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Aging and vascular dysfunction: beneficial melatonin effects

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Abstract Aging is characterized by a progressive deterioration of physiological functions and metabolic processes. In aging and in diseases associated with the elderly, the loss of cells in vital structures or organs may be related to several factors. Sirtuin1 (SIRT1) is a member of the sirtuin family of protein deacetylases involved in life span extension; however, its involvement in the aging is not yet completely defined. Recently, melatonin, a pleiotropic molecule, shown to activate SIRT1 in primary neurons of young animals, as well as in aged neurons of a murine model of senescence. Melatonin is known to modulate oxidative stress-induced senescence and pro-survival pathways. We treated 6- and 15-week-old apolipoprotein E (APOE)-deficient mice (APOE 6w and 15w) with two melatonin formulations (FAST and RETARD) to evaluate their anti-aging effect. Morphological changes in vessels (aortic arch) of APOE mice were evaluated SIRT1, p53, endothelial nitric oxide synthase (eNOS), and endothelin-1 (ET-1) markers. We demonstrate that SIRT1 and eNOS decresed in APOE mice between 6 and 15 weeks and that aging induced an elevated expression of p53 and ET-1 in APOE animals. Melatonin improved the impairment of endothelial damage and reduced loss of SIRT1 and eNOS decreasing p53 and ET-1 expression. The RETARD melatonin preparation caused a greater improvement of vessel cytoarchitecture. In summary, we indicate that SIRT1-p53-eNOS axis as one of the important marker of advanced vascular dysfunctions linked to aging. Finally, we suggest that extended-release melatonin (RETARD) provides a more appropriate option for contrasting these dysfunctions compared with rapid release melatonin (FAST) administration.

Keywords Melatonin · Atherosclerosis · Aging · Endothelial cells

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Introduction

It is evident that the increase of human life expectation has important socioeconomic and health consequences and is related to a reduction in the disease load associated with aging. The age-associated infirmities that accompany increased life expectancy involve cardiovascular diseases (CVDs) which include diabetes, stroke, heart attack, heart failure, hypertension, and neurodegenerative disease (Donmez and Guarente 2010; Pallas et al. 2011). Some theories of aging attempt to explain the complex mechanism of disease-associated with

aging process (Balaban et al. 2005). These include physiological changes such as inflammation and oxidative stress as important factors in the development and progression of age-related diseases (Stein et al. 2010).

During inflammation, in early stages of diseases, endothelial cells (ECs) became activated by circulating proinflammatory molecules including cytokines (such as tumor necrosis factor-α) or modified lipoproteins (e.g., oxized LDL). Once activated, inflamed ECs express chemokines, cytokines, and adhesion molecules, which attract and recruit inflammatory cells such as macrophages and T cells (Deanfield et al. 2007). Moreover, they release a variety of vasodilators, including nitric oxide (NO) and prostacyclin as well as vasoconstrictor agents such as endothelin-1 (ET-1) and angiotensin II (Wang et al. 2010). These endothelium-derived vasoactive factors do not only regulate regional blood flow but also influence proliferation and/or hypertrophy of vascular smooth muscle cells (VSMCs) (Köhler and Hoyer 2007; Ivey et al. 2008). Recently, these cells were hypothesized to be involved in vascular pathologies (Higashi et al. 2009).

An increasing body of evidence suggests that increased oxidative stress accounts for a significant proportion of endothelial dysfunction. An elevation of the production of oxygen-derived free radicals such as the superoxide anion has been linked to impair endothelial vasomotor function in experimental models of atherosclerosis (Heitzer et al. 2003). It has been proposed that activated ECs exhibit the morphological features of cellular senescence (Ota et al. 2008) and they show changes in several transcription factors. Moreover, endothelial senescence also is a major risk factor for atherosclerotic CVD (Ito et al. 2010).

Aging promotes endothelial senescence and it is, as reported above, associated with pathways inducing atherosclerosis in the human. However, the mechanisms underlying the aging-induced attenuation of endothelium-dependent functions are not yet clear. At this regard, during the past 10 years, one molecule that has captured much attention of medical practitioners and researchers alike in the burgeoning field of anti-aging medicine is silent information regulator 1 (SIRT1). SIRT1 and other members of the sirtuin family are class III nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases (Tang 2011) that have both histone and non-histone targets. The decetylation reaction catalyzed by sirtuins is coupled to cleavage of NAD into nicotinamide and 1-O-acetyl-ADP ribose (Zu et al. 2010).

SIRT1 and other members of the sirtuin family, considered longevity proteins, are widely distributed and have been shown to regulate a variety of physiopathological processes, including inflammation, cellular senescence/aging, cellular apoptosis/proliferation, differentiation, metabolism, stem cell pluripotency, and cell cycle regulation (Chung et al. 2010). SIRT1 is highly expressed in ECs and regulates their angiogenic function. It exerts protective effects against endothelial dysfunction by reducing stress-induced senescence and regulating cell defenses and survival (Ota et al. 2007; Yu et al. 2009). The overexpression of SIRT1 regulates important effectors like the tumor suppressor p53, endothelial nitric oxide synthase (eNOS), and transcription factor NFkB preventing cells from DNA damage-induced apoptosis, promoting vasodilatory and vascular regenerative functions in ECs and VSMCs, and regulating oxidative stress-induced endothelial senescence (Tajes et al. 2009; Rippe et al. 2010; Borradaile and Pickering 2010; Chen et al. 2010).

