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Chronic melatonin treatment prevents age-dependent cardiac mitochondrial dysfunction in senescence-accelerated mice

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Abstract
Heart mitochondria from female senescence-accelerated (SAMP8) and senescence-resistant (SAMR1) mice of 5 or 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 ATP content. The results show that the age-dependent mitochondrial oxidative damage in the heart of SAMP8 mice was accompanied by a reduction in the electron transport chain complex activities and in ATP levels. Chronic melatonin administration between 1 and 10 months of age normalized the redox and the bioenergetic status of the mitochondria and increased ATP levels. The results support the presence of significant mitochondrial oxidative stress in SAM mice at 10 months of age, and they suggest a beneficial effect of chronic pharmacological intervention with melatonin, which reduces the deteriorative and functional oxidative changes in cardiac mitochondria with age.

Keywords: *Aging, mitochondria, heart, oxidative damage, melatonin treatment*

Introduction

Aging is characterized by a loss of physiological capacities of the organism. Among other theoretical and experimental proposals, the free radical theory of aging [1] suggests that aging is caused by free radicals produced during aerobic respiration; these radicals induce oxidative damage that accumulates over time [2–4]. Mitochondria, the main source of free radicals in the cell, produce reactive oxygen species (ROS) through the univalent reduction of oxygen as electrons escape the respiratory chain. These ROS include superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$) [1,2]. Since mtDNA and proteins are located near the electron transport chain (ETC), these molecules readily sustain oxidative damage from ROS and equally toxic reactive nitrogen species (RNS) [5,6]. 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 cell [7].

The senescence-accelerated mouse (SAMP8) is established as a murine model of accelerated aging [8–11]. SAMP-strain mice show relatively strain-specific age-associated phenotypic pathologies such as a shortened life span and early manifestation of senescence (including loss of activity, alopecia, lack

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of hair glossiness, skin coarseness, periphthalmic lesions, increased lordokyphosis and systemic senile amyloidosis), similar to several geriatric disorders observed in humans [8–11]. Compared with the senescence resistant, long-lived mice (SAMR1), SAMP8 mice display a shorter lifespan [8,9]. One possible mechanism promoting accelerated aging and death in SAMP8 mice is their hyperoxidative status compared with that in the SAMR1 [10,11]. Many of the studies conducted to test this hypothesis have used the brain as the experimental tissue, although different patterns of age-dependent increases in both lipid peroxidation (LPO) and superoxide dismutase (SOD) activity have been reported in other organs [12–14]. While high redox state and increased electron leakage from brain mitochondria were reported in 3-month-old mice, [13], other studies reported no significant differences in mitochondrial respiration between SAMP8 and SAMR1 until 12 months of age [15]. It was proposed that, at 18 months of age, respiratory control may be insufficient to provide the ATP synthesis necessary for normal cell metabolism [16]. These data suggest the existence of a mild deficiency in the mitochondrial ETC in young mice before age-associated mitochondrial dysfunction develops. The reduced (GSH) to disulfide glutathione (GSSG) ratio, a highly sensitive indicator of the cellular redox state [17], shifts toward oxidation during aging in mitochondria from several tissues including heart, liver, lung and kidney of SAMP8 mice [18–21].

Melatonin (aMT) is a ubiquitously acting direct free radical scavenger and also an indirect antioxidant [22,23]. aMT is highly efficient in detoxifying the devastatingly toxic $HO^{\cdot}$ [24,25] and H_2O_2 [26], and it also directly interacts with RNS [27]. aMT also stimulates a host of antioxidative enzymes including SOD, glutathione peroxidase (GPx) and glutathione reductase (GPx); these actions further reduce the oxidation state of cells [23,28]. Multiple lines of recently accumulated evidence suggest that aMT plays a protective role in both initial and advanced stages of conditions whose pathogenesis involves damage by oxygen- and nitrogen-based reactants [29–32]. Considering that some antioxidants have been reported to increase survival of SAMP8 mice [12], the use of aMT as an age-deferring therapy in this species also seems feasible.

