RESEARCH

Testosterone increases bradykinin-induced relaxation in the coronary bed of hypertensive rats

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Abstract

Physiological or supraphysiological levels of testosterone appear to be associated with the development of risk factors for cardiovascular diseases such as hypertension, as this hormone modulates the release of endothelial factors. However, its actions are still controversial, especially in the coronary circulation of hypertensive animals. This study was designed to assess the effects of testosterone treatment (T) on endothelium-dependent coronary vascular reactivity in orchietomized SHR. The animals were divided into SHAM, orchietomized (ORX), ORX+T and ORX+T+aromatase inhibitor (AI). All treatments lasted 15 days. Blood pressure (BP) was measured. Dose–response curves to bradykinin (BK) were constructed using the Langendorff technique, followed by inhibition of endothelium mediators (NO, prostanoids, EETs) and potassium channels. The intensity of eNOS, COX-1, COX-2, Akt, and gp91phox protein expression was quantified by Western blotting. BP was elevated in SHAM, ORX+T, and ORX+T+AI groups. However, we did not observe differences in the ORX group. Baseline coronary perfusion pressure (CPP) remained unaffected. Orchietomy did not change the BK-induced relaxation compared to the SHAM group, whereas testosterone treatment increased it. This response was diminished in the absence of NO, prostanoids, and EETs in the SHAM and ORX groups, while in ORX+T group the relaxation was diminished only in the absence of NO and EETs. There was no difference in eNOS, COX-1, COX-2, and gp91phox protein expression, though Akt expression was increased in ORX and ORX+T groups. These results show that testosterone treatment can modulate endothelial function, especially in the coronary circulation under hypertension conditions, via NO and EETs pathways.

Key Words

- ▶ coronary vascular bed
- ▶ orchietomy
- ▶ testosterone replacement therapy
- ▶ endothelium-dependent hyperpolarization

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Introduction

Cardiovascular diseases (CVDs) are the major cause of death worldwide, being projected to account more than 23 million deaths by 2030 (Benjamin *et al.* 2018). Hypertension is considered one of the most prevalent

risk factors for CVDs, especially coronary artery disease (MacMahon *et al.* 1990, Vasan *et al.* 2001, Chobanian *et al.* 2003, Dregan *et al.* 2013, James *et al.* 2014). The greater incidence of hypertension and cardiovascular

diseases in men and postmenopausal women compared to premenopausal women points to the female sex hormone estrogen having a protective vascular effect (Farhat *et al.* 1996, Benjamin *et al.* 2018), whereas testosterone appears to exacerbate the development of CVDs (Adams *et al.* 1995). It is known that the withdrawal of male sex hormones is associated with an improvement in endothelial function in adult men (Mark Herman *et al.* 1997), with testosterone therapy having been associated with detrimental effects (Vigen *et al.* 2013). However, clinical and epidemiological studies of the relationship between testosterone and CVDs are controversial, for testosterone treatment in men with low levels of this androgen was associated with mortality decrease (Shores *et al.* 2012). In addition, low testosterone levels are associated with an increased risk of death from CVDs (Haring *et al.* 2012). Clinically, male hypogonadism (Buvat *et al.* 2013) shows low testosterone levels and may lead to hypertension (Svartberg *et al.* 2004), suggesting that the testosterone/hypertension relationship is not completely clear, particularly as to its influence in coronary vascular reactivity.

Classical (genomic or nuclear) actions of testosterone are represented by the interaction of this hormone with the androgen receptor (AR), a member of the superfamily of nuclear receptors activated by ligands that, once stimulated, acts as a transcription factor (Mangelsdorf & Evans 1995). It is also known that testosterone can act through a rapid, non-genomic (extra-nuclear) responses on the vascular system. These actions may involve the interaction between the hormone and AR (Deenadayalu *et al.* 2012) but also occur independently of its classical receptor (Jones *et al.* 2004). These actions may begin both in the plasma membrane and in the cytoplasm, generally raising the concentration of intracellular calcium and activating kinase proteins (Deenadayalu *et al.* 2012).

In the cardiovascular system, testosterone is able to relax rat aortic rings (Bucci *et al.* 2009) and pig coronary artery (Deenadayalu *et al.* 2001), being also known to directly modulate vascular smooth muscle in a rapid, non-genomic manner (Deenadayalu *et al.* 2001, Bucci *et al.* 2009). Testosterone has been also shown to promote relaxation in coronary arteries through ionic flow mediated by potassium channels (Deenadayalu *et al.* 2001) and calcium channels (Montaño *et al.* 2008).

