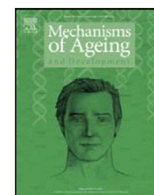
Mechanisms of Ageing and Development 130 (2009) 722–730

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

journal homepage: www.elsevier.com/locate/mechagedev



Autophagy upregulation and loss of NF-κB in oxidative stress-related immunodeficient SAMP8 mice

Beatriz Caballero [a], Ignacio Vega-Naredo [a], Verónica Sierra [a,b], David DeGonzalo-Calvo [a], Pablo Medrano-Campillo [c], Juan M. Guerrero [c], Delio Tolivia [a], María J. Rodríguez-Colunga [a], Ana Coto-Montes [a,*]

[a] Departamento de Morfología y Biología Celular, Facultad de Medicina, Universidad de Oviedo, C/Julián Clavería s/n, 33006 Oviedo, Spain
[b] Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), Villaviciosa, Spain
[c] Departamento de Bioquímica Médica y Biología Molecular, Hospital Virgen de la Macarena, Universidad de Sevilla, Sevilla, Spain

ARTICLE INFO

Article history:
Received 29 March 2009
Received in revised form 19 August 2009
Accepted 4 September 2009
Available online 12 September 2009

Keywords:
Aging
Apoptosis
Autophagy
Oxidative stress
NF-κB

ABSTRACT

Aged spleens from senescence-accelerated prone mice 8 (SAMP8) and senescence-accelerated resistant mice 1 (SAMR1) were examined to determine whether sex or melatonin had an effect on oxidative stress-related immune impairments. We observed that the immunosenescence of SAMP8 mice was associated with a redox imbalance, leading to an age-related increase in oxidative damage, resulting from a decrease in antioxidant defense and protease activity. Moreover, increased apoptotic cell death, a decrease in proliferative activity and the loss of NF-κB activation were also related to the immunodeficiency seen in SAMP8 compared to SAMR1 mice. Females demonstrated higher oxidative stress-related alterations in the immune response, and subsequent, melatonin treatment provided the best protective effects. Pathways involved in autophagy were upregulated in SAMP8 as an adaptive response to oxidative stress, in an attempt to rescue the cell from increased apoptosis and age-related immunodeficiency. However, the NF-κB signaling and autophagic processes were unaffected by treatment with melatonin. Therefore, we propose a key role for NF-κB signaling and autophagy in the oxidative stress-related immunosenescent spleens of SAMP8 mice.

© 2009 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Age-related changes in the immune system increase susceptibility to infectious diseases, autoimmunity and cancer for many elderly individuals. Several alterations of both, innate and adaptive immunity have been described, which present as a deterioration of immunity leading to the use of the term immunosenescence (McGlauchlen and Vogel, 2003; Plackett et al., 2004). This phenomenon is accompanied by a reduction in CD4+ and CD8+ T lymphocytes, a progressive increase in inflammatory status (Vasto et al., 2007) and a decline in antigen presenting cell function (McGlauchlen and Vogel, 2003).

Autophagy has also been implicated in aging, cancer and neurodegeneration, but maintains an essential role in the adaptive immunity for antigen processing, a consequence of their cleavage by lysosomal proteases, such as the cathepsins (Zavasnik-Bergant and Turk, 2007), for major histocompatibility complex type II (MHC-II) molecules (Deretic, 2006). This specialized role for autophagy in immune cells helps defend against intracellular pathogens and is referred to as immunophagy (Deretic, 2006). Recently, it has been suggested that autophagy may be essential for both T lymphocytes survival and proliferation (Pua et al., 2007). Autophagy often precedes apoptosis as a last attempt to rescue the cell before death (Kroemer and Jaattela, 2005). However, pronounced autophagy is actually associated with type II programmed cell death, which is a form of caspase-independent apoptosis (Kurz et al., 2008). Moreover, autophagy is the major mechanism responsible for inflammation restriction, through targeting the nuclear transcription factor κB (NF-κB) (Xiao, 2007), the master mediator of inflammatory and immune responses (Qing et al., 2006). This transcription factor also plays a key role in cell responses against oxidative stress (Michiels et al., 2002).

NF-κB forms homo- and heterodimers with members of the NF-κB/Rel family including p65 (Real A), Rel B, c-Rel, p50 (NF-κB1) and p52 (NF-κB2), with the p65/p50 dimer being the most abundant. NF-κB is normally sequestered in the cytoplasm through interactions with inhibitory κ-B proteins (Iκ-B). After activation by various stimuli, including cytokines, oxidative stress and bacterial or viral products, downstream signaling culminates in the

* Corresponding author. Tel.: +34 985 102779; fax: +34 985 103618.
E-mail address: acoto@uniovi.es (A. Coto-Montes).

0047-6374/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mad.2009.09.001

activation of the Iκ-B kinase (IKK) complex, which is composed of two catalytic subunits (IKK-α and IKK-β) and one regulatory subunit (NEMO/IKK-γ) (Li and Verma, 2002; Michiels et al., 2002). Phosphorylation of the inhibitor κ-B proteins (Iκ-B) by the catalytic subunits of IKK, lead to degradation by the proteasome, thus releasing NF-κB dimers, which are translocated into the nucleus to regulate expression of a wide variety of genes. The NF-κB signaling pathway is part of the innate arm of the immune system (Li and Verma, 2002; Michiels et al., 2002). In addition to the key role of NF-κB in the inflammatory responses, it is well known to function as a cell protector, through its anti-apoptotic effects (Michiels et al., 2002) and antioxidant functions, since NF-κB can increase expression of antioxidant enzymes in response to elevated oxidative stress (Tomas-Zapico and Coto-Montes, 2005).

Senescence-accelerated prone mice 8 (SAMP8) provide a unique model system for studying senescence and aging as they exhibit a marked acceleration of aging (Takeda et al., 1981). We previously demonstrated that oxidative stress is a key factor in accelerated senescence for this strain of mice (Alvarez-Garcia et al., 2006; Lardone et al., 2006; Tomas-Zapico et al., 2006; Gutierrez-Cuesta et al., 2007; Caballero et al., 2008). In this study, we examined male and female spleens from SAMP8 mice at 10 months of age to determine whether relevant processes were altered because of senescence-accelerated immune impairments compared to control samples from SAMR1 mice. We also examined samples from male and female SAMP8 or SAMR1 mice treated with melatonin for 9 months (starting 1 month after birth) to gain insight concerning the antioxidant properties of melatonin associated with age-related changes in immune defenses.

2. Materials and methods

2.1. Animals

SAMP8 and SAMR1 mice breeding pairs were obtained from the Council for SAM Research, Kyoto, Japan, through HARLAN (Barcelona, Spain). They were housed in the Granada' University facility, under a 12/12 h dark/light cycle, temperature controlled (22 ± 1 °C) and bred by brother–sister mating. Animals received tap water and standard pellet diet *ad libitum*. The experimental protocol was approved by the Granada' University Local Animal Care and Use Committee. All experiments were carried out according to the Spanish Government Guide and the European Community Guide for animal care.

2.2. Treatment

Once newborn male and female SAMP8 and SAMR1 mice were separated from their mothers (at 1 month of age), melatonin or vehicle treatments were initiated. Melatonin from SIGMA (St. Louis, MO, USA) was dissolved in a minimum volume of absolute ethanol in bottles protected from light and diluted in the drinking water to yield a dose of 10 mg/kg b.w. during the treatment from 1 to 10 months of age. The concentration of ethanol in the final solution was 0.066%. SAMR1 and SAMP8 mice were decapitated at 10 months of age and they were grouped as follows in each gender ($n = 4$, strain/age/treatment): the R10v group, consisted of SAMR1 mice treated with vehicle from 1 to 10 months of age; the R10m group, included SAMR1 mice treated with melatonin from 1 to 10 months of age; the P10v group, included SAMP8 mice treated with vehicle from 1 to 10 months of age and the P10m group, consisted of SAMP8 mice treated with melatonin from 1 to 10 months of age. The spleen of each mouse was immediately removed, frozen in liquid nitrogen and stored at −80 °C until their use for biochemical experiments. Tissue was homogenized and the protein content was measured in an Ubikon 930 spectrophotometer at 595 nm by the method of Bradford (Bradford, 1976).

