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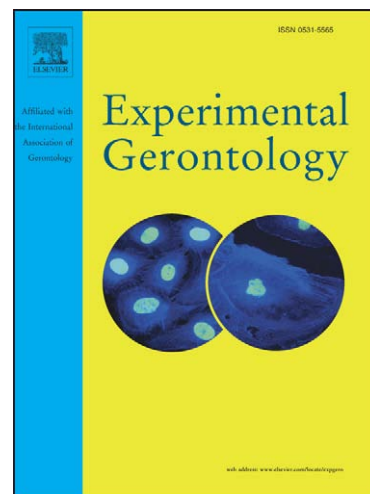
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Improved mitochondrial function and increased life span after chronic melatonin treatment in senescent prone mice

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Running title: Melatonin, mitochondria and longevity

Abstract

We investigated whether chronic melatonin administration influences mitochondrial oxidative stress and life span in mice. Diaphragmatic mitochondria from female senescent prone (SAMP8) and senescent resistant (SAMR1) mice at 5 and 10 months of age were studied. Mitochondrial oxidative stress was determined by measuring the levels of lipid peroxidation, glutathione and glutathione disulfide, and glutathione peroxidase and reductase activities. Mitochondrial function was assessed by measuring the activity of the respiratory chain complexes and the ATP content. The results suggest that the age-dependent mitochondrial oxidative damage in the diaphragm of SAMP8 mice was accompanied by a reduction in the electron transport chain complex activities and in ATP levels. Furthermore, melatonin administration between 1 and 10 months of age normalized the redox and the bioenergetic status of the mitochondria and increased the ATP levels. Melatonin also increased both half-life and longevity, mainly in SAMP8 group. These results suggest an age-related increase in mitochondria vulnerability to oxidation in SAM mice at 10 months of age that was counteracted by melatonin therapy. The effects of melatonin on mitochondrial physiology probably underline the ability of the indoleamine to increase maximal life span in these animals.

Keywords: senescence; oxidative stress; mitochondria; melatonin therapy; life span

I. Introduction

Aging is associated with a loss of muscle mass and strength. Compared with young adults, skeletal muscle from old animals are more susceptible to injury and atrophy (Deschenes et al., 2001; Faulkner et al., 1995). The free radical theory of aging (Harman, 1956) postulates that the production of intracellular reactive oxygen species (ROS) is the major determinant of life span. Mitochondria, the main source of free radicals in the cell, produce ROS through the univalent reduction of oxygen as electrons escape the respiratory chain. These ROS include superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO\bullet$) (Beckman and Ames, 1998; Harman, 1956). These findings connect the general and the mitochondrial theories of aging, with the latter suggesting that senescence should be accompanied by changes in the redox status of the mitochondria (Miquel, 1998). Oxidative stress is described generally as a condition under which increased production on free radical, reactive species, and oxidant-related reactions occur that result in damage.

The senescence-accelerated mouse (SAMP8) is established as a murine model of accelerated aging (Hosokawa, 2002; Mori et al., 1998; Takeda, 1999; Takeda et al., 1991). One possible mechanism promoting accelerated aging is the higher hyperoxidative status found in this mouse strain compared with that in the senescence resistant mouse (SAMR1) (Hosokawa, 2002; Mori et al., 1998). Most of the studies have shown different patterns of age-dependent increase in both lipid peroxidation (LPO) and superoxide dismutase (SOD) activity in different organs of SAMP8 mice (Butterfield et al., 1997; Liu and Mori, 1993; Matsugo et al., 2000). High redox state and increased electron leakage were reported in the brain mitochondria from 3-month-old SAMP8 mice (Liu and Mori, 1993). Some authors have reported the existence of a slight reduction in the respiratory control ratio in SAMP8 mice (Nakahara et al., 1998) that may be significant at the age of 18 months, when these mice may

have an insufficient ATP production (Nishikawa et al., 1998). These data support the existence of a mild deficiency in the mitochondrial electron transport chain (ETC) in young mice before an age-associated mitochondrial dysfunction develops. The reduced (GSH) to disulfide glutathione (GSSG) ratio, a highly sensitive indicator of the cellular redox state (Jones, 2002), shifts toward oxidation during aging in mitochondria from several tissues including heart, liver, lung and kidney of SAMP8 mice (Nakahara et al., 1998; Nishikawa et al., 1998; Park et al., 1996; Rebrin and Sohal, 2004). Anyway, some studies did not report significant differences in mitochondrial respiration between SAMP8 and SAMR1 until 12 months of age (Nomura et al., 1989).

Melatonin (aMT) and its metabolites are natural occurring compound with well-know antioxidant properties. aMT beneficial effects depends on its direct free radical scavenging activity and its indirect antioxidant actions (Reiter et al., 2000; Tan et al., 2000a; Tan et al., 2007). The indoleamine is highly efficient in detoxifying the devastatingly toxic hydroxyl radical (HO•) (Tan, 1993; Tan et al., 1998), and H₂O₂, peroxynitrite anion (ONOO⁻) and, in a lesser extent, nitric oxide (NO•) (Reiter et al., 2000, 2001; Turjanski et al., 2001). However, aMT mainly reduces NO• through the inhibition of its synthesizing enzyme, nitric oxide synthase NOS (Escames et al., 2003). In addition to these scavenging action, aMT also stimulates several antioxidative enzymes including SOD, and glutathione peroxidase (GPx) and reductase (GPx); these actions further reduce the oxidation state in the majority of cells (Antolin et al., 1996; Barlow-Walden et al., 1995; Okatani et al., 2000; Pablos, 1997). Considering that some antioxidants have been reported to increase survival of SAMP8 mice (Butterfield et al., 1997), the involvement of mitochondria in age, and the particular ability of aMT to potentially limit free radical generation in mitochondria, the potential use of aMT to slow down the processes of aging is suggested (Acuna-Castroviejo et al., 2001).

Although mitochondrial production of free radicals increases with advancing age (Nakahara et al., 1998; Nishikawa et al., 1998; Park et al., 1996; Rebrin and Sohal, 2004), it is yet unclear the age at which ROS initiate the senescence stage. Moreover, there are no data regarding the potentially beneficial role of chronic aMT treatment in terms of reducing age-dependent mitochondrial oxidative damage, and whether this treatment influences longevity. To address these questions, we designed a long-term study to evaluate the changes in mitochondrial redox status during age in diaphragmatic mitochondria from female SAMP8 and SAMR1 mice, and the ability of chronic aMT administration to prevent these changes.

