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GATHERING OF AGING AND ESTROGEN WITHDRAWAL IN VASCULAR DYSFUNCTION OF SENESCENT ACCELERATED MICE

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ABSTRACT

The aim of this work was to characterize a mouse model of experimental menopause and cardiovascular aging that closely reflects menopause in women. Senescence accelerated mouse (SAM)-Resistant type 1 (SAMR1, n=30) and SAM-Prone type 8 (SAMP8, n=30) were separated at 5 months of age into three groups: 1) sham-operated (Sham); 2) ovariectomized (Ovx); and 3) ovariectomized chronically-treated with estrogen (Ovx+E2). Contractile responses to KCl (60 mM) and thromboxane A_2 were greater in aorta from SAMP8 mice compared with SAMR1 in all groups. Neither ovariectomy nor estrogen replacement modified the contractile responses from SAMR1 mice. Conversely, in Ovx SAMP8 increased the maximal contractions that were reversed by estrogen treatment. Rings with endothelium from all SAMR1 groups showed a greater relaxation to acetylcholine than SAMP8 groups. In SAMR1, endothelium-dependent relaxation was not altered in Ovx or Ovx+E2 groups. Rings from Ovx SAMP8 showed a decreased maximal response to acetylcholine compared to Sham SAMP8. Estrogen replacement restored the response to acetylcholine altered by ovariectomy. Nitric oxide inhibition by L-NAME marked reduced acetylcholine responses in all groups, but this effect was more pronounced in SAMP8 and Ovx groups (determined by area under the curve reduction). These results indicate that SAMP8 exhibit a significant decreased endothelium-dependent and NO-mediated relaxation and increased vasoconstrictor responses that are potentiated by the lack of estrogen. Because these responses are closely in agreement with vascular dysfunction observed in menopausal women, we propose SAMP8 Ovx as a new model to concomitantly study the effects of aging and menopause in female mice.

1. INTRODUCTION

Aging is widely recognized as a risk factor for vascular disease and involves structural and functional alterations in vasculature. 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).

In woman, besides aging a decline of sex hormones production by menopause has been associated to increased risk of cardiovascular disease. Estrogen has been largely described as cardioprotective in both experimental and observational studies (Cano et al., 2007; Dantas and Sandberg, 2006). Cardiovascular protection by estrogen has been mostly associated to an increase of endothelium-derived vasodilator factors, including nitric oxide (NO) (Mendelsohn, 2009) and prostacyclin (Sobrino et al., 2010). Vasoconstrictor prostanoids, such as thromboxane A_2 (TXA2), have also been implicated in the phatophysiology of vascular dysfunction during estrogen withdrawn (Dantas et al., 1999; Dantas et al., 2004) and aging (Briones et al., 2005; Vanhoutte, 2009) and play an important role in the regulation of vascular tone in the normal systemic female vasculature (Fulton and Stallone, 2002), which involves the TXA2 receptor (TXA2R) (Li et al., 2008).

Senescence accelerated mice (SAM) is a murine model that has been widely used to study age-associated diseases. This model consists on a family of inbred strains with a common genetic background, originally derived from a colony of AKR/J mice, created as a result of selective breeding of mice showing a phenotype of severe exhaustion (SAM-Prone) and inbreeding of a normal phenotype (SAM-Resistant) (Takeda et al., 1981). Most of the clinical features exhibited by SAMP mice correspond to pathophysiological states found in aging humans. Vascular studies using these models

are not abundant, but few studies in male mice have shown morphological alterations, mechanical and endothelial dysfunction (Llorens et al., 2007).

In general, the animal models used to study the cardiovascular effects of estrogen withdrawn are often not associated to aging. In these studies, ovariectomy - the most used surgical model to induce menopause in mice – are performed at early age and may not reflects the physiological state of menopause in women. It is still unclear to what extent the protective effects of estrogen replacement described in young females can be extrapolated to older ones. Because SAM model offers several advantages to study cardiovascular aging in a convenient and standard time course, we have use female SAM to characterize a model of experimental menopause that closely reflects menopause in women. The induction of surgical menopause in an animal model of senescence will allow us to determine how aging affects vascular function and estrogen-mediated effects on nitric oxide and TXA2 responses in females. In this regard, changes in aortic responses from female SAMR (type 1 – SAMR1) and SAMP (type 8 – SAMP8) were determined in an attempt to assess the adequacy of these strains as experimental model to study vascular changes during aging and menopause.

2. MATERAL AND METHODS

2.1 Experimental animals

Female senescence-accelerated mouse-resistant 1 (SAMR1, *n*=30) and senescence-accelerated mouse-prone 8 (SAMP8, *n*=30) were obtained from the breeding stock at Parc Cientific de Barcelona and housed according to institutional guidelines (constant room temperature – 22º C, 12 h light/dark cycle, 60% humidity, standard mice chow and water ad libitum). All protocols were approved by the Institutional Ethics Committee at the University of Valencia, conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Both SAMR1 and SAMP8 were randomly separated at 5 months of age into three groups: 1) sham-operated (Sham); 2) ovariectomized (Ovx); and 3) ovariectomized chronically-treated with estrogen (Ovx + E2).