2.3. Oxidative stress-related assays

Protein carbonyl was determined spectrophotometrically (Uvikon 930, KONTRON INTRUMENTS, Milan, Italia) at 340 nm following the method developed by Levine et al. (1990) with the modifications of Coto-Montes and Hardeland (Coto-Montes and Hardeland, 1999). The chromogene 2,4-dinitrophenylhydrazine reacts with the carbonyl groups of the damaged proteins. Results are expressed as nmol of protein carbonyl per mg of protein.

Lipid peroxidation was determined by malondialdehyde (MDA) and 4-hydroxyalkenal (4HDA) content, using a Lipid Peroxidation Assay Kit from Calbiochem (No 437634), based on the condensation reaction of the chromogene 1-methyl-2-phenylindole with either MDA or 4HDA. The stable chromophores were measured spectrophotometrically (Uvikon 930) at 586 nm. Results are expressed as nmol MDA + 4HDA per mg protein.

Catalase activity (CAT) was assayed spectrophotometrically (Uvikon 930) according to Lubinsky and Bewley (1979) using H_2O_2 as substrate. Data are expressed as μmol of H_2O_2 consumed per mg of protein per min. Glutathione reductase (GR) catalyzes the reduction of the oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH+H^+ as substrate. The assay was carried out spectrophotometrically (Uvikon 930) according to Kum-Tatt et al. (1975) monitoring the oxidation of NADPH. Data are expressed as nmol of NADPH consumed per mg of protein per min.

2.4. Protease activities

Cathepsin B was assayed fluorimetrically (Cytofluor TM 2350. MILLIPORE CORP., MA, USA) according to the method developed by Barrett (1980) with minor modifications of Schreurs et al. (1995) using Z-Arg-Arg-AMC as specific substrate. An excitation wavelength of 360 nm and emission wavelength of 460 nm were used with aminomethylcoumarin solutions (AMC) as standards. The results were expressed as enzymatic milliunits per mg of protein.

Cathepsin D was assayed spectrophotometrically (Uvikon 930) in according to Takahashi and Tang (1981) with minor modifications of Schreurs et al. (1995) using hemoglobin as substrate. The optical densities were read in the supernatants at 230 nm. The cathepsin D results were expressed as enzymatic units per mg protein.

Caspase-3 activity was determined by using a colorimetric assay kit (CASP-3-C) from SIGMA (Munich, Germany). The colorimetric substrate Acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) is hydrolyzed by caspase-3 and the p-NA is released from the substrate. To account for non-specific hydrolysis of the substrate, a control reaction mixture contained tissue homogenates, substrate and the specific caspase-3 inhibitor Ac-DEVD-CHO in assay buffer was also assayed. Both mixtures were monitored at 37 °C by a plate reader ELx800 UV Bio-Tek Instruments (IZASA, Sevilla, Spain). Data obtained using the caspase-3 inhibitor were substracted from the data obtained without caspase-3 inhibitor to correct for any non-specific hydrolysis. Data were normalized by protein content of each sample. The caspase-3 activity was expressed as nmol of pNA releases per minute per mg of protein.

2.5. Western blot detection

Aliquots of tissue homogenate (50 μg of protein per sample) were separated by electrophoresis SDS-PAGE and transferred onto a polyvinylide fluoride sheets (PVDF) (Immobilon TM-P; MILLIPORE CORP., MA, USA). Membranes were blocked overnight with 5% non-fat milk and incubated with the respective primary antibodies against Beclin 1, LC-3, MT-1, cathepsin D, BcL-2, p53 and PCNA from SANTA CRUZ BIOTECHNOLOGY (Santa Cruz, CA, USA). Antibodies of anti-NF-κB signaling pathway were from CELL SIGNALLING. After washing the membranes, they were incubated with a specific peroxidase-conjugated anti-IgG secondary antibodies from SIGMA (Munich, Germany) followed by the enhanced chemiluminescence detection system following the manufacturer's protocol (Western Blotting Luminol Reagent; SANTA CRUZ BIOTECHNOLOGY (Santa Cruz, CA, USA)). Digital images were analyzed with a Quantity one program (BIORAD, Hercules, CA, USA) which permits a semi-quantitation of band intensity in front of actin. Routinely, protein load was monitored using the Bradford method (Bradford, 1976) and phenol red staining of the blot membrane.

2.6. Statistical analysis

Statistical analyses were performed with SPSS 12.0. Data are presented as mean values ± SEM calculated from at least three separate experiments, each performed in triplicate. The normality of the data was analyzed by Kolmogorov–Smirnov test. Mean comparisons were analyzed by one-way ANOVA following Student's *t*-test to compare mean between strains mice (SAMR1 versus SAMP8), treatments (vehicle versus melatonin) and sexes (males versus females). The level of significance accepted was $p < 0.05$.

3. Results

3.1. Oxidative stress markers

There was no statistically significant difference in protein damage (Fig. 1A) between the males groups analyzed (Fig. 1A, left). However, the protein damage in males showed the same tendency as females, where protein damage was higher ($p < 0.01$) in P10v compared to R10v (Fig. 1A, right). Melatonin treatment decreased the protein damage ($p < 0.05$) significantly in female P10m mice (P10v versus P10m) (Fig. 1B, right). Differences between sexes were only found in R10v showing minor protein damage in females ($p < 0.05$).

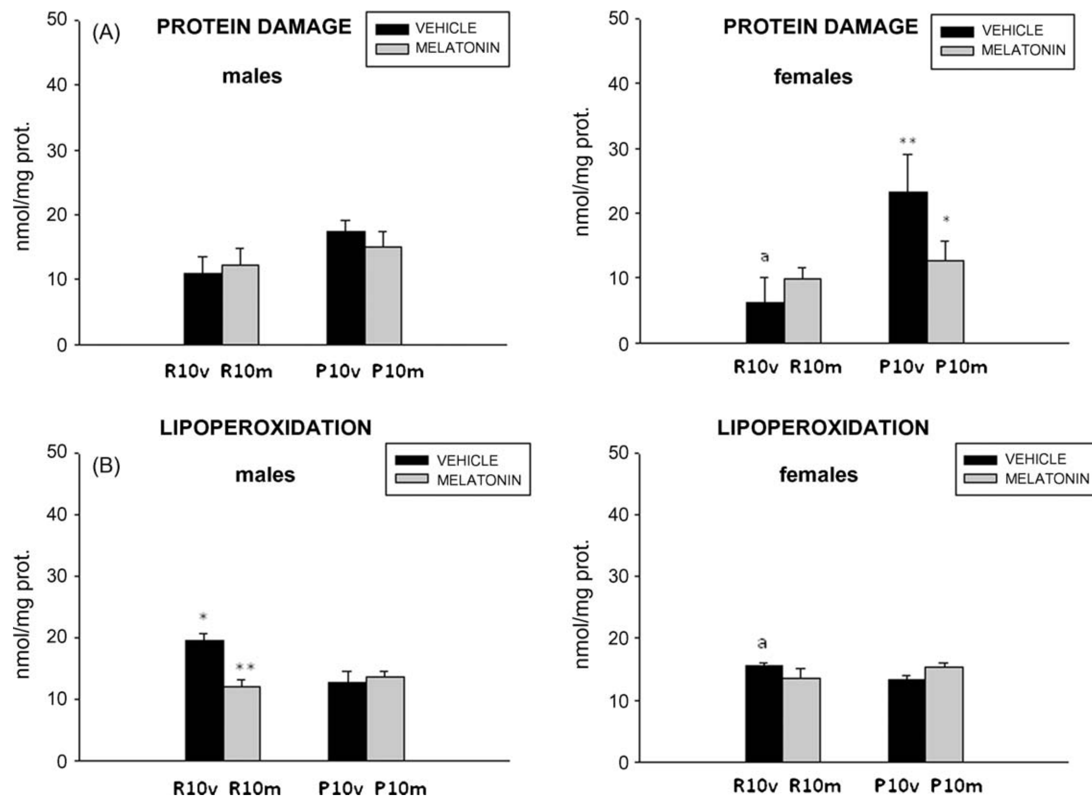


Fig. 1. (A) Protein damage (DP) (nmol/mg prot.) in spleen from male (left) and female (right) SAMR1 (R10) and SAMP8 (P10) mice at 10 months of age treated with the vehicle, ethanol at 0.066%, (R10v and P10v) or melatonin at 10 mg/kg (R10m and P10m) in the drinking water starting at 1 month after birth to 10 months of age. *$p < 0.05$ versus P10v; **$p < 0.01$ versus R10v; $^{a}p < 0.05$ compared with the same group of males. (B) Lipoperoxidation (LPO) (nmol/mg prot.) in spleen from male (left) and female (right) SAMR1 and SAMP8 mice at 10 months of age. *$p < 0.05$ versus P10v; **$p < 0.01$ versus R10v; $^{a}p < 0.05$ compared with the same group of males.

