioproline-supplemented mice (Fig. 3). The relationship between the amount of food taken up and the weight in both experimental groups is analyzed in Fig. 4. The linear relationship between the ratio of food intake, [(g/day × mouse) in thioproline-supplemented mice)/(g/day × mouse) in control mice] and the ratio of weight, [(g/mouse) in thioproline-supplemented mice)/(g/mouse) in control mice], indicates an equal metabolic utilization of energy intake, as g food/day (*i.e.*, as kJ/day), in terms of body weight gain in both mice groups.

The relationship between body weight and survival is analyzed in Fig. 5. The negative hyperbolic correlation between individual body weight and individual survival fits the data of control and thioproline-treated mice, indicating that extended survival largely depended on reduced weight.

Mice were individually tested for neurological performance every 2 weeks, starting at 28 weeks of age and for their entire lives. Neuromuscular coordination was assayed by the tightrope test and exploratory function was tested by the T-shaped maze. Success in both tests decreased continuously with age, but, interestingly, the decline was less marked in thioproline-supplemented animals (Fig. 6). In control mice, success in the tightrope test (taking as reference the about 57% success at 28 weeks of age (young-adult mice), decreased to 36% at 52 weeks (old-adult mice) and to 10% at 78 weeks (senescent mice). This loss in motor coordination was ameliorated in thioproline-supplemented mice, with success values of 41% and 25% at the two time points of 52 and 78 weeks (Fig. 6A). Likewise, success in the T-maze test decreased continuously with age, starting with a value of about

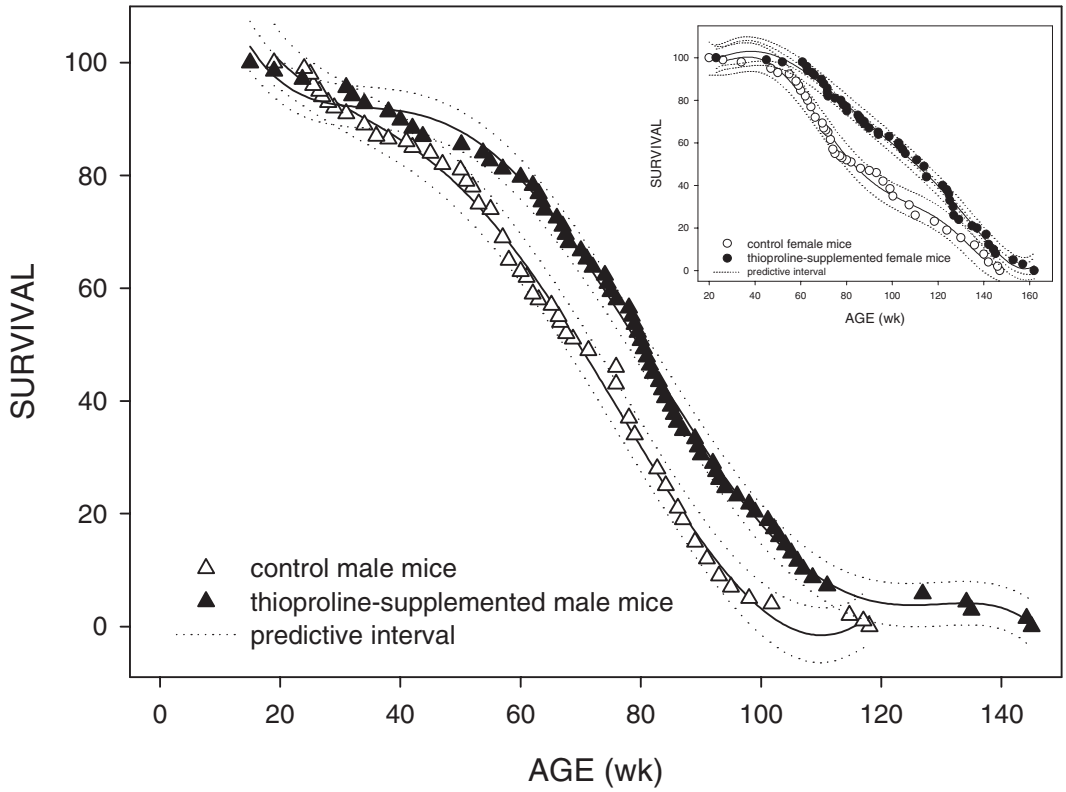

FIG. 1. Mouse survival curves. Control male mice ($n = 50$): median life span, 62 ± 4 weeks; maximal life span, 118 ± 4 weeks; thioproline-supplemented male mice ($n = 70$): median life span, 80 ± 3 weeks; maximal life span, 145 ± 3 weeks. Statistics for equality of survival distributions for male: Log Rank: 9.8, $p < 0.001$; Breslow: 11.5, $p < 0.0001$; Tarone–Ware: 11, $p < 0.0001$. **Inset:** Control female mice ($n = 40$): median life span, 82 ± 5 weeks; maximal life span, 147 ± 4 wk; thioproline-supplemented female mice ($n = 50$): median life span, 99 ± 5 weeks; maximal life span, 162 ± 5 weeks. Statistics for equality of survival distributions for male: Log Rank: 5.6, $p < 0.01$; Breslow: 6.6, $p < 0.01$; Tarone–Ware: 6.3, $p < 0.01$.

