Aging-related endothelial dysfunction in the aorta from female senescence-accelerated mice is associated with decreased nitric oxide synthase expression

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A B S T R A C T
The present study investigated the time-course for aging-associated effects on contractile and relaxing vascular responses and nitric oxide (NO) production in the aorta from female senescence-accelerated resistant (SAMR1) and prone (SAMP8) mice. Both SAMR1 and SAMP8 were studied at three different ages: 3 (young), 6 (middle age) and 10 (old) months. Concentration–response curves to phenylephrine (10−8 to 10−5 M) or acetylcholine (10−8 to 10−5 M) were performed in the aortic rings in the absence or in the presence of NO synthase (NOS) inhibitor L-NAME (10−4 M). Protein and gene expression for endothelial NOS (eNOS) was determined by immunofluorescence, Western blot and real-time PCR. Although we have not seen any difference in vascular responses when comparing both strains at 3 months old, we found a significant aging-associated impairment of vascular reactivity that follows a distinct time-course in SAMR1 and SAMP8. In SAMR1, increases in phenylephrine contraction and decreases in acetylcholine relaxation were only seen at 10 months old, while SAMP8 displays altered responses at 6 months that are further impaired at 10 months old. L-NAME treatment enhanced phenylephrine contractions and completely inhibited acetylcholine relaxations in all age groups of SAMR1 and SAMP8. However, the magnitude of increase in phenylephrine contraction by L-NAME was markedly reduced by aging and followed a faster pace in SAMP8. Similar pattern of responses was observed in the time course for changes of eNOS expression, suggesting an earlier and more pronounced aging-associated decrease of NO production and eNOS expression in SAMP8. These results reveal that aging enhances contractile responses to phenylephrine and decreases endothelium-dependent relaxation to acetylcholine in the aorta from female mice by a mechanism that involves a decrease of NO production. This process occurs earlier in the aorta from SAMP8 mice, establishing these mice as suitable model to study cardiovascular aging in a convenient and standard time course.

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1. Introduction

Aging is a physiological process associated with structural and functional alterations in vasculature that increase cardiovascular morbidity and mortality even in the absence of known cardiovascular risk factors (Lakatta and Levy, 2003). Vascular aging is associated with endothelial dysfunction, arterial stiffening and remodeling, impaired angiogenesis, defective vascular repair, and with an increasing prevalence of atherosclerosis (Erusalimsky, 2009; Lakatta and Levy, 2003).

Data from clinical and basic research have established that vascular aging in women does not follow the same chronology as in men (Pereira et al., 2010; Shaw et al., 2006; Takenouchi et al., 2009). Among young and middle ages women are at lower risk of cardiovascular disease as compared to their male peers. If known risk factors that influence cardiovascular aging are excluded (e.g., smoking, cholesterol, hypertension), males still display a pattern of progressive vascular aging at a faster pace than do females (Pereira et al., 2010; Takenouchi et al., 2009). Despite the great amount of studies to describe the pathophysiology of vascular aging, most studies were conducted in the male vasculature. Little information is available on the vascular effects of aging in females.

It is well established that vascular tone is shifted toward vasoconstriction and endothelial dysfunction, yielding a hypertensive state with aging (Schutzer and Mader, 2012). Large and growing segments of the general population are age 65 or older, most of them are women, and this percentage will continue to rise. Primary care of this specific population is becoming a priority for clinicians and a major public health issue. Hypertension, orthostatic hypotension, arterial insufficiency, and atherosclerosis are common disorders in the elderly.

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that lead to significant morbidity and mortality. A fundamental understanding of why α-adrenergic receptor-mediated contraction is impaired with age will provide new insights and innovative strategies for the management of these disorders.

The senescence accelerated mouse prone strain (SAMP8) has been shown to be one of the most appropriate models to study aging and age-associated diseases (Takeda et al., 1997). It shows an age-related deterioration of learning and memory at an earlier age, compared with the senescence-accelerated resistant strain (SAMR1) (Butterfield and Poon, 2005; Miyamoto, 1997). Vascular studies using these models are not abundant, but few studies in male (Llorens et al., 2007) and female (Novella et al., 2010, 2013; Novensa et al., 2011) mice have shown morphological alterations and mechanical and endothelial dysfunction. To clarify the evolution of vascular dysfunction with chronological aging and accelerated senescence, we sought to determine the time-course dependent evolution of acetylcholine-induced endothelium-dependent relaxation and phenylephrine-induced contraction in mice at different ages. We further investigated a possible causal relationship between altered phenylephrine-induced contractions and the expression levels of endothelial nitric oxide synthase (eNOS), a purported essential component of vascular function in aging aorta.