Lipoperoxidation (Fig. 1B) was less in P10v mice compared to R10v mice, with statistical differences ($p < 0.05$) only appearing in males (Fig. 1B, left). Melatonin treatment decreased ($p < 0.01$) the lipoperoxidation levels mainly in R10m males (R10v versus R10m) (Fig. 1B, left) with the same tendency in R10m females (Fig. 1B, right). However, melatonin treatment did not produce a significant effect on the level of lipoperoxidation in P10m mice (P10v versus P10m) in either sex (Fig. 1B). A difference between sexes was only found in the case of R10v mice, where lipoperoxidation was lower ($p < 0.05$) in females.

3.2. Antioxidant enzymes

We observed less CAT activity in P10v versus R10v, with statistical differences ($p < 0.05$) only appearing in females (Fig. 2A, right). Melatonin treatment produced a SAM strain-dependent effect; decreasing CAT activity in R10m mice, mainly in females (R10v versus R10m) ($p < 0.01$) (Fig. 2A, right) and increasing CAT activity in males ($p < 0.01$) and females ($p < 0.001$) P10m mice (P10v versus P10m) (Fig. 2A, right). Differences between sexes were uncovered in the groups R10v, P10v ($p < 0.05$) and P10m ($p < 0.01$) with a higher CAT activity in females. Glutathione reductase (GR) activity (Fig. 2B) also decreased in male ($p < 0.01$) and female ($p < 0.05$) P10v mice compared to R10v mice. Melatonin treatment reduced GR activity in male ($p < 0.01$) and female ($p < 0.05$) R10m mice (R10v versus R10m) (Fig. 2B). However, GR activity was increased after melatonin treatment in P10m mice (P10v versus P10m), mainly in females ($p < 0.001$) (Fig. 2B, right). Differences between sexes were found in P10v and P10m groups with a higher GR activity ($p < 0.05$) observed in females.

3.3. Protease activities

Cathepsin D aspartyl-protease activity was lower in P10v compared to R10v mice, mainly in males ($p < 0.001$) (Fig. 3A, left). Melatonin treatment increased CD activity in both R10m and P10m mice (R10v versus R10m; P10v versus P10m), both in males and females (Fig. 3A). However, cathepsin D activity was consistently higher in females than in males ($p < 0.05$) (Fig. 3A, right). Moreover, we did not observe strong variations in cathepsin D protein expression between SAM mice, treatments or sexes (Fig. 3B) except for elevated levels of procathepsin D isoforms (of 46 and 52 kDa) in all groups studied. Cathepsin B cysteine-protease activity was decreased in P10v compared to R10v, males ($p < 0.001$) and females ($p < 0.05$) (Fig. 4). Melatonin treatment further decreased cathepsin B activity in male ($p < 0.001$) and female ($p < 0.05$) R10m mice (R10v versus R10m), but only in females ($p < 0.01$) in the case of P10m mice (P10v versus P10m) (Fig. 4, left). Cathepsin B activity was consistently higher in females ($p < 0.05$) of either group studied compared to males, except for R10v mice.

3.4. Autophagic proteins

Beclin 1 protein (Fig. 5A, up), a marker of autophagic processes, was consistently upregulated in P10v compared to R10v in both sexes. Melatonin treatment decreased Beclin 1 levels in all groups (R10v versus R10m; P10v versus P10m) except for P10m males, in which the melatonin treatment increased the levels of Beclin 1 (P10v versus P10m). Differences between sexes were observed in both SAM mice with minimal Beclin 1 detection in females. The conversion of LC-3 I (soluble unlipidated form) to LC-3 II

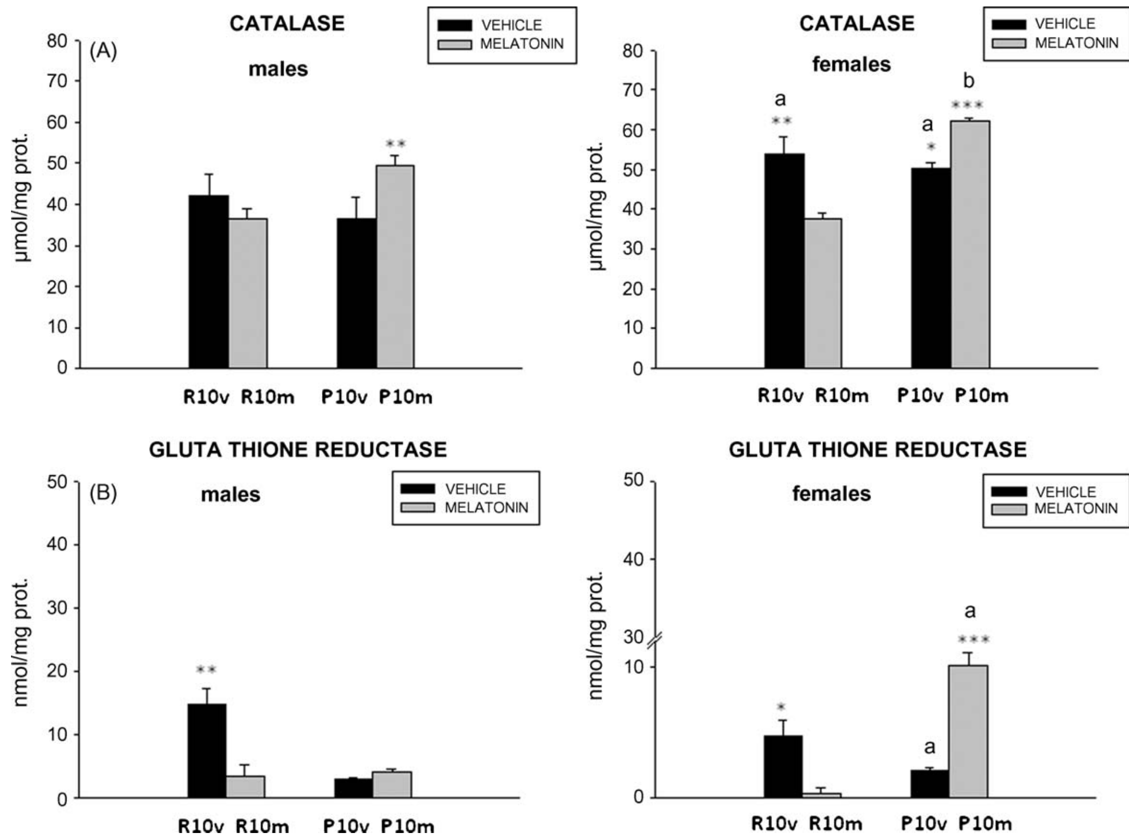


Fig. 2. (A) Catalase (CAT) activity (μmol/mg prot.) in spleen from male (left) and female (right) SAMR1 (R10) and SAMP8 (P10) mice at 10 months of age treated with the vehicle, ethanol at 0.066%, (R10v and P10v) or melatonin at 10 mg/kg (R10m and P10m) in the drinking water stating at 1 month after birth to 10 months of age. $^{*}p < 0.05$ versus R10v; $^{**}p < 0.01$ versus R10m; $^{***}p < 0.001$ versus P10v and R10m; $^{a}p < 0.05$, $^{b}p < 0.01$ compared the same group of males. (B) Glutathione reductase (GR) activity (nmol/mg prot.) in spleen from male (left) and female (right) SAMR1 and SAMP8 mice at 10 months of age. $^{*}p < 0.05$ versus R10m and P10v; $^{**}p < 0.01$ versus P10v and R10m; $^{***}p < 0.001$ versus P10v and R10m; $^{a}p < 0.05$ compared to the same group of males.