88%. In control mice, success values were 50% and 41% at 52 and 76 weeks of age, respectively. The neurological loss was again less pronounced in thioproline-supplemented mice with success values of 72% and of 53% at 52 and 78 weeks, respectively (Fig. 6B). The success in both neurological tests, tightrope and T-maze, was inversely related to mouse weight, and single straight lines fitted the data of the animals of both groups (Fig. 7).

The content of the oxidation products of mitochondrial constituents, due to free-radical mediated reactions, increased in mouse brain and liver with age and was partially prevented by thioproline supplementation (Table 1). Oxidation of brain mitochondrial proteins in control mice, considering protein carbonyls as markers and taking the value at 28 weeks as reference, augmented by 38% and by 79% at 52 and 78 weeks of age, respectively. This increase was significantly prevented in thioproline-supplemented mice, by 50% and by 58%, at the two considered ages. The TBARS content in brain mitochondria showed a similar pattern with aging and with thioproline supplementation; the contents were 32% and 86% increased at 52 and 78 weeks in control animals, but only 12% and 41% increased in thioproline-supplemented mice at the same time points. Liver mitochondria exhibited similar changes in both markers of oxidative damage, albeit less marked (Table 1).

The activities of the mitochondrial enzymes NADH-cytochrome *c* reductase, cytochrome *c* oxidase, and mitochondrial nitric oxide synthase (mtNOS), three constitutive proteins of the inner mitochondrial membrane, decreased almost linearly between 28 and 78 weeks of mouse age, in the range of 17% to 61% in brain and of 4% to 37% in liver (Table 2). A decreased NADH-cytochrome *c* reductase activity with an unaffected succinate-cytochrome c reductase activity is interpreted as a selectively decreased NADH-dehydrogenase (complex I) activity (32, 34). Complex I activity decreased in brain and liver, taking 28 weeks as reference point, by 17% and 20% at 52 weeks, and by 33% and 30% at 78 weeks. Cytochrome *c* oxidase (complex IV) activity similarly decreased in brain and liver by 26% and 27% at 52 weeks and by 38% and 41% at 78 weeks. The activity of mtNOS was more markedly decreased: in control mice the activity decreased in brain and liver to 66% at 52 weeks and to 39–43% at 78 weeks. Thioproline supplementation was effective in preventing this age-associated decrease in mtNOS activity in brain and liver; with activities decreasing only to 91–96% at 52 weeks and to 70–63% at 78 weeks. Mitochondrial enzymic activities were found correlated to mouse weight and a single straight line fitted the data of the three enzymes and of the animals of both groups (Fig. 8).