Although mitochondrial production of free radicals increases with advancing age [18–21], it is yet unclear the age at which ROS initiate the senescence stage in SAMP8 mice. Because at 3–5 months of age SAMP8 mice show early signs of oxidative stress compared with SAMR1 mice, and at the age of 11 months these signs are more evident [11–13], we considered it worthwhile to assess the differences in the mitochondrial oxidative stress between 5 and 10 months of age, a period of time that may mark the difference between SAMP8 and SAMR1 mice in terms of mitochondrial oxidative damage. 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 has any yet-undiscovered side effect. To address these questions, we designed a long-term study to evaluate the changes in mitochondrial redox status during age in heart mitochondria from female SAMP8 and SAMR1 mice. Animals were treated with vehicle or aMT from 1 to 10 months of age, and the mitochondrial redox status and ETC activity were assessed. We chose heart mitochondria since this muscle has a high bioenergetic requirement, and their involvement in aging of SAMP8 mice is yet unclear.

Materials and methods

Reagents

N-(1-naphthyl) ethylenediamine dihydrochloride, EGTA, EDTA, Hepes, bovine serum albumin (BSA), proteinase K, Percoll, sulfanilamide, phosphoric acid, 1,4-dithio DL-threitol (DTT), NADPH, methanesulfonic acid, disulfide and reduced glutathione, glutathione reductase, 5-sulfosalcylic acid, 2-vinylpiridine, 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.

Animals and treatments

Female SAMP8 and their control SAMR1 mice breeding pairs were obtained from the Council for SAM Research, Kyoto, Japan, through Harlan (Barcelona, Spain). The animals were maintained in the University's facility under a 12–12 h light–dark cycle (lights on at 07:00 h) at 22 ± 1°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 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 ($n = 35$ 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 0.066%. 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.

Isolation of mitochondria

Animals were killed by cervical dislocation and heart mitochondria were immediately isolated [29]. All procedures were carried out at 0–4°C. Briefly, the heart 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 fraction with a density of 1.052–1.075 g/ml, corresponding to a 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 [33].

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 [33] or used for LPO measurements. For this purpose, a commercial LPO assay kit that estimated both malondialdehide (MDA) and 4-hydroxyalkenals (4HDA) was used (Bioxytech LPO-568 assay kit, OxisResearch, Portland, OR, USA) [34]. LPO concentration is expressed in nmol/mg prot.

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) [35]. GRd activity was measured in samples (35 μl) added to 465 ml 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.

Measurements of GSH and GSSG

Both GSH and GSSG were measured by a slight modification of an established fluorometric method [36]. Mitochondrial fractions were deproteinized 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, 5 mM EDTA-Na2, 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 alkalinized with NaOH 0.1 N. Aliquots of 45 μl were then incubated with 10 μl ophthalaldehyde solution and 145 μl NaOH 0.1 N 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.

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 [33]. 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 μM decylubiquinone, 0.8 μM 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 μM) at 340 nm in a UV1603 Shimadzu spectrophotometer (IZASA, Seville, Spain) [37]. The activity of complex II (succinate: DCIP oxireductase, 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, 0.5 M succinate, 0.8 μM antimycin, 50 μM rotenone, 2 μM KCN, 50 μM DCIP, pH 7.4. The reaction was initiated by the addition of 50 μM 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 [38]. 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 $MgCl_2$, 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 μM 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 [38]. 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 μM cytochrome *c* previously reduced with sodium borohydride and measuring the absorbance at 550 nm [38].

Measurements of mitochondrial content of adenine nucleotides

Adenine nucleotides were determination by HPLC with a ProPac PA1 column (4 × 250 mm: Dionex) and a binary gradient of 0.3 M ammonium carbonate and water [39]. 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 [33] 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 supernants 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 50–100% B and then maintained 100% B during 25 min); 5 min 100–50% B and then another 5 min with 50% B [39]. For calibration, water was used as blank and 3.125, 6.250, 12.5 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.

Statistics

Data are expressed as the mean ± SEM of at least six animals analyzed in duplicate. An ANOVA followed by Student's *t*-test was used to compare the means between groups. A *p*-value of less than 0.05 was considered statistically significant.