2. Experimental procedures

2.1. Chemicals

N-(1-naphthyl) ethylenediamine dihydrochloride, EGTA, EDTA, HEPES, bovine serum albumin (BSA), proteinase K, Percoll, mannitol, sulfanilamide, phosphoric acid, 1,4-dithio DL-threitol (DTT), NADPH, methanesulfonic acid, disulfide and reduced glutathione, glutathione reductase, 5-sulfosalicylic acid, 2-vinylpyridine, cumene hydroperoxide, 5,5'-dithiolbis (2-nitrobenzoic acid) (DTNB), and mannitol were purchased from Sigma-Aldrich (Madrid, Spain). All other reagents were of the highest purity available.

2.2. Animals and treatments

Female SAMP8 and their control SAMR1 mice breeding pairs were obtained from Harlan (Barcelona, Spain). The animals were maintained in the University's facility under a 12 h light/12 h dark cycle at 22 ± 2 °C and they were given regular chow and tap water, under the supervision of veterinarians. All experiments were performed according to the Spanish Government Guide and the European Community Guide for animal care.

Animals for the biochemical and mitochondrial measures were used at 5 and 10 months of age. Once newborn mice were separated from their mothers (at the age of 1 month), aMT or vehicle treatments were initiated. The animals were separated into the following groups (n = 35 female animals/group): a) R5v group, consisting of SAMR1 animals treated with vehicle from 1 to 5 months of age; b) P5v group, included SAMP8 mice treated with vehicle from 1 to 5 months of age; c) R10v group, included SAMR1 mice treated with vehicle from 1 to 10 months of age; d) P10v group, consisted of SAMP8 mice treated with vehicle from 1 to 10 months of age; e) R10m group, included SAMR1 mice treated with aMT from 1 to 10 months of age, and f) P10m group, consisting of SAMP8 mice treated with aMT from 1 to 10 months of age. aMT was dissolved in a minimum volume of absolute ethanol and then diluted in the drinking water to yield a dose of 10 mg/kg b.w. during the months of treatment. The concentration of ethanol in the final solution was 0.66%. Water bottles were covered with aluminum foil to protect from light, and the drinking fluid was changed twice weekly. All mice were killed at the end of their respective treatment period, i.e., 5 or 10 months. For survival experiments, 50 female SAMR1 and 50 female SAMP8 mice were used.

2.3. Isolation of mitochondria

Animals were killed by cervical dislocation and diaphragms were immediately isolated (Escames et al., 2006). All procedures were carried out at 0-4 °C. Briefly, the diaphragm was excised, washed with saline, treated with proteinase K (1 mg/mL) for 30 s, washed with buffer A (250 mM mannitol, 0.5 mM EGTA, 5 mM Hepes and 0.1% fatty acid free BSA, pH 7.4, at 4 °C), and homogenized (1/10, w/v) in buffer A at 800 rpm with a Teflon pestle. The homogenate was centrifuged at 600 g for 5 min at 4 °C (twice), and the supernants were mixed and centrifuged at 10 300 g for 10 min at 4 °C. Then, the mitochondrial pellets were suspended in 0.5 mL buffer A and poured in ultracentrifuge tubes containing 1.4 mL buffer B

(225 mM mannitol, 1 mM EGTA, 25 mM Hepes and 0.1% BSA, and pH 7.4, 4 °C) and 0.6 mL Percoll. The mixture was centrifuged at 105 000 g for 30 min at 4 °C. The pure mitochondrial fraction was collected, washed twice with buffer A at 10 300 g for 10 min at 4 °C to remove the Percoll, and frozen to -80 °C. Mitochondrial protein content was determined in an aliquot of homogenized cardiac mitochondria without BSA (Lowry et al., 1951).

2.4. LPO determination

Mitochondrial fractions were thawed, suspended in ice-cold 20 mM Tris-HCl buffer, pH 7.4, and sonicated to break mitochondria membranes. Aliquots of these samples were either stored at -80 °C for total protein determination (Lowry et al., 1951) or used for LPO measurements. For this purpose, a commercial LPO assay kit that estimated both malondialdehyde (MDA) and 4-hydroxyalkenals (4HDA) was used (Bioxytech LPO-568 assay kit, OxisResearch, Portland, OR, USA) (Esterbauer and Cheeseman, 1990). LPO concentration is expressed in nmol/mg prot.

2.5. Measurements of GPx and GRd activities

Mitochondrial fractions were thawed and suspended in 200 µL of buffer A (potassium-phosphate 50 mM and EDTA-K2 1 mM, pH 7.4) and sonicated. To measure GPx activity, 10 µL of each sample were added to 240 µL of a working solution containing buffer A plus 4 mM sodium azide, 4 mM GSH, 0.2 mM NADPH and 0.5 U/mL GRd. After incubation for 4 min at 37 °C, the reaction was started by adding 10 µL of cumene hydroperoxide (0.3%) and the GPx activity was determined following the oxidation of the NADPH for 3 min at 340 nm in an UV spectrophotometer (Shimadzu Deutschland GmbH, Duisburg, Germany) (Griffith, 1999). GRd activity was measured in samples (35 µL) added to 465 µL of a working solution containing buffer A plus 2 mM GSSG. After incubation for 4 min at 37 °C, the reaction was

started by adding 8.5 μL of 9.5 mM NADPH solution, and the GRd activity was measured following the oxidation of NADPH for 3 min at 340 nm (UV-1603 Shimadzu spectrophotometer). GPx and GRd activities are expressed as nmol/min/mg prot. In both cases, non-enzymatic NADPH oxidation was subtracted from the overall rates.

2.6. Measurements of GSH and GSSG

Both GSH and GSSG were measured by a fluorometric method (Hissin and Hilf, 1976) slightly modified. Mitochondrial fractions were desproteinized with ice-cold 10% TCA and centrifuged at 20 000 g for 15 min. For GSH measurement, 10 μL supernatant was incubated with 10 μL of an ethanol ophthalaldehyde solution (1 mg/mL) and 180 μL phosphate buffer (100 mM sodium phosphate, 2.5 mM EDTA- Na_2 , pH 8.0) for 15 min at room temperature. Then, the fluorescence of the samples was measured at 340 nm excitation and 420 nm emissions in a plate-reader spectrofluorometer (Bio-Tek Instruments, Inc., Winooski, USA). For GSSG measurement, 30 μL aliquots of supernatants were preincubated with 12 μL N-ethylmaleimide solution (5 mg/mL in distilled water) for 40 min at room temperature, and then alkalized with NaOH 0.15 N. Aliquots of 45 μL were then incubated with 10 μL ophthalaldehyde solution and 145 μL NaOH 0.1 for 15 min at room temperature. The fluorescence was then measured. GSH and GSSG concentrations were calculated according to standard curves prepared accordingly. The levels of GSH and GSSG are expressed in nmol/mg prot.