2.2 Surgical Procedures and Hormonal Treatment

Ovariectomy was performed under isoflurane anesthesia. Briefly, a single 1-cm dorsal midline incision was made in the skin and the ovaries were removed. At the moment of the surgery, half of the Ovx mice received estrogen replacement by subcutaneous implant of an osmotic pump (Model 2004, Alzet Osmotic Pumps®; Durect Corporation, Cupertino, CA, USA) containing 17β-estradiol in 50% DMSO at a dose of 10 µg/Kg/day (Ovx + E2). Untreated Ovx was implanted with a pump filled with vehicle only (50% DMSO in saline). Sham female mice were also included. To control the efficacy of ovariectomy and estrogen treatments, uterine weight was evaluated. Four weeks after surgical procedures, body weight was measured, and mice were euthanized.

2.3 Isolated mouse aorta preparation

Thoracic aorta was excised, placed immediately in ice cold Krebs-Henseleit solution and cleaned of surrounding tissue. Arteries were dissected into 4-mm rings, mounted between 2 stainless steel holders (100 μm inner diameter), and placed in a 4 ml tissue baths containing modified Krebs-Henseleit (in mM: NaCl 115; KCl 4.6; KH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25; glucose 11.1; EDTA 0.01, pH 7.3-74) at 37°C and aerated with 95% O_2/5% CO_2 for isometric force measurements (Grass FT03, Grass Instruments Division Astromed, Inc., West Warwick, RI, U.S.A.). In some experiments, the endothelium was mechanically removed by gently rubbing the arterial lumen with a roughened stainless-steel wire. Changes in isometric force were recorded by use of Chart *v.* 3.4/s software and a MacLab/8e data acquisition system (ADInstruments, East Sussex, U.K.). To establish the resting tension for maximal force development, a series of preliminary experiments was performed on arterial rings of similar length and outer diameter by repeatedly challenging them to 60 mM KCl. Basal tension was increased gradually until contractions reaches its maximal level (data not shown). Once the optimal resting tension was reached (1 g), aortic rings were allowed to attain a steady level of tension during a 2-hour equilibration period before testing. Functional integrity of the endothelium was confirmed routinely by the presence of relaxation induced by acetylcholine (10^{-6} M) during contraction obtained with serotonin (10^{-5} 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 the TXA2 mimetic U46619 (10^{-9} to $3x10^{-7}$ M). To test the endothelium-dependent responses, aortic rings were precontracted with serotonin (10^{-5} M) and cumulative relaxation curves to acetylcholine (10^{-9} to $3x10^{-6}$ M) were obtained in each ring. The responses to

acetylcholine were carried out in rings with and without endothelium. In another series of experiments, the preparations with endothelium were preincubated with L-NAME (10^{-4} M) 15 min prior to the concentration-responses curves to acetylcholine.

2.4 Immunofluorescence

Thoracic aorta from all groups of mice were excised and mounted in an optimum cutting temperature Tissue-Tek® OCT compound, mould, snap frozen in dry ice, and stored at –80°C. Arteries were sectioned (4 μm) and thaw mounted onto polylysine covered slides. Sections were fixed in acetone (15 min) and blocked for 30 min with horse serum. Sections were then incubated overnight at 4°C with 1:200 anti-TXA2R rabbit polyclonal antibody (sc-30036, Santa Cruz Biotech). The following day, the tissues were washed with PBS (3 x 5 min) and costained with phalloidin (10μM) and 1:500 Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen) at room temperature. The tissues were then washed with PBS (3 x 5 min) and coverslips were mounted on slides using ProLong Gold antifade reagent with DAPI (Invitrogen). Aortic sections were visualized through a fluorescent microscope (Olympus SX-31) with a 40X objective lens (Olympus) by using a digital color charge-coupled device (CCD) camera (Olympus). To elicit fluorescent images, tissue-mounted slides were illuminated with a 200-W mercury lamp. For each image, the light was passed through a different excitation filter: 1) 350nm (for DAPI); 2) 490 nm (for Alexa 488); and 3) 590 nm (for phalloidin). Each aorta was photographed in 3 different regions and results were expressed as an average of fluorescence elicited (using Mac Biophotonic ImageJ Software).

2.5 Drugs

The following drugs were used: serotonin hydrochloride, acetylcholine chloride, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) and 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) were obtained from Sigma (Sigma Chemical Co, St. Louis, MO, USA). All drugs for vascular reactivity studies were dissolved in Krebs solution except U46619, which was dissolved initially in ethanol, and further diluted in Krebs solution to the proper final concentration. 17β-estradiol (Sigma Chemical Co, St. Louis, MO, USA) was dissolved in 50% DMSO and saline. Stock solutions of each drug were freshly prepared at the day of experiment, and kept on ice throughout the experiment.