Results

Mitochondrial oxidative stress

Figure 1 shows the changes in LPO levels in SAMR1 and SAMP8 mice. Age did not change the cardiac mitochondria levels of LPO in SAMR1 mice, but

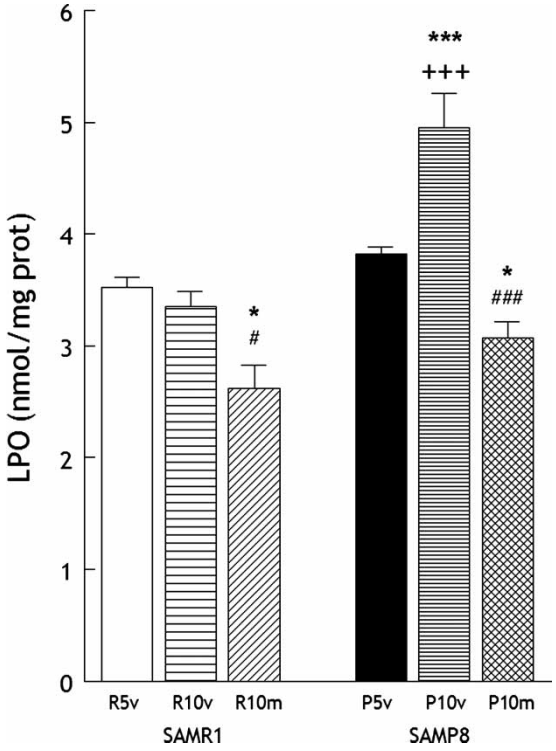

Figure 1. Effect of age and aMT treatment on the LPO levels in heart mitochondria from female SAMR1 and SAMP8 mice. Animals were given vehicle (0.066% ethanol) or aMT (10 mg/kg) in the drinking water from 1 month after birth, and sacrificed 4 or 9 months later (at 5 and 10 months of age, respectively). Results are expressed as a mean ± 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. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with R5v or P5v; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared with R10v or P10v; $^{+++}p < 0.001$ compared with the same group of SAMR1 mice.

increased them in SAMP8 animals ($p < 0.001$). After aMT treatment, the levels of LPO in SAMR1 mitochondria decreased significantly ($p < 0.05$), reaching lower levels than those found at the age of 5 months. aMT also counteracted the age-dependent increase of LPO levels in SAMP8 mice ($p < 0.001$), and reduced them below the levels detected in mitochondria harvested at 5 months of age ($p < 0.05$).

The activity of GPx did not change in cardiac mitochondria SAMR1 mice with age, whereas it decreased in SAMP8 mice ($p < 0.001$) (Figure 2A). aMT treatment reduced the activity of GPx in SAMR1 mice below its activity at 5 months of age ($p < 0.01$), and partially counteracted the age-dependent GPx activity reduction in SAMP8 mice ($p < 0.01$). The activity of GPx after aMT treatment was significantly higher in mitochondria of SAMP8 than in SAMR1 mice ($p < 0.01$). At 5 months of age, SAMP8 mice showed higher activity of mitochondrial GPx than SAMR1 mice ($p < 0.05$). Age induced significant reductions in GRd activity in both SAMR1 cardiac mitochondria ($p < 0.001$) and SAMP8 ($p < 0.05$) mice (Figure 2B). Treatment with aMT, which partially counteracted the age-dependent reduction in GRd activity in SAMR1 mice, elevated the GRd activity above its activity at 5 months of age in SAMP8 mice ($p < 0.01$).

Age reduced the GSH:GSSG ratio SAMR1 cardiac mitochondria ($p < 0.01$), an effect counteracted by aMT treatment (Figure 3A). In SAMP8 mice, aMT increased the mitochondrial GSH:GSSG ratio above the values at 5 months of age. The GSG:GSSG ratio in SAMP8 was lower than in SAMR1 mice. Levels of mitochondrial total glutathione were similar in all experimental groups except for the SAMP8 mice treated with aMT, where a significant rise in total glutathione was measured ($p < 0.001$); this increase was mainly due to an elevation in GSH (3.18 ± 0.1 vs. 1.19 ± 0.05 nmol/mg prot, $p < 0.001$) (Figure 3B).

