

Melatonin administration prevents cardiac and diaphragmatic mitochondrial oxidative damage in senescence-accelerated mice

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Abstract

Cardiac and diaphragmatic mitochondria from male SAMP8 (senescent) and SAMR1 (resistant) mice of 5 or 10 months of age were studied. Levels of lipid peroxidation (LPO), glutathione (GSH), GSH disulfide (GSSG), and GSH peroxidase and GSH reductase (GRd) activities were measured. In addition, the effect of chronic treatment with the antioxidant melatonin from 1 to 10 months of age was evaluated. Cardiac and diaphragmatic mitochondria show an age-dependent increase in LPO levels and a reduction in GSH:GSSG ratios. Chronic treatment with melatonin counteracted the age-dependent LPO increase and GSH:GSSG ratio reduction in these mitochondria. Melatonin

also increased GRd activity, an effect that may account for the maintenance of the mitochondrial GSH pool. Total mitochondrial content of GSH increased after melatonin treatment. In general, the effects of age and melatonin treatment were similar in senescence-resistant mice (SAMR1) and SAMP8 cardiac and diaphragmatic mitochondria, suggesting that these mice strains display similar mitochondrial oxidative damage at the age of 10 months. The results also support the efficacy of long-term melatonin treatment in preventing the age-dependent mitochondrial oxidative stress.

Journal of Endocrinology (2007) **194**, 637–643

Introduction

Oxidative stress could be related to aging through variations in reactive oxygen species (ROS) generation, ROS elimination, or both. Although antioxidants do not determine the rate of aging, their negative correlation with maximum longevity indicates that the endogenous rate of free radical production should be lower in long-lived than in short-lived animals (Barja 2004). Starting from the free radical theory of aging (Harman 1956), there is increasing evidence supporting the participation (Barja 2004) of free radicals not only in aging but also in many age-associated diseases (Beckman & Ames 1998, Sohal *et al.* 2002). A revised hypothesis of aging (Miquel 1998), has focused on the role of ROS-dependent mutations of the mitochondrial genome (mtDNA) in post-mitotic senescence. According to this theory, ROS production, mtDNA damage, and respiratory chain dysfunction are linked in a vicious cycle generating a progressive decline of mitochondrial function and cell viability. These processes may be responsible for the loss of physiological performance in senescent cells and many age-related degenerative diseases (Shigenaga *et al.* 1994, Miquel 1998, Barja & Herrero 2000).

The senescence-accelerated strain of mice (SAM) shows senescence acceleration. It was proposed that a high hyperoxidative stress status could be responsible for aging acceleration in these mice when compared with the accelerated

senescence-resistant mice (SAMR1; Ames *et al.* 1993, Takeda *et al.* 1997, Mori *et al.* 1998, Takeda 1999, Hosokawa 2002). Most of the studies conducted for the assessment of the oxidative stress status in SAMP8 mice were done in the brain, which shows an increase in lipid peroxidation (LPO) and superoxide dismutase (SOD) activity with age (Takeda *et al.* 1997). However, there is no agreement yet on the age of onset at which these changes take place. In fact, some authors reported changes in brain LPO levels in 11-month-old SAMP8 males (Hosokawa 2002), whereas others showed transient changes in brain lipid peroxidation (LPO), protein carbonyl content, and SOD and catalase activities between 3 and 11 months of age in SAMP8 mice (Butterfield *et al.* 1997, Takeda *et al.* 1997). Moreover, it was reported that 2-month-old SAMP8 males had higher redox state with increased mitochondrial electron leakage and ROS production (Liu & Mori 1993, Butterfield *et al.* 1997, Matsugo *et al.* 2000). Mitochondria from other tissues of SAMP8 mice showed that the ratio between reduced glutathione (GSH) and oxidized GSH disulfide (GSSG) glutathione, the GSH:GSSG ratio, a highly sensitive indicator of the cellular redox state (Nomura *et al.* 1989), shifts toward oxidation during aging (Yarian *et al.* 2005). These data, and the fact that some antioxidants increased SAMP8 survival, suggest that the mechanism of senescence acceleration in SAMP8 mice is related to free radical damage (Park *et al.* 1996, Nishikawa *et al.* 1998).

