STIM1/Orai1 contributes to sex differences in vascular responses to calcium in spontaneously hypertensive rats

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ABSTRACT

Sex differences in Ca^{2+}-dependent signalling and homoeostasis in the vasculature of hypertensive rats are well characterized. However, sex-related differences in SOCE (store-operated Ca^{2+} entry) have been minimally investigated. We hypothesized that vascular protection in females, compared with males, reflects decreased Ca^{2+} mobilization due to diminished activation of Orai1/STIM1 (stromal interaction molecule 1). In addition, we investigated whether ovariectomy in females affects the activation of the Orai1/STIM1 pathway. Endothelium-denuded aortic rings from male and female SHRSP (stroke-prone spontaneously hypertensive rats) and WKY (Wistar–Kyoto) rats and from OVX (ovariectomized) or sham female SHRSP and WKY rats were used to functionally evaluate Ca^{2+} influx-induced contractions. Compared with females, aorta from male SHRSP displayed: (i) increased contraction during the Ca^{2+}-loading period; (ii) similar transient contraction during Ca^{2+} release from the intracellular stores; (iii) increased activation of STIM1 and Orai1, as shown by the blockade of STIM1 and Orai1 with neutralizing antibodies, which reversed the sex differences in contraction during the Ca^{2+}-loading period; and (iv) increased expression of STIM1 and Orai1. Additionally, we found that aortas from OVX-SHRSP showed increased contraction during the Ca^{2+}-loading period and increased Orai1 expression, but no changes in the SR (sarcoplasmic reticulum)-buffering capacity or STIM1 expression. These findings suggest that augmented activation of STIM1/Orai1 in aortas from male SHRSP represents a mechanism that contributes to sex-related impaired control of intracellular Ca^{2+} levels. Furthermore, female sex hormones may negatively modulate the STIM/Orai1 pathway, contributing to vascular protection observed in female rats.

INTRODUCTION

The coupling process between the ER (endoplasmic reticulum) and plasma membrane to mediate SOCE (store-operated Ca^{2+} entry) remained a mechanistic mystery until the recent discovery of STIM1 (stromal interaction molecule 1). The Ca^{2+} sensor STIM1 was identified by two independent research groups as

Key words: aorta, calcium, hypertension, Orai1, stromal interaction molecule 1 (STIM1), sex difference, vascular protection.

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; ACh, acetylcholine; CRAC, Ca^{2+} release-activated Ca^{2+}; EMEM, Eagle's minimum essential medium; ER, endoplasmic reticulum; OVX, ovariectomized; PE, phenylephrine; PSS, physiological salt solution; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; SHRSP, stroke-prone SHR; SOCE, store-operated Ca^{2+} entry; SR, sarcoplasmic reticulum; STIM1, stromal interaction molecule 1; VSMC, vascular smooth muscle cell; WKY, Wistar–Kyoto.

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an essential component of the Ca^{2+}-store-depletion-triggered Ca^{2+} influx [1,2]. Later, it was demonstrated that, in addition to being an ER Ca^{2+} sensor, STIM1 functions within the plasma membrane to control the operation of Ca^{2+} entry through CRAC (Ca^{2+} release-activated Ca^{2+}) channels [3]. These observations were better understood after the report of a new plasma membrane protein Orai1 [also known as CRACM1 (CRAC modulator 1)]. It was shown that Orai1 is essential for SOCE [4]. Accordingly, STIM1 and Orai1 accumulate and co-localize in specific areas where the ER comes into close proximity to the plasma membrane, the so-called puncta formations [5]. Orai1 gathers at discrete sites in the plasma membrane directly opposite to STIM1, resulting in local CRAC channel activation [6]. Mutation of Orai1 in patients with a hereditary severe combined immune deficiency syndrome results in defective CRAC channel function [7].

Ca^{2+} plays a central role in vascular contraction. Abnormalities in Ca^{2+} handling have been implicated in the increased response to constrictor stimuli and augmented myogenic tone in VSMCs (vascular smooth muscle cells) in hypertension [8–11]. We have reported previously that augmented activation of STIM/Orai is a contributing mechanism that leads to impaired control of intracellular Ca^{+2} levels in hypertension [12].

Sex-associated differences in hypertension have been repeatedly observed in epidemiological studies. However, the mechanisms conferring vascular protection in females, compared with males, are not completely elucidated. Because Ca^{+2} triggers VSMC contraction and its regulation is highly controlled, differences in Ca^{2+}-handling mechanisms have been proposed to explain sex-related differences in vascular function in hypertension [13,14].

Therefore we hypothesized that vascular protection in females reflects decreased Ca^{2+} mobilization due to lower activation of Orai1/CRAC channels, via its interaction with the intracellular Ca^{2+} sensor STIM1. In addition, we investigated whether ovariectomy affects activation of the Orai1/STIM1 pathway.

MATERIALS AND METHODS

Animals

Male and female SHRSPs [stroke-prone SHR (spontaneously hypertensive rats)] at 5–6 months of age were obtained from the breeding colony at Michigan State University, East Lansing, MI, U.S.A. Age-matched male and female WKY (Wistar–Kyoto) rats were purchased from Harlan. Rats were maintained on a 12-h light/dark cycle, housed two per cage and allowed access to normal chow and free water intake. SBP (systolic blood pressure) was measured in non-anaesthetized animals by tail cuff using a RTBP1001 blood pressure system (Kent Scientific).

All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education.

Ovariectomy

Ovariectomy was performed in 6-month-old WKY rats and SHRSP. Briefly, under aseptic conditions, rats were anaesthetized with isoflurane (5 % in O_2). A small incision was made in the lower abdomen, and both ovaries were removed [OVX (ovariectomized) rats] or not (sham rats). After 12 weeks, SBP was assessed. Body and uterus (dry) weight were evaluated, and blood was collected to measure hormone levels by RIA (Diagnostic Systems Laboratories) in order to confirm the effectiveness of ovariectomy.