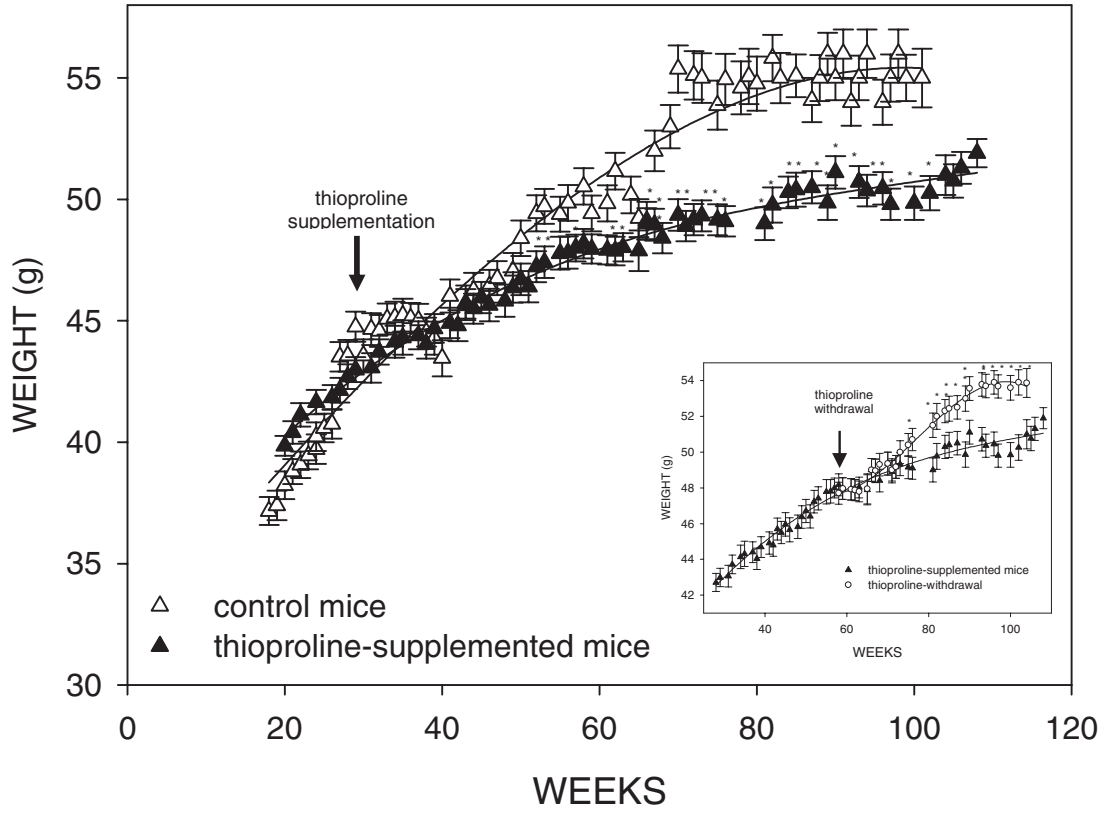

FIG. 2. Male mice weight. Hyperbola adjustment: control, $r^2 = 0.96$; thioproline-supplemented, $r^2 = 0.97$; *t*-test $*p < 0.05$ from 50 weeks of age. Thioproline-supplemented mice with survival higher than 110 weeks (included in Fig. 1) were excluded from this graph. **Inset:** A group of 8 thioproline-supplemented mice were deprived of thioproline at week 52. $*p < 0.05$.

Thioproline added *in vitro* to mouse brain mitochondria in the concentration range of 10–50 μM did not elicit any effect on the mitochondrial respiratory rates of metabolic states 3 and 4 (Table 3). Brain mtNOS functional activity (*i.e.*, the rates of mitochondrial O_2 uptake that are regulated by the intramitochondrial production of NO by mtNOS), were not affected by thioproline *in vitro.* (Table 3). Similar results were observed with mouse liver mitochondria (data not shown). It is worth comparing these data with the one in Table 2. Chronic thioproline supplementation has a clear *in vivo* ef-

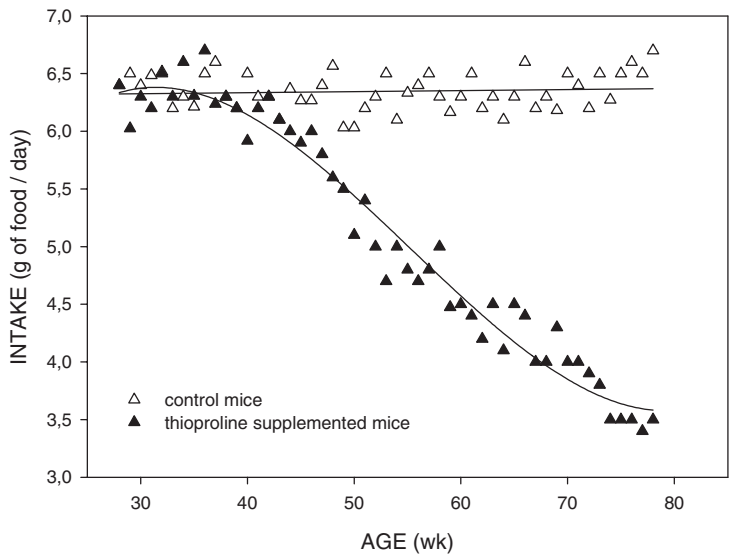

FIG. 3. Spontaneous food intake of control thioproline-supplemented mice: control mice, R = 0.99 ± 0.16, $p < 0.5$, and thioproline-supplemented mice, R = 0.99 ± 0.18, $p < 0.01$.