Melatonin (*N*-acetyl-5-methoxytryptamine, aMT) is an ubiquitous direct free radical scavenger and also an indirect antioxidant (Reiter *et al.* 1999, 2000). The aMT is highly efficient in detoxifying the toxic hydroxyl radical ($\bullet\text{OH}$), and probably H_2O_2 (Tan *et al.* 1993, 1998, 2000), and it also directly interacts with singlet oxygen ($^1\text{O}_2$), peroxyxynitrite anion (ONOO^-), and nitric oxide (Reiter *et al.* 2000, 2001, Turjanski *et al.* 2001). Indirect actions of aMT involve the stimulation of antioxidative enzymes, including SOD, GSH peroxidase (GPx), and GSH reductase (GRd; Antolin *et al.* 1996, Okatani *et al.* 2000). Recent evidence suggests that aMT is a highly potent antioxidant in specifically protecting against mitochondrial oxidative damage (Acuna-Castroviejo *et al.* 2001, Cuzzocrea *et al.* 2001, Montilla *et al.* 2001, Wu *et al.* 2001).

Thus, we considered it worthwhile to evaluate the age-dependent changes in the redox status of heart and diaphragmatic mitochondria, two muscles with high bioenergetic requirements showing important dysfunctions with age, in SAMP8 and SAMR1 mice. We also assessed whether chronic aMT treatment, from 1 to 10 months of age, can prevent mitochondrial oxidative stress in aged mice.

Materials and Methods

Reagents

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

Animals

Male 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 in a ratio of 12 h light:12 h darkness cycle (lights on at 07:00 h) at $22 \pm 1^\circ\text{C}$ 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 the newborns were separated from their mothers (at the age of 1 month), aMT or vehicle treatments were started. The animals were distributed among the following groups ($n=35$ animals/group): a) R5v group, comprising 5-month-old SAMR1 animals treated with vehicle from 1 to 5 months of age; b) P5v group, comprising 5-month-old SAMP8 mice treated with vehicle from 1 to 5 months of age; c) R10v group, comprising 10-month-old SAMR1 mice treated with vehicle from 1 to 10 months of age;

d) P10v group, comprising 10-month-old SAMP8 mice treated with vehicle from 1 to 10 months of age; e) R10m group, comprising 10-month-old SAMR1 mice treated with aMT from 1 to 10 months of age; and f) P10m group, comprising 10-month-old SAMP8 mice treated with aMT from 1 to 10 months of age. The 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 9 months of the treatment. Daily consumption of drinking water was calculated before the treatments in all groups of mice. The amount of water consumed per mouse per day was similar in every mouse strain and it is calculated every month to adjust the dose of melatonin. The concentration of ethanol in the final solution was 0.066%. Drinking water bottles were covered with aluminum foil to protect from light, and the drinking fluid was changed twice weekly.

Isolation of mitochondria

Animals were killed by cervical dislocation and heart and diaphragm mitochondria were immediately isolated (Escames *et al.* 2006). All procedures were carried out at $0-4^\circ\text{C}$. Briefly, heart and diaphragm were 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 r.p.m. with a Teflon pestle. The homogenate was centrifuged at 600 *g* for 5 min at 4°C (twice), and the supernatants 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 and the fraction 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 mitochondria without BSA (Lowry *et al.* 1951).

LPO determination

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

Measurements of GPx and GRd activities

Mitochondrial fractions were thawed and suspended in 200 μl buffer A (potassium phosphate 50 mM and EDTA- K_2 1 mM,

pH 7.4) and sonicated. To measure GPx activity, 10 μ l of each sample was added to 240 μ l 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 μ l cumene hydroperoxide (0.3%), and the GPx activity was determined following the oxidation of the NADPH for 3 min at 340 nm in a u.v. spectrophotometer (Shimadzu Deutschland GmbH, Duisburg, Germany; Jaskot *et al.* 1983). For GRd activity measurement, 35 μ l of each sample was added to 465 μ l of a working solution containing buffer A plus 2 mM oxidized glutathione (GSSG). After incubation for 4 min at 37 °C, the reaction was started by adding 8.5 μ l of 9.5 mM NADPH solution, and the GRd activity was measured following the oxidation of NADPH for 3 min at 340 nm in an u.v. spectrophotometer. GPx and GRd activities were expressed as nmol/min per mg prot. In both cases, nonenzymatic NADPH oxidation was subtracted from overall rate.

Measurement of GSH and GSSG

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

Statistical analysis

Data are expressed as the mean \pm S.E.M. of at least six animals analyzed in duplicate. An ANOVA followed by Dunnett's *t*-test was used to compare the means between groups. A *P* value of <0.05 was considered statistically significant.