Melatonin, an endogenously produced indoleamine, is a remarkably functionally pleiotropic molecule (Reiter et al. 2010a) which acts as a highly effective antioxidant and free radical scavenger (Tan et al. 1993; Reiter et al. 2009) with beneficial actions against CVDs (Rodella et al. 2010; Reiter et al. 2010b; Dominguez-Rodriguez et al. 2010). Moreover, there are a number of data noting the effects of melatonin on aging processes (Poeggeler 2005; Bubenik and Konturek 2011). It is known that endogenous melatonin production diminishes in elderly persons (Reiter 1992) and that the total antioxidative capacity of serum correlates well with its melatonin levels in humans (Benot et al. 1999). Moreover, melatonin shows beneficial anti-aging effects in rats, prevents lipid peroxidation, and other destructive processes related to oxidative stress (Poeggeler 2005; Paredes et al. 2009; Paradies et al. 2010). Therefore, the age-related reduction in serum melatonin levels may play a role in the elevated oxidative damage observed in the elderly population (Reiter et al. 2002).

In light of these observations and considering that melatonin increases the level of SIRT1 in young primary neurons, as well as in aged neurons of murine

model of senescence (Tajes et al. 2009), this indoleamine may be beneficial in reducing the atherosclerotic process. Moreover, understanding the role and the mechanisms of melatonin in SIRT1 modulation and cellular function could help to identify promising therapeutic strategies for the treatment of aging-related diseases. To address this issue, we used apolipoprotein E (APOE)-deficient mice, a model that spontaneously develops hypercholesterolemia and atherosclerotic lesions in the aorta in a time-dependent manner (Rodella et al. 2007; Zhang et al. 2008). We wanted to test whether melatonin reduces atherosclerotic lesions, via an action on SIRT1, considered a longevity protein, the indolamine would aid in the aging process. Moreover, we used two different melatonin formulations (kindly provided by Nathura s.r.l, Reggio Emilia, Italy): Armonia Fast (FAST) and Armonia Retard (RETARD). FAST is an immediate release melatonin formulation, while RETARD was formulated to release a low dose of melatonin rapidly and a higher dose over a longer period of time. One obvious peculiarity of RETARD formulation is the prolonged release of melatonin in vivo. This latter feature could represent an important point of melatonin administration since the findings could provide a new delivery system of the indoleamine which could have greater efficacy in clinical situations.

Materials and methods

Animals

APOE-deficient mice are widely used as animal models of atherosclerosis as reported above. Seventy male mice (Charles River Laboratories S.r.l, Lecco, Italy and Harlan Laboratories S.r.l, Udine, Italy) were housed in an animal experimental unit with 12 h alternating light–dark cycle and constant temperature. Protocols were approved by the Italian Ministry of Health and complied with "Guiding Principles in the Use of Animals in Toxicology" which were adopted by the Society of Toxicology in 1989.

Study design

Mice were randomly divided into seven groups (ten animals each): group I, C57BL6 mice 15 weeks old at sacrifice; group II, C57BL6 mice treated with ARMONIA FAST from the 6th to 15th week of life; group III, C57BL6 mice treated with ARMONIA RETARD from the 6th to 15th week of life; group IV, APOE mice without treatment and 6 weeks old at sacrifice (APOE 6w); group V, APOE mice without treatment and 15 weeks old at sacrifice (APOE 15w); group VI, APOE mice treated from the 6th to 15th week of life with melatonin ARMONIA FAST (APOE FAST); and group VII, APOE mice treated from 6th to 15th week of life with melatonin ARMONIA RETARD (APOE RETARD). The melatonin (kindly provided by Nathura s.r.l, Reggio Emilia, Italy) was administrated at a dose of 10 mg/kg/day. In particular, melatonin was administered orally in two different ways to mimic the different Nathura formulations: ARMONIA FAST (FAST) and ARMONIA RETARD (RETARD). FAST is an immediate release formulation and the treatment was carried out by direct oral administration via gastric gavage, every day before the light in the animal room went off. By comparison, RETARD was formulated to release a low dose of melatonin rapidly and a higher dose over a longer period of time (these dose are in a ratio of 1 to 3). In this case, the administration was available in the drinking fluid every night (from 18.00 to 06.00 hours). The melatonin-containing water bottles were removed at 06.00 hours and were replaced with bottles of water until noon. From noon to 18.00 hours, no drinking water was given to stimulate mice to drink more during the dark period. This device was adopted only in RETARD melatonin-treated groups for miming the slow release of ARMONIA RETARD formulation. APOE mice from 6 to 15 weeks were studied since we previously documented early vascular changes and advanced atherosclerotic lesions, respectively, during this interval as described by Jawien et al. (2004) and by our group in a previous work (Rodella et al. 2007).

At the end of the study, all the animals were killed by dislocation and vessels (aortic arch) were carefully removed. This section was used since it provides an excellent measure of plaque development since it is the area of greatest plaque formation (Coleman et al. 2006; Nakashima et al. 1994). Each sample was fixed in 4% paraformaldehyde and a part was embedded in paraffin wax according to standard procedures and serial sections were cut using a microtome (5 μm thickness). Other parts of the samples were store at −20°C and cut using a cryostat (8 μm thickness).

Histopathological examination

Hematoxylin–eosin staining

Embedded paraffin sections were deparafinized, rehydrated, and finally stained with hematoxylin–eosin staining. A minimum of 20 fields for each slide was examined and evaluated for severity of changes by two observers blinded to the treatments.

Oil red staining

The sections cut using a cryostat were fixed in 10% formalin and were stained with Oil Red O solution for 30 min at room temperature, after rising with 50% isopropyl alcohol and then with hematoxylin for 30 s, modified from Yatera et al. (2010). Digital images were taken using a light microscope and then analyzed with a software program (Image Pro Plus, Milan, Italy). Lipid deposition was evaluated by the ratio of the percentage of Oil Red O-stained areas to the total aortic area randomly selected in a total of five slides per animal, according to Song et al. (2011), Kato et al. (2009), and Inanaga et al. (2010).

Blood withdrawal

The blood withdrawal from heart was made during the dark period. For the FAST-treated mice, blood withdrawal was made at 1900, 2000, and 0000 hours; while for the mice treated with RETARD, it was made at 1900, 2200, 0000, 0100, and 0200 hours. Samples were centrifuged at 11.000 rpm for 10 min at 4°C and plasma was processed for radioimmunoassay (RIA).