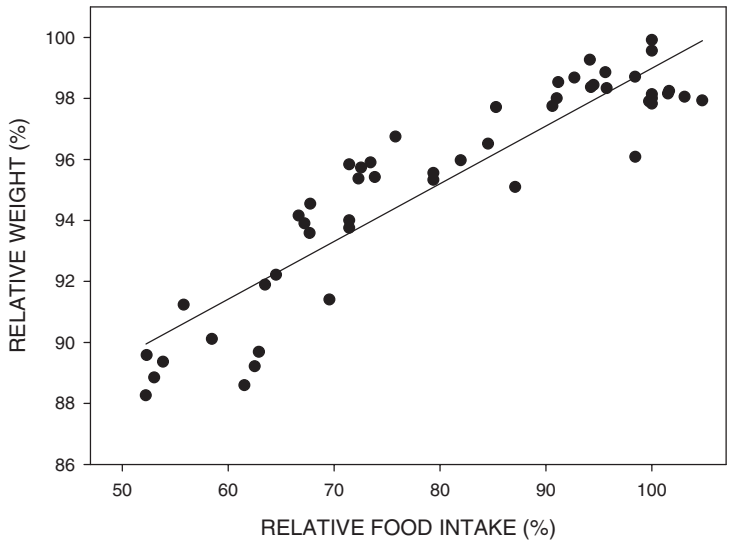

FIG. 4. Correlation between relative food intake (percentage of food intake (g/day) of thioproline-supplemented in relation to control mice) and relative weight (percentage of weight of thioproline-supplemented and control mice) from 28 weeks to 78 weeks of age. R = 0.99 ± 1.4, $p < 0.5$.

fect, shown by the protection of oxidative damage and the enzymatic activities of complexes I and IV and of mtNOS.

The thioproline molecule is not endowed with antioxidant properties, as determined by the ABTS test. The antioxidant capacities (expressed in trolox equivalent units) were: thioproline = 0.00; proline = 0.00; methionine = 0.00, cysteine = 0.50 ± 0.02 units, and trolox 1.00.

DISCUSSION

Dietary supplementation with thioproline was capable of increasing mice median and maximal life span, in the quantitative range of 10–29%, in male and female mice, and in agreement with reports of increased median life span in *Drosophila melanogaster* treated with this compound (29). Female mice were less affected than male mice by dietary thioproline with a lower increase in survival, a situation analogous to other conditions in which male mice show a greater increase in survival than females (32–34).

The senescence accelerated mice strain CD-1/UCadiz used in this study has been also used to show the beneficial effects of moderate exercise (32), spontaneous neurological activity (34), and high doses of vitamin E (33) on survival, brain function, and brain mitochondrial oxidative damage, and electron transfer. The CD 1/UCadiz ages without large anomalies, with accumulation of amyloid bodies in brain, liver, heart, and kidney, and with a decrease in neurological function at the end of their lives that is comparable to normal mice (16).

The level of thioproline dietary supplementation used in this study, 2.0 g/kg food is similar to the ones used by de la Fuente *et al*. (0.7–1.0 g/kg) in mice immunological studies (12) and by Suo *et al*. (5.0 g/kg) in rats (40). In *Drosophila melanogaster*, the biological effects were observed with a thioproline at 3.0 g/kg food (29). In studies with isolated

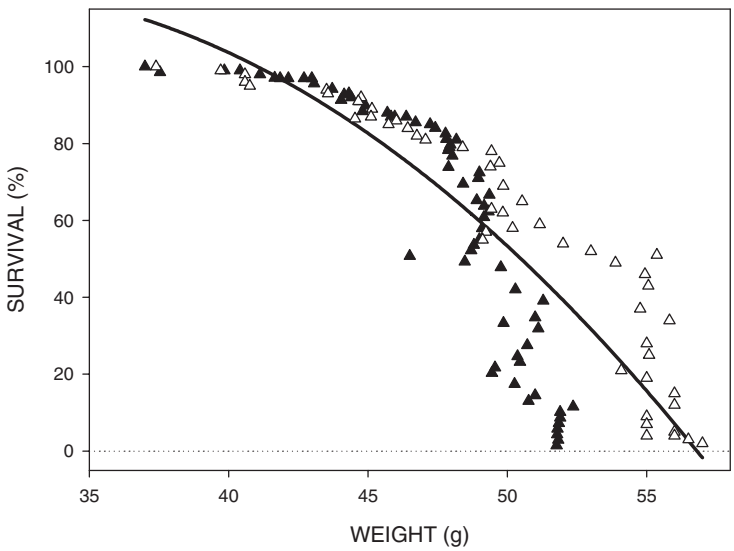

FIG. 5. Correlation between mice weight and survival. △, control mice; ▲, thioproline-supplemented mice. Hyperbola adjustment of the data in the plot, $r^2 = 0.74$. Control, $r^2 = 0.99$, $p < 0.001$; thioproline-supplemented, $r^2 = 0.99$, $p < 0.001$. Paired *t*-test $p < 0.0001$.