Vascular functional studies

After the rats were killed by CO_2 inhalation, thoracic aortas were rapidly excised and placed in ice-chilled (≈4 °C) PSS (physiological salt solution; 130 mM NaCl, 14.9 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4{\cdot}7H_2O$, 5.5 mM glucose, 1.56 mM $CaCl_2{\cdot}2H_2O$ and 0.026 mM EDTA). Segments of thoracic aorta were carefully dissected and the endothelium was removed by gently rubbing the lumen side of the vessels with a metallic pin. Aortic rings (4 mm in length) were mounted on two stainless steel wires in standard organ chambers (model 610M; Danish MyoTech) for isometric force recording, as described previously [15,16]. After stabilization, arterial integrity was assessed first by stimulation of vessels with 120 mM KCl and, after washing and a new stabilization period, by contracting the segments with PE (phenylephrine; 1 μM). Endothelium denudation was assessed by the absence of a relaxation response to ACh (acetylcholine; 1 μM) during the PE-induced contraction. At the end of the experimental protocol, aortas were allowed to stabilize in PSS for 10 min and then contracted with 120 mM KCl in order to evaluate membrane integrity. No significant differences between initial and final KCl-induced contractions were observed.

Experimental protocol

Force development in response to a Ca^{2+}-handling experimental protocol was evaluated in aortas from both rat groups, as described in Figure 1. Briefly, aortic rings were contracted with PE (1 μM). When the contraction reached a plateau, aortas were washed in Ca^{2+}-free EGTA buffer for 20 min in order to deplete intracellular Ca^{2+} stores (depletion period). During the depletion period, aortas were incubated with vehicle or thapsigargin (10 μM; a selective Ca^{2+}-ATPase inhibitor) and some rings were treated with non-selective CRAC channel blockers 2-APB (2-aminoethoxydiphenyl borate; 100 μM)

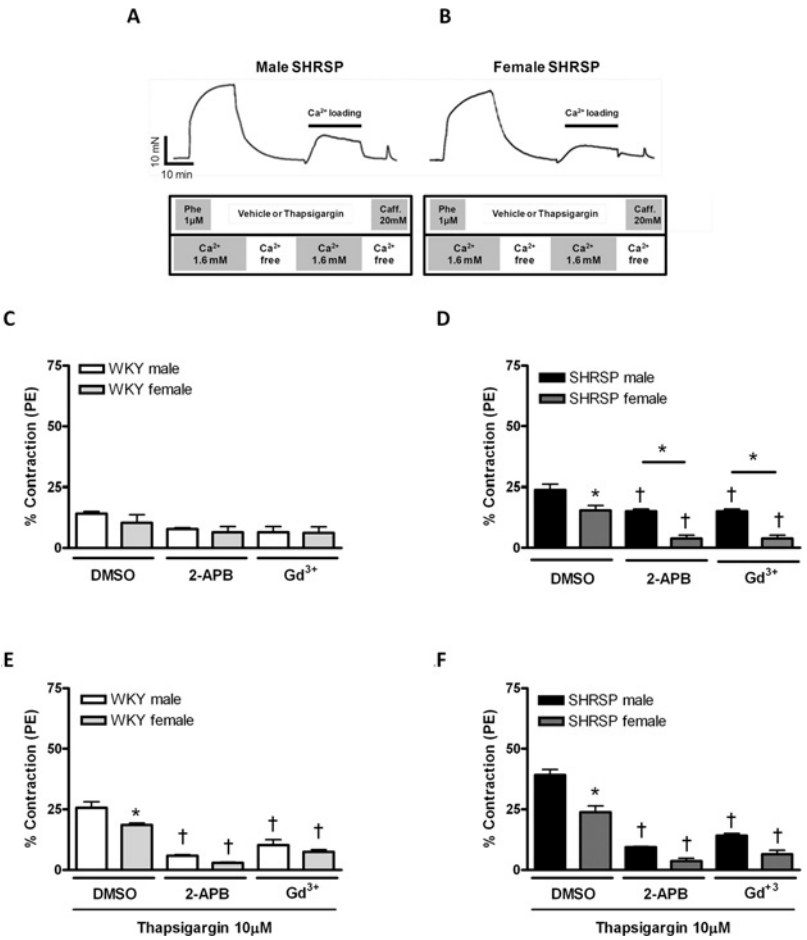

Figure 1 Increased contraction during the Ca^{2+}-loading period in aortas from male compared with female SHRSP is normalized after Orai1/CRAC channel inhibition

(A and B) The tracings illustrate the protocol used to evaluate force in response to Ca^{2+} influx after depletion of intracellular Ca^{2+} stores (loading period) in aortas from (A) male and (B) female SHRSP. Aortic rings were contracted with PE (1 μM) and then washed in Ca^{2+}-free EGTA buffer in the presence or absence of thapsigargin (10 μM). After 20 min, 1.6 mM Ca^{2+} buffer was added and the aortic rings were incubated for an additional 15 min without or with thapsigargin. Aortic rings were washed with Ca^{2+}-free EGTA buffer for 2 min and then stimulated with caffeine (20 mM). This protocol was performed in aortas from (C and E) male (white bars) and female (grey bars) WKY rats or in aortas from (D and F) male (black bars) or female (hatched bars) SHRSP in the (C and D) absence or in the (E and F) presence of thapsigargin. Note that the contractions were significantly inhibited after CRAC channel blockade with 2-APB and Gd^{3+}. Values are means $\pm$ S.E.M. $^*P < 0.05$ compared with the respective male rats; $\dagger P < 0.05$ compared with respective DMSO vehicle.

and Gd^{3+} (100 μM). After Ca^{2+} depletion, intracellular Ca^{2+} stores were loaded (Ca^{2+}-loading period) by placing aortas in 1.6 mM Ca^{2+} buffer, for 15 min, and the contractile responses were determined. The bathing medium was then replaced with Ca^{2+}-free buffer and, after 2 min, the aortic segments were stimulated with caffeine (20 mM) in order to release intracellular Ca^{2+} stores, which resulted in a transient contraction [15,16].