Mitochondrial respiratory chain activity and ATP content

The activities of the mitochondrial respiratory chain complexes I, II, III and IV are shown in Figure 4. The activity of the complex I (Figure 4A) increased after aMT treatment ($p < 0.01$). aMT treatment also stimulated the activity of the complex I above the values found at 5 months in SAMP8 mice ($p < 0.05$). The activity of complex II significantly decreased with age in SAMR1 and SAMP8 mice, although in this case, aMT treatment was unable to modify these values (Figure 4B). In the case of complex III, its activity dropped with age in SAMR1 and SAMP8 mice, an effect counteracted by aMT administration (Figure 4C). Advancing age was also associated with a reduction in the activity of complex IV in SAMP8 but not in SAMR1 mice (Figure 4D). The activity of complex IV did not change in SAMR1 mice after aMT treatment. However, aMT counteracted the age-dependent decrease in complex IV activity in SAMP8 mice ($p < 0.05$). Increasing age was accompanied by a loss of cardiac mitochondrial ATP content in SAMR1

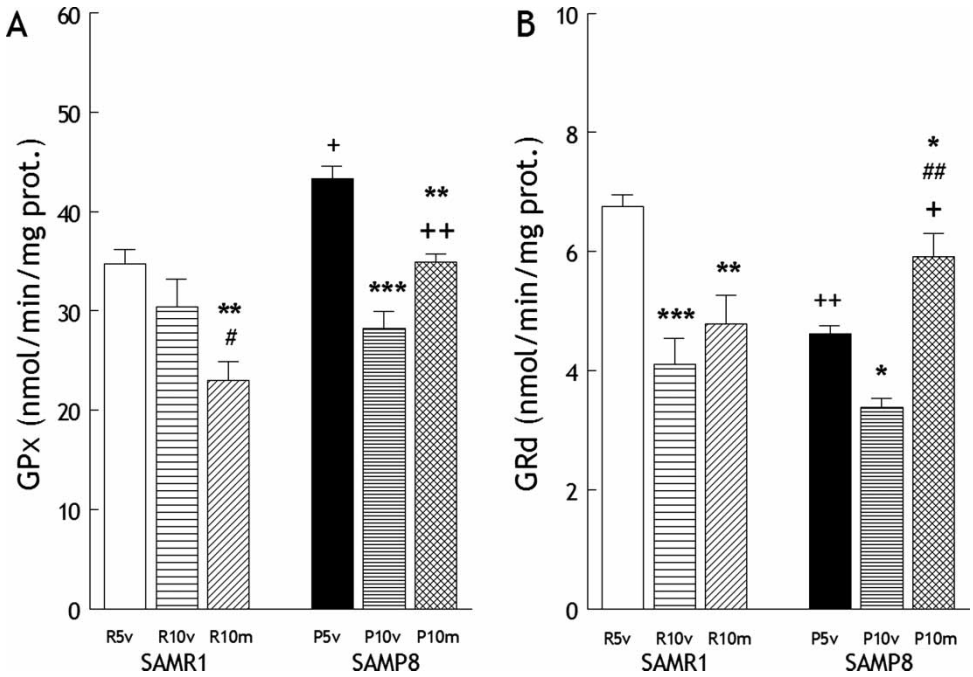

Figure 2. Effect of age and aMT treatment on the glutathione peroxidase (GPx) (A) and glutathione reductase (GRd) (B) activities in heart mitochondria from female SAMR1 and SAMP8 mice. See legend of Figure 1 for additional information. Results are expressed as a mean ± SEM value of six experiments measured in duplicate. $^{\star}p < 0.05$, $^{\star\star}p < 0.01$ and $^{\star\star\star}p < 0.001$ compared with R5v or P5v; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with R10v or P10v; $^{+}p < 0.05$ and $^{++}p < 0.01$ compared with the same group of SAMR1 mice.