2.7. Determination of mitochondrial complexes I, II, III and IV activities

Mitochondrial pellets were thawed, suspended in 350 μL of the incubation medium corresponding to the complex to be measured and immediately sonicated to prepare submitochondrial particles. Mitochondrial protein concentration was measured using BSA as

standard (Lowry et al., 1951). To determine the complex I activity, submitochondrial fractions (0.6 mg/mL) were incubated for 3 min in a medium containing 250 mM sucrose, 50 mM potassium-phosphate, 1 mM KCN, 50 μ M decylubiquinone, 0.8 μ M antimycin, pH 7.4. The reaction was initiated by the addition of NADH, and the activity of the complex I (NADH CoQ oxidoreductase, expressed as nmol oxidized NADH/min/mg prot) was measured following the rate of the oxidation of NADH (100 μ M) at 340 nm in a UV1603 Shimadzu spectrophotometer (IZASA, Seville, Spain) (Barrientos, 2002). The activity of complex II (succinate: DCIP oxidoreductase, expressed in nmol reduced DCIP/min/mg prot) was measured in 1 ml medium containing submitochondrial particles (0.03 mg/mL), 100 mM potassium-phosphate, 20 mM succinate, 0.8 μ M antimycin, 50 μ M rotenone, 2 μ M KCN, 50 μ M DCIP, pH 7.4. The reaction was initiated by the addition of 50 μ M decylubiquinone. The activity of complex II was measured following the rate of reduction of 2, 6-DCIP at 600 nm with 520 nm as reference wavelength (Hissin and Hilf, 1976). The activity of complex III (ubiquinol: cytochrome *c* reductase, expressed in nmol reduced cytochrome *c*/min/mg prot) was measured in 1 mL medium containing submitochondrial particles (0.03 mg/mL), 35 mM potassium-phosphate, 5 mM MgCl₂, 2.5 mg/ml BSA, 1.8 mM KCN, 50 mM rotenone and 2 mM decylubiquinone, pH 7.5. The reaction was started by adding 125 μ M cytochrome *c* and the activity of complex III was measured following the rate of reduction of cytochrome *c* at 550 nm with 580 nm as the reference wavelength (Hissin and Hilf, 1976). The activity of complex IV (cytochrome *c* oxidase, expressed as nmol oxidized cytochrome *c*/min/mg prot) was measured in 1 ml medium containing submitochondrial particles (0.1 mg prot/ml) and 50 mM potassium-phosphate, pH 6.8. The reaction was initiated by adding 75 μ M cytochrome *c* previously reduced with sodium borohydride and measuring the absorbance at 550 nm (Hissin and Hilf, 1976).

2.8. Measurements of mitochondrial content of adenine nucleotides

Adenine nucleotides were determined by HPLC with a ProPac PA1 column (4 x 250 mm: Dionex) and a binary gradient of 0.3 M ammonium carbonate and water (Krauss *et al.*, 1997). Purified mitochondria were rapidly resuspended in ice-cold 0.5 M perchloric acid, mixed during 120 s in vortex (to break the mitochondrial membranes) and centrifuged at 25 000 g for 15 min at 2 °C to precipitate proteins. Pellets were frozen to -80 °C to determine protein concentration (Lowry *et al.*, 1951) and the supernatants were mixed with 8 µL 5 M potassium carbonate to neutralize the acid and centrifuged at 12 000 g for 10 min at 2 °C. The resultant supernatants were used for HPLC measurements. After stabilizing the column with the mobile phase, 20 µL of each sample were injected onto the HPLC system. The mobile phase consisted in water (phase A) and 0.3 M ammonium carbonate pH 8.9 (phase B), and the following time schedule for the binary gradient (flow rate 1 mL/min) was used: 5 min, 50 % A and 50% B; 5 min 50 to 100 % B and then maintained 100% B during 25 min); 5 min 100 to 50% B and then another 5 min with 50% B (Krauss *et al.*, 1997). For calibration, water was used as blank and 3.125 µg/mL, 6.250 µg/mL, 12.5 µg/mL and 25 µg/mL of each nucleotide (AMP, ADP and ATP) were used for constructing the standard curves. Absorbance of the samples was measured with an UV detector at 254 nm wavelength and the concentration of each nucleotide in the sample was calculated based on the peak area.

2.9. Statistical analysis

Results are presented as means ± SEM of at least 6 animals analyzed in duplicate. An ANOVA followed by Student's t test was used to compare the means between groups. Prism 4 software (GraphPad Software, Inc. San Diego, Ca, USA) was used for the analysis of

survival, using the product limit method of Kaplan and Meier to create survival curves, and comparing these curves using the logrank test. Statistical significance for all analyses was set at $p < 0.05$.

3. Results

3.1. Mitochondrial oxidative stress

Figure 1 shows the changes in LPO levels in SAMR1 and SAMP8 mice. Age did not change the diaphragmatic mitochondria levels of LPO in SAMR1 mice, but increased them in SAMP8 animals ($p < 0.001$). After aMT treatment, the levels of LPO, mainly in SAMP8 mice, were reduced to the levels detected at 5 months of age ($p < 0.001$).

The activity of GPx decreased with age in both mice strains ($p < 0.001$) (Fig. 2, left), and effect counteracted by aMT administration, that was significantly higher in SAMP8 mice ($p < 0.001$). The activity of GPx after aMT treatment was significantly higher in mitochondria of SAMP8 than in SAMR1 mice ($p < 0.001$). Age also induced a significant reduction in GRd activity in SAMR1 and SAMP8 mice ($p < 0.01$) (Fig. 2 right). Treatment with aMT, that partially recovered the GRd activity in SAMR1 mice, was able not only to counteract the age-dependent reduction of GRd activity in SAMP8 mice ($p < 0.001$), but increased it above its activity at 5 months of age ($p < 0.001$).

Mitochondrial oxidative stress was also assessed by the significant reduction in the GSH/GSSG ratio, mainly in SAMR1 ($p < 0.05$). This effect was counteracted by aMT treatment ($p < 0.05$) (Fig. 3, left). In SAMP8 mice, aMT administration increased the mitochondrial GSH/GSSG ratio above its value found at 5 months of age ($p < 0.001$). Total glutathione levels decreased in groups of vehicle in SAMR1 at 10 months of age ($p < 0.001$) and treatment with aMT increase in this case ($p < 0.05$) (Fig. 3, right).

3.2. Mitochondrial respiratory chain activity and ATP content

The activities of the respiratory complexes are shown in Table 1. The activity of the complexes I, II, III and IV were not modified by age in any mice strain. Treatment with aMT produced a slight increase in complex I activity in both mice strains. Chronic administration of aMT also increased significantly complex II activity in SAMR1 ($p < 0.05$) and SAMP8 ($p < 0.001$), complex III activity in SAMR1 ($p < 0.001$) and SAMP8 ($p < 0.001$), and complex IV activity in SAMP8 mice ($p < 0.01$).