Antibody delivery using the Chariot™ technique

Antibodies against STIM1 and Orai1 (ProSci) were intracellularly delivered using the Chariot™ technique (Chariot™ Protein Delivery Reagent; Active Motif). This transfection reagent is able to deliver antibodies into cells while preserving their ability to localize to the proper cellular compartment and to recognize antigens within the cell [17–19]. Chariot™–antibody complexes were prepared and used according to the manufacturer's instructions. Briefly, aortic rings were incubated in EMEM (Eagle's minimum essential medium) containing 1 % L-glutamine, 10 % fetal bovine serum, 0.5 % penicillin and 0.5 % streptomycin for 30 min at 37 °C. For each transfection, 12 μl of Chariot™ in 100 μl of 40 % DMSO was mixed with 6 μg of antibody in 100 μl of PBS and incubated at room temperature (25–28 °C) for 30 min to allow the complex to form. Aortas were transferred to a sterile 24-well cell culture plate, overlaid with 200 μl of

Chariot™–antibody complex and mixed gently. EMEM (400 μl) was added and the tissues were incubated for 1 h at 37 °C. Then, EMEM medium (750 μl) was added and the tissues were incubated further for 2 h at 37 °C. After this period, rings were mounted on the myograph and functional studies were performed. Chariot™–anti-(mouse IgG) complexes were incubated with aortas and used as additional controls, but they did not produce any significant effects compared with empty Chariot™ (results not shown).

Western blot analysis

Aortas from hypertensive and control rats were isolated, cleaned of fat and endothelium-denuded prior to freezing in liquid nitrogen. Extracted proteins (40 μg) were separated by electrophoresis on a 10 % polyacrylamide gel and transferred on to a nitrocellulose membrane. Non-specific-binding sites were blocked with 5 % skimmed milk in Tris-buffered saline with 0.1 % Tween 20 for 1 h at 24 °C. Membranes were then incubated with antibodies overnight at 4 °C. Antibodies were as follows: anti-STIM1 and anti-Orai1 (1:1000 dilution; ProSci) and β-actin (1:1000 dilution; Sigma–Aldrich). Human ovary and mouse thymus tissue lysates (ProSci) were used as positive controls for STIM1 and Orai1 respectively. After incubation with secondary antibodies, signals were revealed with chemiluminescence and quantified densitometrically. Results were normalized by β-actin expression and are expressed as units of change from the control.

Drugs and solutions

2-APB, Gd^{3+}, PE, thapsigargin and ACh were purchased from Sigma–Aldrich. All reagents were of analytical grade. Stock solutions were prepared in deionized water. Control solutions containing vehicle (DMSO) were also used throughout the experimental protocols.

Data analysis

Results are means ± S.E.M. Contractions during the Ca^{2+}-loading period and upon caffeine stimulation were recorded as changes in the displacement (mN) from baseline, and are expressed as a percentage from the initial PE-induced contraction. Statistically significant differences were calculated using a two-way ANOVA with a Bonferroni post-hoc test. $P < 0.05$ was considered significant.

RESULTS

Sex differences in SBP

At 24 weeks, no differences were observed in SBP between male WKY rats and female WKY rats (116 ± 3 and 110 ± 2 mmHg respectively, $n = 8$). However, male SHRSP displayed higher SBPs in comparison with female SHRSP (234 ± 10 and 168 ± 4 mmHg respectively, $n = 8$). Additionally, both male and female SHRSP had higher SBPs when compared with male and female WKY rats respectively.

Sex differences in force development during the Ca^{2+}-loading period

Figures 1(A) and 1(B) illustrate the protocol used to evaluate (i) the contractile response during the Ca^{2+} loading, generated as a consequence of the intracellular Ca^{2+} stores depletion; and (ii) the transient contraction induced by caffeine, which reflects the functional capacity of the SR (sarcoplasmic reticulum) to release Ca^{2+} (see the legend of Figure 1 for further details).

PE-induced contraction was similar between aortas from male WKY rats and female WKY rats (20.2 ± 0.9 and 16.9 ± 1.8 mN respectively, $n = 6$). However, contraction in response to PE was increased in aortas from male SHRSP compared with female SHRSP (24.4 ± 1.2 and 18.5 ± 2.3 mN, respectively, $n = 6$). Contractile responses induced during theCa^{2+}-loading period or upon caffeine stimulation are represented as a percentage of the initial PE-induced contraction.

During the Ca^{2+}-loading period, contraction was not changed in aortas from male WKY rats and female WKY rats (11.3 ± 2.4 and 11.78 ± 0.3 % respectively, $n = 6$). Pre-incubation with 2-APB (100 μM) or Gd^{3+} (100 μM), used to inhibit CRAC channels, did not affect contraction induced during the Ca^{2+}-loading period in aortas from either male or female WKY rats (Figure 1C).

Contraction-induced by Ca^{2+} loading was greater in aortas from male SHRSP compared with female SHRSP (23.9 ± 2.1 compared with 15.9 ± 2.2 % respectively, $n = 6$). CRAC channel blockade with 2-APB or Gd^{3+} significantly inhibited contractions in aortas from both male and female SHRSP (Figure 1D). However, sex differences observed before CRAC channel inhibition persisted even after 2-APB and Gd^{3+} incubation (Figure 1D).