Radioimmunoassay procedure

Melatonin plasmatic concentration was measured by RIA, according to Welp et al. (2010). In particular, we used a specific rabbit antiserum antimelatonin and [^{125}I]-labeled melatonin (80,000 cpm).

Immunofluorescence analyses of SIRT1/p53, eNOS/ET-1

Alternate sections were deparaffinized, rehydrated, and incubated in 1% BSA for 2 h at room temperature. To obtain a double staining, the sections were incubated for 1 h at room temperature and after overnight at 4°C, with rabbit polyclonal antibody against SIRT1 (1:50; Santa Cruz Biotechnology, CA), with mouse monoclonal antibody against p53 (1:600; Genetex, Irvine, USA), or with rabbit polyclonal antibody against eNOS (1:70; AnaSpec, San Josè, CA) or with goat polyclonal antibody against ET-1 (1:250; Santa Cruz Biotechnology, CA). Thereafter, the sections were labeled using anti-rabbit Alexa Fluor 488, anti-mouse Alexa Fluor 488, anti-goat Alexa Fluor 488, or anti-rabbit Alexa Fluor 546-conjugated secondary antibodies (1:200, Invitrogen, UK). Finally, the samples were counter-stained with DAPI, mounted, and observed with a confocal microscope (510 Meta Zeiss, Germany) at a final magnification of ×400. The microscope is placed on a vibration isolation table in an air-conditioned room kept at constant temperature. We set the pinhole at one airy unit, for best compromise between depth discrimination and efficacy. Moreover, we used the following as laser unit: argon (wavelength 488, maximum power at 30.0 mW), HeNe 543 (wavelength 543, maximum power at 1.2 mW), and Diode 405-30 (wavelength 405, maximum power at 30.0 mW). The immunofluorescent control was performed by omitting the primary antibody and in presence of isotype-matched IgGs.

The samples mentioned above were also evaluated by two observers blinded to the treatments for semiquantitative and quantitative analysis using an optical fluorescent microscope. For semiquantitative analysis, the results were expressed as no staining (−), weak (+), moderate (++), and strong (+++) positivity. Additionally, for quantitative evaluation, staining intensity was computed as immunopositivity and measured in six samples for each experimental group. Digitally fixed images of the slices (×400 magnification) were analyzed using an optical fluorescent microscope (Olympus, Germany) equipped with an image analyzer (Image Pro Plus, Milan, Italy). The immunopositivity was calculated for arbitrary areas, measuring 20 random fields with the same area for each sample. The data were pooled to represent a mean value and a statistical analysis was applied to compare the results obtained from the different experimental groups. The levels of immunopositivity are expressed as arbitrary unit (AU).

Statistical analyses

Results are expressed as mean ± S.E.M. Statistical significance of differences among the experimental

groups was evaluated by analysis of variance corrected by Bonferroni test with significance set at $P<0.05$.

Results

Biochemical analysis of plasma melatonin showed different kinetics between FAST and RETARD melatonin formulations. Immediately, after FAST melatonin administration, its concentration peak was evident, declining rapidly in 1 h (Fig. 1a). Instead, after RETARD melatonin administration, plasma melatonin levels remained elevated 4–5 h and only subsequently gradually decreased (Fig. 1b).

Histopathological studies revealed that vessels of APOE mice showed vascular damage with endothelial cell detachment in tunica intima and remarkable disorganization of VSCMs in tunica media (Fig. 2a, b). These alterations were more evident in APOE 15w relative to those in APOE 6w. The atherosclerotic lesions developed gradually over time with the atherosclerotic plaques being evident in APOE 15w mice. On the contrary, in control mice not treated and treated with FAST or RETARD melatonin and in APOE-treated mice with both formulations of melatonin, the vessels showed a normal morphology (Fig. 2c, e). It is important to note that RETARD melatonin treatment, compared to FAST melatonin treatment, was more efficient in terms of reducing vascular damages, restoring tunica intima and media cytoarchitecture as one can see in Fig. 2d, e.

Oil Red O staining showed that in APOE 15w mice there was a markedly aortic lipid deposition that are characteristic of atheromatous plaques, while in the other groups, the staining was negative (Fig. 2f, g). Moreover, the lipid lesion size at the aortic arch was very small in control group, treated and not with melatonin, in APOE 6w mice, and in APOE mice treated with both melatonin formulations. On the contrary, in APOE 15w, the intimal thickening and accumulation of lipid became significant (Fig. 2h).

Immunofluorescence staining revealed that the vessels of APOE 6w and 15w mice were negative for SIRT1 protein (Fig. 3a, b). On the contrary, SIRT1 expression was found in control treated and non-melatonin-treated mice as well as in APOE FAST or RETARD melatonin-treated animals (Fig. 3c–e). In particular, a moderate reaction for SIRT1 was localized in the cytoplasm of ECs and in the cytoplasm and nuclei of tunica media cells of control treated and non-melatonin-treated mice (Fig. 3c). Moreover, SIRT1 was expressed in cytoplasm and nuclei of tunica intima cells of APOE FAST- and RETARD-treated mice. This positive reaction was moderate for APOE mice treated with FAST melatonin and strong for APOE RETARD melatonin-treated mice. VSMCs revealed no positivity in APOE 6w and 15w in APOE FAST-treated mice. In control-treated and non-melatonin-treated mice and in APOE RETARD-treated mice, these cells exhibited moderate reactivity. Tunica