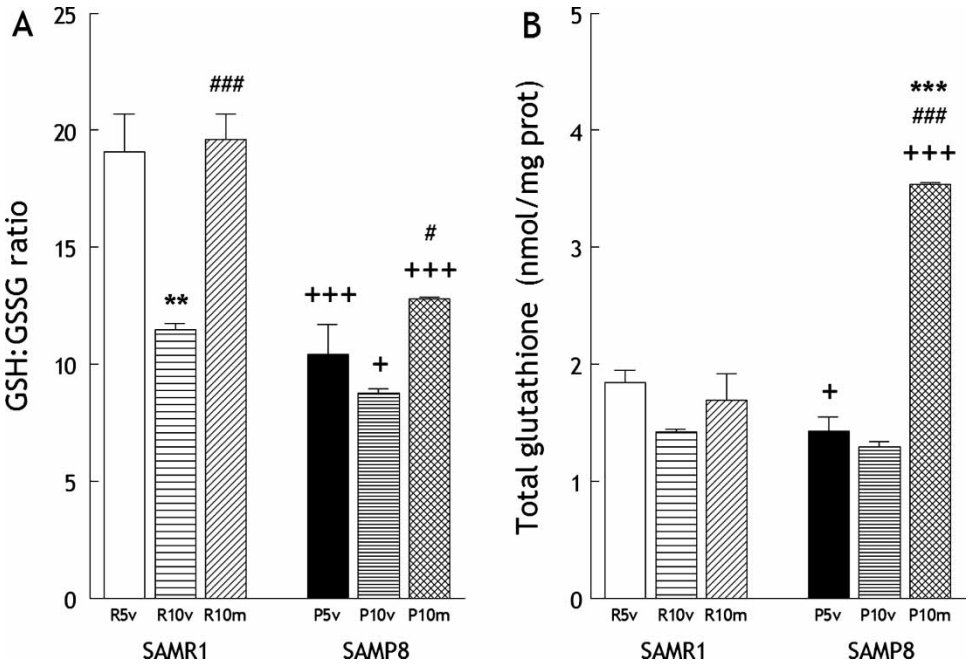

Figure 3. Effect of age and aMT treatment on the reduced to disulfide glutathione (GSH:GSSG) ratio (A) and total glutathione content (B) in heart mitochondria from female SAMR1 and SAMP8 mice. See legend of Figure 1 for additional information. Results are expressed as a mean ± SEM value of six experiments measured in duplicate. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with R5v or P5v; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared with R10v or P10v; $^{+}p < 0.05$ $^{+++}p < 0.001$ compared with the same group of SAMR1 mice.

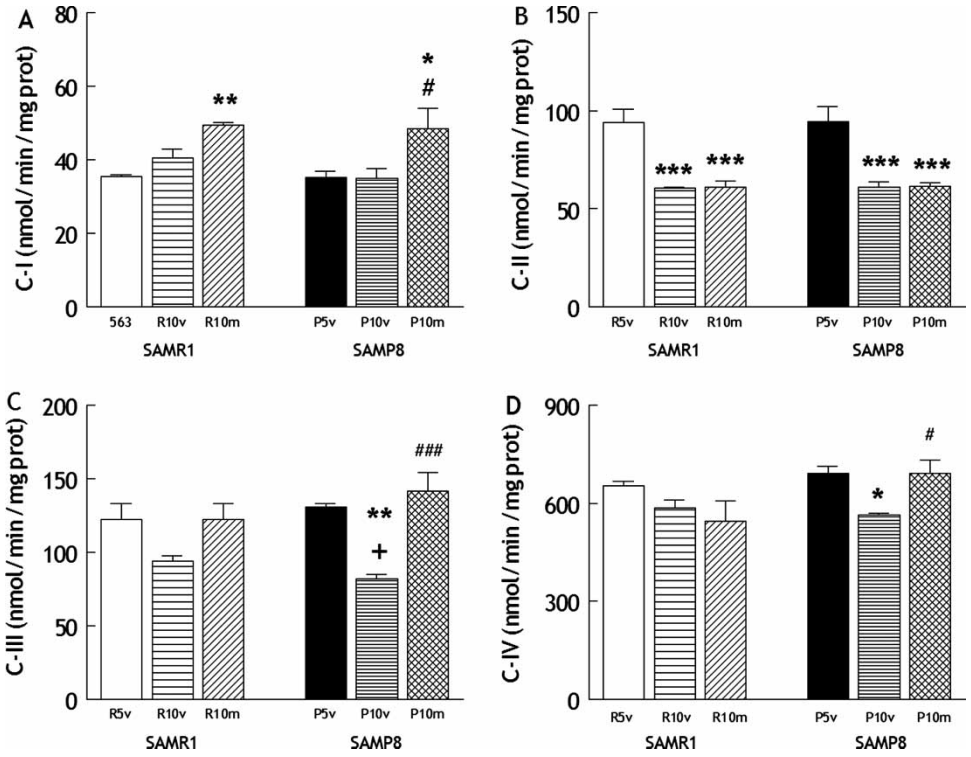

Figure 4. Effect of age and aMT treatment on the complex I (A), II (B), III (C) and IV (D) activities in heart mitochondria from female SAMR1 and SAMP8 mice. See legend of Figure 1 for additional information. Results are expressed as a mean ± SEM value of six experiments measured in duplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with R5v or P5v; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared with R10v or P10v.

mice that was more conspicuous in SAMP8 mice (Figure 5A, $p < 0.001$). Administration of aMT reversed, in part, the reduction of cardiac mitochondrial ATP in SAMP8 mice ($p < 0.01$). The ATP/ADP ratio also diminished in SAMP8 with age ($p < 0.01$), an effect counteracted by aMT treatment (Figure 5B, $p < 0.01$).