Thapsigargin (10 μM) was used to inhibit the SR Ca^{2+}-ATPase and promote depletion of intracellular Ca^{2+} stores. This pharmacological approach resulted in continuous stimulation of the SR Ca^{2+} sensor STIM1 and, consequently, amplified the activation of SOCE through CRAC channels. Accordingly, after thapsigargin incubation, increased contraction was observed during the Ca^{2+}-loading period in all of the experimental groups.

After thapsigargin incubation, augmented contraction was observed in aortas from male WKY rats compared with female WKY rats (25.6 ± 2.4 compared with 18.6 ± 0.7 % respectively, $n = 6$). In the presence of thapsigargin, CRAC channel blockade with 2-APB or Gd^{3+} resulted in a significant reduction in contraction in aorta from both male and female WKY rats (Figure 1E).

Thapsigargin resulted in a greater contractile response in aortas from male SHRSP compared with female

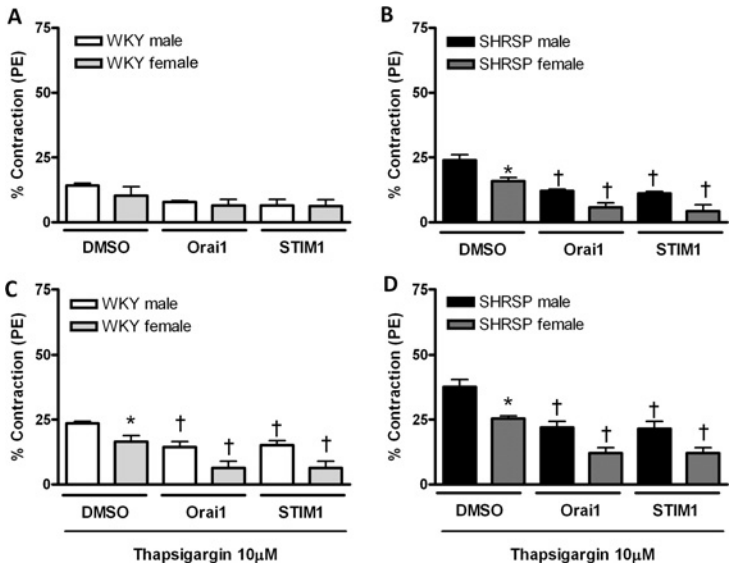

Figure 2 Neutralizing antibodies against STIM1 and Orai1 prevented sex differences in contractile responses during the Ca^{2+}-loading period in aortas from SHRSP

Contractions during the Ca^{2+}-loading period were evaluated in aortas from (A and C) male (white bars) and female (grey bars) WKY rats or in aortas from (B and D) male (black bars) or female (hatched bars) SHRSP in the (A and B) absence or in the (C and D) presence of thapsigargin. Values are means ± S.E.M. $^*P < 0.05$ compared with the respective male rats; †$P < 0.05$ compared with the respective DMSO vehicle.

SHRSP during the Ca^{2+}-loading period (39.2 ± 2.2 compared with 23.8 ± 2.5 % respectively). Simultaneous incubation of thapsigargin with 2-APB or Gd^{3+} reduced contraction during the Ca^{2+}-influx period in aortas from male and female SHRSP, and abolished sex differences within these groups (Figure 1F).

Because 2-APB or Gd^{3+} can be used to block other store-operated Ca^{2+} channels, we used transfection with Orai1 or STIM1 antibodies as a strategy to neutralize these enzymes and to evaluate better the role of Orai1-CRAC/STIM1 during the Ca^{2+}-loading period.

Neutralizing antibodies against Orai1 or STIM1 did not modify contractile responses in aortas from male or female WKY rats during the Ca^{2+}-loading period (Figure 2A). However, antibodies against Orai1 and Stim1 resulted in decreased contraction during the Ca^{2+}-loading period in aortas from male and female SHRSP. In addition, after transfection of the antibodies, sex differences observed in aortas from SHRSP were abolished (Figure 2B).

Simultaneous incubation of thapsigargin with anti-Orai1 or anti-STIM1 antibodies reduced contraction during the Ca^{2+}-loading period in aortas from male and female WKY rats. Furthermore, sex differences in aortas from WKY rats, observed after thapsigargin incubation, were abolished by the neutralizing antibodies (Figure 2C). Similar results were observed when aortas from male and female SHRSP were simultaneously incubated with thapsigargin and transfected with neutralizing antibodies (Figure 2D).

Intracellular Ca^{2+} store depletion in male and female SHRSP

Caffeine stimulation (20 mM) was used to evaluate the SR Ca^{2+}-buffering capacity. Aortas from male WKY rats and female WKY rats displayed similar contractile responses upon caffeine stimulation (13.2 ± 0.5 and 12.1 ± 2.0 % respectively, $n = 6$). Neutralizing antibodies did not affect caffeine-induced contraction in aortas from either male or female WKY rats (Figure 3A).

No sex differences were observed in the contractile responses induced by caffeine in aortas from male or female SRHSP (21.2 ± 0.3 and 20.0 ± 1.7 % respectively, $n = 6$). Neutralizing antibodies against Orai1 and STIM1 resulted in reduced contractions in response to caffeine stimulation in aortas from both male and female SHRSP (Figure 3B).

When caffeine stimulation was performed in the presence of thapsigargin, contractions were almost abolished in all of the experimental groups and no differences among the groups were observed. In this condition, transfection of Orai1 or STIM1 antibodies had no further effect (results not shown).

Sex differences in Orai1 and STIM1 expression

Aortas from male SHRSP and male WKY rats displayed augmented levels of Orai1 when compared with their respective female groups. Additionally, Orai1 protein expression was augmented in aortas from male SHRSP compared with male WKY rats (Figure 4A).