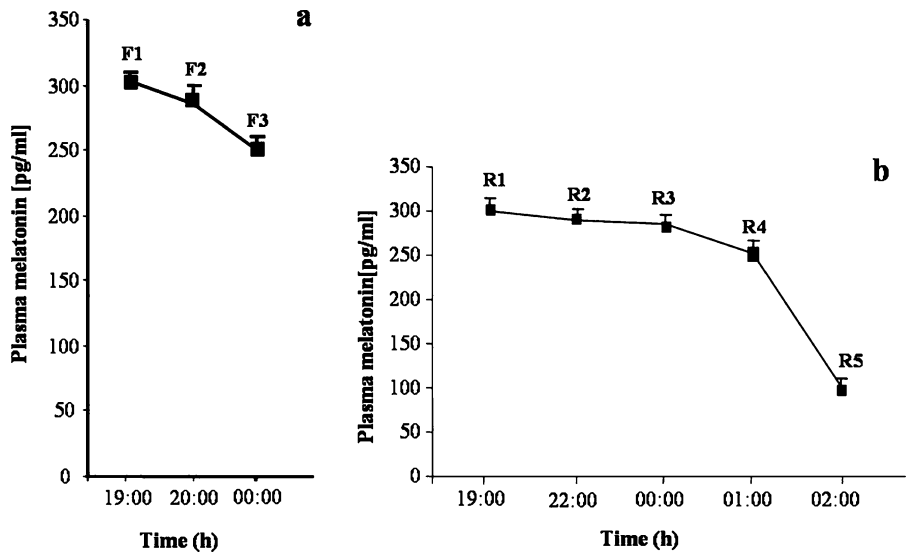

Fig. 1 a–b Radioimmunoassay analysis of melatonin in plasma samples of APOE mice treated with melatonin FAST (*F1*, *F2*, and *F3*) (**a**) or with melatonin RETARD (*R1*, *R2*, *R3*, *R4*, and *R5*) (**b**). The graphs represent the plasma content expressed in picograms per milliliter

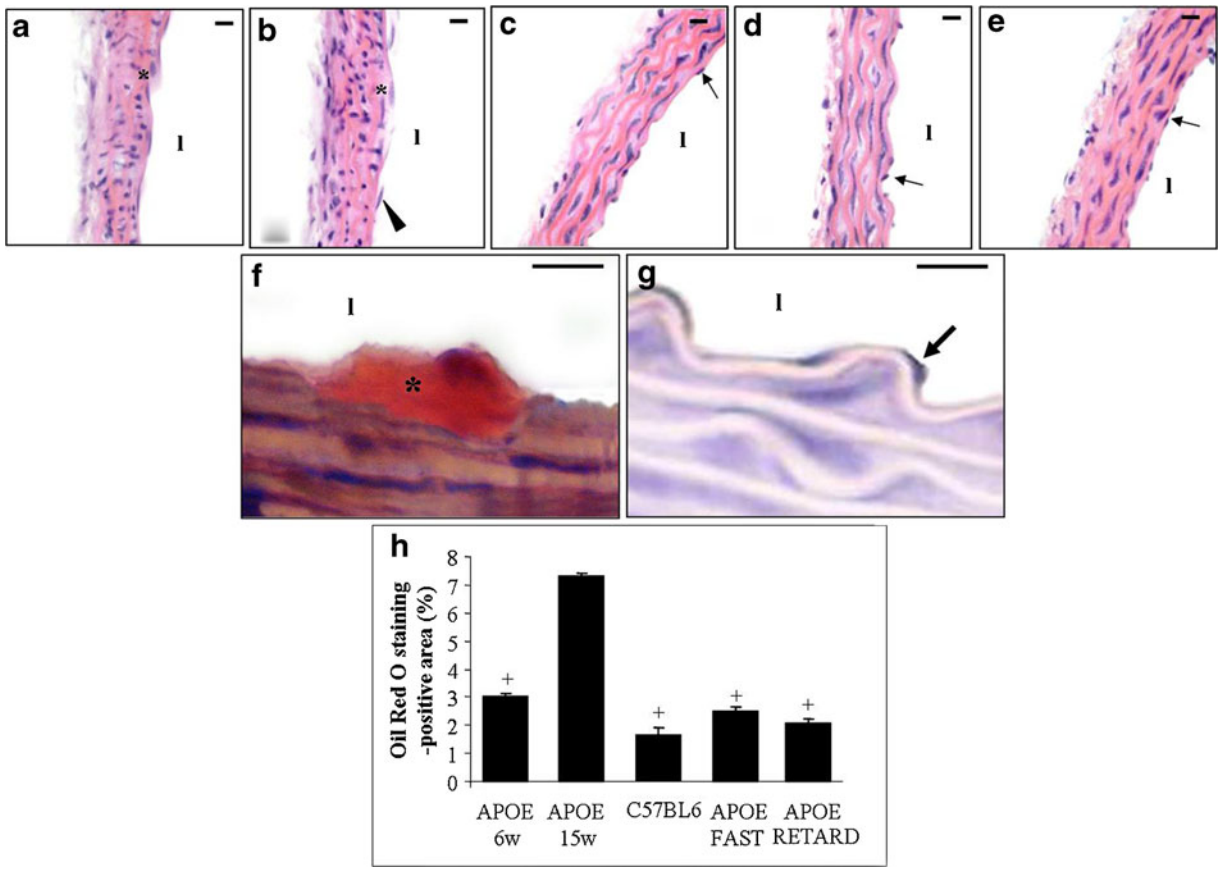

Fig. 2 **a**–**e** Photomicrographs of aorta morphology in APOE mice at 6 weeks (**a**), APOE mice at 15 weeks (**b**), C57BL6 (**c**), APOE mice treated with melatonin FAST (**d**), and APOE mice treated with melatonin RETARD (**e**). Hematoxylin–eosin staining. **f**–**g** Photomicrographs representative of Oil Red O-stained aortic section in APOE mice at 15 weeks (**f**) and in APOE mice treated with melatonin RETARD (**g**). **h** The *graph* shows the percentage of Oil Red O staining-positive area. *Asterisk* indicates the atherosclerotic plaque. *Arrow* indicates the endothelial cells and the *arrowhead* shows the endothelial detachment. $^{+}P<0.05$ vs. APOE 15w. *Bar* 20 μm

adventitia cells showed no positive reaction in any mice. The semiquantitative data of SIRT1 immunofluorescence are summarized in Table 1 and the differences are shown in Fig. 3f.