2.6 Data analysis

Data are expressed as means ± SEM. Contractions to KCl and U46619 are shown as absolute tension (mg). Relaxation is expressed as the percentage to the precontraction in response to serotonin (10^{-5} M). pD_2 (negative logarithm of the molar concentration at which half-maximum contraction occurs) was determined from individual concentration-response curves by non-linear regression analysis. Area under the concentration-response curve (AUC) was calculated from each individual concentration–response curve to acetylcholine and was expressed as arbitrary units. The contribution of NO to the vascular relaxation induced by acetylcholine was calculated by subtracting from the AUC for acetylcholine, the AUC for acetylcholine in the 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 treatment groups (i.e, Sham *vs.* Ovx *vs.* Ovx + E2) 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 Morphometry

Body weight of SAMP8 mice was significantly lower than that of SAMR1 (Figure 1A). In SAMR1, ovariectomy significantly increased body weight ($P<0.05$), an effect that was attenuated by estrogen treatment. On the other hand, neither ovariectomy or estrogen replacement affected body weight in SAMP8. Changes on uterine weight by ovariectomy and estrogen treatment were similar in both strains (Figure 1B). As expected, uterine weight decreased with ovariectomy ($P<0.05$), assuring the efficacy of the surgical process. Estrogen treatment prevented the loss in uterine mass by ovariectomy to similar levels as seen in Sham females. These data demonstrate the effectiveness of estrogen dose used to treat Ovx mice.

3.2 Effects of ovariectomy and estrogen treatment on contractile responses

Contractile responses to KCl (60 mM) were greater in aorta from SAMP8 mice compared with SAMR1 in all groups studied (Figure 2A). Neither ovariectomy nor estrogen treatment modified the contractile responses to KCl in aortic rings from SAMR1 mice. Conversely, in SAMP8, ovariectomy significantly increased the maximal contraction to KCl (Sham: 669 ± 25 vs Ovx: 831 ± 44 mg; $P<0.05$), an effect that was completely reversed by estrogen treatment (Figure 2A). Similarly, contractile responses to the TXA2 analogue U46619 were marked increased in all groups of SAMP8 in comparison to SAMR1 (Figure 2B and Table 1). In Sham SAMR1 mice, U46619 caused concentration-dependent contractions with a pD_2 of 7.92 ± 0.08 and maximal contraction of 1182 ± 67 mg (Figure 2B and Table 1). In SAMR1, neither maximal contraction nor pD_2 values to U46619 were affected by ovariectomy and estrogen treatment (Figure 2B and Table 1). In aortic rings of SAMP8 mice, maximal contraction

to U46619 was marked increased in Ovx group compared to Sham and Ovx + E2 groups (Figure 2B and Table 1).

3.3 Effects of ovariectomy and estrogen treatment on endothelium-dependent and nitric oxide (NO)-mediated relaxation.

In aortic rings with endothelium, all SAMR1 groups showed a greater relaxation to acetylcholine than SAMP8 groups (Figure 3A and Table 2). Despite that most groups have shown similar maximal relaxation, the sensitivity to acetylcholine (evidenced by pD_2 values) was marked increased in aortic rings from SAMR1 (Table 2). In SAMR1, endothelium-dependent relaxation was not altered by ovariectomy or estrogen treatment. On the other hand, aortic rings from Ovx SAMP8 showed a significantly decrease on maximal response to acetylcholine compared with those from sham-operated SAMP8 (Figure 3A and Table 2). Estrogen replacement restored the vasodilator response to acetylcholine in Ovx SAMP8 (Figure 3A and Table 2). The removal of endothelium and nitric oxide production blunted acetylcholine-induced relaxation in all groups studies (Table 2). However, when the areas under the curve were analyzed, there was a greater attenuation of the NO-mediated relaxation in all groups of SAMR1 mice in comparison to the respective SAMP8 groups (Figure 3B), suggesting a more pronounced NO production by young females. Ovariectomy aggravates the responses observed in aged animals, an effect that is improved by estrogen treatment. Interestingly, ovariectomy also induced an attenuation in NO-mediated responses in SAMR1, but to a much lower extent than the responses observed in SAMP8 (Figure 3B).