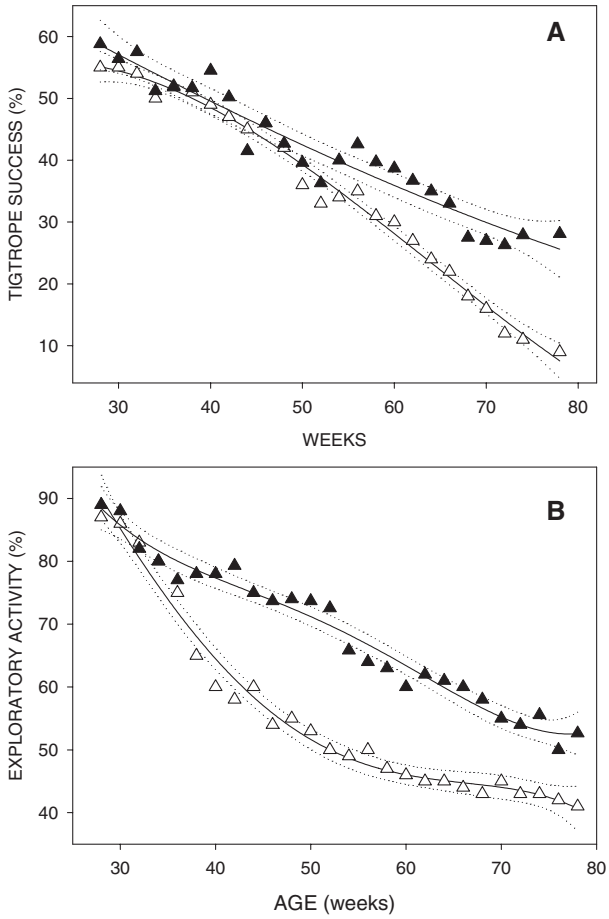

FIG. 6. Effect of thioproline supplementation on neuromuscular and exploratory activities of male mice as a function of age. △, control mice; ▲, thioproline-supplemented mice. (**A**) Tightrope test: control, $r^2 = 0.99$; thioproline-supplemented mice, $r^2 = 0.93$; *t*-test $p < 0.05$. (**B**) T-maze test: control, $r^2 = 0.98$; thioproline-supplemented mice, $r^2 = 0.97$; *t*-test $p < 0.001$.

cells, thioproline was added to the suspension medium in the 0.1–5 m*M* range (11). In humans, thioproline was given at a dose of 5–60 mg/kg daily (19), a dosage that is comparable to other anorexic drugs, such as Sibutramine (10 mg/day) and Orlistat (360 mg/day) (49).

The decrease in food intake, albeit available *ad libitum*, in thioproline-supplemented mice can be interpreted either as an olfactory or gustatory aversion or as a cumulative effect of thioproline on the appetite control areas of the central nervous system. A partial answer is given by the data in Fig. 2; it can be garnered that dietary thioproline did not produce a difference in weight gain in the period of 28–40 weeks, which would be consistent with the sensorial aversion interpretation, but the weight difference was evident from 50 weeks onwards. Mice supplemented with thioproline showed neither decrease in weight nor in food intake for the first 2 months, which indicates no preference in terms of both diets. However, after 50 weeks, mice restricted themselves in the intake of available food.

The negative correlation between body weight and survival that fitted both control and thioproline-supplemented mice (Fig. 3) is in clear agreement with the concept of increased mammalian survival in caloric restriction (15, 45). It may be surmised that dietary thioproline provides a model of decreased food intake and anorexia. Early reports indicated that diets added with antioxidants, such as 2-mercaptoethylamine (23), ethoxiquin (10), and 2-ethyl-6-methyl-3-hydroxypyridine (14), increased rat and mice median life span in the range of 13–29%. The data presented here opens the possibility that such substances share with thioproline a physiological effect based on the reduction of spontaneous food intake. The anorexic effect is similar to the physiological action of leptins and to the pharmacological effect of anorexic drugs that regulate food intake by acting on the hypothalamic centers that control feeding behavior and hunger (1, 22, 49).

Interestingly, thioproline-supplemented mice showed an improvement in the age-associated declines of neurological functions, with increased success in the tightrope and the T-maze tests at old and senescent ages. Decreases in neuromus-

