Biogerontology (2007) 8:663–672
DOI 10.1007/s10522-007-9108-4

RESEARCH ARTICLE

The senescence-accelerated mouse (SAM-P8) as a model for the study of vascular functional alterations during aging

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Received: 7 May 2007 / Accepted: 16 August 2007 / Published online: 5 September 2007

Abstract We studied vascular function in quiescent aortas from senescence-accelerated resistant (SAM-R1) and prone (SAM-P8) mice. Myographical studies of thoracic aorta segments from 6–7 month-old mice showed that the contractility of SAM-P8 aortas was markedly higher than that of SAM-R1 after KCl depolarization or phenylephrine addition. Acetylcholine dose-response relaxation curves revealed that SAM-R1 vessels were slightly more sensitive than those of SAM-P8. In the presence of the NO synthase inhibitor, L-NAME, all vessels displayed contractions to acetylcholine, but these were more distinct in the SAM-R1. Phenylephrine plus L-NAME displayed stronger contractions in both animal strains, but were markedly more pronounced in SAM-R1. The cyclooxygenase inhibitor, indomethacin did not change the vessel responses to acetylcholine or phenylephrine. These data indicate that NO synthase, not cyclooxygenase, was responsible for the differences in contractility. Standard histology and immunohistochemistry of endothelial NO synthase revealed no differences in the expression of this protein. In contrast, increased levels of malondialdehyde were found in SAM-P8 vessels. We conclude that SAM-P8 vessels exhibit higher contractility than those of SAM-R1. Furthermore, our results suggest that the endothelium of SAM-P8 vessels is dysfunctional and lacks normal capability to counteract smooth muscle contraction. Therefore, our findings support SAM-P8 as a suitable model for the study of vascular physiological changes during aging.

Keywords SAM · Vascular function · Aging · Vessels · Endothelium · NO · Lipid peroxidation

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Introduction

Within the realm of general age-related deterioration, quality of life is particularly affected by the functional decline of the cardiovascular system. Blood vessels, in particular, show mechanical and

morphological alterations as well as endothelial dysfunction (For review see: Ferrari et al. 2003). A loss in the distensibility of the aorta (Hallock and Benson 1937) is one sign of aging that can be detected in senescent mice (Reddy et al. 2003). The morphological bases for these mechanical alterations are the well known vascular transformations that occur during senescence: arteries become tortuous and elongated with intimal and medial thickening together with an increase in collagen accumulation in the media, and elastin fragmentation (Ferrari et al. 2003). Aged mice also display an increase in the intimal and medial thickness (Goyal 1982). Finally, aging causes a decline in endothelium-dependent relaxation both in humans (Egashira et al. 1993) and mice (Blackwell et al. 2004).

Unfortunately, studies on the vascular physiological alterations during senescence are scarce, likely due to the cost and difficulty in maintaining animals until they reach senescence (Zhou et al. 2003). Moreover, these types of studies have been performed in animals grown to a certain age, questionably considered to be the equivalent of human senescence (Folkow and Svanborg 1993; Llorens et al. 2005). In fact, some studies on the cardiovascular system during senescence have yielded conflicting results (Llorens et al. 2005; Zieman et al. 2001), possibly due to the disparity of ages used. An alternative approach, never before attempted in vascular functional studies, is to use a standard model of genetic senescence.

One of the standard models of animal aging is the senescence-accelerated mouse (SAM). This strain was initially created by Takeda et al. (1981) as a result of selective inbreeding of mice showing a phenotype of severe exhaustion (SAM-prone) and inbreeding of a normal phenotype (SAM-resistant). Vascular studies employing this model are not abundant, but do include histopathological (Fenton et al. 2004; Lee et al. 2000; Ueno et al. 2001; Yagi et al. 1995; Zhu et al. 2001) and blood pressure determinations (Zhu et al. 2001). However, functional studies on the cardiovascular system or on the role of NO, the key component of cardiovascular regulation (Llorens et al. 2002), have not been performed to date.

The main aim of the present work is to explore the possibility of using the SAM-P8 mouse as a model for performing vascular functional studies. We analyzed the isometric tension changes of aortic segments obtained from 6–7 month-old mice. These procedures were accompanied by analytical and immunohistochemical studies of oxidative stress and NO synthase in these vessels.