Results

Figure 1 shows the levels of LPO in heart A) and diaphragmatic B) mitochondria from male mice. In heart mitochondria, LPO levels increased significantly in SAMR1 and SAMP8 mice at 10 months of age. The aMT treatment

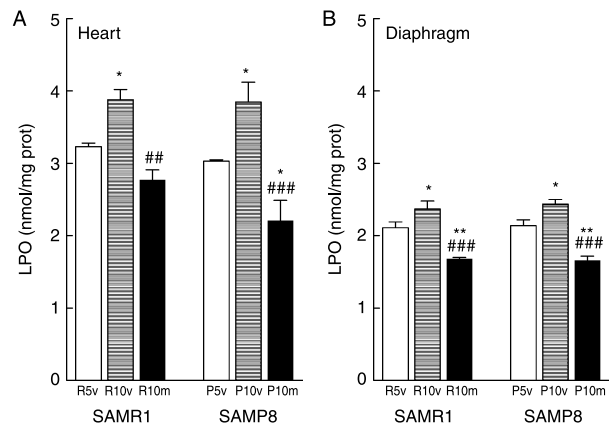


Figure 1 Effect of age and aMT treatment on the LPO levels in cardiac (A) and diaphragmatic (B) mitochondria from male SAMR1 and SAMP8 mice. Animals were maintained with vehicle (0.066% ethanol) or aMT (10 mg/kg) in the drinking water from 1 month after birth, and killed 4 and 9 months later (at 5 and 10 months of age respectively). Each point is the mean \pm S.E.M. value of six animals measured in duplicate. R5v and R10v, SAMR1 animals treated with vehicle and killed at 5 and 10 months of age; P5v and P10v, SAMP8 mice treated with vehicle and killed at 5 and 10 months of age; R10m and P10m, SAMR1 and SAMP8 animals respectively treated with aMT and killed at 10 months of age. **P*<0.05, ***P*<0.01 versus R5v or P5v; ##*P*<0.01, ###*P*<0.001 versus R10v or P10v.

counteracted the age-dependent increase in LPO levels in both SAMR1 and SAMP8 mice, reaching lower levels than those found at the age of 5 months in the latter. Diaphragmatic mitochondria content of LPO also increased in both mice strains at 10 months of age, whereas aMT administration counteracted the effect of age, reducing the levels of LPO below those found at 5 months of age (Fig. 1). Although the levels of LPO at 5 months of age were higher in the heart than in the diaphragm mitochondria, the effects of age and of aMT treatment were similar in both tissues.

The GSH:GSSG ratios in cardiac and diaphragmatic mitochondria are shown in Fig. 2. In cardiac mitochondria, age did not modify the GSH:GSSG ratio in SAMR1 mice but reduced it in SAMP8 animals (Fig. 2A). Administration of aMT significantly increased these ratios in both mice strains, increasing the total GSH content (Table 1). Diaphragmatic mitochondria of SAMR1 and SAMP8 mice showed a significant reduction in the GSH:GSSG ratio at 10 months of age which was counteracted by aMT treatment (Fig. 2B), an effect also accompanied by an increased total GSH pool (Table 2).

The activities of cardiac mitochondrial GPx and GRd are shown in Fig. 3. No significant changes in GPx or GRd activities were detected in 10-month-old mice when compared with mice at 5 months of age. After aMT treatment, however, the activity of both enzymes increased in SAMP8 mice, whereas aMT had no effect of SAMR1 mice. Age did not modify GPx and GRd activities in diaphragmatic mitochondria of SAMR1 mice, whereas aMT treatment reduced GPx and increased GRd activity in this

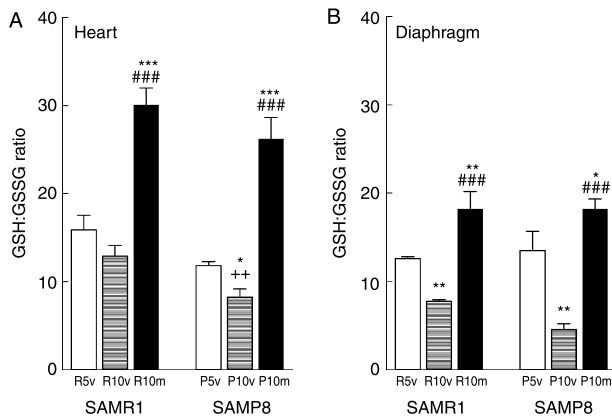


Figure 2 Effect of age and aMT treatment on the GSH:GSSG ratios in cardiac (A) and diaphragmatic (B) mitochondria from male SAMR1 and SAMP8 mice. See legend of Fig. 1 for additional information. Results are expressed as a mean \pm S.E.M. value of six animals measured in duplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus R5v or P5v; ### $P < 0.001$ versus R10v or P10v; ++ $P < 0.01$ versus the same group of SAMR1 mice.