Discussion

Damage to mitochondria as a result of the intrinsic generation of free radicals is theoretically involved in the process of cellular aging. Our study documents the existence of age-dependent oxidative stress in heart mitochondria of SAMR1 and SAMP8 mice. Accordingly, with most of the oxidative stress markers measured, SAMP8 mice showed significantly higher mitochondrial oxidative damage than the corresponding SAMR1 groups of mice. Moreover, aMT given in the drinking water beginning at 1 month and continuing for 9 months, counteracted the age-dependent oxidative damage and mitochondrial dysfunction in SAMP8 mice, and also corrected the minor alterations present in the cardiac mitochondria of SAMR1 mice. Finally, aMT treatment restored the ability of cardiac mitochondria to produce ATP. The data document that exogenously administered aMT for long intervals protects against age-dependent oxidative stress and cardiac mitochondrial dysfunction. No side-effects of aMT treatment were noted in this study.

Several lines of evidence document an increased susceptibility of SAMP8 mice to oxidative damage; the resulting dysfunction is associated with accelerated aging [11–16,18–21]. Thus, SAM mice represent a good model to study the age-dependent induction of oxidative stress and its role on mitochondrial function in a variety of organs including the heart. Our results show that, especially at the age of 10 months, SAMP8 mice exhibited greater cardiac oxidative stress than did 10-month-old SAMR1 mice. The presence of oxidative stress in mitochondria would be expected to be accompanied by changes in LPO and GSH, damage to the mitochondrial ETC and deficits in ATP production. The present findings show that LPO levels increased in cardiac mitochondria of SAMP8 mice at the age of 10 months, whereas they remained unchanged in SAMR1 mice at the same age. This difference very likely reflects the higher ROS generation in the former due to the accelerated aging [13–15]. The increased LPO is also consistent with the reduced mitochondrial membrane fluidity found in aging female SAMP8 mice [40]. Moreover, it was recently found that MDA, a product of LPO, induces oxidative damage to aconitase and ATP synthase in heart and skeletal muscle [16], a finding also consistent with the reduction in the ATP content in mitochondria from 10-month-old SAMP8 mice as reported herein.

The redox status of a tissue is reliably reflected by the GSH:GSSG ratio [17,41]. Rises in GSSG reflects the gap between the rates of GSSG formation and its