Methods

Preparation of the animals

Male SAM-R1 (senescence-accelerated resistant) (n = 14) and SAM-P8 (senescence-accelerated prone) (n = 16) were obtained from Harlan UK Ltd (Blackthorn, UK) through Harlan Interfauna Ibérica (Barcelona, Spain) and maintained until they reached 6–7 months. SAM-R1 weighed 31.4 ± 0.6 g and SAM-P8, 31.1 ± 1.1 g, when sacrificed. Housing and procedures were performed in accordance with the Declaration of Helsinki and with the European Community (86/609/EEC) regulations. Finally, these procedures were approved by the Ethical Committee of the University of Castilla-La Mancha. Husbandry conditions in our animal facility meet the European standards: open system room with a one-way airflow system (temperature, 20–22°C; light period, 12/12; humidity, 45%). Mice were given a commercial diet (PanLab, Barcelona, Spain) and tap water *ad libitum*. SAM-P8, but not SAM-R1, showed early signs of advanced aging, such as hair coarseness and various skin injuries at the time they were sacrificed. SAM-P8 have a lifespan of 12.1 months and SAM-R1 of 18.9 months, which are within the range reported elsewhere (Takeda et al. 1981; Tanaka et al. 2004).

Preparation of the vessels

A thoracotomy was performed, followed by a careful dissection of the thoracic aorta down to the diaphragm. Pharmacological studies were performed as previously described (Llorens et al. 2005). Sequential 3.0 mm segments were briefly immersed in a 5 ml organ bath containing 37°C Krebs-Henseleit solution bubbled with 95% O_2 and 5% CO_2. Two steel pins (200 μm in diameter) were introduced through the lumen and isometric tension recorded by means of two 4-channel myographs (Danish MyoTechnology, Aarhus, Denmark). An optimal passive tension of

1.5 g was applied to the arterial segments, and they were allowed to equilibrate for a period of 60 min before the experiments were started. Tension was readjusted when necessary and the bath fluid was changed every 15 min.

Experimental protocols

Protocol 1. Unspecific vascular contractility

The contractility of the arterial segments was examined by depolarization with a single dose of KCl (5×10^{-2} M). The contractile force to KCl also served to normalize the forces attained by the other drugs tested. Thus, every result is expressed as a percentage of KCl contraction.

Protocol 2. Vascular smooth muscle contraction to phenylephrine. Role of endothelium

Responses to cumulative doses of phenylephrine (10^{-9}–10^{-5} M) were analysed. To test the NO and prostaglandin component of the endothelial modulation of vessel contraction, segments were incubated for 20 min. with L-NAME (10^{-5} M) or indomethacin (10^{-5} M, 20 min), respectively.

Protocol 3. Endothelium-dependent relaxation

A single dose of phenylephrine (10^{-5} M) was added to the bath to provide a contractile tone. When tension was stable, acetylcholine (10^{-9}–10^{-5} M) was added. Vessels failing to achieve at least 60% relaxation were assumed to be damaged and were discarded.

Determination of lipid peroxidation

Malondialdehyde levels were determined as described by Gomez Lazaro et al. 2007. Each sample was briefly homogenized in a 10-fold volume of ice-cold 20 mM BTris–HCl buffer, pH 7.4. After centrifugation for 6 min at 3000 rpm at 4°C, amounts of MDA were determined in supernatant using a Lipid Peroxidation Assay Kit from Calbiochem (No. 437634).

Immunohistochemistry and standard histology

All samples were fixed by immersion in formalin and embedded in paraffin using a Tissue Embedding System apparatus (TES99; Medite, Hannover, Germany). Heat-induced epitope retrieval in 10 mmol l^{-1} citrate buffer, pH 6.0, was used for all formalin-fixed, paraffin-embedded tissue sections. Sections were stained immunohistochemically with the standard avidin–streptavidin method using an automated platform (Dako autostainer; Dako, Glostrup, Denmark). A polyclonal IgG antibody raised against a synthetic peptide corresponding to amino acids 1181–1194 of human eNOS/NOS III (10/11 identical amino acids in mouse and rat) (Upstate, Lake Placid, NY) was used as a primary antibody (dilution, 1:200). Diaminobenzidine was used as the chromogen and sections were counterstained with haematoxylin. Additional serial sections from the same samples obtained at various levels throughout the entire thoracic aorta were stained using standard Masson's trichrome, elastica-van Gieson, and haematoxylin-eosin staining procedures.