mice strain (Fig. 4). In SAMP8 mice, however, mitochondrial GPx and GRd activities increased at 10 months of age. The effect of aMT administration was similar to that found in SAMR1 mice, i.e. the indoleamine reduced GPx activity but increased GRd activity (Fig. 4).

Discussion

The findings of this study indicate the existence of a significant age-dependent oxidative damage in cardiac and diaphragmatic mitochondria. Mitochondrial oxidative stress, however, was similar in SAMR1 and SAMP8 mice, suggesting that SAMP8 mice did not show signs of accelerated aging at least up to 10 months of age in the mitochondria studied. Our study demonstrates that oxidative stress increases from 5 to 10 months of age, thus supporting

Table 1 Age- and aMT-dependent changes in glutathione (GSH), glutathione disulfide (GSSG), and total glutathione in cardiac mitochondria of senescence-accelerated strain of mice (SAM) mice. Data are the mean \pm S.E.M.

	GSH (nmol/mg prot)	GSSG (nmol/mg prot)	GSH + GSSG (nmol/mg prot)
SAMR1			
R 5v	1.87 \pm 0.07	0.12 \pm 0.02	1.99 \pm 0.07
R 10v	1.81 \pm 0.07	0.14 \pm 0.01	1.95 \pm 0.06
R 10m	2.21 \pm 0.10 [§]	0.07 \pm 0.01 ^{*,}	2.28 \pm 0.10 ^{*,§}
SAMP8			
P 5v	1.30 \pm 0.03 ^a	0.11 \pm 0.01	1.42 \pm 0.03 [†]
P 10v	1.81 \pm 0.31 [†]	0.22 \pm 0.04 [*]	2.03 \pm 0.15 [†]
P 10m	2.36 \pm 0.37 ^{*,}	0.09 \pm 0.04 [§]	2.45 \pm 0.14 ^{*,§}

* $P < 0.05$, [†] $P < 0.01$, and ^a $P < 0.001$ versus R5v or P5v; [§] $P < 0.05$, and ^{||} $P < 0.01$ versus R10v or P10v; [†] $P < 0.05$, and ^a $P < 0.01$ versus equal group in SAMR1.

Table 2 Age- and aMT-dependent changes in glutathione (GSH), glutathione disulfide (GSSG), and total glutathione in diaphragmatic mitochondria of senescence-accelerated strain of mice (SAM) mice. Data are the mean \pm S.E.M.

	GSH (nmol/mg prot)	GSSG (nmol/mg prot)	GSH + GSSG (nmol/mg prot)
SAMR1			
R 5v	1.23 \pm 0.05	0.10 \pm 0.004	1.32 \pm 0.06
R 10v	1.24 \pm 0.03	0.16 \pm 0.01 [†]	1.40 \pm 0.03
R 10m	1.64 \pm 0.15 ^{*,§}	0.09 \pm 0.01	1.73 \pm 0.13 ^{*,§}
SAMP8			
P 5v	0.81 \pm 0.003 ^b	0.07 \pm 0.001	0.87 \pm 0.01 ^b
P 10v	0.87 \pm 0.01 ^a	0.17 \pm 0.03 [*]	1.04 \pm 0.03 ^a
P 10m	1.44 \pm 0.15 ^{*,}	0.09 \pm 0.02 [§]	1.53 \pm 0.17 ^{*,}

* $P < 0.05$, [†] $P < 0.01$, and ^a $P < 0.001$ versus R5v or P5v; [§] $P < 0.05$, ^{||} $P < 0.01$, and ^b $P < 0.001$ versus R10v or P10v; ^a $P < 0.05$, ^b $P < 0.01$ versus equal group in SAMR1.

the mitochondrial theory of aging elsewhere proposed (Miquel 1998). Another interesting finding of this work was the efficacy of chronic aMT administration to prevent the age-dependent oxidative stress.