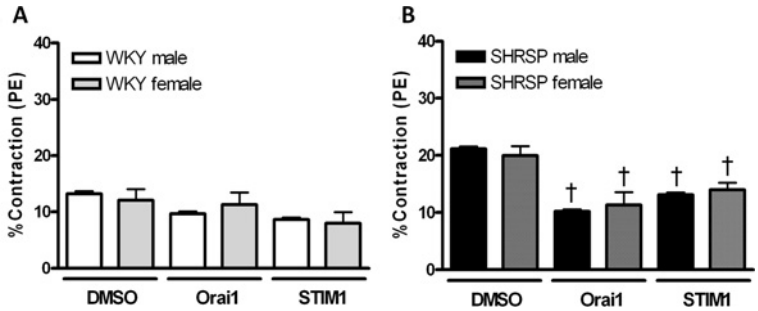

Figure 3 No sex differences in Ca^{2+} release from the SR upon caffeine stimulation in aortas from SHRSP or WKY rats

Transient contractions upon caffeine stimulation, after incubation in Ca^{2+}-free buffer, were measured in aortas from (A) male (white bars) and female (grey bars) WKY rats or in aortas from (B) male (black bars) or female (hatched bars) SHRSP rats. Values are expressed as means ± S.E.M. †$P < 0.05$ compared with the respective DMSO vehicle.

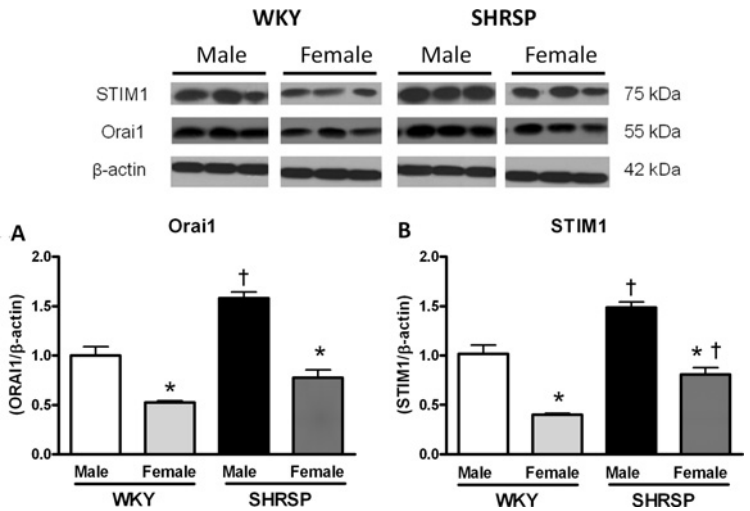

Figure 4 Aortas from male SHRSP display increased Orai1 and STIM1 protein levels compared with those from female SHRSP

Upper panels, representative Western blot images; lower panels, quantification of the Western blots demonstrating that (A) Orai1 and (B) STIM1 proteins are augmented in aortas from male SHRSP compared with male WKY rats or female SHRSP. Densitometric analyses were performed and values were normalized to β-actin protein expression. *$P < 0.05$ compared with the respective control; †$P < 0.05$ compared with the respective male rats.

Augmented levels of STIM1 were observed in male SHRSP and male WKY rats when compared with their respective female groups. Hence STIM1 protein expression was increased in aortas from male and female SHRSP compared with male and female WKY rats (Figure 4B).

Effect of ovariectomy on SBP, body weight and sex hormones

After 12 weeks of ovariectomy, the SBP was increased in female OVX-WKY rats compared with female sham-WKY rats (128 ± 3 compared with 113 ± 3 mmHg respectively, $n = 6$), as well as in female OVX-SHRSP compared with female sham-SHRSP (206 ± 2 compared with 184 ± 4 mmHg respectively, $n = 6$).

Body weight was augmented after ovariectomy in both female OVX-WKY rats and female OVX-SHRSP (256 ± 3 g and 238 ± 5 g respectively, $n = 6$) compared with their respective sham groups (238 ± 5 g in sham-WKY rats and 218 ± 5 g in sham-SRSHP, $n = 6$; $P < 0.05$).

After ovariectomy, uterus weight was reduced in the female OVX-SHRSP compared with female sham-SHRSP (0.053 ± 0.009 compared with 0.118 ± 0.009 g respectively, $n = 6$; $P < 0.001$) and in female OVX-WKY rats compared with female sham-WKY rats (0.004 ± 0.005 compared with 0.087 ± 0.003 respectively, $n = 6$; $P < 0.01$).

The efficiency of ovariectomy was also determined by measuring hormonal levels. After ovariectomy, 17β-oestradiol, progesterone and testosterone plasma levels were reduced in both female OVX-WKY rats and OVX-SHRSP (Table1).

Table 1 Plasma sex hormone levels in sham-WKY rats, OVX-WKY rats, sham-SHRSP and OVX-SHRSP

Values are means ± S.E.M., $n = 5$–6. *$P < 0.05$ compared with the respective WKY rats; †$P < 0.05$ comapred with the respective sham.