Preparation of the drugs and chemicals

All compounds in this study, except those obtained as indicated, were purchased from Sigma Aldrich (Alcobendas, Spain). Indomethacin was freshly prepared in ethanol for every experiment. Other compounds were prepared in distilled water.

Statistical analyses

Significance was determined by the unpaired two-tailed Student's *t*-test. All differences were considered significant at $P < 0.05$.

Results

SAM-P8 mice display higher vascular smooth muscle contractility compared to SAM-R1

In the first set of experiments, we ascertained the ability of the aortic segments to contract by means of KCl. As expected, vessels from both mouse strains did contract

when challenged with 5×10^{-2} M KCl. It is notable that SAM-P8 vessels contracted with a force 90% stronger than that demonstrated by SAM-R1 (Fig. 1).

Next, we analyzed the endothelial modulation of vascular smooth muscle contractions to phenylephrine. The contractile force exhibited by SAM-P8 segments was significantly higher than that of SAM-R1 (Fig. 2A and Table 1), but not sensitivity to phenylephrine. To examine the extent to which NO inhibits phenylephrine contractions, vessels were incubated with the NOS inhibitor, L-NAME. As Fig. 2A illustrates, 10^{-5} M L-NAME showed a marked increase in the force of these contractions and also in the sensitivity to phenylephrine (Table 1), both in SAM-R1 and SAM-P8 mice. It is noteworthy that the increase in force caused by L-NAME was markedly higher in SAM-R1 vessels ($P < 0.01$). Subsequently, we assayed the role of prostaglandins in the endothelium-dependent modulation of contractility by inhibiting prostaglandin synthesis using the cyclooxygenase inhibitor, indomethacin. Indomethacin (10^{-5} M) elicited no effects on phenylephrine-induced contractions (Fig. 2B and Table 1).

Endothelium-dependent relaxations in the SAM-P8 versus SAM-R1

Vessels from both mouse strains relaxed to acetylcholine, reaching a similar maximal effect (Fig. 3).

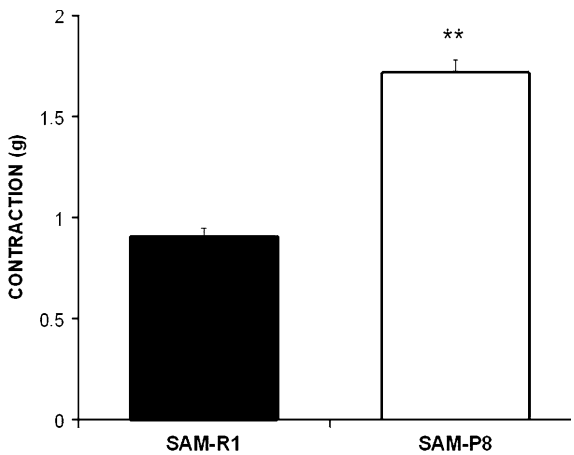

Fig. 1 Response of quiescent vessels to depolarization with KCl (50 mM). SAM-P8 aortic segments displayed markedly stronger contractile forces compared to SAM-R1. Results express mean ± s.e.m. from $n = 5$–6 mice. $^{**}P < 0.01$ compared with SAM-R1

The sensitivity to acetylcholine was slightly, albeit significantly lower in SAM-P8 vessels compared to the SAM-R1 (Fig. 3 and Table 1). To assess the NO component of endothelium-dependent relaxation, we assayed the effects of L-NAME. At 10^{-5} M, L-NAME completely abolished acetylcholine-induced relaxations in vessels from both strains. In turn, vessels displayed a contraction that was significantly weaker in SAM-P8 mice (Fig. 3A and Table 1). After acetylcholine stimulation, there is a release of thromboxane A2 and prostacyclin as a result of cyclooxygenase stimulation (Behrendt et al. 2002). Thus, we studied the role of this pathway. As shown in Fig. 3B, indomethacin failed to cause variations in the maximal effects of the acetylcholine-induced relaxations. Indomethacin reduced the sensitivity to this drug in SAM-R1 vessels (Fig. 3B and Table 1).