p53 immunofluorescence, in the vessels of both APOE groups (6w and 15w) showed a high positivity for this protein compared to vessels of control mice and those treated with either melatonin formulation (Fig. 4a–c). In particular, p53 expression was weak and moderate respectively in the cytoplasm of the tunica media of APOE 6w and 15w and moderate in the adventitial cells of the same animals (Fig. 4a, b). Some endothelial cells of the tunica intima of both groups exhibited a weak to moderate positive reaction for p53. On the contrary, intima, media, and adventitial cells were negative in the vessels of control mice and in those given either APOE FAST or RETARD melatonin (Fig. 4c–e).

For eNOS expression, the vessels of APOE 6w and 15w showed a low positivity for this protein in ECs (Fig. 4f, g). On the other hand, eNOS staining had strong positivity in nuclei and cytoplasm of tunica intima cells of control mice and those treated with either melatonin formulation (Fig. 4h). Moreover, melatonin treatments restored significantly the expression of this protein after either in APOE FAST or RETARD melatonin treatment. This positivity was moderate in ECs of APOE FAST-treated animals and strong in APOE RETARD-treated mice (Fig. 4i–l). The tunica media and tunica adventitia were always negative.

ET-1 expression was moderate in APOE 6w and strong in APOE 15w in tunica media and tunica

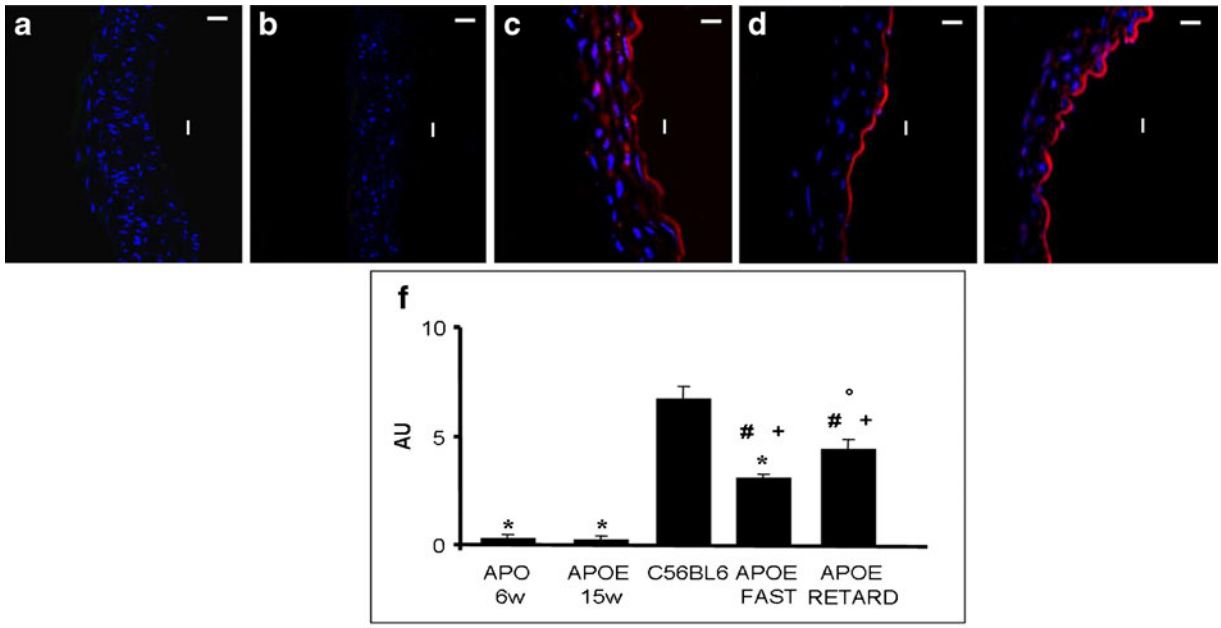

Fig. 3 **a**–**e** Immunofluorescence images of SIRT1 (*red staining*) expression of aortas in APOE mice at 6 weeks (**a**), APOE mice at 15 weeks (**b**), C57BL6 mice (**c**), APOE mice treated with melatonin FAST (**d**), and APOE mice treated with melatonin RETARD (**e**). **f** The graph shows the histomorphometrical analyses, expressed in arbitrary units (AU), of SIRT1 immunopositivity in mice aorta samples. $*P<0.05$ vs. C57BL6; $^{\#}P<0.05$ vs. APOE 6w, $^{+}P<0.05$ vs. APOE 15w and $^{\circ}P<0.05$ vs. FAST. Nuclei were stained with DAPI (*blue*). *l* identifies the lumen of the aorta (*Bar* 20 μm)

adventitia cells (Fig. 4m, n) compared to control vessels and those of mice treated with FAST or RETARD melatonin where it was always negative (Fig. 4o). The positivity was uniformly present in the cytoplasm of VSMCs and in the cytoplasm of tunica adventitia cells of APOE 6w and 15w (Fig. 4m, n). Tunica intima cells exhibited a weak or moderate positive reaction (Fig. 4m, n). The same results observed in all control groups were also noted in the vessels of APOE mice treated with FAST or RETARD melatonin (Fig. 4p, q). With regard to the tunica intima, media, and adventitial cells, they were negative in all groups except in APOE 6w and 15w.

The semiquantitative data of the immunofluorescence procedures are summarized in Tables 1 and 2 and the statistical analyses of the data are shown in Fig. 5. It is important to note that a very high and statistically significant immunostaining of all studied proteins in APOE 6w and 15w was observed with respect to control vessels and in the vessels of treated animals with different melatonin formulations. We noted that there were no differences due to the administration of both melatonin formulations since all the parameters reported above have been studied in C57BL6 mice treated and not with FAST or RETARD melatonin and they showed similar results among these groups.