In addition to the important role of testosterone by acting directly on blood vessels, studies have also focused on demonstrating the effects of testosterone deprivation in the cardiovascular system. Orchiectomy can reduce the expression of voltage-dependent potassium channels in rat aorta, which can be reversed through androgen

replacement (Zhou *et al.* 2008). In a previous study, we showed that testosterone replacement therapy was able to prevent damage in coronary vascular reactivity caused by orchiectomy-induced hormonal deficiency in normotensive rats (Rouvier *et al.* 2015). Taken together, the aforementioned findings attribute to testosterone a possible protective action on the cardiovascular system. However, the mechanisms by which this hormone modulates coronary vascular reactivity when induced by an endothelium-dependent vasodilator agonist (bradykinin), remain to be described. Our hypothesis is that testosterone treatment could modulate endothelial function in isolated heart under hypertension conditions. Thus, this study was designed to assess the effects of testosterone treatment on endothelium-dependent coronary vascular reactivity in orchiectomized spontaneously hypertensive rats (SHR).

Materials and methods

Animal procedure

Male SHR with 8 weeks old (200–300 g) were used. The animals were supplied by the Health Sciences Center of the Federal University of Espirito Santo. All procedures were conducted in accordance with recommendations in the Brazilian Guidelines for the Care and Use of animals for the Scientific Purpose and Didactics and the Guidelines Euthanasia Practice and approved by the Institutional Ethics Committee for the Use of Animals (CEUA-UFES, protocol # 062/2017). The animals were maintained in the group (four animals per cage) with free access to water and food (Nuvilab CR-1), under controlled conditions of temperature (22–24°C) and humidity (40–60%), with a 12 h light:12 h darkness cycle. The animals were randomly divided into four experimental groups: control (SHAM), orchiectomized (ORX), orchiectomized and treated with testosterone (ORX+T), and orchiectomized and treated with testosterone plus aromatase inhibitor (ORX+T+AI).

After anesthesia with ketamine (70 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), the animals were fixed with surgical tape over a surgical bed. Before incision, disinfection of the testicular sac was performed with iodized alcohol. Surgery was performed through an incision in the midline of the testicular sac. After exposure of the testicles, the *tunica vaginalis* was opened, and spermatic cord was ligated and the testes were removed. The incision was closed and the animals received antibiotic (Enrofloxacin, 10 mg/kg, i.m., Chemitec®, São Paulo, Brazil), and analgesic (Flunixin meglumine, 2.5 mg/kg i.m., Chemitec®). The control group underwent

sham surgery (SHAM), except that the testicles were not removed.

Testosterone replacement (bioidentical testosterone–IMAFAR) was performed for 15 days via subcutaneous injection of a dose of 0.5 mg/kg/day, mimicking a physiological concentration, as previously described (Moysés *et al.* 2001). For groups that did not receive testosterone treatment, the same volume of vehicle (sunflower oil) was administered (Moysés *et al.* 2001). The group that was treated with anastrozole (AI, 0.1 mg/kg/day, diluted in saline solution at 0.9%) received it orally, in a maximum volume of 0.2 mL (Altintas *et al.* 2010). The animals treated with testosterone received the first dose on the day of orchiectomy to avoid low hormonal levels post-surgery.

The rats were submitted to an adaptation period (3 days) before starting the records. The animals were placed in an external chamber at 37°C for 10 min. They were then transferred to a heated chamber (IITC INC/Life Science) at the same temperature, and then a pneumatic cuff was placed in the proximal tail region on which was inflated and deflated automatically. After adaptation for 5 min, the systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded. We considered records that variations were not greater than 5 mmHg. Records associated with animal movements were discarded (Ibrahim *et al.* 2006, Fritz & Rinaldi 2008). The pressure measurements were performed one day before the surgical procedure (day 0), and at 7 and 15 days.