2. Material and methods

2.1. Experimental animals

The SAM model is a murine model of aging widely used in experimental aging research (Takeda et al., 1997). Among the strains of SAM mice, senescence-accelerated mice-resistant 1 (SAMR1) serves as a control exhibiting a normal aging phenotype. Senescence-accelerated mouse-prone 8 (SAMP8), on the other hand, exhibits an accelerated aging phenotype with atherogenesis (Fenton et al., 2004) and endothelial dysfunction (Llorens et al., 2007; Novella et al., 2010).

Female SAMR1 (n = 48) and SAMP8 (n = 48) were obtained from the breeding stock at Research Unit at School of Medicine, University of Valencia and housed according to institutional guidelines (constant room temperature 22 °C, 12 h light/dark cycle. 60% humidity, standard mouse chow and water ad libitum). All protocols were approved by the Institutional Ethics Committee at the University of Valencia and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Both SAMR1 and SAMP8 mice at 3 (n = 16 of each strain), 6 (n = 16 of each strain), and 10 (n = 16 of each strain) months old were euthanized with anesthesia with isoflurane and the thoracic aorta was harvested from the aortic arch to the diaphragm.

2.2. Determination of nitrates and nitrites

In the anesthetized mice, blood samples (0.5 ml) were collected by cardiac puncture and plasma was separated by centrifugation at 1200 x g for 10 min. The levels of nitric oxide (NO) metabolites (NO2/NO3) were determined in the plasma by a commercial colorimetric assay kit (Cayman Chemical Company) following the supplier’s instructions.

2.3. Isolated mouse aorta preparation

For functional studies, the thoracic aorta (n = 10 mice per group) was excised, placed immediately in iced-cold Krebs–Henseleit solution and cleaned of surrounding tissue. Rings (4-mm long) were cut for iso- metric recording of tension. Outside diameter of the rings was measured using an ocular micrometer within a Wild M8 zoom microscope (Heerbrugg, Switzerland) and ranged from 0.8 to 1 mm. Typically, four rings per mouse were studied.

Two stainless-steel holders (100 µm in diameter) were introduced through the arterial lumen and placed in a 4 ml tissue bath containing modified Krebs–Henseleit (in mM: NaCl 115; KCl 4.6; KH2PO4 1.2; MgCl2 1.2; CaCl2 2.5; NaHCO3 25; glucose 11.1; EDTA 0.01, pH 7.3–7.4) kept at 37 °C and aerated with 95% O2/5% CO2 for isometric force measurements (Grass FT03, Grass Instruments Division Astro-Med, Inc., West Warwick, RI, U.S.A.). Changes in isometric force were recorded by the use of the Chart v. 3.4/s software and a MacLab/8e data acquisition system (ADInstruments, Castle Hill, Australia). Once the optimal resting tension was reached (10 mN), the aortic rings were allowed to attain a steady level of tension during a 1-hour equilibration period before testing. Functional integrity of the endothelium was confirmed routinely by the presence of relaxation induced by acetylcholine (10−7–10−6 M) during contraction obtained with phenylephrine (10−8 M). Following the equilibration period, arterial segments were exposed to receptor-independent depolarizing agent KCl (60 mM) until the contraction reached a stable plateau (10 to 20 min). After washout and return to stable baseline, contractile responses were determined by cumulative concentration–response curves to phenylephrine (10−8 to 10−5 M).

In some experiments, rings were incubated for 15 min with Nω-nitro-L-arginine methyl ester (L-NAME, 10−4 M) before starting concentration–response curves to phenylephrine.