Figure 4 shows the ATP levels (left) and ATP/ADP ratio (right) determined in diaphragmatic mitochondria of SAMR1 and SAMP8 mice. Treatment with aMT counteracted the reduction in ATP levels and ATP/ADP ratio in SAMP8 mice ($p < 0.01$). SAMR1 mice, that did not show ATP and ATP/ADP ratio changes with age, also responded to aMT treatment ($p < 0.01$).

3.3. Animal survival

To determine whether the improvement in mitochondrial function after aMT treatment correlated with an increase in longevity, mice survival was analyzed. Figure 5 shows the survival curves obtained from vehicle- and aMT-treated SAMR1 and SAMP8 mice. The half-lives in vehicle- and aMT-treated SAMR1 mice were 20 and 23 months, respectively, whereas maximal longevity increased from 25 to 26 months after aMT treatment ($p < 0.01$, Figure 5, left). Interestingly, aMT therapy increased both the half-life of SAMP8 mice from 16 to 22 months, and the longevity from 23 to 27 months ($p < 0.0001$, Figure 5, right). The beneficial effects of aMT to increase both half-life and longevity were significantly higher in SAMP8 than in SAMR1 mice ($p < 0.0001$). Vehicle-treated SAMR1 survival was significantly higher than vehicle-treated SAMP8 mice ($p < 0.001$).

4. Discussion

Mitochondrial dysfunction and subsequent bioenergetics failure are closely linked to aging process. The diaphragm is the most important inspiratory muscle in mammals and plays a critical role in the maintenance of adequate alveolar ventilation. Unfortunately, diaphragmatic force-generating capacity decreases with age (Bou-Resli et al., 1991; Criswell et al., 1997; Gosselin et al., 1994; Polkey et al., 1997; Tolep et al., 1995). Mitochondrial function in diaphragm may be reduced with age due to the high metabolic activity of this muscle, producing high ROS generation and subsequent oxidative damage. Diaphragmatic mitochondria do not only provide the ATP required by this muscle but also produces ROS. If the redox balance between ROS and antioxidants is disrupted, the equilibrium tilts toward an oxidized state (Sies, 1985). Accordingly, our results show the existence of an age-dependent oxidative stress in diaphragmatic mitochondria of SAMR1 and SAMP8 mice that was more significant at 10 months of age. In general, SAMP8 mice showed significantly higher mitochondrial oxidative damage than the corresponding SAMR1 group of mice. Importantly, aMT given in the drinking water for 9 months, counteracted the age-dependent oxidative damage and mitochondrial dysfunction in SAMP8, and also corrected the minor alterations present in the diaphragmatic mitochondria of SAMR1 mice. These changes reflected an improvement of the mitochondrial function by aMT, because the indoleamine treatment also increased the ATP production.

Multiple lines of evidence suggest that SAMP8 mice may have an increased vulnerability to oxidative damage (Karbownik et al., 2000). Our results show that SAMP8 mice exhibited an increased diaphragmatic oxidative stress with age. The higher LPO levels and lower ATP content in mitochondria from SAMP8 compared with SAMR1 mice suggest the presence of higher oxidative stress in the former. Liu and Mori (Nomura et al., 1989) observed that the

levels of LPO increased at 11 months of age in the brains of SAMP8 but not in SAMR1 mice compared to those at 3 months. A similar time-dependent effect on LPO levels was detected in our hands, since they increased in diaphragmatic mitochondria of SAMP8 mice at 10 months, whereas they remained unchanged in SAMR1 mice at the same age. These findings may correlate with the accelerated aging in this mice strain (Liu and Mori, 1993; Matsugo et al., 2000; Nomura et al., 1989). The increased LPO is also consistent with the reduced mitochondrial membrane fluidity found in female SAMP8 mice (Yarian et al., 2005). Moreover, it was recently found that MDA, a product of LPO, induces oxidative damage to aconitase and ATP synthase in heart and skeletal muscle (Yarian et al., 2005), a finding consistent with the age-dependent reduction in the mitochondrial ATP content here reported.

Mitochondrial antioxidative defense includes SOD and the GSH redox cycling system. Whereas the former dismutates $O_2^{\cdot-}$ to H_2O_2 , GPx transforms H_2O_2 into water. In this process, GSH is oxidized to GSSG and the enzyme GRd restores reduced GSH levels. The redox status of a tissue is reflected by two main factors: the amount of GSH and the ratio between GSH and GSSG (Droge, 2002; Jones, 2002). Rises in GSSG reflects the gap between the rates of GSSG formation and its reconversion to GSH, whereas the GSH/GSSG ratio is indicative of the overall redox state. GSH is a ubiquitous tripeptide thiol amino acid that is a major reductive force for maintaining intracellular redox equilibrium and regulating cellular defenses. Under normal conditions, a low oxidant load and a high biosynthetic activity are associated with high GSH levels. This situation typically occurs under conditions of caloric restriction, which attenuates the rates of mitochondrial $O_2^{\cdot-}$ and H_2O_2 production (Rebrin et al., 2003; Sohal et al., 1994). In this study at 5 months of age, SAMP8 already exhibited a lower mitochondrial GSH/GSSG ratio than age-matched SAMR1 mice. These findings reflect an oxidizing tendency of the mitochondria due to higher ROS production in SAMP8 (Rebrin and Sohal, 2004).

Total glutathione content in the diaphragmatic mitochondria was lower in SAMP8 mice at than in SAMR1 at 5 months. This difference was mainly due to the lower mitochondrial GSH levels in SAMP8. Since mitochondria do not synthesize GSH, its reduction is likely due to a reduced transport of cytosolic GSH into mitochondria, a finding also reported for SOD in SAM mice (Park et al., 1996). At 5 months of age, diaphragmatic mitochondria from SAMP8 showed a tendency to increase GPx activity compared to SAMR1, reflecting a condition of higher peroxide production in the former. GRd activity was lower in SAMP8 than in SAMR1 mitochondria at 5 months of age, and it showed a tendency to further decrease at 10 months in the former. GRd is highly sensitive to oxidative stress, which may irreversibly damage the enzyme, reducing its ability to recycle GSH (Martin et al., 2000). Again, our data suggest that SAMP8 mice were under higher oxidative stress than were SAMR1 animals.

Together with the age-dependent mitochondrial oxidative stress, we found a tendency to the reduction in the activity of the respiratory complexes with age. This reduction was more evident in complex III and IV activities. These results newly support that the age-associated damage is greater in mitochondria of SAMP8 than in SAMR1 mice, which may result from uncoupling caused by higher membrane damage reported in the former (Nakahara et al., 1998) and assessed by the increased LPO levels in these mice here reported.