Isolated heart preparation (modified Langendorff method)

The experiments were performed on isolated perfused hearts from male SHR that were anesthetized with ketamine (70 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and injected i.v. with heparin (100 IU) (Bell *et al.* 2011). Five minutes after heparin injection, rats were euthanized, and hearts were excised. Analyses of the coronary vascular bed were performed on whole hearts using the Langendorff preparation as previously described (Santos *et al.* 2010). Briefly, using a Langendorff apparatus (Hugo Sachs Electronics, March-Hugstetten, Germany), the isolated hearts were perfused with modified Krebs solution containing the following (in mM): 120 NaCl, 1.26 CaCl₂·2H₂O, 5.4 KCl, 2.5 MgSO₄·7H₂O, 2 NaH₂PO₄·H₂O, 27 NaHCO₃, 1.2 Na₂SO₄, 0.03 EDTA, and 11.0 glucose. The Krebs solution was equilibrated with a mixture of 95% oxygen and 5% carbon dioxide at a controlled pressure of 100 mmHg to bring the pH to 7.4.

The hearts were perfused at a rate of 10 mL/min with a peristaltic pump (MS-Reglo 4 channels, Hugo Sachs Electronics) and were kept at 37°C. A fluid-filled balloon was introduced into the left ventricle through a steel cannula connected to a pressure transducer (AD Instrument MLT0380/A Reusable BP Transducer) to measure the isovolumetric cardiac force. The balloon was pressurized by a spindle syringe until it reached a preload of 10 mmHg.

The baseline coronary perfusion pressure (CPP) was assessed by means of a pressure transducer (AD Instrument MLT0380/A), connected immediately near the aortic perfusion cannula, through which the coronary bed was perfused, attached to a digital data acquisition system (PowerLab System). LabChart 7.3.1 software was used to read the records obtained by the data acquisition system (ADInstruments). The CPP changes were directly related to changes in vascular resistance. After stabilization of the system for 40 min, the baseline CPP was obtained and a dose–response curve of bradykinin (BK, Sigma) was constructed, which was administered *in bolus* (0.1 mL) to increasing concentrations (10^{−10} to 10^{−6} M) before and after perfusion with 100 μM of N^ω-nitro-L-arginine methyl ester (L-NAME, non-specific inhibitor of the enzyme nitric oxide synthase – NOS) or 2.8 μM of indomethacin (INDO, non-specific inhibitor of cyclooxygenase – COX), or inhibition combined with 100 μM of L-NAME+2.8 μM of INDO or 100 μM of L-NAME+2.8 μM of INDO+0.75 μM of clotrimazole (CLOT, CYP epoxigenase inhibitor) or 100 μM of L-NAME+2.8 μM of INDO+0.75 mM of tetrabutylammonium (TBA, non-specific blocker of potassium channels) (Moazed *et al.* 2009).

All inhibitors were perfused for at least 20 min until the *in bolus* injection of BK was repeated. The relaxing response was calculated by the following equation:

$$\Delta(\%) = 100 - \left(\frac{\text{CPP}_{\text{after infusion}} \times 100}{\text{CPP}_{\text{before infusion}}} \right)$$

Dissection of the coronary arteries

The animals were anesthetized with ketamine (70 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and killed by decapitation. The thorax cavity was opened, the heart was removed and placed in a Petri plate with a cold Krebs solution modified (in mM): 120 NaCl, 1.25 CaCl₂·2H₂O, 5.4 KCl, 2.5 MgSO₄·7H₂O, 2 NaH₂PO₄·H₂O, 27 NaHCO₃, 1.2 Na₂SO₄, 0.03 EDTA, and 11 glucose at a pH 7.4 for the dissection procedure. The anterior descending branch of the left coronary artery and the septal branch were

isolated in a dissection microscope (M900, F. Vasconcelos) and then freed from surrounding cardiac tissue, snap-frozen in liquid nitrogen and stocked at -80°C until use.