To test the endothelium-dependent responses, the aortic rings were pre-contracted with phenylephrine (10−7–3 × 10−8 M). These concentrations of phenylephrine produced a stable contraction, corresponding to 75–90% of the response induced by 60 mM of KCl and cumulative relaxation curves to acetylcholine (10−5 to 10−5 M) were obtained in each ring. In another series of experiments, the preparations with endothelium were pre-incubated with L-NAME (10−5 M) 15 min prior to the concentration–response curves to acetylcholine. The effects of sodium nitroprusside (10−11 to 10−4 M), an endothelium-independent vasorelaxant and NO donor, were studied in vessels contracted with 3 × 10−6 M of phenylephrine. After a stable contraction was obtained, concentration–response curves were recorded. In all experiments the concentration of phenylephrine required to precontract the arteries was titrated to produce a similar level of precontraction.

2.4. Protein expression

In another set of animals (n = 6 mice in each experimental group) half of the thoracic aorta was gradually frozen in Tissue-Tek OCT compound (for protocols in the aortic section), and the other half was snap frozen into liquid nitrogen (for mRNA and protein expression analyses). Protein expression of eNOS was determined in the aortic sections from SAMR1 and SAMP8 mice by two semi-quantitative methods: immunofluorescence and Western blot as previously described (Novella et al., 2010). For immunofluorescence, the aortic sections (4 µm) were thaw-mounted onto polylysine covered slides, fixed in acetone (15 min) and blocked for 30 min with horse serum. Sections were incubated overnight at 4 °C with anti-eNOS (Abcam — ab5589). Following washes, sections were co-stained with 10 µM phalloidin (Sigma) and Alexa Fluor 488 conjugated goat anti-rabbit (Invitrogen). Coverslips were mounted on slides using the ProLong Gold antifade reagent with DAPI (Invitrogen). Coverslips were mounted on slides using the ProLong Gold antifade reagent with DAPI (Invitrogen), and sections were visualized through a confocal microscope (Axiovert 200, Carl Zeiss Inc.) with a 40× objective lens (Zeiss). For each image, light was passed through a different excitation filter: 1) 350 nm (for DAPI); 2) 490 nm (for Alexa 488); and 3) 590 nm (for phalloidin). Each aorta was recorded in 3 different regions and results were expressed as an average of fluorescence elicited using Mac Biophotonic ImageJ software.

For Western blot analysis, equal amount of protein from each sample (50 µg) was resolved by SDS-PAGE on 4–12% gels and electroblotted onto nitrocellulose membranes. After 1 h blocking with 5% milk in phosphate-buffered saline with 0.1% (v/v) Tween 20 (PBST), membranes were incubated for 1 h in PBST containing 5% milk and the specified primary antibody: monoclonal anti-eNOS, 1:1000 (BD Transduction Laboratories). After 4 washes with PBST, the membranes were incubated for 1 h with a horseradish peroxidase-labeled goat anti-mouse at a 1:2000 dilution in PBST containing 1% milk. After 4 additional washes, the membranes were incubated with a...
chemiluminescent reagent according to the manufacturer’s protocols (SuperSignal West Pico, Pierce Chemical Co.), and chemiluminescent signal was visualized by LAS-3000 Imaging System (Fujiﬁlm). Densitometric analyses of Western blots were performed using a Mac Biophotonic ImageJ software. All membranes were re-blotted using a monoclonal antibody anti-GAPDH (1:2500; Santa Cruz Biotechnology) as loading control. Data were normalized to corresponding values of GAPDH densitometry.

2.5. Gene expression by quantitative real-time PCR (qRT-PCR)

Total RNA was isolated and reverse transcribed as previously described (Novensa et al., 2010). mRNA encoding eNOS was quantiﬁed by qRT-PCR based on SYBR® Green ﬂuorescence, using the GAPDH mRNA as internal control. The speciﬁc primer sequences for mice were: eNOS (NM_0087134) 5′-TGT CAC TAT GCC AAC CAG CGT-3′, 5′-GGGCAA TGT GAG TCC GAA AA-3′ and GAPDH (NM_008084.2) 5′-ACC CCA GCA AGG ACA CTG AGC AAG-3′. Real-time PCR reactions were set following the manufacturer’s conditions (Applied Biosystems). Ct values obtained for each gene were normalized to Ct of housekeeping gene GAPDH (ΔCt) and converted to the linear form using the term 2−ΔCt.

2.6. Drugs

The following drugs were used: phenylephrine hydrochloride, aetacincholine chloride, sodium nitroprusside dehydrate, and Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME) were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA). All drugs for vascular reactivity studies were dissolved in Krebs solution. Stock solutions of each drug were freshly prepared at the day of the experiment, and kept on ice throughout the experiment.