Discussion

APOE mice showed many morphological vascular alterations that are similar to those observed in human CVDs linked to aging as reported by Pereira et al.

Table 1 Semiquantitative analyses of SIRT1 immunofluorescence data

SIRT1			
Study design	Tunica intima	Tunica media	Tunica adventitia
APOE 6w	–	–	–
APOE 15w	–	–	–
C57BL6 treated and not treated mice	++	++	–
APOE FAST-treated mice	++	–	–
APOE RETARD-treated mice	+++	++	–

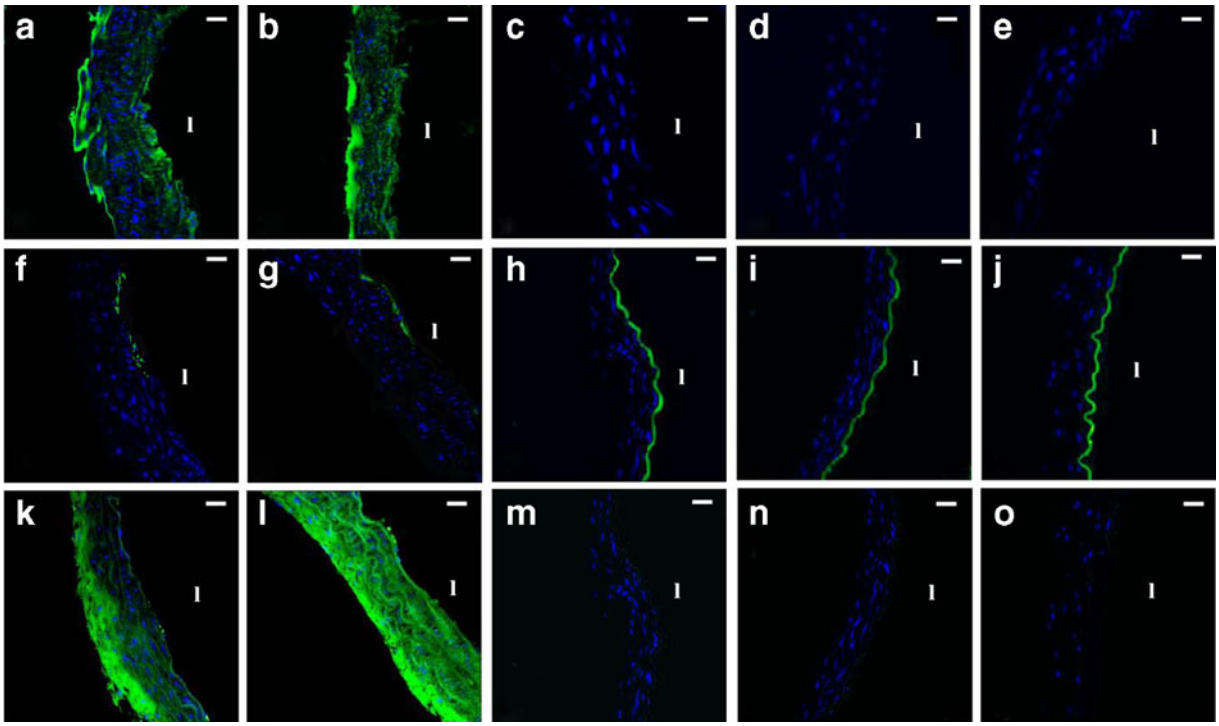

Fig. 4 **a**–**q** Immunofluorescence images of p53 (**a**–**e**), eNOS (**f**–**l**), and ET-1 (**m**–**q**) expression of aortas in APOE mice at 6 weeks (**a**), APOE mice at 15 weeks (**b**), C57BL6 mice (**c**), APOE mice treated with melatonin FAST (**d**), and APOE mice treated with melatonin RETARD (**e**), Nuclei were stained with DAPI (*blue*). *l* identifies the lumen of the aorta. *Bar* 20 μm

(2010). Thus, this was the starting point to verify the morphological alterations in vessels of these animals an attempt to clarify the cardiovascular processes underlying the biological mechanisms related to aging.

In developed countries, the aged population will increase fivefold in the next several decades (Pallàs et al. 2008) and it is therefore imperative that the knowledge be increased to aid in early diagnosis and effective treatments of age-related diseases to either slow or modify the progression of disorders related to the present-day increase in life expectancy. Such information could be useful to improve treatment of life-threatening illnesses as or CVDs. Herein, we studied the protective role of melatonin in vascular dysfunction linked to aging as summarized in earlier

Table 2 Semiquantitative analyses of p53, eNOS and ET-1 immunofluorescence data

Study design	p53			eNOS			ET-1		
	Tunica intima	Tunica media	Tunica adventitia	Tunica intima	Tunica media	Tunica adventitia	Tunica intima	Tunica media	Tunica adventitia
APOE 6w	+/++	+	+	−/+	–	–	+/++	++	++
APOE 15w	+/++	++	++	+	–	–	+/++	+++	+++
C57BL6 treated and not treated mice	–	–	–	+++	–	–	–	–	–
APOE FAST-treated mice	–	–	–	++	–	–	–	–	–
APOE RETARD-treated mice	–	–	–	+++	–	–	–	–	–

The intensity of the immunofluorescence staining was evaluated as no staining (−), weak (+), moderate (++), and strong to very strong positivity (+++)

APOE apolipoprotein E, *eNOS* endothelial nitric oxide synthase, *ET-1* endothelin-1