	WKY rats		SHRSP	
Hormone	Sham	OVX	Sham	OVX
17β-Oestradiol (pmol)	16.1 ± 1.4	5.7 ± 0.7†	10.0 ± 0.2*	4.9 ± 1.2†
Progesterone (pmol)	27.2 ± 2.3	16.4 ± 0.8†	35.2 ± 7.3*	10.9 ± 3.7†
Testosterone (pmol)	0.16 ± 0.03	0.02 ± 0.01†	0.22 ± 0.02	0.02 ± 0.001†

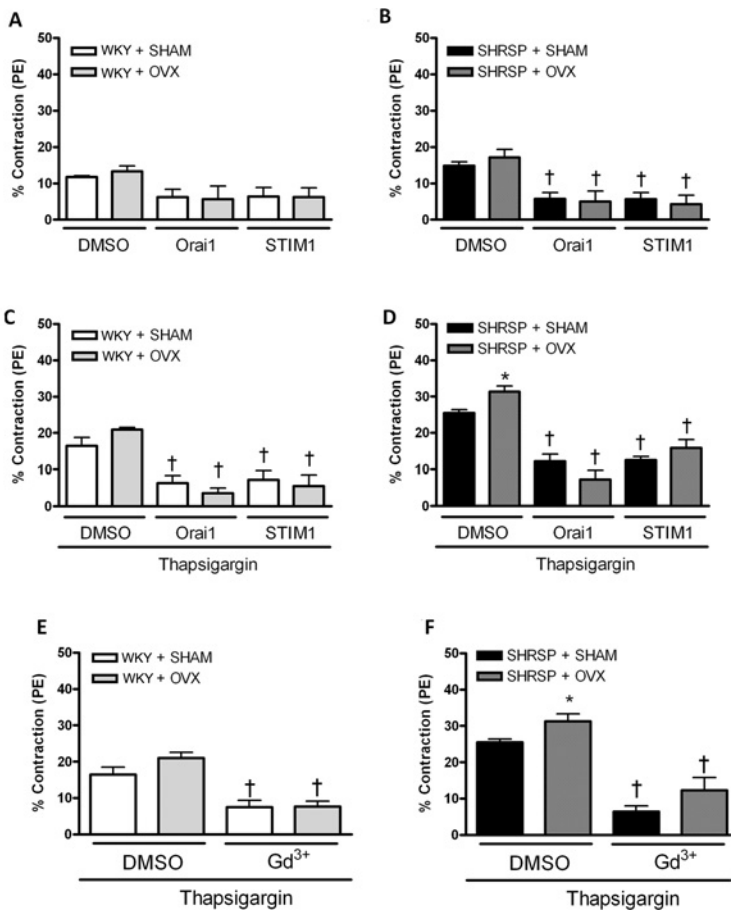

Figure 5 Ovariectomy increased the contractile response during Ca^{2+} influx in aortas from SHRSP upon thapsigargin stimulation

Contractions during the Ca^{2+}-loading period were evaluated in aortas from (A, C and E) sham-WKY rats (white bars) and OVX-WKY rats (grey bars) or in aortas from (B, D and F) sham-SHRSP (black bars) or OVX-SHRSP (hatched bars) in the (A and B) absence or in the (C–F) presence of thapsigargin. Values are means ± S.E.M. *$P < 0.05$ compared with the respective sham rats; †$P < 0.05$ compared with the respective DMSO vehicle.

Effect of ovariectomy in force development during the Ca^{2+}-loading period

The results described above showed sex differences in vascular responses during the Ca^{2+} influx period, which were enhanced after thapsigargin incubation. Therefore female rats underwent ovariectomy in order to investigate whether sex hormones play a protective role in the vasculature of female hypertensive rats.

PE-induced contraction was similar in aortas from sham-WKY and OVX-WKY rats (17.2 ± 0.4 and 18.7 ± 2.8 mN respectively, $n = 6$), as well as in aortas from sham-SHRSP and OVX-SHRSP (19.1 ± 1.8 and 21.7 ± 1.6 mN respectively, $n = 6$).

During the Ca^{2+}-loading period, contractions were similar in aortas from sham-WKY or OVX-WKY rats, and neutralizing antibodies against Orai1 and STIM1 did not affect the contractile response (Figure 5A).

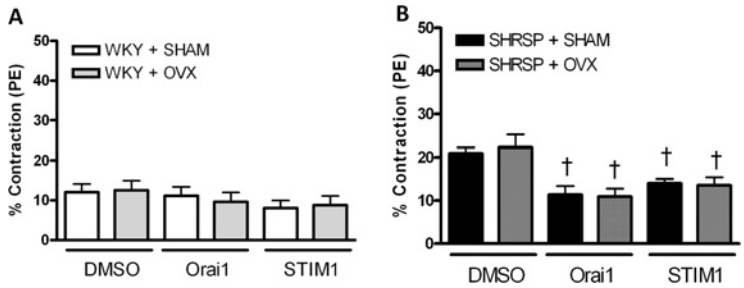

Figure 6 Ovariectomy did not interfere with Ca^{2+} release from the SR

Contractions in response to caffeine after incubation in Ca^{2+}-free buffer were evaluated in aortas from (A) sham-WKY rats (white bars) and OVX-WKY rats (grey bars) or in aortas from (B) sham-SHRSP (black bars) or OVX-SHRSP (hatched bars). Values are expressed as means $\pm$ S.E.M.; $\dagger P < 0.05$ compared with the respective DMSO vehicle.

Aortas from sham-SHRSP and OVX-SHRSP displayed similar contractile responses during the Ca^{2+}-loading period. Neutralizing antibodies against Orai1 and STIM1 significantly reduced vascular contractions during the Ca^{2+}-loading period in aortas from both sham-SHRSP and OVX-SHRSP (Figure 5B).

After thapsigargin incubation, all experimental groups displayed increased contractile responses during the Ca^{2+}-loading period. No significant differences were observed between aortas from sham-WKY and OVX-WKY rats (16.4 ± 2.3 and 20.96 ± 0.6% respectively, $n = 6$). Simultaneous thapsigargin-incubation and antibody transfection reduced contractile responses in aortas from sham-WKY and OVX-WKY rats during the Ca^{2+}-loading period (Figures 5C and 5E).

However, under thapsigargin incubation, aortas from OVX-SHRSP displayed increased contraction during the Ca^{2+}-loading period when compared with sham-SHRSP (31.3 ± 1.9 compared with 25.5 ± 0.8% respectively, $n = 6$). When thapsigargin was used in aortas transfected with neutralizing antibodies against Orai1 and STIM1 or with Gd^{3+} a reduced contraction response during the Ca^{2+}-loading period was observed (Figure 5D and 5F). Under these conditions, differences in the contractile responses observed between aortas from sham-SHRSP and OVX-SHRSP were abolished.