2.7. Data analysis

Data are expressed as means ± SEM. Contractions to phenylephrine are shown as absolute tension (mN). Relaxation is expressed as the percentage to the precontraction in response to phenylephrine (3 × 10−6 M). The pD2 (negative logarithm of the molar concentration at which half-maximum contraction occurs) was determined from individual concentration–response curves by non-linear regression analysis. Differences in the vascular responses were compared by calculating the difference between the areas under the control curves (AUC). NO bioavailability was evaluated by comparing the AUC for phenylephrine or acetylcholine obtained in the absence and presence of L-NAME. In each experimental group n indicates the number of animals. Differences between mouse strains (i.e. SAMR1 vs. SAMP8) and by experimental groups (i.e., 3 vs. 6 vs. 10 month-old groups) were analyzed by two-way ANOVA, and then Bonferroni’s test was performed. Statistical signiﬁcance was accepted at P < 0.05. The statistical analysis was carried out using the Prism 4 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Age-dependent increase of contraction in response to phenylephrine

Phenylephrine caused a concentration-dependent contraction in the isolated aortic rings from all groups (Fig. 1). The values for pD2 were not signiﬁcantly different between the SAMR1 and SAMP8 and in all aging groups (Table 1). When SAMR1 and SAMP8 were analyzed independently, we found a chronological aging-associate increase in contractile responses. In SAMR1, the maximal contraction to phenylephrine was signiﬁcantly increased only when mice were 10 months old, while SAMP8 displays augmented responses at 6 months that are further increased at 10 months old (Table 1). Differences between strains were also observed. At 6 months, maximal contractions in response to phenylephrine were signiﬁcantly enhanced in the aorta from SAMP8 compared to SAMR1. The difference between maximal responses of the SAMP8 and SAMR1 groups was greater at 10 months old. Interestingly, the magnitude of contractile responses in 10-month-old SAMR1 was comparable to that obtained from 6-month-old SAMP8 (Fig. 1 and Table 1).

3.2. Effects of NOS-inhibition on contractile responses to phenylephrine

In the aortic rings from all groups of both SAMR1 and SAMP8 mice, treatment with the NOS inhibitor L-NAME (10−4 M) markedly increased contractile responses to phenylephrine (Fig. 2A and Table 1), although it did not modify the sensitivity to phenylephrine (evidenced by pD2 values). The indirect evaluation of NO contribution to phenylephrine-mediated contractions was evidenced by calculating the difference between the areas under the curves obtained in L-NAME-treated and untreated (control) curves (Fig. 2B). Both SAMR1 and SAMP8 mice presented an age-dependent reduction of the differences in the area under the curve, suggesting an attenuation of NO release by phenylephrine. This aging-associated reduction was faster in SAMP8, starting at 6 months and further increasing at 10 months old. Conversely, the

![Fig. 1. Concentration–response curves to phenylephrine in the aortic rings of 3-, 6-, and 10-month-old SAMR1 and SAMP8 mice. Each data point shows the mean ± SEM from n = 10 mice for each group.](image-url)
aging-associated decrease in NO contribution in SAMR1 was only at 10 months old (Fig. 2B).

3.3. Time course of the endothelium-dependent and NO-mediated relaxation to acetylcholine

Endothelium-dependent relaxation to acetylcholine did not differ between the SAMR1 and SAMP8 groups at 3 months old (Fig. 3A and Table 2). In SAMR1, decreases in acetylcholine-induced relaxation were observed only in 10-month-old group. On the other hand, SAMP8 displayed decreased endothelium-dependent responses at 6 months, which were further decreased at 10 months (Fig. 3A and Table 2). Differences between strains were observed at 6 and 10 months. In these aging groups, maximal relaxations and sensitivity (evidenced by pD2 values) in response to acetylcholine were significantly reduced in the aorta from SAMP8 compared to SAMR1 (Table 2). Responses to acetylcholine in 10-month-old SAMR1 were comparable to those obtained in 6-month-old SAMP8 (Fig. 3A and Table 2). When the areas under the curve were analyzed, the NO-mediated relaxation was attenuated in 6-month-old group of SAMP8 mice and further reduced in 10-month-old group (Fig. 3B). In SAMR1, attenuation only was observed at 10-month-old group (Fig. 3B). In SAMP8 mice, the reduction of NO-mediated responses was faster in comparison to the respective SAMR1 groups (Fig. 3B), suggesting a more pronounced NO reduction in SAMP8 females with aging. The treatment with L-NAME completely blunted acetylcholine-induced relaxation providing evidence that the aging- and senescence-associated impairment of endothelial function is mostly dependent on changes of NO bioavailability.