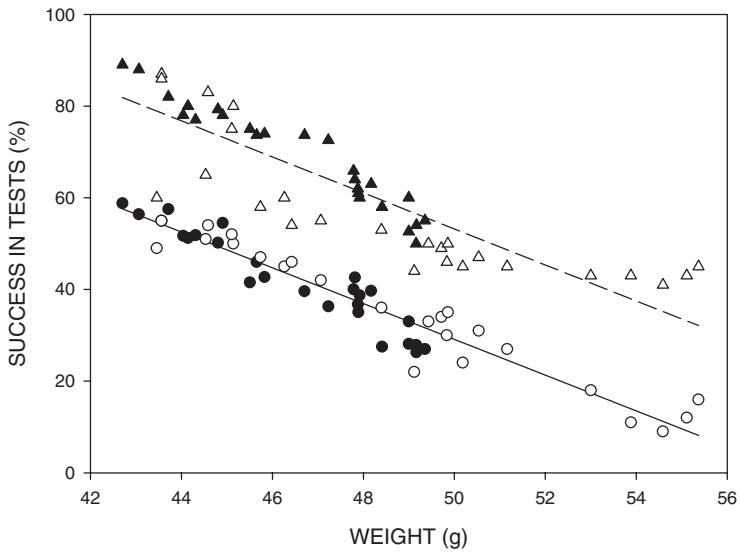

FIG. 7. Correlation between successes in neuromuscular (—) and **exploratory activities** (**- - -**) **and weight of male mice.** Control mice, ○ and △; thioproline-supplemented mice, ● and ▲; tightrope test, ○ and ● ($r^2 = 0.92$; *t*-test $p < 0.01$); T-maze, △ and ▲ ($r^2 = 0.76$; *t*-test $p <$

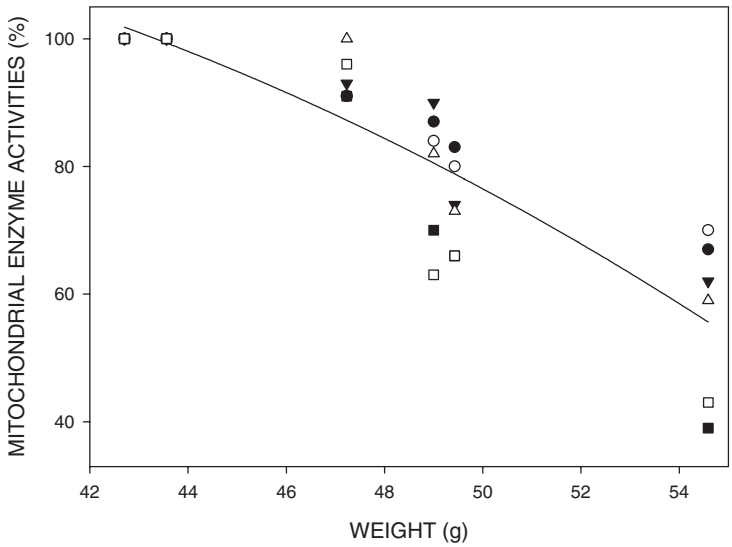

FIG. 8. Correlation between of brain and liver mitochondrial activities (complex I, complex IV, and mtNOS, expressed as percentage of the activities at 28 weeks of age) and body weight of male mice. $r^2 = 0.80$, *t*-test $p < 0.01$. Complex I: brain ● and liver ○; Complex IV: brain ▼ and liver △; and mtNOS: brain ■ and liver □.

cular coordination and maze performances were linearly correlated with increased weight, a fact that expands the beneficial effects of restricted food intake to neurological function. A similar partial prevention of the age-associated decline of neurological functions was reported for dietary supplementations with acetyl-carnitine and lipoic acid (20, 26), flavonoid-rich vegetable extracts (6), and vitamin E at high doses (33); effects that were interpreted as due to protection or amelioration of oxidative damage.

It was shown that neurological performance is linearly related to brain complex I and IV electron transfer rates (32, 33) and negatively correlated with the mitochondrial content of brain lipid and protein oxidation products (17, 32, 34). Applying the concept of rate limiting step in complex systems, it follows that decreased rates of electron transfer and a limited energy supply by brain mitochondria may be considered important factors in the neurological dysfunction inherent in physiological aging.

Accumulation of oxidative damage and decrease of mitochondrial energetic competence (ATP production) in the organs and tissues of aged mammals are the two underlying concepts of the mitochondrial hypothesis of aging (5, 39, 47).

In this study, brain and liver mitochondria isolated from aging mice showed an increased content of oxidation products, as previously reported (5, 32–34), in agreement with the report that the anorexic drug phentermine reduced food intake associated to a decrease in oxidation products in rat brain and liver (3).