Western blotting

The coronary arteries of each group (SHAM, ORX and ORX+T) were macerated in 25 μL of RIPA supplemented with protease and phosphate inhibitors (65.2 mM Tris-HCl, 154 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.8 mM EDTA, 1 mM PMSE, 10 mM sodium orthovanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate and protease inhibitor). The homogenates were centrifuged at 9.5 g for 10 min at 4°C for the removal of debris. The protein concentration of the samples was determined by colorimetric method (Bio Rad DC Protein assay). Aliquots of the lysate, containing 20 μg of total protein, were mixed with sample buffer to final concentration 1x. They were then boiled for 5 min and applied to the gel. Protein separation was done by electrophoresis on 10% polyacrylamide gel at 4°C for approximately 2 h at 150 V. The molecular weight standard used was the Prestained Protein Ladder – extra broad molecular weight (Abcam). After electrophoresis, the proteins were electrotransferred to nitrocellulose membrane and blocked with 5% nonfat milk in TBS-T (0.13 M NaCl, 20 mM Tris, 0.1% Tween 20 and pH 7.6) 2 h. The blocking solution was withdrawn and the membranes were washed with TBS-T 36 times for 5 min. They were then incubated with primary antibody diluted in TBS-T with 3% BSA. Mouse antibody to endothelial NOS (eNOS) (1:1000 dilution, Abcam; ab76198), COX-2 (1:1000 dilution, BD; 603203), gp91phox (1:1000 dilution, Abcam; ab80897) and β -actin (1:5000; Abcam; ab6276); rabbit antibody to COX-1 (1:1000 dilution, Abcam; ab109025), and Akt (1:1000 dilution, Abcam; ab8805). Membranes were incubated overnight at 4°C . Six washings of 5 min were followed with TBS-T. Anti-mouse (1:2000, Cell Signaling; 7076) or anti-rabbit (1:2000, Santa Cruz Biotechnology; sc-2357) conjugated secondary antibodies were diluted in 1% BSA and applied

to the membrane for 1 h at room temperature. Then another six washes of 5 min were made and then the chemiluminescence detection was performed using the luminol reagent (Santa Cruz Biotechnology) followed by exposure to radiographic film. Protein quantification was done by densitometry using ImageJ software (NIH). Protein expression was calculated based on the ratio of the specific density of each protein band vs the corresponding density of β -actin.

Statistical analysis

Data analysis was performed with the statistical software GraphPad Prism 7. The data were expressed as the mean $\pm$ S.E.M., *n* represents the number of animals studied. For each data set, D'Agostino–Pearson omnibus normality test was also performed. The comparisons between the groups were made using the one-way ANOVA. The relaxing response to BK was calculated by the two-way ANOVA. In all cases, the *post hoc* Tukey test was used, and a significance level of $P < 0.05$ was established.

Results

Ponderal data

The body weight was not affected among the studies groups (SHAM, ORX, and ORX+T). The weight of the prostate gland (mg) and seminal vesicle decreased in the ORX group. In addition, the testosterone treatment increased the weight of the prostate and seminal vesicle when compared to ORX group (Table 1).

Non-invasive assessment of blood pressure (BP)

By the 15th day of follow-up, SBP was found to be elevated – by age, when compared to day 0 – in SHAM animals (from 181 ± 2 to 205 ± 6 mmHg) and in the groups receiving testosterone treatment, ORX+T (from 179 ± 3 to 203 ± 4 mmHg) and ORX+T+AI (from 167 ± 2 to 213 ± 3 mmHg) (Fig. 1A). However, SBP remained

Table 1 Body weight and ratio of weight of glands (mg) with the length of the tibia (mm) in the SHAM, ORX and ORX+T groups after 15 days of treatment with a physiological dose of testosterone.

Parameter	<i>n</i>	SHAM	<i>n</i>	ORX	<i>n</i>	ORX+T
Body weight (g)	22	226 ± 6	20	209 ± 7	20	215 ± 5
Prostate gland (mg/mm)	22	6.22 ± 0.346	20	0.87 ± 0.210^a	20	$3.38 \pm 0.348^{a,b}$
Seminal gland (mg/mm)	22	15.43 ± 0.869	20	2.68 ± 0.205^a	20	$7.77 \pm 0.618^{a,b}$

The values are expressed as the mean $\pm$ S.E.M. One-way ANOVA and post-test Tukey were used.

^a $P < 0.05$ vs SHAM group; ^b $P < 0.05$ vs ORX group.

Figure 1

Blood pressure (BP) assessment in SHR. (A) Systolic blood pressure (SBP) and (B) diastolic blood pressure (DBP). SHAM ($n = 13$), ORX ($n = 18$), ORX+T ($n = 22$) and ORX+T+AI ($n = 15$) groups. Data are expressed as mean $\pm$ S.E.M. Two-way ANOVA and Tukey's post-tests were used. * $P < 0.05$ compared to SHAM, # $P < 0.05$ compared to ORX+T and + $P < 0.05$ compared to ORX+T+AI.

stable in the ORX group, with no differences having been observed after 15 days (187 ± 3 mmHg) when compared to day 0 (178 ± 2 mmHg). DBP (Fig. 1B) followed the same response pattern by the end of 15 days of follow-up. We observed that, compared to day 0, DBP was elevated in SHAM (from 128 ± 2 to 152 ± 5 mmHg), ORX+T (from 126 ± 3 to 150 ± 4 mmHg) and ORX+T+AI (from 121 ± 4 to 160 ± 4 mmHg) groups, while remaining stable in the ORX group (from 125 ± 3 mmHg to 134 ± 3 mmHg).