Supporting the data on vascular reactivity, analysis of eNOS protein (Fig. 4A and B) and gene (Fig. 4C) expression revealed an earlier and more pronounced aging-associated decrease eNOS expression in SAMP8 when compared to SAMR1. Similar pattern of responses was observed when measurement of NO metabolites, NO2−/NO3−, was performed. Plasma samples of SAMR1 and SAMP8 mice reveal a progressive decrease on NO production in SAMP8, while SAMR1 mice show a significant decrease on NO production only at 10 months old (Fig. 4D).

3.4. Time course of the endothelium-independent and NO-mediated relaxation to sodium nitroprusside

Concentration–response curves to sodium nitroprusside were performed to evaluate smooth muscle sensitivity to NO in the aortic rings. In phenylephrine-contracted aortic rings from both SAMR1 and SAMP8, sodium nitroprusside induced a maximal relaxation of 100% in all groups and the sensitivity was not altered by the age of mice or strain (Fig. 5).

4. Discussion

Several studies have examined vascular function associated with aging; however, most of these studies have not examined the progression of vascular dysfunction as aging develops. This is a time-course study that focuses on the female senescent-accelerated mouse model during development of aging. The present study brings new insights in the comprehension of the physiological role of adrenergic and endothelium-dependent vasorelaxation to acetylcholine responses in the aorta from female mice with accelerated senescence. Our results indicate that both chronological aging and accelerated senescence induce 1) an increment in contractile responses to α1-adrenergic receptor stimulation, 2) a decrease in endothelium-dependent relaxation to acetylcholine and 3) a decrease of eNOS expression and NO production. Although aging-associated effects were obtained in both SAMR1 and SAMP8, they were accelerated in SAMP8, suggesting this strain as an appropriate model to study vascular effects of aging in a more convenient standard time. At 3 months old, both strains exhibited a similar, receptor-dependent contractile response induced by phenylephrine, thus discarding differences due to strain-specific changes. Nevertheless, contractile responses in SAMP8 mice were higher than those of SAMR1 at 6 months old, and further increased at 10 months.

Phenylephrine-induced constriction is mediated primarily via activation of α1-adrenoceptors that play important roles in control of systemic blood pressure and are involved in age-related vasospasms in human subjects (DiNenno et al., 2001). The finding that female SAMP8 aorta is hyper-responsive to phenylephrine agrees with previous studies on catecholamine vascular reactivity in the aged animals (Erac et al., 2010; Kung and Luscher, 1995; Llorens et al., 2007). Such enhanced contractions may have important clinical implications. α1-Adrenergic receptors are prime mediators of vasoconstriction in the human vasculature (Leech and Faber, 1996; Rudner et al., 1999) and thus affect arterial and venous vascular tone, peripheral resistance and blood pressure, and cardiac filling (Docerty, 2010). Thus, increased α1-adrenergic-mediated contractions induced by aging may contribute to the enhanced cardiovascular responses in SAMP8 compared with SAMR1 mice.

 Nonetheless, even though our results suggest a potential aging-associated modulation of sympathetic pathway, in a previous study we demonstrated an aging- and senescence-associated increased contraction to another non-sympathetic agonist, thromboxane A2 (TXA2) (Novella et al., 2010, 2013). In this regard, we can speculate that there is an aging- and senescence-associated modulation of key regulators of vascular contraction. It is well known that endothelial cells contribute to the regulation of vascular tone and modulate the response to different agonists by releasing several vasoactive factors (Deanfield et al., 2007). Therefore, the enhanced contractile responses to phenylephrine observed in the present study could be due to decrease of endothelial-derived vasodilators and/or increased of vasoconstrictors. We have previously reported that a lower NO release in response to TXA2 receptor activation modulates the greater contractions to this agonist in SAMP8 (Novella et al., 2013). In the present study, the use of NOS inhibitor L-NAME augmented the contractile responses in the aorta from both strains at all aging groups studied, suggesting that NO is a key contributor to attenuate phenylephrine-induced contraction. However, the magnitude of L-NAME-induced potentiation in vasoconstriction was diminished by aging, suggesting an age-associated decrease in the contribution of NO to attenuate contractile responses by phenylephrine in female mice. Moreover, we observed that the reduction of NO contribution by aging was accelerated in SAMP8, starting at 6 months old, and parallels the changes in phenylephrine responses.