SAM-P8 exhibit significant increases in lipid peroxidation

An increase in oxidative stress has been described in different tissues obtained from SAM-P8 (Alvarez-Garcia et al. 2006). In order to evaluate the levels of oxidative stress in the aortic segments, we analyzed the levels of MDA, a marker of oxidative stress, in mice aortas. Oxidative stress, assessed by measuring MDA accumulation within the vessel wall, was remarkably increased in aortas from SAM-P8 mice compared to SAM-R1 (Fig. 4).

Morphological studies revealed no differences between SAM-P8 and SAM-R1 vessels

Histological examination of all the tunicae was performed employing Masson's trichrome, Van Gieson (data not shown) and haematoxylin-eosin (Fig. 5A–D) staining techniques. No significant differences between strains were found in the appearance, thickness or presence of collagen or elastic fibres for any of the layers of the vessel wall. The number of smooth muscle cell layers was 3–4 in both SAM-R1 and SAM-P8 aortas. The entire aorta was carefully inspected for signs of atherosclerosis. No abnormal thickening, overproduction of collagen (Masson's trichrome), foam macrophages or cholesterol was detected. No disruptions of laminae or any

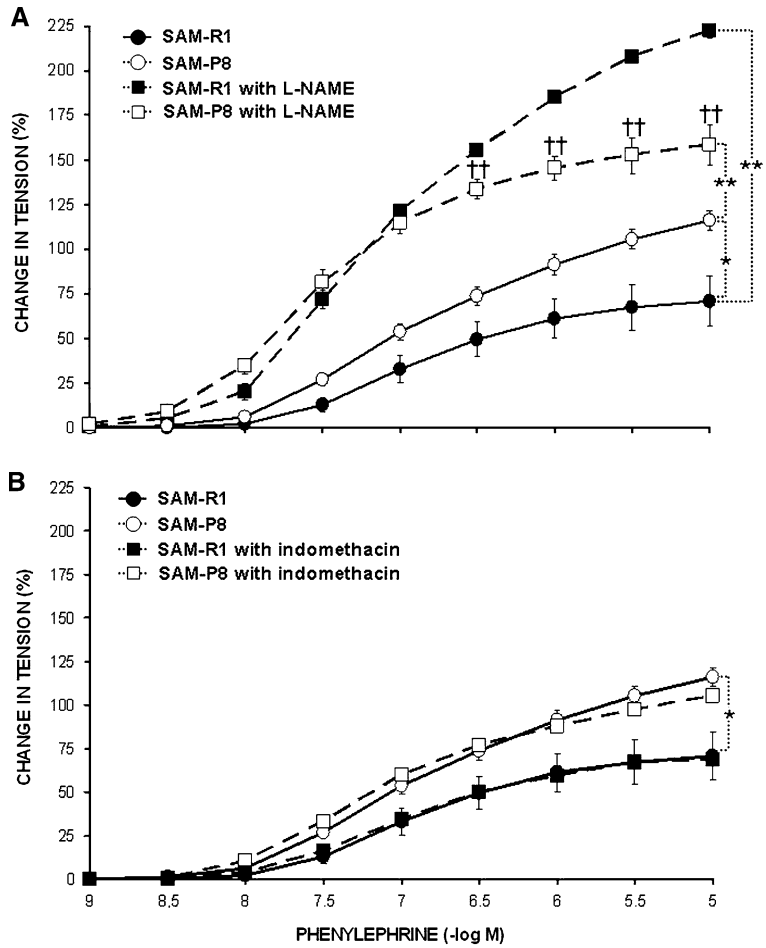

Fig. 2 Responses of quiescent vessels to increasing concentrations of phenylephrine**.** The role of NO or prostanoids in the endothelial modulation of aortic smooth muscle contractions elicited by phenylephrine were studied by means of L-NAME (10^{-5} M) (**A**) or indomethacin (10^{-5} M) (**B**). Results are shown as a percentage of KCl contraction and express mean ± s.e.m. from n = 5–6 mice. *, ** $P < 0.05$ and $P < 0.01$. $^{\dagger}P < 0.01$ SAM-R1 with L-NAME versus SAM-P8 with L-NAME

Table 1 Effects of cumulative concentrations of phenylephrine (PHE) and acetylcholine (Ach) in the presence or absence of L-NAME and indomethacin on quiescent aortic segments of SAM-R1 and SAM-P8 mice