The most important finding in the present study is the ability of chronic aMT administration in preventing the age-associated mitochondrial dysfunction. Several studies have reported that acute aMT administration, an antioxidant and free radical scavenger (Antolin et al., 1996; Tan et al., 2000a, 2000b, 2002; Turjanski et al., 2001), counteracted mitochondrial deficiencies in SAM mice (Okatani et al., 2001, 2002a, 2002b). The indoleamine and several of its metabolites are scavengers of free radicals and it is now considered a component of the endogenous antioxidant system (Reiter, 1998, 2000; Tan et al., 2007). One molecule of aMT may scavenge two molecules of free radicals, both a HO[•] and a

$O_2^{\bullet-}$ or two HO^{\bullet} (Castillo et al., 2005; Nishikawa et al., 1998). Additionally, the lipophilicity of aMT permits that the indoleamine crosses cell membranes, reaching all intracellular structure (Costa et al., 1997; Menendez-Pelaez et al., 1993). *In vitro* and *in vivo* experiments have shown that aMT influences mitochondrial homeostasis, reducing mitochondrial hydroperoxide levels and stimulating the activity of GPx and GRd (Acuna-Castroviejo et al., 2001; Martin et al., 2000).

Our data regarding chronic aMT administration support the concept that the indoleamine exerts profound protective effects against mitochondrial dysfunction resulting from age-dependent oxidative stress. In fact, aMT reduced LPO in diaphragmatic mitochondria of SAMP8 mice to values below those found at 5 months of age not only in SAMP8 mice but also in SAMR1. Due to its lipophilicity, aMT also accumulates in the mitochondria at high concentrations (Martin et al., 2000), and interacts with lipid bilayers (Costa et al., 1997) stabilizing mitochondrial inner membranes and protecting them from oxidative damage (Garcia et al., 1999). The antioxidative capacity of aMT was also reflected by the significant increase in the GSH/GSSG ratio in both mice strains. These effects of aMT were followed by an increase in total glutathione, which probably reflects the stimulation of the gamma-glutamylcysteine synthetase activity, the regulatory enzyme of GSH synthesis and thus, the GSH content (Barrientos, 2002). Additionally, aMT increased the activity of GPx and GRd mainly in SAMP8 mice, increasing the bioavailability of GSH to the mitochondria. Along with the improvement of mitochondrial status in terms of reduction in their oxidative stress by aMT treatment, the indoleamine also increased the activity of the ETC complexes, especially in SAMP8 mice. In these conditions, diaphragmatic mitochondria increase their ATP content, thus yielding more ATP for muscle contraction, avoiding diaphragmatic dysfunction. These findings are well supported by other data in the literature (Escames et al., 2006; Lopez et al., 2006; Martin et al., 2002).

Treatment with aMT significantly increased both half-life and life span in SAM mice. Although aMT treatment increased survival in SAMR1 mice, the main effect of the indoleamine was produced in SAMP8 mice, which, increasing their survival by 4 months, reached the same longevity than SAMR1 mice. This is an interesting finding, and support experimental data from other pathophysiological conditions, reporting that aMT is more efficient in unregulating mitochondrial function when it is impaired. In this regard, using the same experimental paradigm, we recently showed that age is responsible for similar mitochondrial oxidative failure in other tissues such as heart, whereas chronic aMT administration prevented cardiac mitochondrial dysfunction in a similar extent than here reported in diaphragmatic mitochondria (Rodríguez et al., 2007a). Moreover, aMT reduced the age-dependent increase in serum pro-inflammatory cytokines in SAM mice (Rodríguez et al., 2007b). Aging also disturbs the mitochondria of many other tissues such as liver and lungs (Escames et al., 2003, 2006). In all cases, aMT administration prevented mitochondrial dysfunction restoring their normal homeostasis. With these data in mind, we analysed survival

In general, our results suggest that oxidative stress due to free-radical generation combined with a less effective system of defense against oxidative stress may result in the mitochondrial alterations found in SAMP8 mice. This hypothesis is consistent with the predictions of the mitochondrial theory of aging (Miquel, 1998). Due to the beneficial effects of aMT treatment on SAM mice, the age-dependent reduction of the aMT levels in different tissues of this mice strain recently reported (Lardone et al., 2006), may facilitate mitochondrial dysfunction in these tissues. Preventive therapy with aMT may certainly protect these mitochondria from age, maintaining their bioenergetic ability, thus matching senescent mice to control mice in terms of longevity. These effects suggest that pharmacological intervention with aMT might help to ameliorate age and age-related disorders.

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Figure legends

Fig. 1: Effect of age and aMT treatment on the LPO levels in diaphragmatic mitochondria from female SAMR1 and SAMP8 mice. Animals were given vehicle (0.66% ethanol) or aMT (10 mg/kg) in the drinking water from 1 month after birth, and sacrificed 4 or 9 months later (at 5 and 10 months of age, respectively). Results are expressed as mean \pm SEM of six experiments measured in duplicate. R5v and R10v, SAMR1 animals treated with vehicle and sacrificed at 5 and 10 months of age; P5v and P10v, SAMP8 mice treated with vehicle and sacrificed at 5 and 10 months of age; R10m and P10m, SAMR1 and SAMP8 animals, respectively, treated with aMT and sacrificed at 10 months of age. *** p < 0.001 compared with R5v or P5v; ### p < 0.001 compared with R10v or P10v; +++ p < 0.001 compared with the same group of SAMR1 mice.

Fig. 2: Effect of age and aMT treatment on the glutathione peroxidase (GPx) (left) and glutathione reductase (GRd) (right) activities in diaphragmatic mitochondria from female SAMR1 and SAMP8 mice. See legend of Fig. 1 for additional information. Results are expressed as a mean \pm SEM value of six experiments measured in duplicate. ** p < 0.01 and *** p < 0.001 compared with R5v or P5v; ### p < 0.001 compared with R10v or P10v; +++ p < 0.001 compared with the same group of SAMR1 mice.

Fig. 3: Effect of age and aMT treatment on the reduced to disulfide glutathione (GSH:GSSG) ratio (left) and total glutathione content (right) in diaphragmatic mitochondria from female SAMR1 and SAMP8 mice. See legend of Fig. 1 for additional information. Results are expressed as a mean \pm SEM value of six experiments measured in duplicate. * p < 0.05; ** p < 0.01 and *** p < 0.001 compared with R5v or P5v; + p < 0.05 and ++ p < 0.01 compared with the same group of SAMR1 mice.

Fig. 4: Effect of age and aMT treatment on the ATP content (left) and the ATP/ADP ratio (right) in diaphragmatic mitochondria from female SAMR1 and SAMP8 mice. See legend of Fig. 1 for additional information. Results are expressed as a mean \pm SEM value of six experiments measured in duplicate. * $p < 0.05$ and ** $p < 0.01$ compared with R5v or P5v; # $p < 0.05$ and ## $p < 0.01$ compared with R10v or P10v; + $p < 0.05$ compared with the same group of SAMR1 mice.