Concerning the decreased capacity to produce energy, we have recently ruled out a reduction in mitochondrial mass in brain and liver upon aging (31), and pointed out to decreased rates of electron transfer, with conservation of F_1-ATP synthase phosphorylating capacity (31) as the main mechanism of mitochondrial dysfunction in aging. Accordingly, the activities of mitochondrial complexes I and IV were found decreased with aging and thioproline supplementation de-

TABLE 1. EFFECT OF DIETARY THIOPROLINE ON OXIDATIVE DAMAGE MARKERS IN MOUSE BRAIN AND LIVER MITOCHONDRIA

ORGAN/marker/group	*Age 28 weeks*	*Age 52 weeks*	*Age 78 weeks*
BRAIN/Protein carbonyls			
Control	42 ± 4	58 ± 4*	75 ± 5*
Thioproline-supplemented	—	50 ± 4	56 ± 4†
TBARS			
Control	4.9 ± 0.4	6.5 ± 0.4*	9.1 ± 0.4*
Thioproline-supplemented	—	5.5 ± 0.4	6.9 ± 0.4†
LIVER/Protein carbonyls			
Control	119 ± 7	148 ± 7*	205 ± 7*
Thioproline-supplemented	—	136 ± 6	145 ± 7†
TBARS			
Control	4.3 ± 0.4	5.2 ± 0.3	6.4 ± 0.4*
Thioproline-supplemented	—	4.8 ± 0.4	5.3 ± 0.4†

Values in pmol/mg mitochondrial protein. 10 mice in each group. * $p < 0.05$ for aging, compared with 28 weeks old mice. † $p < 0.05$ for thioproline-supplemented mice, compared with control group. ANOVA parameters, for brain: protein carbonyls $F_{(4,45)} = 8.4$, $p < 0.001$; and TBARS $F_{(4,45)} = 13.9$, $p < 0.001$; and for liver: protein carbonyls $F_{(4,45)} = 24.2$, $p < 0.001$; and TBARS $F_{(4,45)} = 4.5$, $p < 0.01$.

TABLE 2. EFFECT OF DIETARY THIOPROLINE ON ENZYMATIC ACTIVITIES OF MOUSE BRAIN AND LIVER MITOCHONDRIA

ORGAN/marker/group	*Age 28 weeks*	*Age 52 weeks*	*Age 78 weeks*
BRAIN/NADH-cytochrome c reductase			
Control	317 ± 11	265 ± 10*	213 ± 10*
Thioproline-supplemented	—	290 ± 10	277 ± 11†
Succinate-cytochrome c reductase			
Control	132 ± 9	126 ± 10	130 ± 9
Thioproline-supplemented	—	135 ± 10	129 ± 10
Cytochrome oxidase			
Control	114 ± 9	84 ± 9*	71 ± 9*
Thioproline-supplemented	—	106 ± 9	103 ± 9†
mtNOS			
Control	0.64 ± 0.06	0.42 ± 0.05*	0.25 ± 0.03*
Thiop;Thioproline-supplemented		0.58 ± 0.05†	0.45 ± 0.06†
LIVER/NADH-cytochrome c reductase			
Control	452 ± 15	362 ± 15*	316 ± 15*
Thioproline-supplemented	—	410 ± 15	380 ± 15†
Succinate-cytochrome c reductase			
Control	154 ± 11	163 ± 11	162 ± 11
Thioproline-supplemented	—	159 ± 11	166 ± 11
Cytochrome oxidase			
Control	125 ± 8	91 ± 7*	74 ± 7*
Thioproline-supplemented	—	125 ± 7	102 ± 7†
mtNOS			
Control	0.90 ± 0.06	0.59 ± 0.06*	0.39 ± 0.05*
Thioproline-supplemented	—	0.86 ± 0.06	0.57 ± 0.05†

NADH- and succinate-cytochrome c reductase, and cytochrome oxidase activities are expressed in nmol cytochrome c (reduced or oxidized)/min. mg protein (32; 34); mtNOS in nmol NO/min. mg protein. 10 mice in each group. *$p < 0.05$ for aging, compared with 28 weeks old mice. †$p < 0.05$ for thioproline-supplemented mice, compared with control group. ANOVA parameters, for brain: NADH-cytochrome c reductase $F_{(4,45)} = 13.9$, $p < 0.001$; succinate-cytochrome c reductase $F_{(4,45)} = 0.09$ NS; cytochrome oxidase $F_{(4,45)} = 3.8$, $p < 0.05$; and mtNOS $F_{(4,45)} = 7.6$, $p < 0.001$; and for liver: NADH-cytochrome c reductase $F_{(4,45)} = 10.6$, $p < 0.001$; succinate-cytochrome c reductase $F_{(4,45)} = 0.2$ NS; cytochrome oxidase $F_{(4,45)} = 9.8$, $p < 0.001$; mtNOS $F_{(4,45)} = 14.2$, $p < 0.001$.