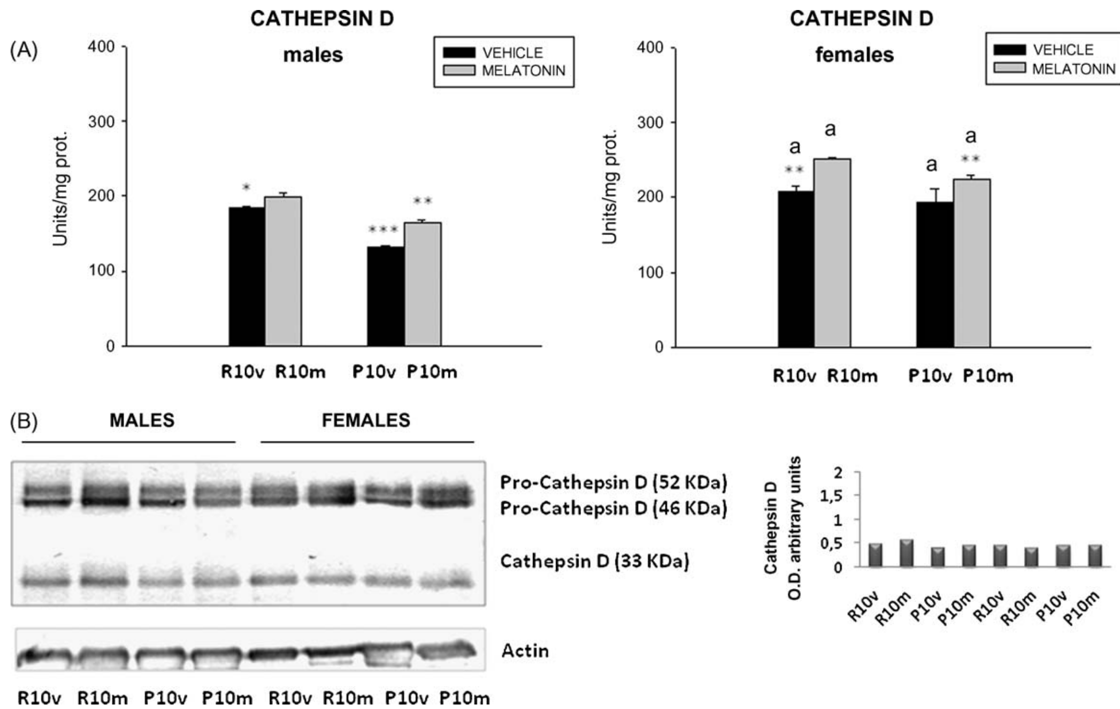


Fig. 3. (A) Cathepsin D activity (Units/mg prot.) in spleen from male (left) and female (right) SAMR1 (R10) and SAMP8 (P10) mice at 10 months of age treated with the vehicle, ethanol at 0.066%, (R10v and P10v) or melatonin at 10 mg/kg (R10m and P10m) in the drinking water starting at 1 month after birth, to 10 months of age. $^{*}p < 0.05$ versus R10m; $^{**}p < 0.01$ versus P10v and R10m; $^{***}p < 0.001$ versus R10v; $^{a}p < 0.05$ compared to the same groups of males. (B) Cathepsin D protein detection by western blot using 50 μg of protein from male and female spleen of SAMR1 and SAMP8 mice at 10 months of age. Right panels: Bar chart showing semi-quantitative optical density (arbitrary units of blot bands) normalized to actin.

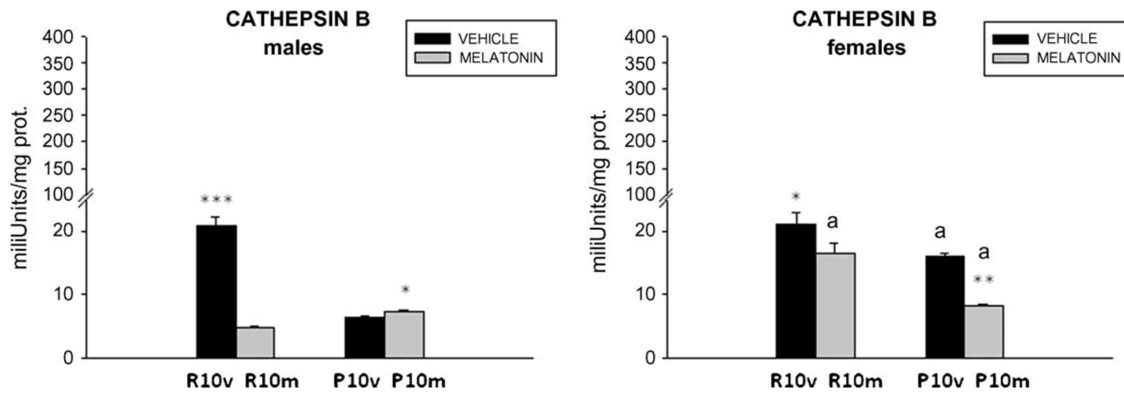


Fig. 4. Cathepsin B activity (milliunits/mg prot.) in spleen from male (left) and female (right) SAMR1 (R10) and SAMP8 (P10) mice at 10 months of age treated with the vehicle, ethanol at 0.066%, (R10v and P10v) or melatonin at 10 mg/kg (R10m and P10m) in the drinking water starting at 1 month after birth, to 10 months of age. $^{*}p < 0.05$ versus R10m and P10v; $^{**}p < 0.01$ versus P10v and R10m; $^{***}p < 0.001$ versus R10m and P10v; $^{a}p < 0.05$ compared to the same groups of males.

(membrane-bound form-phospholipid conjugated) is closely correlated with completed autophagosomes and autolysosomes (Kabeya et al., 2000). We detected LC-3 I (Fig. 5A, down) in all groups, although representing the lower levels in P10v compared to R10v mice in either sex. LC-3 I was slightly increased with melatonin treatment in P10m mice (P10v versus P10m) of both sex. We only detected LC-3 II (Fig. 5A, down) protein in P10v and P10m mice, with little detected in R10v mice, of either sex. Melatonin treatment only slightly increased LC-3 II levels in female P10m mice (P10v versus P10m). Differences between sexes were observed including minimal LC-3 II detection in females.

3.5. Proliferative activity

PCNA levels (Fig. 5B) were decreased in P10v mice of both sex (P10v versus R10v), but more so in females. Melatonin treatment decreased PCNA levels in all groups (R10m and P10m) of either sex, except for male P10m mice.