3.4 Effects of ovariectomy and estrogen treatment on aortic expression of thromboxane A_2 receptors (TXA2R).

Changes in the expression of TXA2R were determined in aortic rings by immunofluorescence analysis. Figure 4A shows representative immunofluorescent images of thoracic aorta from Sham SAMR1 (top) and Sham SAMP8 (bottom). Images represent staining of nucleus (blue, DAPI), actin fibers (red, phalloidin), TXA2R (green), and merged images. No significant differences were observed on phalloidin-induced fluorescence. As illustrated on Figure 4B, TXA2R expression is marked increased in aortic rings from SAMP8 in comparison to respective SAMR1 groups, although no significant differences were observed on Ovx+E2 groups. Ovariectomy increases TXA2R fluorescence in SAMP8, but not in SAMR1 mice. Despite a trend of increase observed in aortas from Ovx SAMR1, there was no statistical significance (Figure 4B). Estrogen treatment completely abolished ovariectomy-induced TXA2R up-regulation in SAMP8 and induced a slightly (yet not significant) decrease in SAMR1.

4. **DISCUSSION**

In these studies we provide a suitable murine model to simultaneously determine the effects of aging and estrogen withdrawn on vascular function, conditions that closely mimic menopause in women. As we have demonstrated, aging is associated with a decline in endothelium-dependent relaxation and an increase of vasoconstrictor responses in aorta of female senescent mice. Moreover, our findings support an important role for estrogen to regulate vascular function in aged, but not in young, blood vessels, suggesting that the protective effects of estrogen can be more noticeable in certain pathophysiological conditions in which some level of vascular damage has already occurred. This must be the case with aging.

Aging, per se, is known to cause a series of alterations in the endogenous mechanisms that control cardiovascular function leading to subsequent increase of risk of cardiovascular disease (Ferrari et al., 2003; Lakatta, 2003; Lakatta and Levy, 2003). In women, aging includes an aggravating risk factor in comparison to men. The decrease in estrogen production by menopause is thought to contribute to increased cardiovascular risk (Kannel, 2002). For this reason it is particularly difficult to distinguish what would be the contribution of aging and the lack of estrogen in the control of vascular function in menopausal women.

Although reproductive senescence and loss of ovarian function is a common phenomena among mammals, there are relevant differences between species (Wu et al., 2005). Contrary to women, the beginning of ovarian decline in rodents is characterized by periods of constant estrous, elevated levels of estrogen and lack of ovulation (Felicio et al., 1984; Lu et al., 1979). Only later in life, old rodents will experience low levels of estrogen and progesterone and little or no remaining ovarian follicle (Felicio et al., 1984; Lu et al., 1979). In this regard, the cardiovascular effects of estrogen in

experimental studies have been determined mostly in young ovariectomized females, while the increase of cardiovascular risk during estrogen withdrawn is observed mostly in middle aged women.

Unfortunately, little information is available on whether the vascular effects of estrogen are modified with aging in females. Few recent studies have addressed this issue using rodent models at middle and advanced age. In female rodents that normally do not exhibit cardiovascular complications, aging-associated changes in vascular function are not evident in middle aged animals, becoming more apparent only when animals are senile (LeBlanc et al., 2009; Lekontseva et al., 2010; Moien-Afshari et al., 2003). Others, conversely, have described significant effects of aging on vascular function in middle aged female rats with risk for cardiovascular disease (Fortepiani et al., 2003; Wynne et al., 2004). However, the fact that those animal models normally display increased risk for cardiovascular disease at early stage, when they are young adults, they may not represent a good model to determine aging- and menopause-associated vascular modifications in women.

In our studies we have used female senescent-accelerated mouse (SAM) model to determine the progressive effects of aging into the female vasculature. SAM is a murine model that has been increasingly used to study aging-associated diseases, as it ages fast and predictably, allowing experimental work to be performed in a convenient and standard time course (Llorens et al., 2007). Moreover, the absence of vascular damage at early age (Butterfield and Poon, 2005) establishes SAM as an optimal model to study vascular aging. Functional studies on vascular function system and the role of NO, the key component of cardiovascular regulation, have been recently performed in male SAM (Llorens et al., 2007). Our studies provide, for first time, a description of changes in vascular reactivity in female, using SAM as a model of aging.

Like other rodents, SAM mice do not reach reproductive senescence until later in life (Yuan et al., 2005). According to uterine weight data in Sham animals, we can suggest that at 6 months SAMR1 and SAMP8 exhibit similar hormonal status. Besides, vaginal smear performed for one week prior sacrifice have revealed no significant modification on estrous cycle on both strains (data not shown), confirming this hypothesis. For this reason, the SAM model still could not be considered, as such, as a good model to study natural menopause, although it is a good model to study progressive aging. In this regard, we have performed a surgical menopause on SAM mice by the time they start to be at middle age and to present impairment of vascular function (5-6 months).