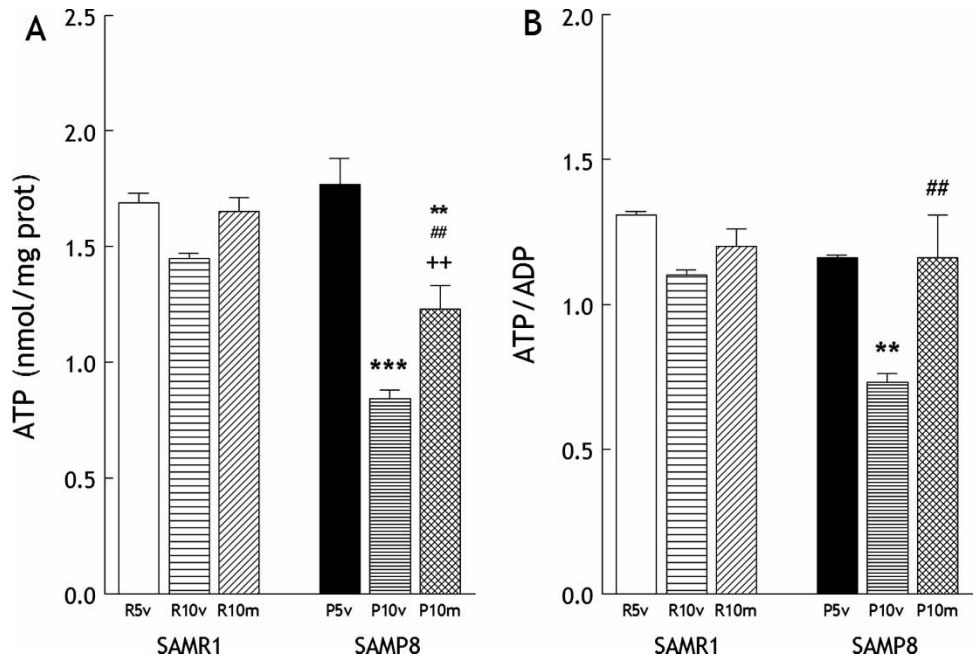

Figure 5. Effect of age and aMT treatment on the ATP content (A) and the ATP/ADP ratio (B) in heart mitochondria from female SAMR1 and SAMP8 mice. See legend of Figure 1 for additional information. Results are expressed as a mean ± SEM value of six experiments measured in duplicate. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with R5v or P5v; $^{\#\#}p < 0.01$ compared with R10v or P10v; $^{++}p < 0.01$ compared with the same group of SAMR1 mice.

reconversion to GSH, whereas the GSH:GSSG ratio is indicative of the overall redox state. Under normal conditions, a lower oxidant load and a higher biosynthetic activity are associated with high GSH levels. This occurs under conditions of caloric restriction which attenuates the rates of mitochondrial $O2\cdot-$ and H_2O_2 production [42,43]. Our data show that 5-month-old SAMP8 mice already exhibited a lower mitochondrial GSH:GSSG ratio than age-matched SAMR1 mice with this difference being maintained when the animals reached 10 months of age. These findings reflect an oxidizing tendency of the mitochondria due to higher ROS production in SAMP8 [18].

Total glutathione content of the heart mitochondria was generally reduced in SAMP8 mice at 5 and 10 months of age compared with levels in SAMR1 mice; this change was mainly dependent on a reduction in mitochondrial GSH levels. Since mitochondria do not synthesize GSH, its reduction was likely due to a reduced transport of cytosolic GSH into mitochondria, a finding also reported for SOD in SAM mice [19]. During detoxification of H_2O_2, GPx oxidizes GSH to GSSG. Thereafter, GRd reduces GSSG to GSH to maintain the GSH pool. At 5 months of age, SAMP8 heart mitochondria showed higher GPx activity than did SAMR1 animals, reflecting a condition of higher peroxide production in the former. The subsequent deficit in GSH in 10-month-old SAMP8 mice determined the reduction in GPx activity found in these mice. GRd activity was lower in SAMP8 than in SAMR1 mitochondria at 5 and 10 months of age. GRd is highly sensitive to oxidative stress, which may irreversibly damage the enzyme, reducing its ability to recycle GSH [28]. Again, our data suggest that SAMP8 mice were under higher oxidative stress than were SAMR1 animals.

Major questions remaining are whether the signs of oxidative stress present in cardiac mitochondria of SAMP8 mice are a prelude to mitochondrial failure and, if so, whether they may be in part responsible for the shorter life span in SAMP8 compared to SAMR1 mice. Accompanying ROS induction in mitochondria, the concomitant protein thiolation may represent a subsequent event responsible for many of the alterations including bioenergetics failure that appear later [18]. These alterations may also underlie the age-related impairment of mitochondrial respiration observed in liver mitochondria as well. It has been calculated that, at the age of 18 months, hepatic mitochondria from SAMP8 animals are unable to produce sufficient amounts of ATP for normal cell metabolism [21]. In the present study, we found an age-dependent reduction in the activity of the respiratory complexes II, III and IV in heart mitochondria from SAMP8 mice, whereas only complex II activity was reduced in SAMR1 animals. At 5 months of age, the activities of the four ETC complexes were similar in SAMR1 and SAMP8 mice. The age-associated damage is greater in mitochondria of SAMP8 mice than in this organelle of SAMR1 animals, possibly as a result of uncoupling caused by membrane damage [21]. The increased LPO levels in 10-month-old SAMP8 mitochondria found herein further support this hypothesis. Subsequent to mitochondrial ETC impairment was the significant reduction in the synthesis of mitochondrial ATP measured in SAMP8 mice at 10 months of age, a finding absent in SAMR1 mice.