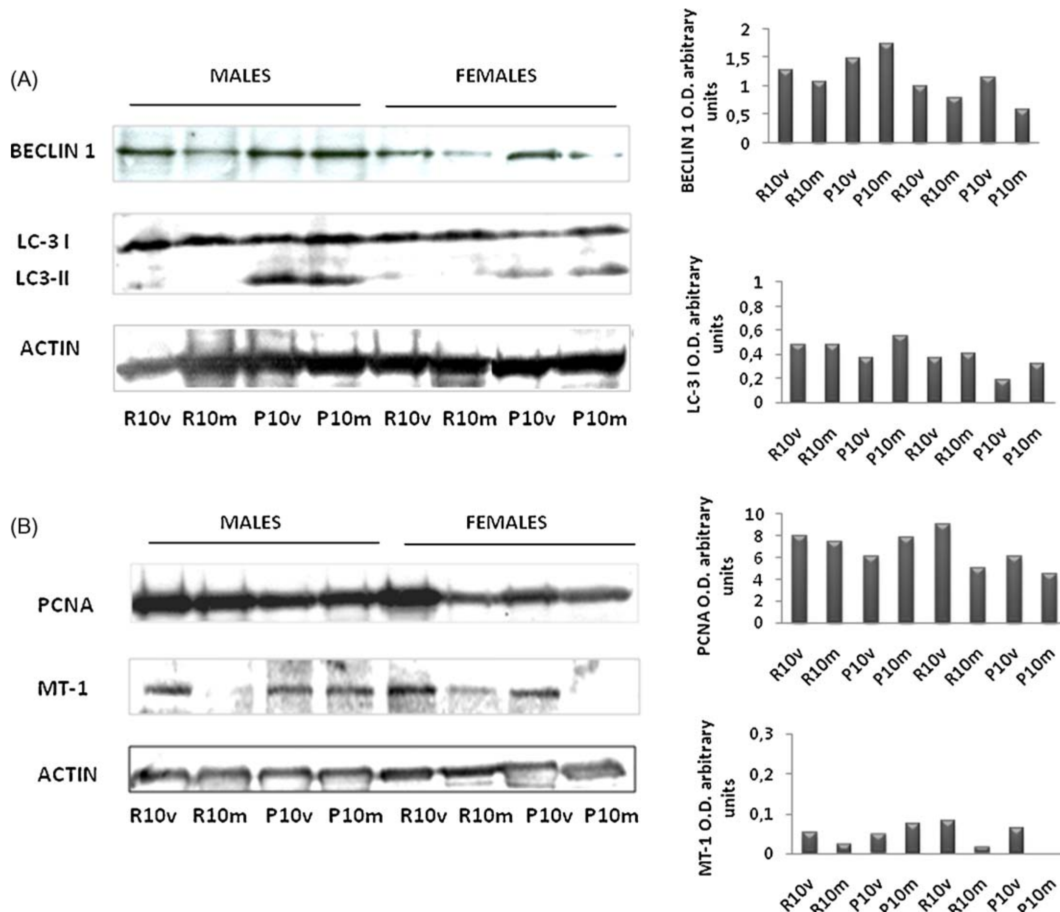


Fig. 5. Detection by western blot of Beclin 1 and LC-3 I/II proteins (A), proliferating cellular nuclear antigen (PCNA) and MT-1 melatonin receptor (B), using 50 μg of protein from male and female spleen of SAMR1 (R10) and SAMP8 (P10) mice at 10 months of age treated with the vehicle, ethanol at 0.066%, (R10v and P10v) or melatonin at 10 mg/kg (R10m and P10m) in the drinking water starting at 1 month after birth, to 10 months of age. Right panels: Bar chart showing semi-quantitative optical density (arbitrary units of blot bands) normalized to actin.

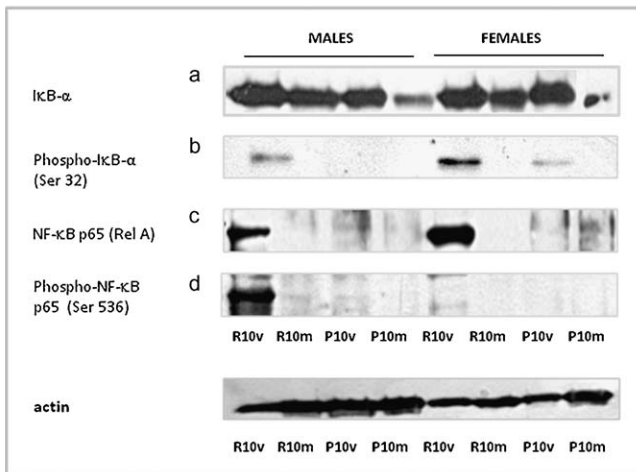


Fig. 6. Detection of proteins from NF-κB signaling pathway by western blot using 50 μg of protein from male and female spleen of SAMR1 (R10) and SAMP8 (P10) mice at 10 months of age treated with the vehicle, ethanol at 0.066%, (R10v And P10v) or melatonin at 10 mg/kg (R10m and P10m) in the drinking water starting at 1 month after birth, to 10 months of age; IκB-α (a), IκB-α phosphorylated at Ser 32 (b), p65/Rel A (c) and p65/Rel A phosphorylated at Ser 536 (d). Actin was used as a loading control.

3.6. Melatonin receptor

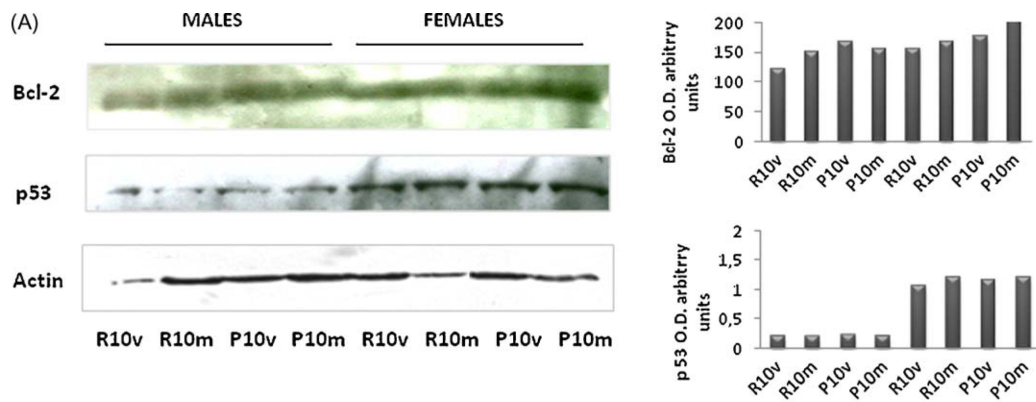
We observed a clear decrease in MT-1 protein (Fig. 5B) in P10v compared to R10v in both sexes, with a more marked decrease in females. MT-1 levels were markedly decreased in SAM mice treated with melatonin (R10m and P10m), except for male P10m mice, where no change in MT-1 levels was detected.

3.7. NF-κB signaling

We observed high levels of Iκ-B (Fig. 6A) in all groups studied, with less detected in males P10v compared to R10v mice, and no expression level differences between both SAM mice in females. Melatonin treatment decreased Iκ-B levels in R10m and P10m mice of both sexes, with the lowest expression levels reached in P10m mice. Phosphorylation of Iκ-B leads to its proteasome-dependent degradation, resulting in release and nuclear translocation of NF-κB dimers (Viatour et al., 2005). However, phosphorylated Iκ-B (Fig. 6B) was only found in R10v mice of both sexes and female P10v mice, with high expression levels in R10v females. Phosphorylation of p65 regulates activation, nuclear localization and transcriptional activity of NF-κB (Viatour et al., 2005). P65 protein was only found (Fig. 6C) in R10v mice of both sexes, with higher expression in R10v females. However, phosphorylated-p65 was (Fig. 6D) mainly detected in the case of R10m males. Levels of p65 and phosphorylated-p65 were almost undetectable in P10v and P10m mice in both sexes (Fig. 6C and D).

3.8. Apoptotic markers

Anti-apoptotic protein Bcl-2 (Fig. 7A, up) was expressed at high levels in male and female R10v and P10v mice, with a slight upregulation in P10v mice. Melatonin treatment slightly increased Bcl-2 levels in both sexes of SAM mice (R10v versus R10m; P10v versus P10m), except for P10v males. P53, a pro-apoptotic transcription factor (Fig. 7A, down), did not show any expression level differences between SAM strains (R10v versus P10v) or between treatments (R10v versus R10m; P10v versus P10m) in either sex. However, higher p53 levels were always observed in female mice (R10v, R10m, P10v and P10m). Capase-3 activity (Fig. 7B) was detected in R10v males and both sexes of P10v mice, but it was higher in P10v females ($p < 0.01$). No caspase-3 activity



(B) CASPASE-3 ACTIVITY (nmol/ mg prot.*min)

	SAMR1 VEHICLE	SAMR1 MELATONIN	SAMP8 VEHICLE	SAMP8 MELATONIN
Males	0,0068 ± 0,003***	ND	0,3618 ± 0,07 [b]	ND
Females	ND	ND	0,8917 ± 0,17	ND

ND: caspase- 3 activity was not detected

Fig. 7. (A) Detection by western blot of Bcl-2 (top) and p53 (middle) proteins, using 50 μg of protein from male and female spleen of SAMR1 (R10) and SAMP8 (P10) mice of 10 months of age treated with the vehicle, ethanol at 0.066%, (R10v and P10v) or melatonin at 10 mg/kg (R10m and P10m) in the drinking water starting at 1 month after birth to 10 months of age. Right panels: Bar chart showing semi-quantitative optical density (arbitrary units of blot bands) normalized to actin. (B) Caspase-3 activity (nmol/(mg prot. min)) in male and female spleen from male and female SAMR1 and SAMP8 mice at 10 months, vehicle group and treated with melatonin $^{***}p < 0.001$ versus P10v; $^{a}p < 0.001$ compared to the same group in females.

was observed in either sex of R10m and P10m mice treated with melatonin.