Baseline coronary perfusion pressure (CPP)

The baseline CPP was not altered in any groups (SHAM, 115 ± 7 mmHg; ORX, 114 ± 5 mmHg; ORX+T, 110 ± 3 mmHg, and ORX+T+AI, 98 ± 4 mmHg), as shown in Fig. 2.

Reactivity of the coronary vascular bed in response to bradykinin (BK)

Endothelium-dependent vasodilation stimulated by BK was observed in all groups (Fig. 3A). However, orchiectomy did not change the vascular relaxation induced by BK compared to the SHAM group, whereas in ORX+T and ORX+T+AI groups, it was increased. As we did not observe differences in CPP or vasodilation among ORX+T and ORX+T+AI animals, which would be indicative of the non-conversion of testosterone to

Figure 2

Baseline coronary perfusion pressure (CPP) in SHR. SHAM ($n = 13$), ORX ($n = 11$), ORX+T ($n = 10$) and ORX+T+AI ($n = 9$) groups. Data are expressed as mean $\pm$ S.E.M. One-way ANOVA and Tukey's post-tests were used.

estrogen, we opted proceed only with the SHAM, ORX and ORX+T groups.

Perfusion with the NOS inhibitor (L-NAME) attenuated the relaxant response to BK in all groups when compared to their respective baseline curve to BK. Furthermore, BK-induced vasodilation, under NOS inhibition, was higher in the ORX+T group (Fig. 3B). In the presence of a COX inhibitor, the BK-induced vasodilation response was reduced in SHAM and ORX animals when compared to their respective baseline conditions, with no differences having been observed in the ORX+T group (Fig. 3C). The combined inhibition with L-NAME+INDO decreased the relaxant response to BK in all groups, with the ORX+T group showing a higher response curve to BK when compared to SHAM or ORX (Fig. 3D). Furthermore, in the presence of CYP or potassium channel inhibitors (CLOT and TBA, respectively), the endothelium-dependent vasodilation response to BK was completely inhibited in all groups, even when combined to NOS and COX inhibitors, confirming the important participation of epoxyeicosatrienoic acids (EETs) and potassium channels in this response in all studied groups (Fig. 3E and F, respectively).

Protein expression

Figure 4 shows the expression of proteins in coronary arteries in SHR. The expression of eNOS, COX-1, COX-2, gp91phox were not different in any groups studied, as shown in Fig. 4A, B, C and D, respectively. In addition, the expression of Akt, was higher in the ORX and ORX+T groups when compared to the SHAM group (Fig. 4E).

Discussion

The main finding of our study was that testosterone treatment increased BK-induced vasodilation in the coronary vascular bed of orchiectomized SHR. Nitric oxide

Figure 3

Dose-response curves to bradykinin in the coronary vascular bed of SHR. SHAM ($n = 12$), ORX ($n = 10$), ORX+T ($n = 9$) and ORX+T+AI ($n = 8$) groups before (A) and after infusion with L-NAME (B, $n = 8$); Indomethacin (INDO) (C, $n = 8$); L-NAME + INDO (D, $n = 8$); L-NAME + INDO + Clotrimazole (CLOT) (E, $n = 8$); L-NAME + INDO + Tetrabutylammonium (TBA) (F, $n = 8$). Data were expressed as mean $\pm$ S.E.M. Two-way ANOVA and Tukey's post-hoc tests were used. * $P < 0.05$ vs SHAM; # $P < 0.05$ vs ORX.

(NO), arachidonic acid metabolites (EETs) and potassium channels participated substantially in this response.

Our results on blood pressure showed that orchiectomy prevented the progress of hypertension. These findings corroborate other studies (Masubuchi *et al.* 1982, Jenkins *et al.* 1994), suggesting an important role of testosterone in the increase of BP in SHR. In addition, orchiectomy-induced prostate and seminal glands' atrophy, suggesting that testosterone plays an important role in the function of these glands. Both results (BP and accessories gland atrophy) confirm our model of hypertension and hormonal deficiency. In relation to the baseline CPP, it was not altered by orchiectomy or testosterone treatment. Similar results in normotensive rats were obtained in our laboratory (Moysés *et al.* 2001, Rouver *et al.* 2015), suggesting that the testosterone does not modulate the baseline CPP in both normotensive and spontaneously hypertensive rats.