The presence of oxidative stress in mitochondria should be reflected by changes in LPO and GSH and, if ROS are produced sufficiently, the enzymes of the redox cycle of GSH may be damaged (Martin *et al.* 2000). Our data indicate a significant increase in LPO levels in cardiac and diaphragmatic mitochondria with age, reflecting the oxidative damage to membrane lipids during aging (Nomura *et al.* 1989, Liu & Mori 1993, Matsugo *et al.* 2000). The age-dependent LPO increase was similar in control and senescence mice. One of the consequences of the LPO increase is the oxidative damage to aconitase and ATP synthase (Yarian *et al.* 2005), which may account for the reported reduction of oxidative phosphorylation efficiency with age (Yarian *et al.* 2005). Our results also show that the GSH:GSSG ratio, indicative of the overall redox state (Griffith 1999, Droge 2002, Jones 2002),

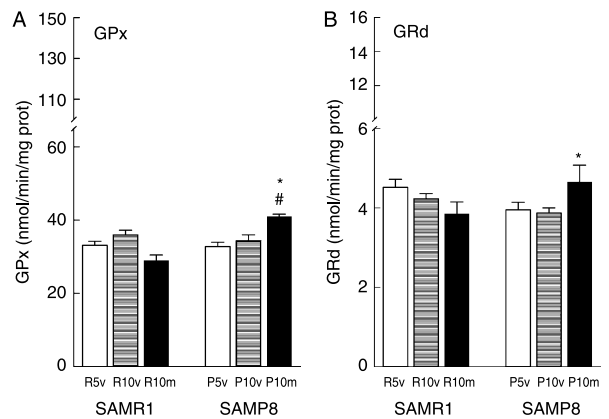


Figure 3 Effect of age and aMT treatment on GPx (A) and GRd (B) activities in cardiac mitochondria from male SAMR1 and SAMP8 mice. See legend of Fig. 1 for additional information. Results are expressed as a mean \pm S.E.M. value of six animals measured in duplicate. * $P < 0.05$ versus R5v or P5v; [†] $P < 0.05$ versus P10v.

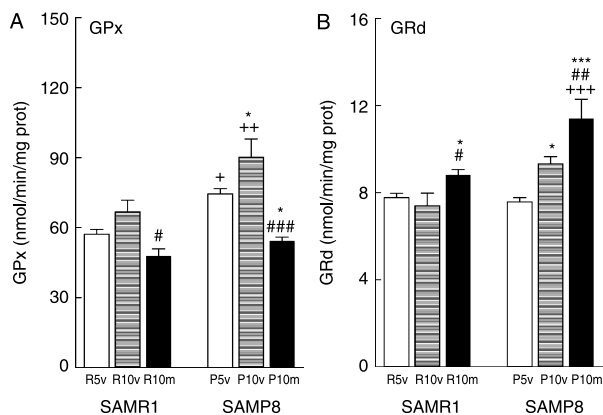


Figure 4 Effect of age and aMT treatment on GPx (A) and GRd (B) activities in diaphragmatic mitochondria from male SAMR1 and SAMP8 mice. See legend of Fig. 1 for additional information. Results are expressed as a mean \pm S.E.M. value of six animals measured in duplicate. * $P < 0.05$, *** $P < 0.001$ versus R5v or P5v; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus R10v or P10v; + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$ versus the same group of SAMR1 mice.

decreased at 10 months of age in both SAMR1 and SAMP8 mice, reflecting an oxidizing tendency due to ROS generation. The GSH:GSSG ratio reduction was mainly due to an increase in GSSG production, probably reflecting an ineffective recycling of GSSG to GSH. Moreover, diaphragmatic mitochondria showed lower GSH:GSSG ratios than cardiac mitochondria, a finding that probably depends on a better GSSG to GSH recycling system in the latter. To analyze the efficiency of the GSH redox cycle in cardiac and diaphragmatic mitochondria, the activities of GPx and GRd were measured. Mitochondria from SAMR1 and SAMP8 hearts did not show significant changes of these enzymes with age. Changes in GPx and GRd activities were more significant in diaphragmatic mitochondria. In this organelle, age did not modify the activity of GPx and GRd in SAMR1 mice, whereas the activity of both enzymes increased significantly at the age of 10 months in SAMP8 mice.