	Control			With L-NAME			With Indomethacin		
	E_{max} (%)	pD2	n	E_{max} (%)	pD2	n	E_{max} (%)	pD2	n
PHE SAM-R1	70 ± 14	6.88 ± 0.05	10	222 ± 11**	7.12 ± 0.06**	7	69 ± 15	6.95 ± 0.08	7
PHE SAM-P8	116 ± 5†	6.86 ± 0.06	8	158 ± 4**††	7.51 ± 0.06**††	7	105 ± 8†	7.09 ± 0.09*	7
ACh SAM-R1	–55 ± 10	7.43 ± 0.14	10	24 ± 5*	7.03 ± 0.13	7	–57 ± 8	6.93 ± 0.18*	6
ACh SAM-P8	–67 ± 6	7.06 ± 0.06†	7	11 ± 2**†	6.81 ± 0.15	6	–67 ± 5	6.97 ± 0.06	6

The maximum effect (E_{max}) is expressed as a percentage of KCl contraction. For clarity relaxations are shown with negative figures. The concentration of the substance which produced 50% of the E_{max}, (EC_{50}) is expressed as pD2 (–log EC_{50}). All E_{max} and pD2 results are shown as the means ± s.e.m. *, ** $P < 0.05$, $P < 0.01$ compared horizontally with the corresponding value in the control columns. †, $^{\dagger\dagger}P < 0.05$, $P < 0.01$ SAM-P8 versus SAM-R1. "n" stands for the number from segments for at least different 5 animals

of the elastic layers within the tunica media could be found.

Since the functional data suggested the existence of alterations in the NO pathway, immunohistochemical studies using antibodies specifically directed to endothelial NO synthase (eNOS) were carried out. Aortas from both SAM-R1 and SAM-P8 showed similarly intense and uniform labelling for e-NOS immunoreactivity in their endothelial cells (Fig. 6A, B).

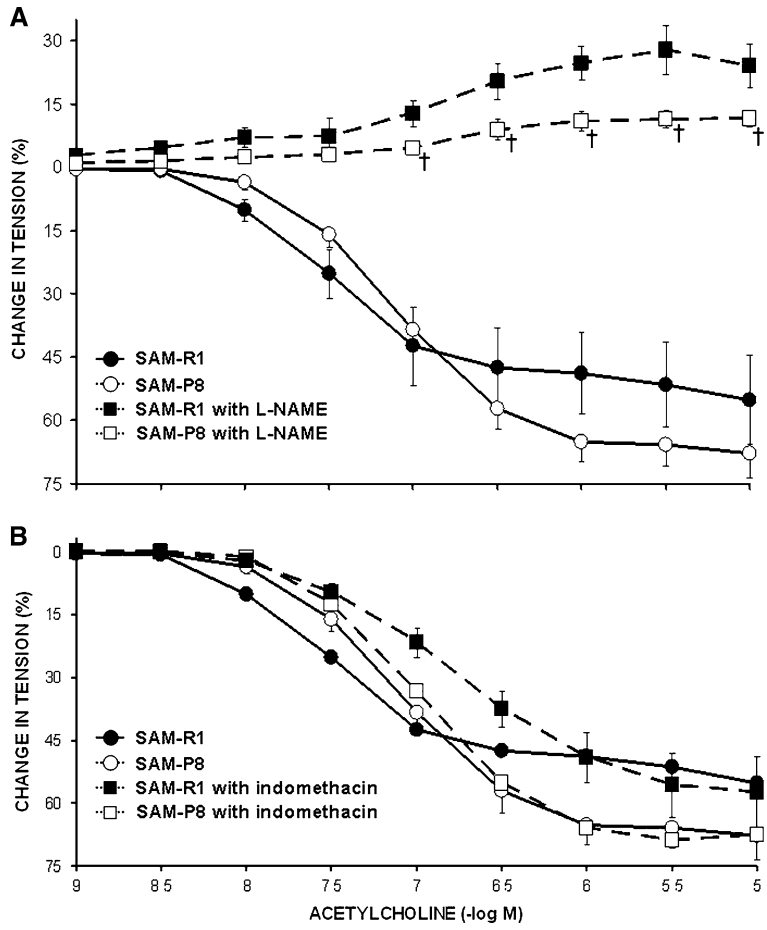

Fig. 3 Responses of quiescent vessels to increasing concentrations of acetylcholine**.** The role of NO or prostanoids was assessed with L-NAME (10^{-5} M) (**A**) or indomethacin (10^{-5} M) (**B**). Results are shown as a percentage of KCl contraction and express mean ± s.e.m. from $n = 5$–6 mice. $^{\dagger}P < 0.05$ SAM-R1 with L-NAME versus SAM-P8 with L-NAME