We analyzed coronary vascular reactivity in spite of no differences having been found in baseline CPP

between groups. All groups showed BK-induced relaxation, which remained unaltered by orchiectomy. Treatment with testosterone for 15 days increased endothelium-dependent vasodilation induced by BK in the coronary vascular bed from orchiectomized SHR. This response was independent of aromatization. This response highlights a probable benefit of testosterone treatment in the coronary vascular bed from SHR. Similarly, Rouver *et al.* demonstrated that replacement therapy with testosterone increased relaxation to BK in the coronary circulation from normotensive rats (Rouver *et al.* 2015).

Following the endothelium-dependent vasodilation analysis, we assessed the participation of possible endothelial mediators involved in the relaxation response. After perfusion with the NO synthesis inhibitor, the vasodilator response to BK decreased in all groups, suggesting the participation, at least in part, of NO as an endothelium mediator in the coronary vascular bed. In the presence of the prostanoids synthesis inhibitor,

Figure 4

Western blot and densitometric analysis of coronary arteries in the SHAM ($n = 8$), ORX ($n = 8$) and ORX+T ($n = 8$) groups. In the upper part, the expression of eNOS (A), COX-1 (B), COX-2 (C), gp91phox (D) and Akt (E) is shown. In the lower part, the densitometric analysis of the expression of each protein respectively is shown. The results (mean $\pm$ s.e.m.) are expressed as the relationship between the signal of the protein under study and the signal of the β -actin. One-way ANOVA and Tukey's post-tests were used. * $P < 0.05$ vs SHAM.

we observed a decrease in the relaxation to BK in SHAM and ORX animals, suggesting the participation of a vasodilator metabolite from the COX pathway, such as prostacyclin (PGI_2). In contrast, inhibition with indomethacin increased the relaxation to acetylcholine in SHR aorta (Luscher & Vanhoutte 1986), which suggests the participation of a vasoconstrictor metabolite from the prostanoids pathway. These differences may occur due to the type of vessel studied. It has been reported that treatment with testosterone did not affect the synthesis of PGI_2 in middle cerebral arteries from male rats (Gonzales *et al.* 2004), while it decreased the synthesis of PGI_2 in rat aortic smooth muscle cells in culture (Nakao *et al.* 1981); pointing to the complexity of testosterone actions on the synthesis of prostanoids.

In SHAM and ORX groups, individual perfusion with L-NAME or INDO, or combined perfusion with L-NAME+INDO decreased relaxation to BK. This result suggests the independent participation of both NO or

prostanoids metabolites in the coronary vascular bed of both groups. Nevertheless, in the ORX+T group, the perfusion with L-NAME or L-NAME+INDO decreased relaxation to BK, whereas the perfusion with INDO did not modify the relaxation to BK, suggesting the participation of NO in relaxation response to BK in the coronary vascular bed in the ORX+T group. Moreover, perfusion with L-NAME+INDO+CLOT, almost abolished BK relaxation in all groups. It suggests also a participation of an endothelium mediator independent of the NOS and COX activity, such as metabolites of CYP, that is, EETs, in all groups. Similarly, a previous study from our laboratory demonstrated the participation of EETs in the relaxation to BK in the coronary bed of normotensive rats (Rouver *et al.* 2015). These metabolites of CYP, would act as possible candidates for endothelium-dependent hyperpolarization (EDH) in the relaxation to BK in rat coronary artery, which were already mentioned by Fulton *et al.* (1995). Fisslthaler *et al.*, in turn, indicated that 11,12-epoxyeicosatrienoic acid,

a product of CYP 2C8/34, is essential in relaxation mediated by EDH in porcine coronary artery (Fisslthaler *et al.* 1999). It is known that a factor able to promotes EDH must exert endothelium-relation responses that does not are inhibits after the use of L-NAME or INDO but are inhibits by potassium channels blockers (Campbell & Harder 2001). We also analyzed the participation of potassium channels, one of the main mediators of EDH. After perfusion with TBA, in the presence of L-NAME+INDO the relaxation to BK was almost abolished in all groups, suggesting the participation of potassium channels in the relaxation to BK in the coronary bed from SHR. Therefore, it seems that treatment with testosterone in the ORX+T group positively modulates the participation of EDH (EETs stimulating potassium channels). It has been suggested a cross-talk between NO and EDH, which could occur by an inhibitory (Nishikawa *et al.* 2000, Liu *et al.* 2002), stimulatory (Qiu & Quilley 2001), synergistic (Cohen *et al.* 1997) or compensatory (Kilpatrick & Cocks 1994) type. Also, several studies suggested that the main pathways of EDH (i.e. potassium channels) would be downregulated in SHR (Fujii *et al.* 1992, Weston *et al.* 2010). Thus, we suggest a possible cross-talk between NO and EDH, since the participation of both NO and EDH was found in this study.