4. Discussion

It is well known that SAMP8 mice display specific age-related changes, characterized by learning, memory and emotional disorders, as well as impairments in immune responses (Takeda et al., 1997). Aging of the immune system is accompanied by changes that lead to a decrease in both cellular and humoral responsiveness (Hodes, 1997). Oxidative stress, altered cytokines patterns, lifelong antigenic burden and apoptotic deregulation represent the hallmarks of immune system remodeling associated with aging (De Martinis et al., 2007). We found a decrease in the proliferative activity of spleen tissue from SAMP8 mice, as shown by a clear decrease in PCNA levels at 10 months of age in both sexes compared to SAMR1 mice. Because deterioration of the immune response is mainly associated with a dramatic decrease in the proliferative capacity of lymphocytes during aging (Tian et al., 1995), a clear difference in immune function between SAM mice is shown, being mainly defective in SAMP8 mice. Our findings further suggest no effect in restoring cell proliferation after melatonin treatment, since PCNA levels were mainly decreased in the groups treated with melatonin, in accordance with another study revealing an inhibitory effect of melatonin on cell proliferation (Liang et al., 2008).

We have previously demonstrated signs of neurodegeneration and oxidative damage in the brain of SAMP8 mice (Alvarez-Garcia et al., 2006) as well as early oxidative damage in other organs of the same strain of mice (Tomas-Zapico et al., 2006; Caballero et al., 2008, 2009). Therefore, oxidative stress is one of the mechanisms contributing to accelerated aging in SAMP8 mice and their age-related impairments. We found high concentrations of protein damage from oxidative stress in the SAMP8 spleen compared to SAMR1 mice, mainly in females, which maintained the highest levels of damaged proteins and showed the least proliferative activity. In fact, melatonin treatment produced the best protective effects in female SAMP8 mice, where melatonin clearly decreased oxidative damage. Therefore, we observed an oxidative stress-related decline in immune function, as a consequence of accelerated senescence, that was more pronounced in female SAMP8 mice.

Accumulation of massive amounts of oxidized proteins (Stadtman, 1992; Stadtman and Levine, 2000) can explain the strong decrease observed in antioxidant activity, (CAT and GR) in the spleen in both sexes of SAMP8 mice, which contributes to the increase in age-related oxidative stress. Therefore, it is clear that redox imbalance in splenocytes can affect adaptive immune processes. Interventions that restore the redox balance might benefit the ailing immune system (Daynes et al., 2003). In fact, SAM mice treated with melatonin demonstrate a decrease in oxidative stress level in the spleen, since antioxidant activity was increased in SAMP8 mice and protein damage decreased, particularly in female SAMP8 mice. These data, corroborate the positive role of melatonin in improving immune functions especially, in situations where the immune system is depressed, as previously shown by Guerrero and Reiter (2002). In contrast, CAT and GR activities were decreased in SAMR1 mice treated with melatonin. Therefore, we observed a SAM strain-dependent effect of melatonin in the antioxidant activity of the aged spleen: ROS levels are reduced in SAMR1 mice which represents a direct antioxidant effect of melatonin. However, an indirect effect of melatonin was observed in the case of SAMP8 mice, since melatonin specifically increased antioxidant enzymes activities. This different antioxidant effect of melatonin could be related to different oxidative stress levels observed in each SAM strain, with the most pronounced damage in SAMP8 females, which also experience a drop in estrogen levels (Castillo et al., 2005). Similar antioxidant effect of melatonin was previously observed in the brain of the same SAM mice after melatonin treatment (Caballero et al., 2008).

Furthermore, the effects of exogenous melatonin on the antioxidant activities of older SAMP8 spleens were independent of the MT-1 receptor, as MT-1 levels were undetectable in the treated SAMP8 mice. Minimal expression of the MT-1 receptor was also observed in the spleen of males and females SAMP8 mice, corroborating recent results revealing an age-related decrease in MT-1 protein for several aged organs (Sanchez-Hidalgo et al., 2008).

Lipid peroxidation is known to affect the membrane fluidity and its physical state, which can eventually result in the decline of lymphocyte proliferation (Eze, 1992). An age-dependent increases in lipid peroxide levels in peripheral organs was also observed in senescence-accelerated mice (Matsugo et al., 2000). This effect was also observed by our laboratory, specifically in the brain and liver of SAMP8 mice (Alvarez-Garcia et al., 2006; Tomas-Zapico et al., 2006; Gutierrez-Cuesta et al., 2007). Contrary to these previous results, we observed less lipid peroxidation in the spleens of SAMP8 males and females. There are many reports concerning lipid peroxidation as a result of aging, where an increase, decrease or unchanging lipid peroxidation level has been showed (Rikans and Hornbrook, 1997). Therefore, the oxidative stress-related increase in lipid damage is clearly tissue-dependent in the aging of SAMP8 mice. In spleen, our results demonstrate that melatonin treatment was only able to decrease lipid damage in SAMR1 males, where lipid peroxidation was the highest among all the groups analyzed. Therefore, taking into account the antioxidant effect of melatonin on protein and lipid damage, melatonin shows a damage level-related effect, which we also observed in the brain of older SAMR1 and SAMP8 mice also treated with melatonin (Caballero et al., 2009).

Strong oxidative stress in spleens from SAMP8 mice also promoted a decrease in the protease activities of cathepsins, which constitute the major portion of the endosomal/lysosomal proteolytic system. These cysteine-proteases are essential for immune response in antigen processing with foreign and self-proteins prior to their binding to MHC-II molecules and subsequent display to specific T-cell receptors (Zavasnik-Bergant and Turk, 2006, 2007). The loss of proteolytic activity of CD and CB, in addition to the antioxidant system impairments, observed in SAMP8 mice, could compromise lymphocyte activation in aged SAMP8 mice, contributing to the immune response alterations observed for this strain. It is important to note that CD and CB have different functions in the regulation of immune responses (Abbas et al., 1996), which could explain the melatonin-induced effect observed on their protease activities. In contrast to CB activity, CD is able to induce activation of both types of CD4+ T helper (Th) lymphocytes (Th1 and Th2), and melatonin specifically increases CD activity improving antigen presentation.

Moreover, increased caspase-3 activity indicates an oxidative stress-related increase in apoptosis for SAMP8 spleens, though the effect is more pronounced in females These data agree with previous works demonstrating an age-related increase in apoptosis of immune cells, reaching a peak at 2 years of age in rats (Kapasi and Singhal, 1999). Interestingly, high p53 levels were also observed in female SAM mice indicating a higher rate of DNA damage than males. Overall, apoptotic differences observed among SAM mice are directly associated with the oxidative damage in each SAM strain, with an increase noted in SAMP8 mice due to their accelerated senescence and displaying a marked difference in the apoptosis between sexes. Caspase-3 activity was significantly decreased in both treated SAM mice reinforcing the protective role of melatonin against cell death of immune cells.

Cells use to utilize the autophagic recycling system for both, the constitutive removal of cytosolic proteins and organelles, and to maintain quality and nutrient supply under adverse conditions. Apart from the fundamental roles of autophagy for survival as well as tumor suppression (Levine and Klionsky, 2004; Debnath et al., 2005), a new specialized role for autophagy in acquired immunity has been suggested (Deretic, 2006). Autophagy is critical for T-cell survival and proliferation (Pua et al., 2007), central and peripheral tolerance induction and the prevention of unwanted inflammation and autoimmunity (Levine and Deretic, 2007). The decrease in autophagy observed with age observed in most tissues and organisms, contributes to the malfunctioning of older cells (Cuervo and Dice, 2000; Cuervo, 2006); Supporting this idea, we recently found autophagic-related neurodegeneration impairments in SAMP8 mice (Caballero et al., 2008), indicating that compromised autophagic turnover can lead to irreversible cell injury. However, in the study, our results demonstrate increased autophagic processes in the spleen of SAMP8 males and females showing high LC-3 II detection, the main marker of macroautophagy (Kabeya et al., 2000; Tanida et al., 2005) and increased Beclin 1 levels, an important protein of the autophagy pathway (Pattingre et al., 2005). Although, it is not yet well established whether, autophagy is a mechanism of cell death or it is a part of the endogenous protective response (Erlich et al., 2006), it obviously has a cytoprotective role during stress conditions through its action as a control mechanism for organelle quality (Li et al., 2006; Cao and Klionsky, 2007). Previous reports have proposed that increased autophagy, which removes oxidatively damaged organelles and proteins, provides a second level of defense, when antioxidant activities are compromised, providing a selective advantage in conferring resistance to oxidative stress (Moore, 2008). In accordance with this and our data, demonstrating a decrease observed in the LPO and antioxidant defenses in SAMP8 males and females, we suggest a pro-survival function for autophagy, as an adaptive response to oxidative stress. In our data, female SAMP8 mice, with the highest amount of protein damage, elevated apoptosis, low antioxidant defense and scarcely proliferative activity, indicated minimal levels of autophagy, which were slightly restored with melatonin treatment. In light of these findings, we suggest a putative relationship between autophagy and melatonin, although this only incorporates the cytoprotective role of melatonin for the immunodeficiency of SAMP8 mice shown here. We have established apoptosis as the main mechanism of cell death for immune cells with senescence accelerated. We also have identified crosstalk between autophagy and apoptosis since, autophagy functions as an attempt to maintain the successful immune responses in the context of the SAMP8-related immunodeficiencies.