Menopause in women has been largely associated with decreased endothelium-dependent and NO-mediated vasodilation (Taddei et al., 1996; Teede, 2007; Virdis et al., 2000). Because both aging (Erusalimsky, 2009) and changes on hormonal status during menopause (Miller and Duckles, 2008) has been described to modulate both NO production and endothelial function, it remains unclear the specific contribution of each factor to endothelial dysfunction during postmenopause. Our data show that aging per se induces a slightly decrease on the sensitivity to acetylcholine, an effect that is potentiated by the removal of ovaries. NO production was also decreased by aging, as evidenced by the degree in reduction in the area under the curve following L-NAME treatment. The fact that those effects were not as evident on SAMR1 Ovx, suggests an overlap between aging and hormonal status to control endothelial function.

Besides NO, vasoconstrictor prostaglandins have also been implicated in the phatophysiology of vascular dysfunction during aging (Briones et al., 2005; Vanhoutte, 2009) and estrogen withdrawn (Dantas et al., 1999; Dantas et al., 2004). In our studies we have observed a significant increase on vasoconstriction induced by the TXA2 analog U-46619 in SAMP8 that was aggravated by ovariectomy. In a series of

experiments using immunofluorescence to determine the expression of TXA2R, we could observe pattern of modulation of these receptors by both aging and ovariectomy in SAM aorta that corroborates the data on vascular reactivity. However, the fact that contractions to KCl showed similar pattern of responses indicate that intrinsic mechanism, that are not dependent on TXA2R up-regulation, also contribute to the altered vasoconstrictor responses observed herein. KCl-induced constriction is mediated primarily via depolarization-induced opening of voltage-gated Ca^{2+} channels and influx of extracellular Ca^{2+}. Accordingly, the ability of accelerated senescence and ovariectomy in SAMP8 mice to increase constrictor responses to KCl is suggestive of an action on voltage-gated Ca^{2+} channels or distal mechanisms that respond to this influx of Ca^{2+}.

Estrogen has shown to have protective effects by modulating NO production and prostaglandin-mediated contraction in several experimental and clinical studies - for review see (Tostes et al., 2003). In contrast, recent clinical trials have questioned the value of estrogen replacement therapy (ERT) in protecting against vascular disease (Rossouw et al., 2002). The surprising results of those trials have been in part explained by the fact that those studies were primarily carried out in aged women, who were on average, 10 years past the onset of menopause, and therefore may not benefit from ERT anymore. The so-called “Timing Hypothesis” suggests that estrogen-mediated benefits to the cardiovascular system may occur only before the detrimental effects of aging are established in the vasculature (Harman, 2006). In order to address this important issue we have further treated our menopause model (SAM-Ovx) with estrogen for 4 weeks. In those studies, we have found that estrogen replacement restored both vasodilator and vasoconstrictor responses altered by ovariectomy, but did not improve aging-associated responses. Our data are not in agreement with few studies describing that aging

negatively affects estrogen-mediated responses in distinct rodent models (Berezan et al., 2008; Fortepiani et al., 2003; Wynne et al., 2004). These discrepancies may be explained by the fact that those animals were studied at a more advanced age or in the presence of an established cardiovascular disease, while we have determined the effects of estrogens on middle aged female that have not reached a high level of vascular damage. With our data so far, we can not determine if estrogen-mediated responses could be affected by a more advanced age in SAMP8 animals. This hypothesis is of great interest in the field and must be addressed in further studies.

4.1 Conclusions

In summary, our studies have established a role of aging and estrogen withdrawn to impair vascular function in female mice. At age of six months (middle age) SAMP8 exhibit a significant decrease of endothelium-dependent and NO-mediated relaxation, at the same time as increase vasoconstrictor responses. Although aging per se has a detrimental effect in the vasculature of middle aged female, these effects are potentiated by the lack of estrogen, and restored by estrogen replacement. Because those alterations are closely in agreement with vascular dysfunction observe in menopausal women, we propose SAMP8 Ovx as a new model to concomitantly study the effects of aging and menopause in female mice.

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Research Highlights

SAMP8 ovariectomized mice is a new model to study the effects of aging and menopause

SAMP8 exhibit a decrease of endothelium-dependent NO-mediated relaxation

SAMP8 show an increased contraction to U46619 that is aggravated by ovariectomy.

In SAMP8 aging effects in the vasculature are potentiated by the lack of estrogen

Detrimental effect induced by ovariectomy in SAMP8 are restored by estrogens

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FIGURE LEGENDS

Figure 1. Figures present body weight (A) and uterine weight (B) of sham operated (Sham), ovarectomized (Ovx) and ovarectomized treated with estradiol (Ovx + E2) SAMR1 and SAMP8 mice. Bars indicate mean ± SEM (n = 10 animals per group; * P < 0.05 *vs.* same treatment of SAMR1 mice, # P < 0.05 *vs.* sham-operated groups, and § P < 0.05 *vs.* Ovx groups).