The expression of eNOS, COX-1, COX-2 and gp91phox (catalytic subunit of NADPH oxidase) were not altered among the groups, but the expression of Akt was increased in the ORX and ORX+T groups. It has been demonstrated the androgen modulation of NOS expression in vascular tissue of normotensive rats, where orchiectomy did not affect its expression in aorta (Blanco-Rivero *et al.* 2006a) and mesenteric artery (Blanco-Rivero *et al.* 2006b, 2007). However, the expression of neural NOS (nNOS), in the mesenteric artery, was reduced (Martín *et al.* 2005). On the other hand, in SHR, neither orchiectomy nor treatment with testosterone modified the expression of COX-1 and COX-2 but after perfusion with INDO, relaxation to BK decreased in the SHAM and ORX groups, which suggests that the activity of COX seems to be preserved. In other studies in normotensive rats, orchiectomy did not affect the expression of COX-2 in the mesenteric artery (Blanco-Rivero *et al.* 2006b), whereas it increases in aorta (Aina Martorell *et al.* 2008). Hence, testicular androgens would modulate the expression and function of COX in different ways, and would depend on the type of isoform, the type of vessel, and the disease.

We found that the expression of Akt was increased in the ORX group, what could not be prevented by testosterone treatment. It has been reported that orchiectomy decreases Akt expression and increases PI3K

expression in the aorta of normotensive rats, leading to vascular inflammation (Zhao *et al.* 2016). We did not observe that pattern in our study, what could be the result of Akt expression being increased in order to indirectly minimize the effects of possible endothelial dysfunction in ORX groups.

Reactive oxygen species (ROS) can affect vascular relaxation (Liu *et al.* 2002). In males, testosterone activates NADPH oxidase via renin angiotensin system (RAS) (Fischer *et al.* 2002), leading to increased synthesis of ROS, that is, superoxide anion ($O_2^{\cdot-}$) (Chignalia *et al.* 2012). The $O_2^{\cdot-}$ reacts rapidly with NO (reducing its bioavailability), forming peroxynitrite ($ONOO^-$), which impairs the vasodilation mediated by EDH (Liu *et al.* 2002). In this context, the expression of the gp91phox subunit of NADPH oxidase was analyzed, which was not different between the groups, which suggests that testosterone does not modulate its expression in the coronary vascular bed from male SHR.

Finally, treatment with testosterone increased the relaxing response to BK in the coronary bed of orchiectomized SHR, with the participation of NO and EETs. This may be due to the fact find in our study that testosterone, in the absence of others testicular androgens, could modulates the coronary vascular reactivity by the following mechanisms: mainly by a probable upregulation of the synthesis and activity of CYP metabolites (EETs) and their action in potassium channels, and by an improvement in NO bioavailability, which would allow the modulation of a probable cross-talk between NO and EDH.

Conclusions

Although treatment with testosterone did not reduce blood pressure, it led to increased endothelium-dependent relaxation in the coronary vascular bed of spontaneously hypertensive orchiectomized rats. This effect appears to be independent of aromatase activity and to involve the participation of NO and arachidonic acid metabolites (i.e. EETs). Our results point to the androgen having a regional impact. This work unveils the important participation of EETs and potassium channels in the coronary circulation of hypertensive rats. The characterization of these mechanisms could lead to a better understanding of the role played by testosterone, especially in the coronary vascular bed under hypertensive conditions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

JC Arapa-Diaz and WN Rouver involved in acquisition of data and substantial contributions to conception and design; analysis and interpretation of data; drafting the article; revising it critically for important intellectual content; and final approval of the version to be published. MD Grando involved in acquisition of data. LM Bendhack and JAS Giesen and RL Santos involved in substantial contributions to conception and design; analysis and interpretation of data; drafting the article; revising it critically for important intellectual content; and final approval of the version to be published.

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