Fig. 5: Survival curves obtained from vehicle- and aMT-treated SAMR1 (left) and SAMP8 (right) mice. The total number of animals used in this study was 40 in each group.

Figure1

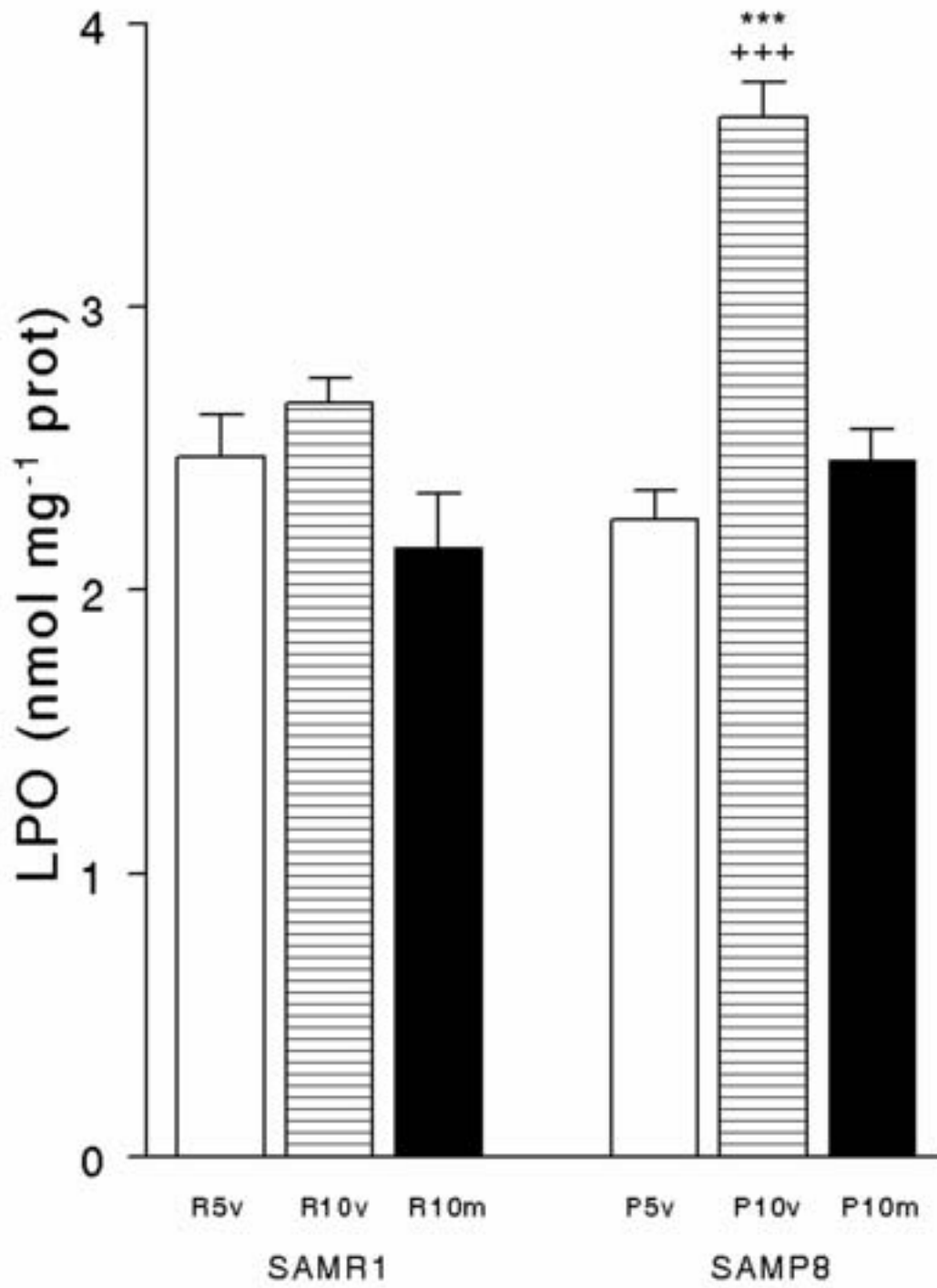


Figure2

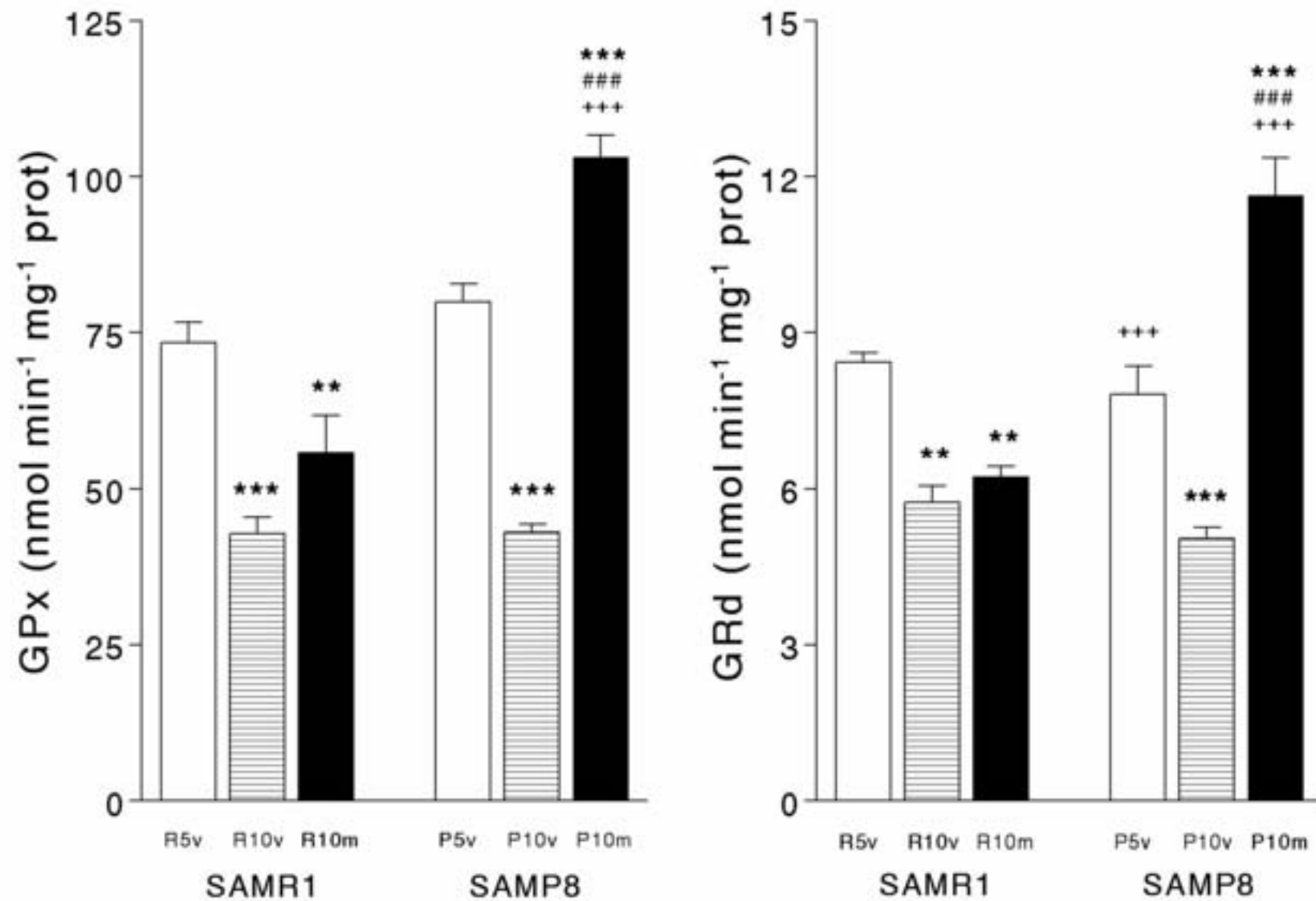


Figure 3

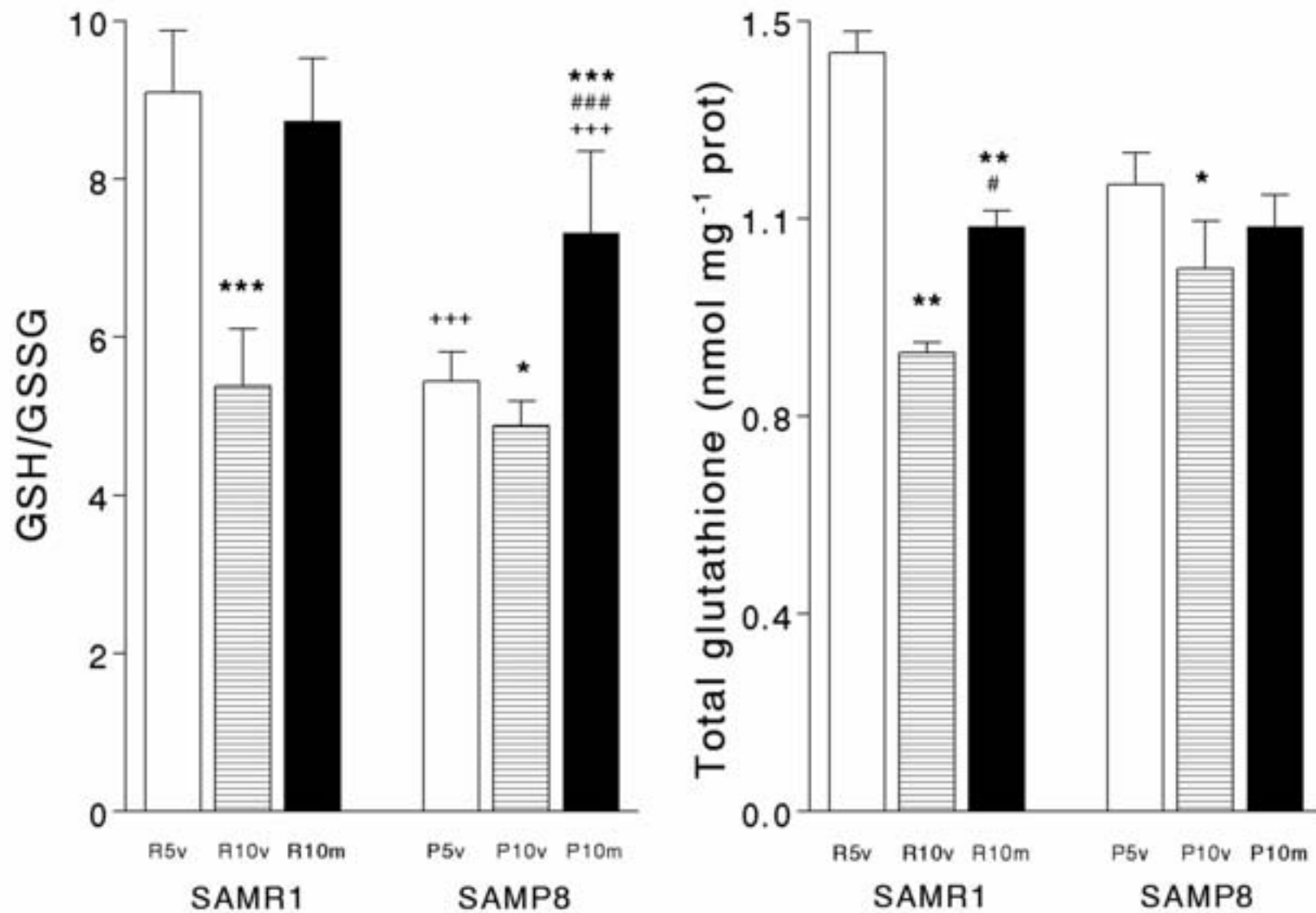


Figure 4

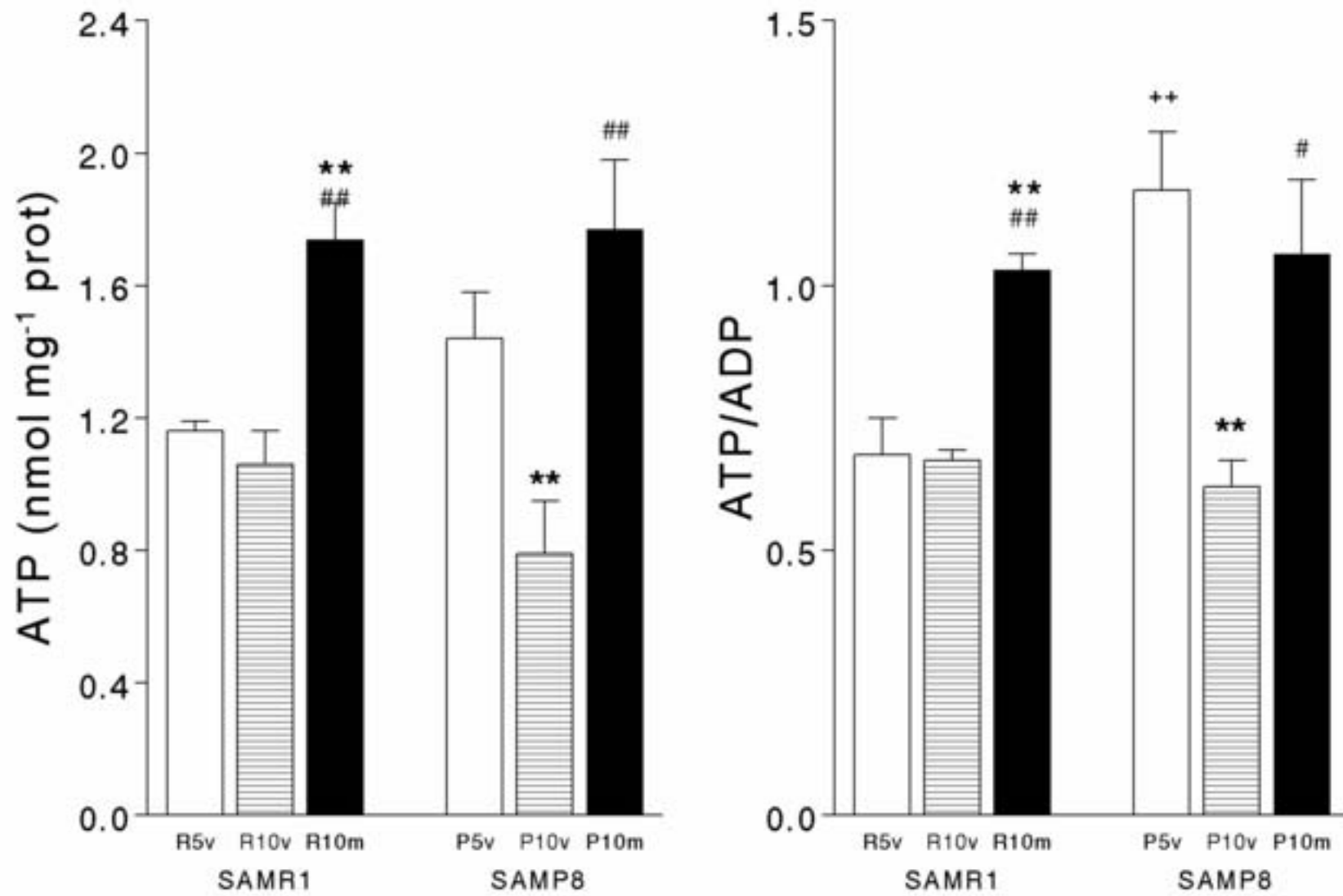


Figure 5

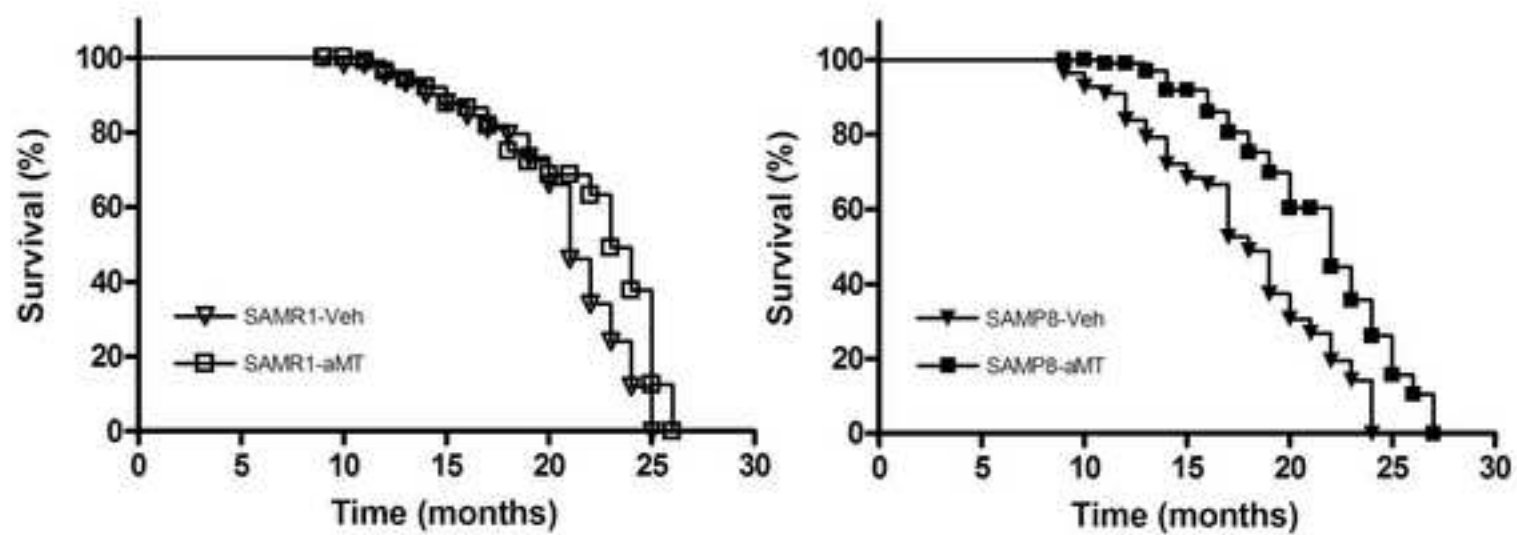


Table 1
Activity of the respiratory complexes I, II, III, and IV in diaphragmatic mitochondria of mice