Figure 2. Contractile responses in aortic rings from sham-operated (Sham), ovarectomized (Ovx) and ovarectomized treated with estradiol (Ovx + E2) SAMR1 and SAMP8 mice. (A) represents maximal contraction to KCl 60mM and (B) concentration-response curves to U-46619. Bars graphs (A) indicate mean ± SEM of maximal contraction to KCl (n = 10 animals per group); each data point (B) show mean ± SEM from n=10 mice for each group. * P < 0.05 vs. same treatment of SAMR1 mice, and # P < 0.05 vs. sham-operated and Ovx + E2 SAMP8 groups.

Figure 3. Concentration-response curves to acetylcholine (A) and NO-dependent relaxation to acetylcholine (B) in aortic rings from sham-operated, Ovx, and Ovx + E2 groups from SAMR1 and SAMP8 mice. Each data point (A) show mean ± SEM from n=10 mice for each group. Bars graphs (B) indicate mean ± SEM of differences between area under curve (AUC) for acetylcholine-induced relaxation in the absence and AUC in the presence of L-NAME 10^{-4} M (n = 10 animals per group). * P < 0.05 vs. same treatment of SAMR1 mice, and # P < 0.05 vs sham-operated groups.

Figure 4. Thromboxane A_2 receptor (TXA2R) expression in thoracic aorta from SAMR1 and SAMP8 mice: **(A)** representative immunofluorescent images of aorta from Sham SAMR1 (top) and Sham SAMP8 (bottom). Images represent staining of nucleus (blue, DAPI), actin fibers (red, phalloidin), TXA2R (green) and merged images. **(B)** Bar graphs show the results of densitometric analyses from pooled data of Sham operated (Sham), ovariectomized (Ovx) and ovarectomized treated with estradiol (Ovx + E2) SAMR1 and SAMP8 mice. Each data represents the mean ± SEM derived from six independent experiments. * $P < 0.05$ vs. same treatment of SAMR1 mice, # $P < 0.05$ vs. sham-operated groups, and § $P < 0.05$ vs. Ovx groups.

Table 1. pD_2 values and maximal contractions (Emax) to U46619 in aortic rings from sham-operated (Sham), ovariectomized (Ovx) and ovariectomized plus estradiol (Ovx +E2) groups

	SAMR1		**SAMP8**	
	pD_2	Emax (mg)	pD_2	Emax (mg)
Sham (*n*=10)	7.92 ± 0.08	1182 ± 67	8.16 ± 0.08*	1626 ± 69*
Ovx (*n*=10)	7.86 ± 0.04	1213 ± 76	8.13 ± 0.02*	1953 ± 46*#
Ovx+E2 (*n*=10)	7.89 ± 0.05	1176 ± 81	8.20 ± 0.05*	1548 ± 44*

Values are means ± SEM. *n* number of mice. * $p < 0.05$ *vs*. SAMR1 group with same treatment, and # $p < 0.05$ *vs*. Sham and Ovx + E2 groups of SAM-P8 mice.

Table 2. pD_2 values and maximal responses (Emax) to acetylcholine in aortic rings with endothelium, without endothelium and with endothelium pretreated with L-NAME (10^{-4} M) from sham-operated (Sham), ovariectomized (Ovx) and ovariectomized plus estradiol (Ovx+E2) groups.

	SAMR1		SAMP8	
	pD_2	Emax (%)	pD_2	Emax (%)
Sham				
With endothelium (*n*=10)	8.3 ± 0.1	95 ± 3	7.8 ± 0.3*	94 ± 3
Without endothelium (*n*=6)	†	4 ± 1	†	3 ± 1
With L-NAME (10^{-4} M) (*n*=6)	†	2 ± 1	†	3 ± 1
Ovx				
With endothelium (*n*=10)	8.1 ± 0.1	96 ± 2	7.2 ± 0.2*#	84 ± 4*#
Without endothelium (*n*=6)	†	6 ± 2	†	5 ± 1
With L-NAME (10^{-4} M) (*n*=6)	†	4 ± 1	†	3 ± 1
Ovx + E2				
With endothelium (*n*=10)	8.1 ± 0.1	97 ± 2	7.7 ± 0.1*	96 ± 2
Without endothelium (*n*=6)	†	7 ± 1	†	6 ± 1
With L-NAME (10^{-4} M) (*n*=6)	†	5 ± 1	†	5 ± 1

Values are means ± SEM. *n* number of mice. Relaxation was expressed as a percentage of the serotonin-induced contraction. † pD_2 value was not calculated as the maximum relaxation to acetylcholine was less than 10%. * $p < 0.05$ *vs.* SAMR1 group with same treatment, and # $p < 0.05$ *vs.* Sham and Ovx + E2 groups of SAMP8 mice.