There is evidence that autophagy can be regulated by ROS (reactive oxygen species) and by the redox-sensible NF-κB transcription factor, the master mediator of inflammatory responses (Xiao, 2007) such that, autophagy can inactivate NF-κB as a tumor suppressor mechanism and control an unwanted inflammatory reaction (Qing et al., 2006). Recently, the activation of NF-κB has emerged as one of the prominent steps in mounting an effective immune response (Kane et al., 2002). In this study, NF-κB activation, by phosphorylation of Iκ-B and subsequent activation of p65 also activated by phosphorylation, was only observed in the spleens of SAMR1 mice, being higher in males. However, males and females spleens from SAMP8 mice showed a loss of NF-κB activation due to a loss of phosphorylated Iκ-B and activated p65 protein. Activation of the NF-κB signaling pathway is specifically required for antigen-induced proliferation, cytokine production and T-cell survival, processes which form the cornerstone of the adaptive immune responses (Kane et al., 2002). Therefore, the loss of activation of NF-κB signaling, associated with accelerated senescence, is consistent with the alterations in lymphocyte activation and immune function in spleens of SAMP8 mice. Moreover, activation or inactivation of NF-κB by melatonin has been published previously (Bruck et al., 2004; Chetsawang et al., 2006). However, in this study, treatment with melatonin did not have a specific effect on the NF-κB signaling pathway due to its anti-inflammatory properties (Rodríguez et al., 2007; Tan et al., 2007). Other NF-κB dimmers should be considered, such as p50/p52, which is also important in survival, inflammation and immune function or p50/Rel B, which plays a key role in lymphogenesis and B cells maturation (Baeuerle and Henkel, 1994; Baeuerle and Baltimore, 1996), in which melatonin might be bigger effect in the case of immune cells.

In conclusion, the immunodeficiencies in older SAMP8 spleens are directly associated with age-related oxidative stress level increases, that lead to an increase in oxidative damage to proteins, alterations in antioxidant and protease activities as well as an increase in apoptotic cell death; being all changes more prominent in females and specifically affected by melatonin. In addition, the loss of NF-κB activation in the SAMP8 spleens contributes to immunodeficiency, with no effect from melatonin treatment. The upregulation of macroautophagy in response to age-related oxidative stress is considered as an adaptive mechanism in the fight against apoptosis associated with senescence accelerated-related immune alterations in SAMP8 mice.

Acknowledgments

The authors thank Gloria Menéndez for her excellent technical assistance. This work was partly supported by the grants: FISS-06-RD06/0013/0011 from the Instituto de Salud Carlos III, INIA-070RTA2007-00087-C02-02 from the Ministerio de Educación y Ciencia and FEDER funds. BC thanks her support from FYCYT and DdG-C is a predoctoral from FICYT from the Gobierno de Principado de Asturias, Spain; IV-N is a predoctoral from The Ministerio de Ciencia e Innovación, Spain; VS is a predoctoral fellow from INIA, Spain.

References

Abbas, A.K., Murphy, K.M., Sher, A., 1996. Functional diversity of helper T lymphocytes. Nature 383, 787–793.

Alvarez-Garcia, O., Vega-Naredo, I., Sierra, V., Gaetani, G.F., 2006. Elevated oxidative stress in the brain of senescence-accelerated mice at 5 months of age. Biogerontology 7, 43–52.

Baeuerle, P.A., Henkel, T., 1994. Function and activation of NF-kappa B in the immune system. Annu. Rev. Immunol. 12, 141–179.

Baeuerle, P.A., Baltimore, D., 1996. NF-κB: ten years after. Cell 87, 13–20.

Barrett, A.J., 1980. Fluorimetric assays for cathepsin B and cathepsin H with methylcoumarylamide substrates. Biochem. J. 187, 909–912.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bruck, R., Aeed, H., Avni, Y., Shirin, H., Matas, Z., Shahmurov, M., Avinoach, I., Zozulya, G., Weizman, N., Hochman, A., 2004. Melatonin inhibits nuclear factor kappa B activation and oxidative stress and protects against thioacetamide induced liver damage in rats. J. Hepatol. 40, 86–93.

Caballero, B., Vega-Naredo, I., Sierra, V., Huidobro-Fernandez, C., Soria-Valles, C., Gonzalo-Calvo, D.D., Tolivia, D., Gutierrez-Cuesta, J., Pallas, M., Camins, A., Rodriguez-Colunga, M.J., Coto-Montes, A., 2008. Favorable effects of a prolonged treatment with melatonin on the level of oxidative damage and neurodegeneration in senescence-accelerated mice. J. Pineal Res. 45, 302–311.

Caballero, B., Vega-Naredo, I., Sierra, V., Huidobro-Fernandez, C., Soria-Valles, C., Gonzalo-Calvo, D.D., Tolivia, D., Pallas, M., Camins, A., Rodriguez-Colunga, M.J., Coto-Montes, A., 2009. Melatonin alters cell death processes in response to age-related oxidative stress in the brain of senescence-accelerated mice. J. Pineal Res. 46, 106–114.

Cao, Y., Klionsky, D.J., 2007. Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. Cell Res. 17, 839–849.

Castillo, C., Ariznavarreta, M.C., Lahera, V., Cachofeiro, V., Gil-Loyzaga, P., Tresguerres, J.A., 2005. Effects of ovariectomy and growth hormone administration on body composition and vascular function and structure in old female rats. Biogerontology 6, 49–60.