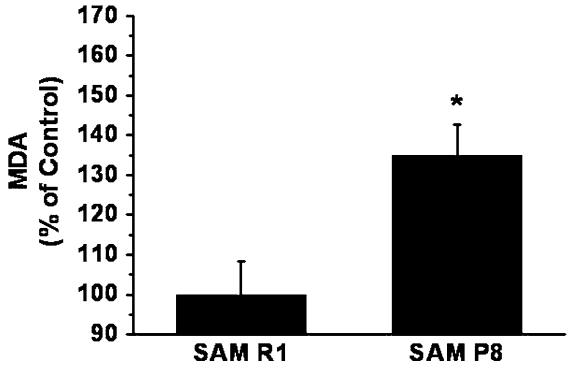

Fig. 4 Levels of malondialdehyde are increased in SAM-P8 aortic tissue. Results are expressed as percentage of MDA levels found in SAM-R1 (control). Data show mean ± s.e.m. from $n = 6$ mice. $^{*}P < 0.05$ compared with SAM-R1

Discussion

The present work is a study of vascular function in aortas from the senescence-accelerated mouse (SAM-P8) in an attempt to assess the adequacy of this strain as a model of vascular physiological alterations during aging. To the best of our knowledge, this is the first study on vascular physiology carried out in this type of animal. SAM-P8 is a mouse strain suffering from an accelerated aging disorder solely of genetic origin and has not been subjected to any experimental manipulation (Takeda et al. 1981). Suspicions that the accelerated senescence of these animals could be caused by a viral disease (Carp et al. 2002) are eclipsed by the large amount of published data supporting the SAM as a model for aging studies (Takeda et al. 1981; Yagi et al. 1995; Lee et al. 2000; Ueno et al. 2001; Zhu et al. 2001; Fenton et al. 2004). One disadvantage of most experiments on aging and vascular physiology performed thus far is that they have been carried out in animals grown to ages which vary from one study to another (Folkow et al. 1993; Küng and Lüscher 1995; Zieman et al. 2001; Zhou et al. 2003; Llorens et al. 2005).