Figure 1

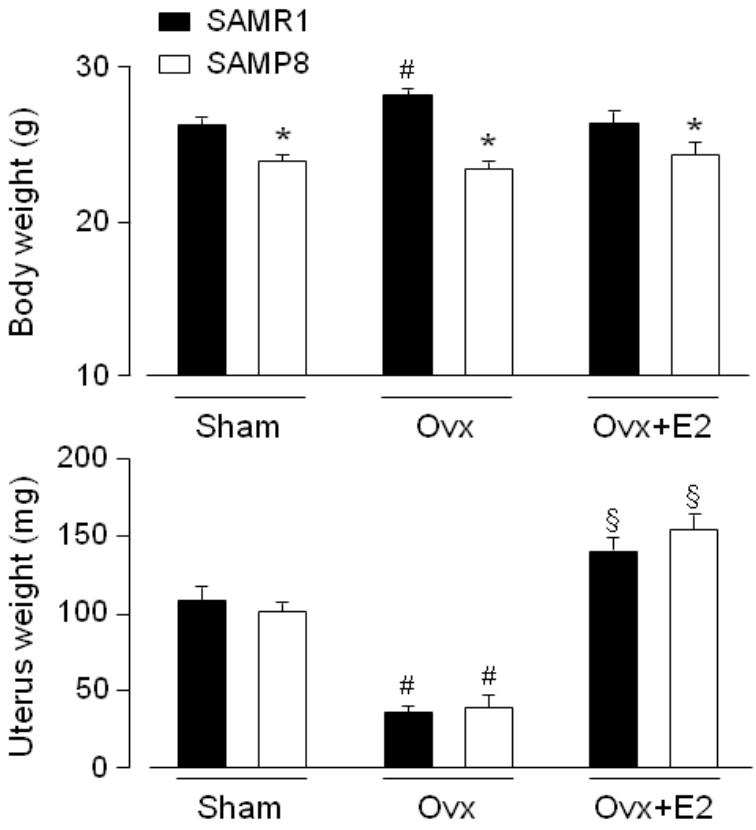

Figure 2