The efficiency of aMT to maintain the mitochondrial homeostasis of GSH was shown in several experimental models (Martin *et al.* 2000, Acuna-Castroviejo *et al.* 2001, Escames *et al.* 2006). *In vitro*, nanomolar concentrations of aMT normalized the GSH pool and increased the activity of the antioxidant enzymes including GPx and GRd in GSH-depleted mitochondria (Martin *et al.* 2000, Acuna-Castroviejo *et al.* 2001). Also *in vivo*, acute administration of aMT protected diaphragmatic and skeletal muscle mitochondria from oxidative/nitrosative damage (Escames *et al.* 2006, Lopez *et al.* 2006b), improving the ability of these mitochondria to produce ATP (Lopez *et al.* 2006a). In SAM mice, aMT administration counteracted mitochondrial deficiencies (Okatani *et al.* 2001, 2002a,b). Recently, an inverse relationship was demonstrated between the reduction in aMT content and the increased oxidative damage in several

tissues of SAM mice including liver, spleen and thymus (Lardone *et al.* 2006).

From these data, we can expect that chronic aMT administration should be beneficial to prevent the age-dependent mitochondrial oxidative damage reported here. Our data confirm that administration of aMT for 9 months inhibits the age-related increase in mitochondrial LPO in both SAMR1 and SAMP8 mice at 10 months of age. These results also indicate that aMT probably reached the mitochondria of the tissues studied, a finding related to the high lipophilicity of the indoleamine. The mechanism of the aMT protection against cell membranes LPO was analyzed elsewhere. When aMT enters cell membranes, it mainly becomes situated in a superficial position in lipid bilayers near the polar heads of membrane phospholipids (Rebrin *et al.* 2005). In this position aMT is well situated to function as a free radical scavenger, and it may be an indirect way for membranes to resist oxidative damage. This mechanism accounts also for the mitochondrial membranes that were protected by the indoleamine. Besides reducing LPO, aMT administration resulted in an improvement of the GSH:GSSG ratio in both mice strains. Whereas aMT increased the activity of GRd in SAMR1 and SAMP8 mice in cardiac mitochondria, the effects of the indoleamine were more evident in the diaphragm. Here, aMT treatment reduced the activity of GPx and increased that of GRd in both SAMR1 and SAMP8 mice. It was described that, in some circumstances, aMT may replace GSH in the defense against free radical attack (Abe *et al.* 1994). Thus, less GSH is expended in the antioxidative defense and GPx activity decreases. These findings may explain the decreased GPx activity after aMT treatment found here. The activity of GRd after aMT administration increased above the values found at 5 months of age, an effect that may account, at least in part, for the recovery of GSH pool and the improvement of the GSH:GSSG ratio found after aMT treatment. Besides these effects, aMT administration also enhanced the total GSH pool in the studied mitochondria. As mitochondria do not synthesize GSH, the data may reflect an improvement in the synthesis (Urata *et al.* 1999) and/or transport of cytosolic GSH to mitochondria, a finding also reported for SOD in SAM mice (Park *et al.* 1996).

In summary, aging produced a significant increase in mitochondrial ROS production and oxidative damage in cardiac and diaphragmatic mitochondria of SAM mice. Up to 10 months of age, SAMR1 and SAMP8 mice show similar mitochondrial oxidative stress, suggesting that 10 months of age is not old enough to detect changes due to accelerated aging in the mitochondria of these tissues. Together, the data agree with the mitochondrial free radical theory of aging and supports the concept that antioxidative therapy may be beneficial to prevent age-dependent oxidative stress. Dietary antioxidants may increase mitochondrial GSH content and GSH:GSSG ratio (Sastre *et al.* 1998), suggesting that a diet enriched with antioxidants is essential to obtain these effects. Only

an enriched diet with different types of antioxidants was found to be useful in increasing the mitochondrial GSH pool (Rebrin *et al.* 2005). Our results showed that the use of aMT as a single antioxidant therapy prevented the age-dependent production of ROS and oxidative damage in cardiac and diaphragmatic mitochondria. Probably, the beneficial effects of aMT may extend to other tissues. Thus, the potential antiaging properties of aMT should be further analyzed for its therapeutical applications in antioxidative therapy.

Acknowledgements

This study was partially supported by grants from the Instituto de Salud Carlos III (grants G03/137 and PI03-0817), and Consejería de Educación, Junta de Andalucía (CTS-101). LCL is a postdoctoral fellow from the Ministerio de Educación (Spain), and J A García and F Ortiz are a predoctoral fellow from the ISCIII (Ministerio de Sanidad, Spain). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 26 June 2007

Accepted 1 July 2007

Made available online as an Accepted Preprint

4 July 2007