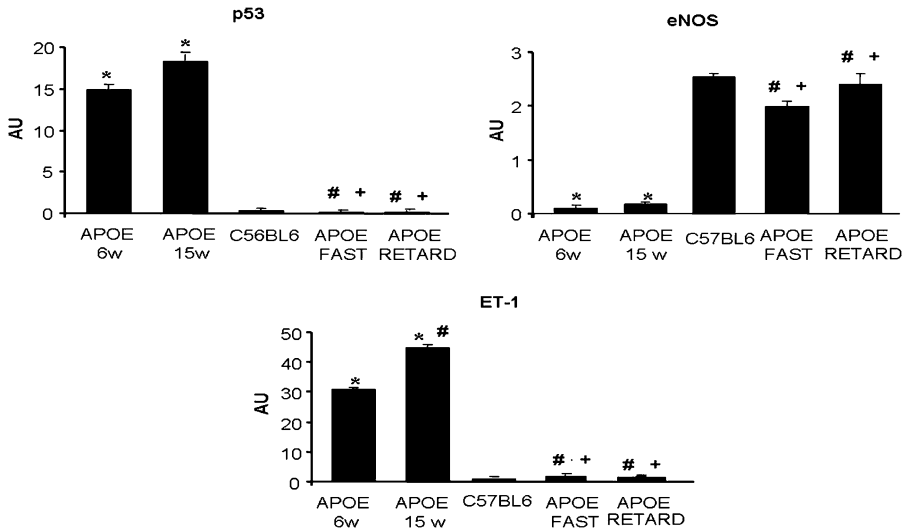

Fig. 5 These graphs show the histomorphometrical analyses, expressed in arbitrary units (AU), of p53, eNOS, and ET-1 immunopositivity in mice aorta samples. *$P<0.05$ vs. C57BL6; $^{\#}P<0.05$ vs. APOE 6w, $^{+}P<0.05$ vs. APOE 15w and $P<0.05$ vs. FAST

investigations (Tajes et al. 2009; Reiter et al. 2010a). With this in mind, we evaluated the biological processes and markers linked to SIRT1, a protein that constitutes a possible association between cardiovascular disorders and aging (Pallàs et al. 2008; Zu et al. 2010).

The present data demonstrate that SIRT1 is implicated in the development of atherosclerosis indicating its role in aging. SIRT1 is not present in the vessels of APOE 6w and 15w mice. This was previously also shown by others in which the levels of SIRT1 are decreased in vitro in lung epithelial cells, ECs, and macrophages in response to cigarette smoke extract as well as in lung of patients with chronic obstructive pulmonary disease (Arunachalam et al. 2010; Chung et al. 2010). It is interesting that not only ECs but VSMCs also exhibited SIRT1 immunoreactivity which may be required for maintaining survival and homeostasis. Our data provide evidence that the presence of SIRT1 in media of control animals seems also to be important for coordinating the activity of key signaling networks that govern postnatal vascular growth and homeostasis to achieve a balanced vascular response as pointed out by to Guarani and Potente (2010). So, the current findings, together the others reported above, provide a new insight into the atheroprotective effects of SIRT1 and imply that SIRT1 may be a potential target for the intervention of VSMC hypertrophy-associated vascular disease. This is interesting since, while SIRT1 has been reported to have a function as an anti-atherosclerosis agent, its role in the inhibition of neointima formation remains unknown (Li et al. 2011).

Previous work demonstrated that SIRT1 increases cell resistance and survival from stress via a number of pathways (Danz et al. 2009), but the mechanisms are not well defined and its presence in nucleus and in the cytoplasm of the cells is unexplained. Regarding biological processes in which it is involved and considering its possible shuttle from nucleus to cytoplasm in several cellular types, we studied its relationship with a number of targets including p53, eNOS, and ET1. We observed an increase of p53 and ET1 expression and a reduction of eNOS when SIRT1 is not present in APOE mice at 6w and 15w.

Considering these findings, we propose that the inhibition of SIRT1 is associated with the induction of apoptosis that is characterized by enhanced expression of the proapoptotic protein p53. It is known that SIRT1-dependent deacetylation of p53 targets (Zhao et al. 2006) leads to either a reduction in the biological activities of this protein, thus influencing a plethora of cellular processes involving p53 including transcriptional silencing, genetic control of aging, cell metabolism, energy homeostasis, DNA repair, and cell survival (Riley et al. 2008; Yang et al. 2011). Moreover, SIRT1-mediated deacetylation antagonizes p53-dependent transcriptional activation and specifically inhibits p53-dependent apoptosis in response to DNA damage and oxidative stress (Vaziri et al. 2001).

In relation to p53 modulation by SIRT1, it is possible that the partial shuttle of SIRT1 from the nucleus probably regulates the acetylated state of p53. Thus, the presence of SIRT1 in the cytoplasm could abolish the nuclear translocation of p53 determining

an inhibition of p53 function as a transcriptional regulator as reported by Han et al. (2008) in mouse embryonic stem cells. Moreover, it is possible that SIRT1 in the cytoplasm also modulates other markers, such as eNOS and ET-1 expression as we observed.

Endothelial-specific overexpression of SIRT1 also attenuated aortic atherosclerotic plaque development in response to the high fat diet in APOE mice; these results suggest that the anti-atherosclerosis effect of SIRT1 is related also to eNOS expression, which is well recognized for increasing NO to promote and improve endothelial survival (Yu et al. 2009). NO production and eNOS expression are severely limited in senescent ECs (Ota et al. 2010). Although NO is known to be involved in reducing oxidative stress and the progression of atherosclerosis (Ota et al. 2008), it exerts protective effects against endothelial dysfunction mediating vasoconstriction of endothelium-dependent vasomotor tone by eNOS deacetylation and increasing NO bioavailability (Ota et al. 2007; Mattagajasingh et al. 2007).