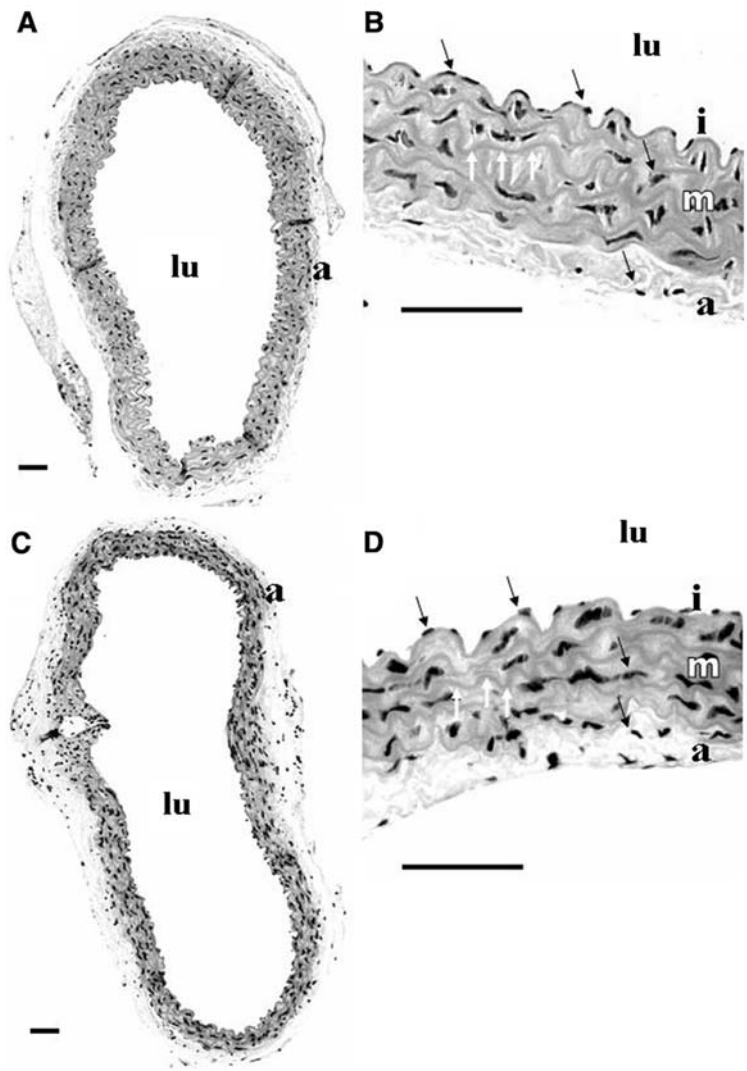

Fig. 5 Histology of SAM-R1 and SAM-P8 aortas. Cross sections of thoracic aortas from SAM-R1 (**A** and **B**) and SAM-P8 (**C** and **D**) stained with haematoxylin-eosin. A and C: 125x; B and D: 640x. Lumen (lu). Black arrows indicate the nuclei (black staining) of endothelial cells in the tunica intima (i), smooth muscle cells in the tunica media (m) and fibroblasts in the tunica adventitia (a). The internal elastic lamina (immediately beneath the endothelium), the external elastic lamina (separating the tunica media from the tunica adventitia) as well as the elastic lamellae in the tunica media (white arrows) are stained in grey. Scale bars: 50 μm

However, SAM age fast and predictably, an important advantage allowing experimental work to be performed in a convenient and standard time course. Moreover, it is known that the aorta of the aged mouse reproduces all the morphological (Goyal 1982), mechanical (Reddy et al. 2003) and endothelial alterations (Blackwell et al. 2004) of the aged human aorta, which makes SAM-P8 an appropriate model for carrying out vascular functional studies.

The aortic specimens from SAM-P8 mice displayed a markedly greater contractility compared to the SAM-R1, both when subjected to depolarization and to increasing concentrations of phenylephrine. These observations could be explained by a possible hypertrophy of the media of SAM-P8 aortas, but this was not the case. Indeed, our histological studies showed that the aortas from both mouse strains displayed a comparable smooth muscle cell layer and no obvious signs of media-to-lumen ratio alterations. Furthermore, we could not find any signs of the atherosclerosis previously reported in SAM-P8 (Fenton et al. 2004) and SAM-P11 (Zhu et al. 2001). This discrepancy is probably due to the fact that the above-cited studies were performed on animals of a much older age than ours and, in the case of Fenton et al. (2004), mice were fed an atherogenic diet. Alterations in the lipid profile have been found in SAM-P1 as early as 3 months of age (Yagi et al. 1995); however, aortic structural alterations were not detectable before 12 months (Fenton et al. 2004; Zhu et al. 2001). At

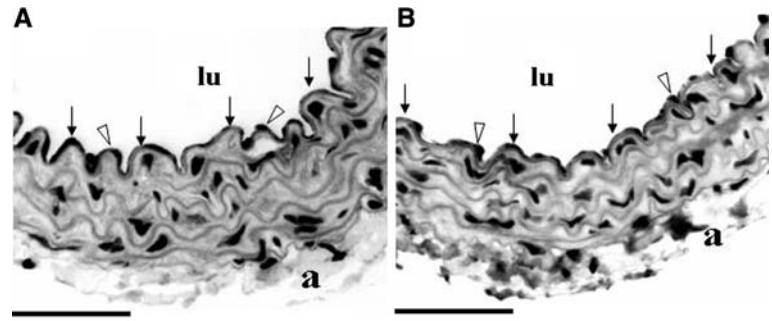

Fig. 6 Detection of eNOS in the endothelium of SAM-R1 and SAM-P8 aortas. Immunoreactivity to eNOS in cross sections from SAM-R1 (**A**) and SAM-P8 (**B**) aortas. Endothelial cells show intense labelling (black arrows) along the surface of the tunica intima in the aortas of both strains. The nuclei from the endothelial cells can be identified protruding towards the lumen (white arrowheads). lu: lumen, a: tunica adventitia. Magnification: 640×. Scale bars: 50 μm