TABLE 3. *IN VITRO* EFFECT OF THIOPROLINE ON THE RESPIRATORY RATES OF MOUSE BRAIN MITOCHONDRIA

	Respiratory rate (ng-at O min.mg protein)			
Substrate/condition	*Thioproline 0 μM*	*Thioproline 10 μM*	*Thioproline 30 μM*	*Thioproline 50 μM*
Succinate				
State 3	139 ± 4	144 ± 4	134 ± 3	150 ± 4
State 4	45 ± 4	46 ± 4	44 ± 4	48 ± 5
Respiratory control	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	3.2 ± 0.1
State 3 + arginine *(a)*	133 ± 4	133 ± 4	125 ± 4	132 ± 4
State 3 + L-NAME *(b)*	166 ± 5	160 ± 6	163 ± 6	164 ± 6
mtNOS functional activity [*b* – *a*]	33 ± 3	33 ± 3	38 ± 3	32 ± 3
mtNOS functional activity [(*b* – *a*)/State 3] × 100	24%	23%	28%	21%
Malate-glutamate				
State 3	69 ± 4	66 ± 5	63 ± 5	64 ± 6
State 4	22 ± 2	22 ± 2	21 ± 2	23 ± 2
Respiratory control	3.1 ± 0.1	3.0 ± 0.1	3.0 ± 0.1	2.8 ± 0.1
State 3 + arginine (a)	60 ± 5	58 ± 5	56 ± 5	58 ± 5
State 3 + L-NAME (b)	74 ± 6	72 ± 6	68 ± 6	70 ± 6
mtNOS functional activity [b – a]	14 ± 2	14 ± 2	12 ± 2	12 ± 2
mtNOS functional activity [(*b* – *a*)/State 3] × 100	20%	21%	19%	19%

Respiratory rates were determined as indicated in Materials and Methods; 2.0 m*M* arginine and 2.0 m*M* L-NAME were added as indicated.

creased the magnitude of these effects. Complexes I and IV (5, 31–34) and mtNOS (31, 33) activities are effective mitochondrial markers of aging. Complex I is the pathway of electron transfer from NAD-dependent dehydrogenases to the respiratory chain, and complex IV the electron pathway from respiratory chain to oxygen. Nitric oxide, the product of mtNOS activity, is currently considered a physiological regulator of cytochrome *c* oxidase activity (4). The association between mtNOS activity and cellular homeostasis has been called the pleiotropic effect of mtNOS and was originally used to describe preserved kidney functions (9). It has been claimed that the effect occurs through NO and H_2O_2 diffusion from mitochondria to the cytosol, a signal that indicates a high mitochondrial energy charge (9, 33). Moreover, NO has been recognized as a causative signal for mitochondrial biogenesis in a process mediated by cGMP and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) (35). The marked decrease in mtNOS activity in aged brain mitochondria is interpreted as an impairment of mitochondrial signaling that hinders a sustained neuronal homeostasis in aging (33). There are claims that caloric restriction acts by a modulation of whole body metabolism, decreasing oxidative damage, and increasing the signaling involved in antioxidant repair (38).

The beneficial effect of thioproline on mitochondrial oxidative damage could be interpreted as due to an antioxidant capacity of this compound, as it was referred before (12). Although thioproline has no antioxidant capacity when assayed *in vitro*, there were reports in which thioproline was able to increase glutathione contents in isolated rodent macrophages (13) and in human hepatocytes (25). However, the effects of dietary thioproline in decreasing accumulation of mitochondrial oxidation products and ameliorating the loss of enzyme activities may be also explained by the anorexic effect with spontaneous caloric restriction. The delay in the process of aging induced by decreased weight (48) encompasses the concept of improved mitochondrial and neurological functions, facts that are well documented at the defined time points of 52 and 78 weeks that describe the stages of old and senescent mice.

It can be concluded that dietary thioproline induces a spontaneous decrease in food intake (*i.e.*, an anorexic effect), associated with improved survival and neurological function through a physiological mechanism that likely involves decreased mitochondrial oxidative damage, modulation of basal metabolism and hypothalamic appetite centers.

The experiments reported here showed no sign of thioproline toxicity, since all indicators improved in thioproline-supplemented mice. Considering that thioproline toxicity in humans is low and that it has been used at 5–60 mg/kg daily (19), it is likely that its use in humans may afford a therapeutic strategy to control weight and age-related neurological functions in humans.

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ABBREVIATIONS

ABTS, 2,2-azo-bis-di-3-ethyl-benzo-thiazolin-sulfonate; cGMP, guanosine 3′,5′-cyclic monophosphate; EDTA, ethylenediaminetetraacetic acid; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; $HO^{\bullet}$, hydroxyl radical; H_2O_2, hydrogen peroxide; mtNOS, mitochondrial nitric oxide synthase; NO, nitric oxide; L-NAME, N_{ω}-nitro-L-arginine methyl ester; HbO_2, oxyhemoglobin; PGC-1α, peroxisome proliferator-activated receptor-α coactivator 1α; ROO•, peroxyl radical; $ONOO^-$, peroxynitrite; $^1O_2$, singlet oxygen; O_2^-, superoxide anion radical; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

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