Effect of ovariectomy on intracellular Ca^{2+} stores

Caffeine-induced contraction was similar between aortas from sham-WKY and OVX-WKY rats (12.08 ± 2 and 12.5 ± 2% respectively, $n = 6$). Transfection with neutralizing antibodies did not affect the caffeine-induced response in aortas from sham-WKY and OVX-WKY rats (Figure 6A).

Aortas from female sham-SHRSP and OVX-SHRSP displayed increased contractile responses upon caffeine stimulation (19.3 ± 3 and 19.93 ± 2% respectively, $n = 6$). No differences between the OVX and sham groups were observed (Figure 6B).

Sex differences in Orai1 and STIM1 expression

Ovariectomy did not change Orai1 expression in aortas from WKY rats. After ovariectomy, Orai1 expression levels were increased in aortas from SHRSP compared with the levels in aortas from sham-SHRSP and OVX-WKY rats (Figure 7A).

Aortas from sham-SHRSP or OVX-SHRSP had increased STIM1 expression; however, ovariectomy did not affect the expression of STIM1 in aortas from SHRSP or WKY rats (Figure 7B).

DISCUSSION

The main findings of the present study are as follows: compared with females, aortas from male SHRSP have (i) increased contraction during the Ca^{2+}-loading period; (ii) similar transient contraction upon Ca^{2+} release from the intracellular stores; (iii) increased activation of STIM1 and Orai1 (as evidenced by blockade of STIM1 and Orai1 with neutralizing antibodies, which reversed sex differences in contraction during the Ca^{2+}-loading period); and (iv) increased expression of STIM1 and Orai1. Additionally, we have found that aortas from OVX-SHRSP exhibit increased contraction during the Ca^{2+}-loading period and increased Orai1 expression, without changes in the SR-buffering capacity or STIM1 expression.

In experimental models of hypertension, compared with females, males develop an earlier and more severe alteration in vascular function, as shown by increased vascular responses upon contractile stimuli, which is a hallmark of hypertension [20,21]. It is widely known that an increased Ca^{2+} concentration is a key marker of hypertension, and deregulation of Ca^{2+} homoeostasis is a major contributor to increased vascular contraction observed during hypertension [8–11,22]. In fact, abnormal handling of Ca^{2+} by vascular myocytes has been suggested previously to account for the sex differences in vascular contraction [13,23,24]

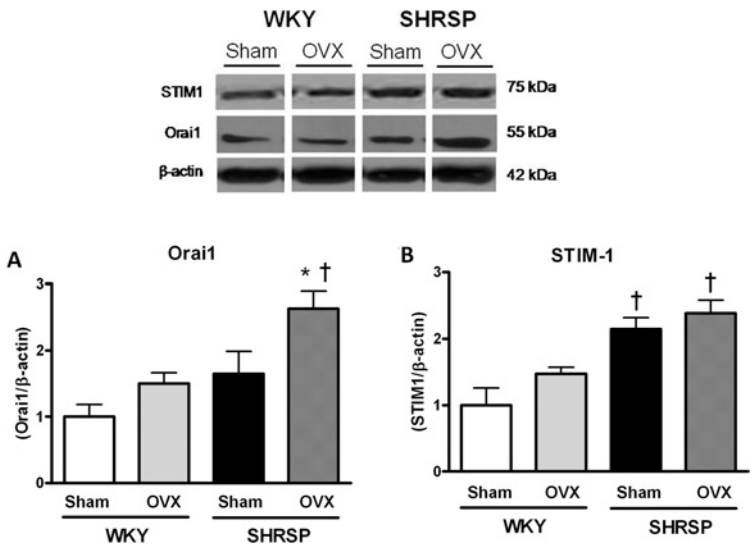

Figure 7 Ovariectomy increased the expression of Orai1 protein levels in aortas from SHRSP

Upper panel, representative Western blot images; lower panels, quantification of the Western blots demonstrating (A) Orai1 and (B) STIM1 protein levels in aortas from sham-WKY rats, OVX-WKY rats, sham-SHRSP and OVX-SHRSP. $^{*}P < 0.05$ compared with the respective sham rats; †$P < 0.05$ compared with the respective WKY rats.

and intracellular Ca^{2+} [14,25–28]. Some of these studies have discussed the importance of sex hormones as modulators of vascular functional differences during the Ca^{2+}-loading period observed between male and female hypertensive rats, but the mechanisms leading to abnormal Ca^{2+} homoeostasis are not completely understood.

In the present study, we used an adapted protocol to indirectly evaluate Ca^{2+} loading and SR-buffering capacity associated with their influence on the contractile response in arteries from male and female rats [15,16]. The contractions triggered by Ca^{2+} influx were increased in aortas from male hypertensive rats. Sex differences were observed both in the presence and the absence of thapsigargin, indicating that the intracellular Ca^{2+} concentration is different between vascular myocytes from males and females in conditions where the SR is either full or empty. These results are in agreement with previous reports showing that Ca^{2+} influx is decreased in both isolated VSMCs and aortas from female rats compared with male rats [25–27]. It has been shown that VSMCs stimulated with AngII (angiotensin II) display augmented Ca^{2+} influx, resulting in increased intracellular Ca^{2+} levels in male SHR compared with female SHR [13,14,28]. These findings show that impaired Ca^{2+} loading is an important contributor to increased vascular contraction in male hypertensive rats, as well as to sex-related vascular dysfunction.