Table 1

<table>
<thead>
<tr>
<th>Phenylephrine</th>
<th>SAMR1</th>
<th>SAMP8</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>pD2 (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>6.5 ± 0.57</td>
<td>6.8 ± 0.60</td>
<td>6.3 ± 0.60</td>
</tr>
<tr>
<td>6 months</td>
<td>7.2 ± 0.60</td>
<td>7.8 ± 0.65</td>
<td>7.4 ± 0.60</td>
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<tr>
<td>10 months</td>
<td>8.0 ± 0.65</td>
<td>8.5 ± 0.60</td>
<td>8.2 ± 0.60</td>
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</table>

Values are means ± SEM. Maximal contraction is expressed as % of initial tension. *P < 0.05 versus SAMR1 group with the same age, #P < 0.05 versus 3-month old group of the same strain and treated group, ‡P < 0.05 versus 6-month old group of the same strain and treated group and §P < 0.05 versus the same age and strain of control group.
Accordingly, relaxations induced by acetylcholine were also reduced with aging in both SAMR1 and SAMP8, an effect that also happened faster in SAMP8 mice. Considering that the endothelium-dependent relaxation to acetylcholine was completely inhibited by L-NAME at all ages, these results reinforce the theory that changes in NO-mediated responses are a major contributor to the aging- and senescence-associated impairment of vascular function in female aorta.

In our studies we have not found any difference in the vascular responses to sodium nitroprusside (NO donor), therefore the reduced vasodilator responses observed in older mice were not caused by a decreased sensitivity of vascular smooth muscle cells to NO, but rather to a reduced endothelium-dependent production or bioavailability of NO. Our results agree with findings of preserved vasorelaxant responses to sodium nitroprusside in humans despite age-associated impairments in acetylcholine-induced endothelium-dependent relaxation (DeSouza et al., 2013).

Fig. 2. (A) Concentration–response curves to phenylephrine (10⁻¹⁰ to 10⁻⁵ M) in the aortic rings from 3-, 6-, and 10-month-old SAMR1 and SAMP8 mice in the absence (control) and in the presence of NOS synthase inhibitor L-NAME (10⁻⁴ M). Each data point shows the mean ± SEM from n = 10 mice for each group. (B) Area under curves (AUC) from concentration–response curves in the presence of L-NAME (10⁻⁴ M) minus AUC in untreated arteries (control). Each data point shows the mean ± SEM from n = 10 mice for each group. *P < 0.05; **P < 0.01; and ***P < 0.001.
et al., 2000; Donato et al., 2007; Eskurza et al., 2004). The fact that endothelium-independent dilation was preserved in the aorta from SAM older animals in the face of reduced endothelium-dependent relaxation represents an advantage of using the SAM mice as a model of vascular aging in humans.

Growing evidences have established that aging results in well-defined genetic and phenotypic changes, which turn the cardiovascular system prone to disease even in the absence of traditional risk factors (Ungvari et al., 2010). Among the genetic changes we can mention an age-related decline in NO synthase expression (Tanabe et al., 2003; Wang et al., 2011). Consistent with vascular reactivity data, no significant differences on eNOS expression were observed when mice were 3 months old. However, when mice reach middle age (i.e. 6 months) a slight decrease of eNOS was observed in SAMR1, while SAMP8 exhibited a much higher reduction on its expression. The levels of eNOS observed in 6-month-old SAMP8 were only seen on SAMR1 when they were 10 months old. Moreover, we observed a decrease in markers of NO metabolism (NO2−/NO3−) that follow similar pattern of modification by aging and senescence observed in vascular reactivity and eNOS expression studies. Therefore, a decrease in NO production by diminished eNOS expression, rather than changes in NO signaling, may be responsible to the impairment of vascular responses by aging and accelerated senescence in female vasculature.