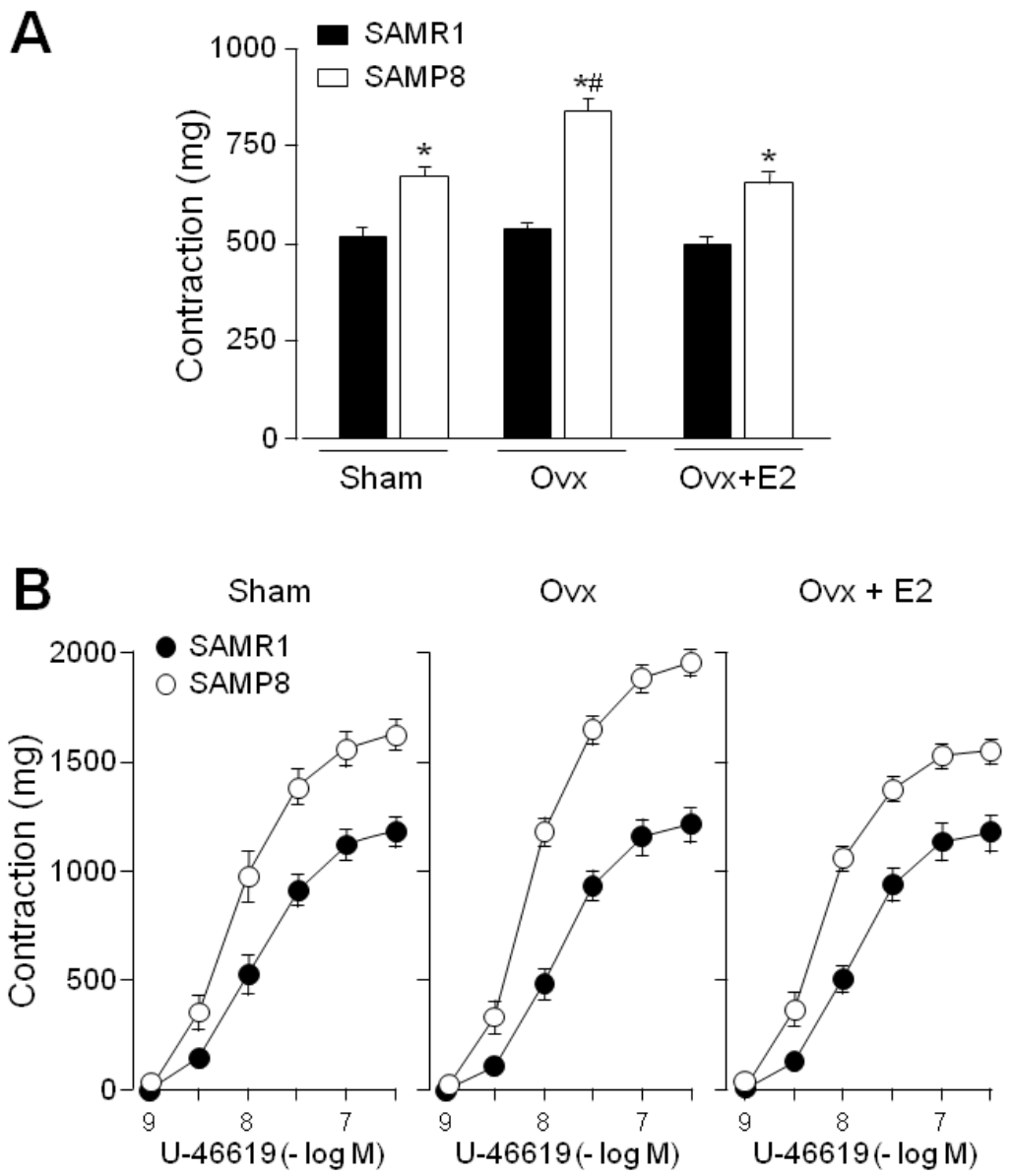

Figure 3

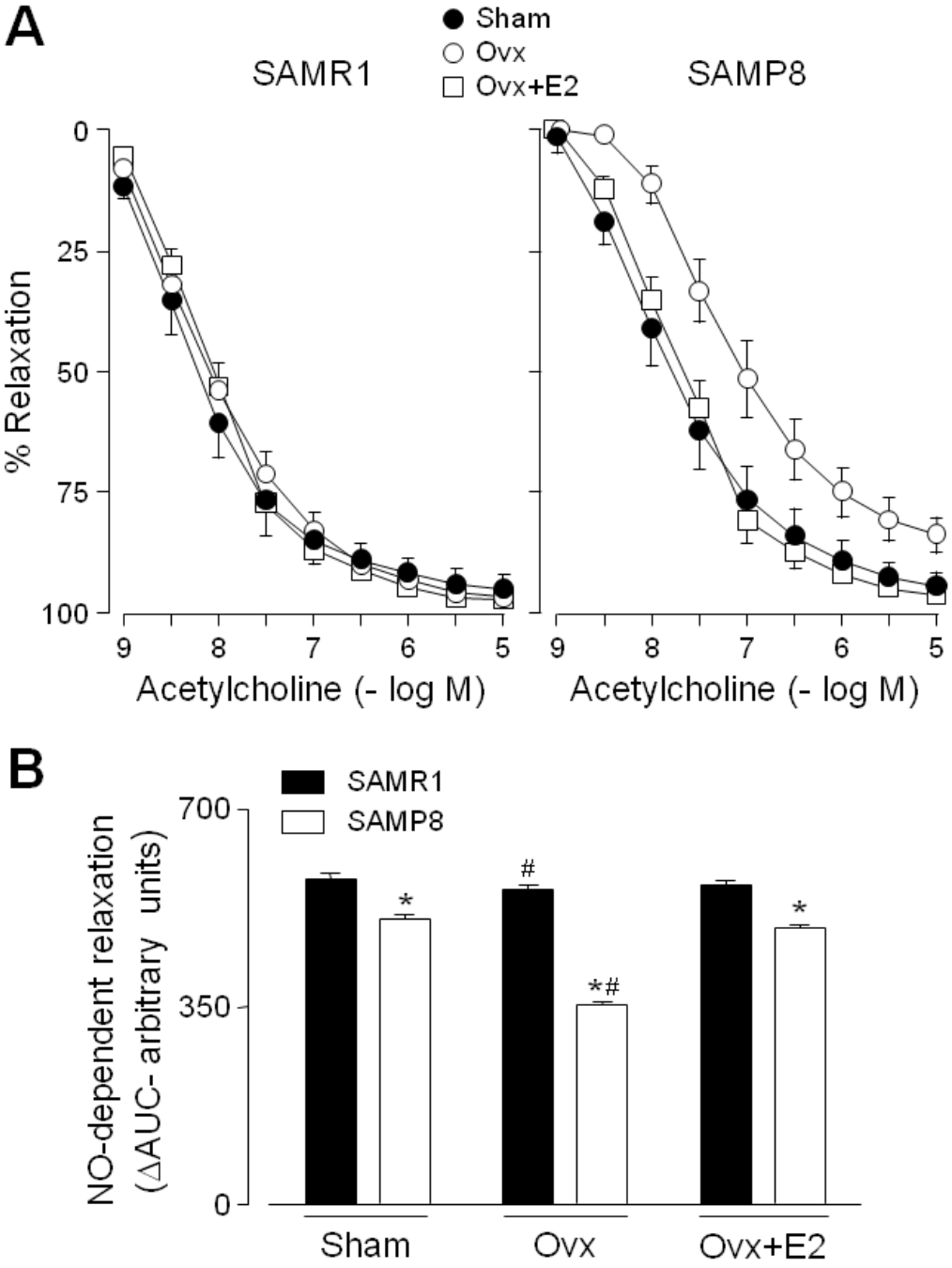

Figure 4