Caffeine-induced contractions, used in the present study as an indicator of Ca^{2+} levels in the SR, were similar between the groups, suggesting that the SR in myocytes from females might have a higher buffering capacity [14,25–27]. In addition, ovariectomy increased contractions during the Ca^{2+}-loading period in aortas from female SHRSP, which is in agreement with previous reports [25–27]. This suggests that the SR Ca^{2+}-ATPase is less functional in vascular myocytes from male SHRSP. Accordingly, agonist-stimulated intracellular Ca^{2+} responses were augmented in VSMCs isolated from male hypertensive rats, resulting in greater contractile responses, reinforcing the suggestion that the SR Ca^{2+}-ATPase is less effective in removing Ca^{2+} after the stimulation of these cells [14]. Therefore sex hormones appear to play a role in sex differences during Ca^{2+} loading, but these differences are not due to Ca^{2+}-release-dependent mechanisms.

It has been shown previously that, in conditions where the intracellular Ca^{2+} stores are depleted, SOCE in smooth muscle cells via CRAC channels involves functional STIM1 proteins. Upon activation, STIM1 translocates to plasma membrane domains and activates Ca^{2+} entry through CRAC/Orai1 channels, similar to the observations made in other tissues [29–32].

Neutralizing antibodies against STIM1 and Orai1 were delivered intracellularly using a protein delivery reagent. This approach was used to complement the pharmacological protocols and to directly inhibit STIM1 and Orai1, making it possible to better understand their contribution to sex differences during the Ca^{2+}-influx period. Our present results demonstrate that the inactivation of STIM1 and Orai1 proteins resulted in smaller contractions triggered by Ca^{2+} loading. Accordingly, STIM1 and Orai1 vascular expression were augmented in aortas from male SHRSP compared with female SHRSP. Additionally, we observed that up-regulation of the expression and function of STIM1 and

Orai1 contributed to augmented contractile responses in hypertension and plays a role in sex differences in vascular function.

We propose that not only the differential expression, but also the differential activation of CRAC/Orai1 channels and the SR Ca^{2+} sensor STIM1 contribute to the sex differences observed during the Ca^{2+}-loading period in aortas from SHRSP. In this regard, some mechanisms have been investigated suggesting that S-glutathionylation [33], O-glyconacylation [34], phosphorylation [35,36] and lipid rafts [37] may play a role in regulating STIM1 activation.

Hence ovariectomy appears to influence the activation of both Orai1 and STIM1 in aortas from female SHRSP. It is conceivable that female sex hormones may exert a protective role in the vasculature in female hypertensive rats. Although these are exciting findings, we do not wish to conclude that this is the only mechanism contributing to the sex differences in increased vascular contractile responses observed in male SHRSP.

An additional point to be addressed is whether increased contractile responses in vessels from male SHRSP are exclusively associated with increased blood pressure levels in these animals. Previous work from Watts et al. [38] indicated that this is not the case. These investigators demonstrated that aortas from SHRSP display increased contractile oscillations in response to cyclopiazonic acid (an SR Ca^{2+}-ATPase inhibitor). Treatment of SHRSP with ramipril, an ACE (angiotensin-converting enzyme) inhibitor, effectively decreased blood pressure, but did not alter the increased contractile oscillations in response to cyclopiazonic acid [38]. Therefore these results suggest that augmented contractile responses, upon depletion of intracellular Ca^{2+}, in vascular smooth muscle from SHRSP may not be primarily associated with elevated blood pressure. In the future, it will be interesting to address whether STIM1/Orai1 represents a direct contributor to blood pressure regulation by evaluating this system in resistance size vessels or by treating animals *in vivo* with STIM1/Orai1 blockers. Currently, we do not have the drugs available to perform such studies. In addition, studies in cell lines may be useful to evaluate further the importance of sex hormones in the STIM1/Orai1 pathway, as well as their effect on intracellular Ca^{2+} levels.

In accordance with other studies [39,40], we have observed that ovariectomy increased SBP in female rats, which reveals the protective effect of sex hormones in blood pressure control. It is known that oestrogen has protective vascular effects. It has been shown that vessels from both normotensive and hypertensive OVX female rats display increased Ca^{2+} influx compared with control (sham-operated) female rats [27] and that oestrogen replacement normalized Ca^{2+} influx [25].

In conclusion, compared with female rats, aortas from male SHRSP display increased contraction during Ca^{2+} influx, indicating augmented intracellular Ca^{2+}, due to the up-regulation of Ca^{2+}-influx mechanisms, including increased activation of STIM1 and Orai1, which results in augmented vascular contraction. In addition, sex hormones seem to negatively modulate the STIM1/Orai1 pathway, contributing to vascular protection observed in female rats.

Recently, we have published a Hypothesis paper [41] analysing evidence for the possible involvement of STIM/Orai signalling in sex differences observed in the Ca^{2+}-handling process, especially in the vasculature [41]. Considering that the STIM1/Orai1 pathway participates in the Ca^{2+}-loading process, it seems plausible that alterations in these proteins may explain differences in Ca^{2+} loading observed in aortas from male and female hypertensive rats, and the findings of the present study are supportive of this concept. Therefore the STIM/Orai system may represent an important contributor to the pathophysiology of cardiovascular diseases and sex-related differences in hypertension.

AUTHOR CONTRIBUTION

Fernanda Giachini performed the vascular reactivity studies and blood pressure analysis, in addition to being responsible for writing the paper. Victor Lima performed the Western blot analysis. Fernando Filgueira ovariectomized the rats and performed some of the blood pressure measurements. Anne Dorrance provided the animals used in the study and, along with Zuleica Fortes and Maria Helena Carvalho, continuously provided ideas and expertise for the project and revisions for the paper. Clinton Webb and Rita Tostes designed the hypothesis and supervised the entire study. All of the authors had full access to the data and take responsibility for its integrity and the accuracy of the analysis. All authors have read and agree to the paper as written.

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