Coto-Montes, A., Hardeland, R., 1999. Antioxidative effects of melatonin in Drosophila melanogaster: antagonization of damage induced by the inhibition of catalase. J. Pineal Res. 27, 154–158.
Cuervo, A.M., 2006. Autophagy in neurons: it is not all about food. Trends Mol. Med. 12, 461–464.
Cuervo, A.M., Dice, J.F., 2000. Age-related decline in chaperone-mediated autophagy. J. Biol. Chem. 275, 31505–31513.
Chetsawang, B., Putthaprasart, C., Phansuwan-Pujito, P., Govitrapong, P., 2006. Melatonin protects against hydrogen peroxide-induced cell death signaling in SH-SY5Y cultured cells: involvement of nuclear factor kappa B, Bax and Bcl-2. J. Pineal Res. 41, 116–123.
Daynes, R.A., Enioutina, E.Y., Jones, D.C., 2003. Role of redox imbalance in the molecular mechanisms responsible for immunosenescence. Antioxid. Redox Signal. 5, 537–548.
De Martinis, M., Franceschi, C., Monti, D., Ginaldi, L., 2007. Apoptosis remodeling in immunosenescence: implications for strategies to delay ageing. Curr. Med. Chem. 14, 1389–1397.
Debnath, J., Baehrecke, E.H., Kroemer, G., 2005. Does autophagy contribute to cell death? Autophagy 1, 66–74.
Deretic, V., 2006. Autophagy as an immune defense mechanism. Curr. Opin. Immunol. 18, 375–382.
Erlich, S., Shohami, E., Pinkas-Kramarski, R., 2006. Neurodegeneration induces upregulation of Beclin 1. Autophagy 2, 49–51.
Eze, M.O., 1992. Membrane fluidity, reactive oxygen species, and cell-mediated immunity: implications in nutrition and disease. Med. Hypotheses 37, 220–224.
Guerrero, J.M., Reiter, R.J., 2002. Melatonin–immune system relationships. Curr. Top Med. Chem. 2, 167–179.
Gutierrez-Cuesta, J., Sureda, F.X., Romeu, M., Canudas, A.M., Caballero, B., Coto-Montes, A., Camins, A., Pallas, M., 2007. Chronic administration of melatonin reduces cerebral injury biomarkers in SAMP8. J. Pineal Res. 42, 394–402.
Hodes, R.J., 1997. Aging and the immune system. Immunol. Rev. 160, 5–8.
Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., Yoshimori, T., 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 19, 5720–5728.
Kane, L.P., Lin, J., Weiss, A., 2002. It's all Rel-ative: NF-kappaB and CD28 costimulation of T-cell activation. Trends Immunol. 23, 413–420.
Kapasi, A.A., Singhal, P.C., 1999. Aging splenocyte and thymocyte apoptosis is associated with enhanced expression of p53, bax, and caspase-3. Mol. Cell Biol. Res. Commun. 1, 78–81.
Kroemer, G., Jaattela, M., 2005. Lysosomes and autophagy in cell death control. Nat. Rev. Cancer 5, 886–897.
Kum-Tatt, L., Tan, I.K., Seet, A.M., 1975. A new colorimetric method for the determination of NADH/NADPH dependent glutathione reductase in erythrocytes and in plasma. Clin. Chim. Acta 58, 101–108.
Kurz, T., Terman, A., Gustafsson, B., Brunk, U.T., 2008. Lysosomes and oxidative stress in aging and apoptosis. Biochim. Biophys. Acta 1780, 1291–1303.
Lardone, P.J., Alvarez-Garcia, O., Carrillo-Vico, A., Vega-Naredo, I., Caballero, B., Guerrero, J.M., Coto-Montes, A., 2006. Inverse correlation between endogenous melatonin levels and oxidative damage in some tissues of SAM P8 mice. J. Pineal Res. 40, 153–157.
Levine, B., Deretic, V., 2007. Unveiling the roles of autophagy in innate and adaptive immunity. Nat. Rev. Immunol. 7, 767–777.
Levine, B., Klionsky, D.J., 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev. Cell 6, 463–477.
Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464–478.
Li, C., Capan, E., Zhao, Y., Zhao, J., Stolz, D., Watkins, S.C., Jin, S., Lu, B., 2006. Autophagy is induced in CD4+ T cells and important for the growth factor-withdrawal cell death. J. Immunol. 177, 5163–5168.
Li, Q., Verma, I.M., 2002. NF-kappaB regulation in the immune system. Nat. Rev. Immunol. 2, 725–734.
Liang, R., Nickkholgh, A., Hoffmann, K., Kern, M., Schneider, H., Sobirey, M., Zorn, M., Buchler, M.W., Schemmer, P., 2008. Melatonin protects from hepatic reperfusion injury through inhibition of IKK and JNK pathways and modification of cell proliferation. J. Pineal Res. 46, 8–14.
Lubinsky, S., Bewley, G.C., 1979. Genetics of catalase in Drosophila melanogaster: rates of synthesis and degradation of the enzyme in flies aneuploid and euploid for the structural gene. Genetics 91, 723–742.
Matsugo, S., Kitagawa, T., Minami, S., Esashi, Y., Oomura, Y., Tokumaru, S., Kojo, S., Matsushima, K., Sasaki, K., 2000. Age-dependent changes in lipid peroxide levels in peripheral organs, but not in brain, in senescence-accelerated mice. Neurosci. Lett. 278, 105–108.
McGlauchlen, K.S., Vogel, L.A., 2003. Ineffective humoral immunity in the elderly. Microbes Infect. 5, 1279–1284.
Michiels, C., Minet, E., Mottet, D., Raes, M., 2002. Regulation of gene expression by oxygen: NF-kappaB and HIF-1, two extremes. Free Radic. Biol. Med. 33, 1231–1242.
Moore, M.N., 2008. Autophagy as a second level protective process in conferring resistance to environmentally-induced oxidative stress. Autophagy 4, 254–256.
Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., Levine, B., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122, 927–939.
Plackett, T.P., Boehmer, E.D., Faunce, D.E., Kovacs, E.J., 2004. Aging and innate immune cells. J. Leukoc. Biol. 76, 291–299.
Pua, H.H., Dzhagalov, I., Chuck, M., Mizushima, N., He, Y.W., 2007. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. J. Exp. Med. 204, 25–31.
Qing, G., Yan, P., Xiao, G., 2006. Hsp90 inhibition results in autophagy-mediated proteasome-independent degradation of IkappaB kinase (IKK). Cell Res. 16, 895–901.
Rikans, L.E., Hornbrook, K.R., 1997. Lipid peroxidation, antioxidant protection and aging. Biochim. Biophys. Acta 1362, 116–127.
Rodríguez, M.I., Escames, G., López, L.C., López, A., García, J.A., Ortiz, F., Acuña-Castroviejo, D., 2007. Chronic melatonin treatment reduces the age-dependent inflammatory process in senescence-accelerated mice. J. Pineal Res. 42, 272–279.
Sanchez-Hidalgo, M., Guerrero Montavez, J.M., Carrascosa-Salmoral, M.D., Naranjo Gutierrez, M.D., Lardone, P.J., de la Lastra Romero, C.A., 2008. Decreased MT1 and MT2 melatonin receptor expression in extrapineal tissues of the rat during physiological aging. J. Pineal Res. 46, 29–35.
Schreurs, F.J., van der Heide, D., Leenstra, F.R., de Wit, W., 1995. Endogenous proteolytic enzymes in chicken muscles. Differences among strains with different growth rates and protein efficiencies. Poult. Sci. 74, 523–537.
Stadtman, E.R., 1992. Protein oxidation and aging. Science 257, 1220–1224.
Stadtman, E.R., Levine, R.L., 2000. Protein oxidation. Ann. N.Y. Acad. Sci. 899, 191–208.
Takahashi, T., Tang, J., 1981. Cathepsin D from porcine and bovine spleen. Methods Enzymol. 80 (Pt C), 565–581.
Takeda, T., Hosokawa, M., Takeshita, S., Irino, M., Higuchi, K., Matsushita, T., Tomita, Y., Yasuhira, K., Hamamoto, H., Shimizu, K., Ishii, M., Yamamuro, T., 1981. A new murine model of accelerated senescence. Mech. Ageing Dev. 17, 183–194.
Takeda, T., Matsushita, T., Kurozumi, M., Takemura, K., Higuchi, K., Hosokawa, M., 1997. Pathobiology of the senescence-accelerated mouse (SAM). Exp. Gerontol. 32, 117–127.
Tan, D.X., Manchester, L.C., Terron, M.P., Flores, L.J., Reiter, R.J., 2007. One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? J. Pineal Res. 42, 28–42.
Tanida, I., Minematsu-Ikeguchi, N., Ueno, T., Kominami, E., 2005. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy 1, 84–91.
Tian, L., Cai, Q., Bowen, R., Wei, H., 1995. Effects of caloric restriction on age-related oxidative modifications of macromolecules and lymphocyte proliferation in rats. Free Radic. Biol. Med. 19, 859–865.
Tomas-Zapico, C., Alvarez-Garcia, O., Sierra, V., Vega-Naredo, I., Caballero, B., Joaquin Garcia, J., Acuña-Castroviejo, D., Rodriguez, M.I., Tolivia, D., Rodriguez-Colunga, M.J., Coto-Montes, A., 2006. Oxidative damage in the livers of senescence-accelerated mice: a gender-related response. Can. J. Physiol. Pharmacol. 84, 213–220.
Tomas-Zapico, C., Coto-Montes, A., 2005. A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. J. Pineal Res. 39, 99–104.
Vasto, S., Candore, G., Balistreri, C.R., Caruso, M., Colonna-Romano, G., Grimaldi, M.P., Listi, F., Nuzzo, D., Lio, D., Caruso, C., 2007. Inflammatory networks in ageing, age-related diseases and longevity. Mech. Ageing Dev. 128, 83–91.
Viatour, P., Merville, M.P., Bours, V., Chariot, A., 2005. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem. Sci. 30, 43–52.
Xiao, G., 2007. Autophagy and NF-kappaB: fight for fate. Cytokine Growth Factor Rev. 18, 233–243.
Zavasnik-Bergant, T., Turk, B., 2006. Cysteine cathepsins in the immune response. Tissue Antigens 67, 349–355.
Zavasnik-Bergant, T., Turk, B., 2007. Cysteine proteases: destruction ability versus immunomodulation capacity in immune cells. Biol. Chem. 388, 1141–1149.