![Graph](image)

**Fig. 3.** (A) Concentration–response curves to acetylcholine (10−9 to 10−5 M) in the aortic rings from 3-, 6-, and 10-month-old SAMR1 and SAMP8 mice in the absence (control) and in the presence of NOS synthase inhibitor L-NAME (10−4 M). Each data point shows the mean ± SEM from n = 10 mice for each group. (B) Area under curves (AUC) from concentration–response curves in the presence of L-NAME (10−4 M) minus AUC in untreated arteries (control). Each data point shows the mean ± SEM from n = 10 mice for each group. *P < 0.05; **P < 0.01; and ***P < 0.001.

**Table 2**

<table>
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<tr>
<th>Acetylcholine</th>
<th>SAMR1</th>
<th>SAMP8</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>7.80 ± 0.09</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>6 months</td>
<td>7.96 ± 0.09</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>10 months</td>
<td>7.16 ± 0.08</td>
<td>76 ± 4</td>
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<tr>
<td>L-NAME 10−4 M</td>
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<td></td>
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<tr>
<td>3 months</td>
<td>6 ± 2</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>6 months</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>10 months</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
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Values are means ± SEM. Maximal relaxation is expressed as the percentage of relaxation from pre-contraction in response to phenylephrine. n = 10 animals per group. *P < 0.05 versus SAMR1 group with the same age. #P < 0.05 versus 3-month old group of the same strain and treated group. ‡P < 0.05 versus 6-month old group of the same strain and †P < 0.05 versus the same age and strain of control group. #P2 value was not calculated as the maximum relaxation to acetylcholine was less than 10%.

The fact that endothelium-independent dilation was preserved in the aorta from SAM older animals in the face of reduced endothelium-dependent relaxation represents an advantage of using the SAM mice as a model of vascular aging in humans.

Growing evidences have established that aging results in well-defined genetic and phenotypic changes, which turn the cardiovascular system prone to disease even in the absence of traditional risk factors (Ungvari et al., 2010). Among the genetic changes we can mention an age-related decline in eNOS expression (Tanabe et al., 2003; Wang et al., 2011). Consistent with vascular reactivity data, no significant differences on eNOS expression were observed when mice were 3 months old. However, when mice reach middle age (i.e. 6 months) a slight decrease of eNOS was observed in SAMR1, while SAMP8 exhibited a much higher reduction on its expression. The levels of eNOS observed in 6-month-old SAMP8 were only seen on SAMR1 when they were 10 months old. Moreover, we observed a decrease in markers of NO metabolism (NO2−/NO3−) that follow similar pattern of modification by aging and senescence observed in vascular reactivity and eNOS expression studies. Therefore, a decrease in NO production by diminished eNOS expression, rather than changes in NO signaling, may be responsible to the impairment of vascular responses by aging and accelerated senescence in female vasculature.
Despite a decreased bioavailability of NO in aged SAM mice, there is evidence that aging is associated with an increased inducible NOS (iNOS) expression in vascular tissues from aged rats (Cernadas et al., 1998; Csiszar et al., 2002) and humans (Rodriguez-Manas et al., 2009). The induction of iNOS within the vascular wall would promote oxidative stress through formation of peroxynitrite from the reaction of iNOS-derived NO with superoxide (Xia and Zweier, 1997) and through superoxide formation following uncoupling of NOS (Xia et al., 1998). Further studies are required to determine the role of the iNOS in vascular dysfunction in SAM mice.
5. Conclusions

This study provides evidence that contractile response to phenylephrine and the endothelium-dependent relaxation to acetylcholine are modified by chronological aging in female mice by a mechanism involving the decreased of eNOS expression and NO production. These effects occur at earlier age in the aorta from female SAMP8 mice (6 months) and are comparable to the effects observed in SAMR1 mice at older age (10 months). Thus we suggest that 6-month-old female SAMP8 mice as a valuable model to study the mechanisms of vascular aging at a convenient standard time course.

Conflict of interest

The authors have no conflicts of interests.

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References


Fig. 5. Concentration–response curves to sodium nitroprusside (10^{-11} to 10^{-7} M) in the aortic rings from 3-, 6-, and 10-month old SAMR1 and SAMP8. Each data point shows the mean ± SEM from n = 10 mice for each group.