In addition to reduced bioavailability of the vasodilator, NO, the imbalance of elevated vasoconstrictors such as ET-1 results in impaired vasorelaxation in early stages of several diseases (Kalani 2008). As the disease progresses, the prolonged loss of the protective effects of NO and activation of the ET-1 lead to structural alterations, thrombosis, and plaque development in the vessel wall (Ergul 2011). Moreover, additional evidence suggests that ET-1 is not only one of the most potent vasoconstrictors, but also stimulates proliferation of VSMCs, promoting fibrosis and inflammation (Schiffrin 2004). Given that the regulation of endothelial function may vary in different vascular beds, we suggest that ET-1 is a key protein in the development and progression of aging disorders. For example, excess ET-1 may facilitate local monocyte migration, and through a local paracrine action, ET-1 may initiate VSMC proliferation and migration. This is in agreement with our data showing an increase of ET-1 positivity in VSMCs of APOE 15w relative to 6-week-old mice.

Interestingly, we observed an increase of ET-1 in fibroblasts, the most prominent cell type in the adventitia, of APOE 6w and 15w and this may indicate and underline the dynamic properties of the adventitia in regulating vessel structure and function. In fact, recently, it has been reported that adventitial fibroblasts, like their neighboring ECs and VSMCs, produce ET-1 after angiotensin II treatment (An et al. 2006). Additionally, emerging evidence suggests that the adventitia may be a potent source of vasoactive hormones such as growth factors and ET-1, which may regulate vascular structure and function via autocrine or paracrine signaling mechanisms which, in turn, would contribute to either the contraction of the VSMCs (Di Wang et al. 2010).

Not less important is the finding that melatonin, which is known to be decreased during aging as well as exhibit a deteriorated rhythm (Reiter et al. 2002; Bubenik and Konturek 2011), increases protein longevity, SIRT1, and to improve cellular survival thereby reducing progression of diseases linked to aging. In particular, we show that RETARD melatonin formulation, which respects the normal circadian rhythm of endogenous melatonin, is especially useful in this regard. Thus, in these animals, SIRT1 expression was similar to that observed in control animals and was present not only in intima cells but also in VSMCs. This stresses the involvement of SIRT1 induction, by melatonin, in tunica media to possibly modulate neointima formation.

It has been reported that melatonin levels reach a maximum at a young age, remain relatively stable until 35–40 year of age, and thereafter diminish

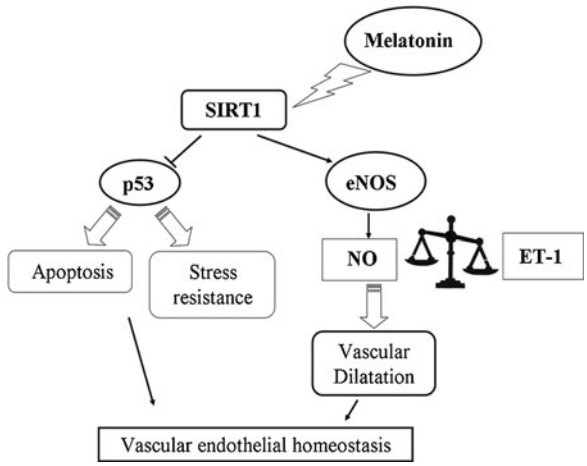

Fig. 6 A proposed mechanism for oxidative stress-induced senescence during aging and the effects of melatonin treatment. Melatonin increases SIRT1, which reduces inflammatory and apoptotic signaling related to p53 and media vasoconstriction due to endothelium-dependent vasomotor tone by eNOS deacetylation and increasing NO bioavailability; so melatonin leads to the downregulation of oxidative stress, inhibition of endothelial damage, and reduction of the apoptotic pathway

gradually reaching levels similar to daytime low concentrations (Jung-Hynes and Ahmad 2009). It has been suggested that the loss of melatonin in the elderly may lead to disorder in the circadian rhythm components, causing a desynchronization of the various genes resulting in a decrease in overall health and possibly an increase in cancer susceptibility and/or progression (Jung-Hynes et al. 2010).

Resveratrol, SRT1720, and melatonin have been reported to be direct activators of SIRT1 protein reducing senescence damage (Baur 2010). Several studies have reported the upregulation of SIRT1 by melatonin, e.g., in the brain of senescence-accelerated SAMP8 mice (Gutierrez-Cuesta et al. 2008) and in neuronal primary cultures from neonatal rat cerebellum (Tajes et al. 2009), or the prevention of SIRT1 decreases in the hippocampus of sleep-deprived rats (Chang et al. 2009). In neuronal cultures from cerebellum, melatonin also enhanced the deacetylation of various SIRT1 substrates, such as FoxO1, NFκB, and p53, effects which were largely reversed by the SIRT1 inhibitor sirtinol (Tajes et al. 2009). Although the experimental basis for a relationship between melatonin and SIRT1 is still rather limited, such an association, if better clarified, would have the potential to become a finding with numerous implications for circadian rhythmicity, aging, and related diseases.

In summary, the current findings indicate the SIRT1-p53-NO axis may be one of the fundamental determinants advancing endothelial dysfunction linked to aging, and the role of SIRT1 as a driver of cellular stress resistance and longevity is noteworthy in the context of its expression profile. However, further studies are required to clarify these relationships. The proposed mechanism by which SIRT1-p53-NO axis and by which melatonin promotes cell survival against senescence is depicted in Fig. 6.

Moreover, the present data indicate that RETARD melatonin in particular, which induces a normal circadian rhythm, could be a useful delivery system of the indoleamine since it seems to have greater efficacy as a protective agent than FAST rapid release melatonin. At this purpose, two other points that can be addressed are as follows: (1) Could FAST melatonin be administered in drinking water? and (2) Could FAST melatonin at higher doses have similar effects of RETARD melatonin? For the first point, to our knowledge, it is not possible, since the dose is strictly linked to water solution. Moreover, it gives a peak rapidly after its administration and so it is necessary to be directly administered. For the second point, higher doses of FAST may provide similar effects as RETARD melatonin, but we hypothesize that this would require a longer time. Obviously, for addressing these hypotheses, more studies are needed.

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