| Group | Complex I (nmol/min/mg prot) | Complex II (nmol/min/mg prot) | Complex III (nmol/min/mg prot) | Complex IV (nmol/min/mg prot) |
|-------|---------------------------------|----------------------------------|-------------------------------------|----------------------------------|
| SAMR1 | | | | |
| R5v | 51.39 ± 3.88 | 192.75 ± 5.58 | 134 ± 1.14 | 999.24 ± 21.30 |
| R10v | 52.13 ± 1.42 | 176.38 ± 6.39 ⁺ | 125.83 ± 1.75 | 954.25 ± 54.12 |
| R10m | 67.70 ± 5.52 [#] | 198.78 ± 8.74 [#] | 163.61 ± 7.09 ^{**,##} | 1067.54 ± 16.91 ^{***} |
| SAMP8 | | | | |
| P5v | 56.38 ± 3.91 | 171.92 ± 5.60 | 120.32 ± 2.39 ⁺ | 978.68 ± 18.02 ⁺⁺⁺ |
| P10v | 59.32 ± 4.58 | 160.85 ± 3.40 | 109.36 ± 1.90 ^{*,+} | 811.79 ± 10.70 ^{**,++} |
| P10m | 69.02 ± 2.27 | 202.15 ± 2.34 ^{**,###} | 144.55 ± 3.63 ^{***,###,++} | 980.61 ± 34.44 ^{##} |

* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with R5v or P5v; ## $p < 0.01$, ### $p < 0.001$ compared with R10v or P10v; + $P < 0.05$, ++ $P < 0.01$ and +++ $P < 0.001$ compared with the same group of SAMR1 mice