The antioxidative properties of aMT and, specifically, its role in reducing mitochondrial oxidative damage, are now apparent. Several studies have reported that acute aMT administration, an antioxidant and free radical scavenger [22–26], counteracted mitochondrial deficiencies in SAM mice [44–46]. *In vitro*, nanomolar concentrations of aMT normalized the GSH pool and increased the activity of antioxidant enzymes including GPx and GRd in GSH-depleted mitochondria [28,32]. aMT also stimulated the activities of the ETC complexes both *in vivo* and *in vitro* [29,47,48], thereby improving the ability of mitochondria to produce ATP [47,49]. Based on careful observations made at the time the animals were killed and considering the beneficial measurements made in cardiac mitochondria, side effects of chronic aMT administration were either non-existent or negligible.

The current findings with chronic aMT administration to SAMP8 mice support the concept that the indoleamine exerts profound protective effects against mitochondrial dysfunction which are results of oxidative stress. In fact, aMT reduced LPO in cardiac mitochondria of SAMP8 mice to values below those found at 5 months of age not only in SAMP8 mice but also in SAMR1. aMT is highly lypophilic, enabling it to readily cross a variety of membranes and enter both cytoplasm and nuclear compartments. When aMT enters cellular membranes, it mainly becomes situated in a superficial position in lipid bilayers near the polar heads of membrane phospholipids [50]. In this position aMT is well situated to function as a free radical scavenger; this may be an important way in which membranes are able to resist oxidative damage. The antioxidative ability of aMT was also reflected by the significant increase in the GSH:GSSG ratio in SAMR1 and SAMP8 mice. These effects of aMT, and its ability to increase total glutathione content in mitochondria, mainly due to an elevation of GSH, may further document a stimulatory action of aMT on the activity of gamma-glutamylcysteine synthetase, the regulatory enzyme of GSH synthesis [51]. Moreover, aMT increased the activity of GPx and GRd in SAMP8 mice, increasing the bioavailability of GSH in the mitochondria.

An unexpected finding was that aMT reduced the activity of GPx in SAMR1 mice. aMT is a direct free

radical scavenger, and several experiments reported that it can substitute for GSH. This is the case of cataract prevention by aMT which occurs in the absence of GSH, after thiol synthesis has been inhibited by buthiomine sulfoximine [52]. Thus, in the presence of low oxidative stress and high amounts of aMT, which was probably the situation in SAMR1 mice treated with the indole, aMT scavenged a sufficient number of free radicals resulting in a reduction of the GPx-GSH system.

An additional consequence of chronic aMT administration at mitochondrial level was an improvement of the ETC activities. Except for complex II, aMT treatment increased the activity of the remaining complexes, especially in SAMP8 mice. These mice also showed an increase in the mitochondrial ATP levels and on ATP/ADP ratio. Other studies also showed that aMT enhances the function of mitochondrial ETC [28,29,47,48]. The mechanism underlying this action is unknown, but its repercussion is that aMT may elevate the efficiency of oxidative phosphorylation and ATP synthesis [47,49]. Thus, the effect of chronic aMT administration against the age-dependent mitochondrial oxidative damage should be accompanied by an improvement of the cardiac function and/or mice survival. However, we did not measure any parameter of heart function after aMT administration at this time and so, the beneficial effects of aMT administration in terms of heart function remain to be clarified. Also, we have not data on the effects of aMT on mice survival, because the animals were sacrificed at the age of 10 months. Anyway, these two questions remain to be addressed.

Overall, the results support the view that SAMP8 mice exhibit a relatively higher level of mitochondrial oxidative stress and mitochondrial respiratory chain impairment than do SAMR1 mice. This is consistent with the predictions of the mitochondrial oxidative stress hypothesis of aging [7]. The age-dependent elevation of oxidative stress possibly resulted from the observed reduction of the aMT levels in different tissues of SAMP8 mice as recently reported [53]. Thus, supplemental treatment with chronic aMT in the drinking water counteracted the age-dependent impairment in cardiac mitochondrial function, which was more evident in SAMP8 mice that in the SAMR1 strain. Previously, dietary antioxidants were shown to increase mitochondrial GSH content and GSH:GSSG ratio [54,55], although the diet enriched with a number of antioxidants is essential to obtain these effects. Only a diet supplemented with different types of antioxidants was found to be useful in increasing the mitochondrial GSH pool [55].

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