one year of age, senescence is fully established and it is not surprising to find morphological alterations in the vessels. It is thus coherent that there were no anatomical differences between our younger, 6–7 month-old groups of mice, even though a clear hyper-responsiveness of SAM-P8 vessels did occur. Morphofunctional mismatching is not uncommon in cardiovascular disease. In hypertension or diabetes, for example, premature signs of vascular endothelial dysfunction are present even before the onset of the disease (Taddei et al.1992), (Caballero et al. 1999). The finding that SAM-P8 vessels are hyper-responsive to phenylephrine concurs with existing studies on catecholamine vascular reactivity in the aged animal (Küng and Lüscher. 1995). It is worth mentioning that this excessive vascular response to catecholamines in the aged individual can be particularly important if we take into account that plasma noradrenaline (Ziegler et al. 1976) and sympathetic activity (Hogikyan et al. 1994) increase with age.

Under NO synthesis blockade with L-NAME, contractions to phenylephrine were stronger in both animal strains than in control conditions. This type of finding is known to be due to a NO releasing response of the endothelium (Yamaguchi et al. 1989). Interestingly, the increase in the force responses to phenylephrine under NO synthase inhibition was remarkably higher in the SAM-R1 aortas. This observation suggests that a much higher release of NO takes place in SAM-R1 vessels in response to a vasoconstriction challenge. Thus, SAM-P8 vasculature seems to be hyper-responsive to catecholamine stimulation and has less bioactive NO to protect itself against vasoconstrictive stimuli.

The data reported above point to the possibility of an existing endothelial dysfunction in SAM-P8 animals. When endothelial function was assessed by means of an acetylcholine dose-response curve (Küng and Lüscher 1995), a significantly lower sensitivity to acetylcholine was indeed detected in the SAM-P8 aortas. The endothelium releases a number of vasoactive substances in response to acetylcholine, including NO and arachidonic acid-derived products (Behrendt et al. 2002). The blockade of NO synthase with L-NAME completely abolished acetylcholine-induced relaxations. This is consistent with the notion that NO is one of the most important substances released by large vessels in response to acetylcholine stimulation (Xu et al. 2007). Moreover, acetylcholine in the presence of the NO synthase inhibitor elicited contractions of the vessels, stronger in SAM-R1 than in SAM-P8 segments. These differences in the force of the L-NAME-induced contractions in vessels challenged with acetylcholine could not be accounted for by prostanoid vasoconstrictors (Shirahase et al. 1987). Indeed, aortas pre-treated with indomethacin presented no detectable changes in the responses to acetylcholine. Most likely, acetylcholine under NO blockade contracted the vessels by direct action on smooth muscle cell receptors. Thus, the observation that acetylcholine contractions in the presence of L-NAME were stronger in SAM-R1 aortas suggests that a higher production of NO took place in these vessels before NO synthase inhibition.

Since the functional data indicated a downregulation of the NO pathway in the SAM-P8 aortic endothelium, we carried out immunohistochemical studies specifically directed towards endothelial NO

synthase. Endothelial cells from SAM-P8 aortas were equally stained with eNOS antibody, as were SAM-R1 endothelial cells. Thus, we could not confirm a downregulation of eNOS in the senescent mice. Subsequently, we analyzed the accumulation of the lipoperoxide marker malondialdehyde. This marker was increased in aortas from SAM-P8, a finding in accordance with the general consensus of increased oxidative stress as the main mechanism responsible for the accelerated senescence observed in the present mouse strain (Alvarez-Garcia et al. 2006). Since it is known that NO avidly reacts with reactive oxygen species (Estevez and Jordan 2002), we cannot exclude that this increased oxidative stress might degrade released NO and therefore account for the results of the present functional studies.

In summary, the SAM-P8 appears to be an adequate model for the performance of vascular functional studies during aging. We found that quiescent vessels from these mice exhibit higher contractility and a diminished ability of the endothelium to compensate for smooth muscle contractions. It is possible that lower bioavailability of NO due to a high rate of degradation underlies the endothelial dysfunction seen in SAM-P8 aortas.

Acknowledgments This work was supported by: The Spanish Ministry of Science (SAF2005-02157 and SAF2005-07919-C02-01), JCCM (04048-00 and 04005-00) and FIS-FEDER (01/3018). A.P-M. and Y.M. were supported by grants from JCCM (JI 03001 and 06016-00). We thank M.A. Olivares and A. Pérez (Complejo Hospitalario Universitario de Albacete) and A.L. Salewski for English revision of the manuscript.

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