Association of testosterone with estrogen abolishes the beneficial effects of estrogen treatment by increasing ROS generation in aorta endothelial cells

Tiago J. Costa,1 Grazia S. Cervalo,1,2 Rosangela A. dos Santos,1 Maria Aparecida de Oliveira,1 Priscila X. Araújo,1 Luciana R. Giaquinto,1 Rita C. Tostes,3 Eliana H. Akamine,1 Zuleica B. Fortes,1 Ana Paula Dantas,4* and Maria Helena C. Carvalho1**

1Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, Brazil; 2Department of Physiological Sciences, State University of Londrina, Londrina, Brazil; 3Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Brazil; and 4Group of Atherosclerosis and Coronary disease, Institut Cliní de Torax, Institut d’Investigaciones Biomédicas August Pi I Sunyer, Barcelona, Spain

Submitted 19 September 2014; accepted in final form 11 January 2015

Costa TJ, Cervalo GS, dos Santos RA, de Oliveira MA, Araújo PX, Giaquinto LR, Tostes RC, Akamine EH, Fortes ZB, Dantas AP, Carvalho MH. Association of testosterone with estrogen abolishes the beneficial effects of estrogen treatment by increasing ROS generation in aorta endothelial cells. Am J Physiol Heart Circ Physiol 308: H723–H732, 2015. First published January 30, 2015; doi:10.1152/ajpheart.00681.2014.—Testosterone has been added to hormone replacement therapy to treat sexual dysfunction in postmenopausal women. Whereas estrogen has been associated with vascular protection, the vascular effects of testosterone are contradictory and the effects of its association with estrogen are largely unknown. In this study we determined the effects of testosterone associated with conjugated equine estrogen (CEE) on vascular function using a model of hypertensive postmenopausal female: ovariectomized spontaneously hypertensive rats. Female spontaneously hypertensive rats were divided into sham-operated, ovariectomized spontaneously hypertensive rats. Female spontaneously hypertensive rats were divided into sham-operated, ovariectomized (OVX), and OVX treated for 15 days with either CEE alone (OVX+CEE) or associated with testosterone (OVX+CEE+T). Angiotensin II (ANG II)-induced contraction was markedly increased in aortic rings from OVX compared with sham-operated rats. CEE treatment restored ANG-II responses, a beneficial effect abrogated by CEE-T. CEE treatment also increased endothelium-dependent relaxation, which was impaired in OVX rats. This effect was lost by CEE-T. Treatment of aortas with losartan (ANG-II type-1 receptor antagonist) or apocynin (NADPH-oxidase inhibitor) restored the endothelium-dependent relaxation in OVX and CEE+T, establishing an interplay between ANG-II and endothelial dysfunction in OVX and CEE+T. The benefits by CEE were associated with downregulation of NADPH-oxidase subunits mRNA expression and decreased reactive oxygen species generation. The association of testosterone with CEE impairs the benefits of estrogen on OVX-associated endothelial dysfunction and reactive oxygen species generation in rat aorta by a mechanism that involves phosphorylation of the cytosolic NADPH-oxidase subunit p47^phox.

testosterone; estrogen; endothelium; ROS; angiotensin II; NADPH oxidase; cardiovascular disease; hypoactive sexual desire disorder

AMONG THE HEALTH ISSUES associated with menopause, hypoactive sexual desire disorder (HSDD) is one of the most common (18, 28). Nearly 10% of postmenopausal women are affected by HSDD worldwide, and in most patients, the exact cause of HSDD is never fully elucidated (28). Specifically to menopausal women, the drop of estrogen levels has been considered as a major cause of HSDD. Nevertheless, hormonal therapy (HT) with estrogen has not demonstrated beneficial effect in sexual dysfunction in many cases of HSDD. In addition to the decline in estrogen levels, ovarian senescence also results in diminished levels of androgens, although transient increases of testosterone levels are observed in several stages of menopause (49). In fact, the combined loss of physiological levels of estrogens and androgens has been pointed as responsible for a variety of signs and symptoms during menopause, including vasomotor symptoms, bone loss, and reduced interest in sex (17). Accordingly, testosterone, which plays an important role in sexual arousal in men (in woman, evidence is less consistent and sometimes contradictory), has been associated with estrogen hormone therapy to treat HSDD (3, 43).

Estrogens are thought to induce cardiovascular protection. Accordingly, estrogens modulate mechanisms that control blood pressure (13) and vascular function, including endothelium-dependent relaxation (7) and contraction (6). Vascular benefits by estrogen have been mostly attributed to increased endothelial nitric oxide (NO) synthase (eNOS) transcription via genomic pathways, as well as increased eNOS activity and NO production via nongenomic activation (14). Estrogen also has antioxidant properties (13). It decreases the expression of NADPH oxidase and the generation of superoxide anion and peroxynitrite and therefore increases NO bioavailability (23). In hypertensive female rats, the lack of estrogen increases blood pressure in association with lower plasma antioxidant levels and increased plasma and vascular free radicals. Treatment with different types of estrogen prevents these effects (6, 13, 23).

Testosterone, on the other hand, has shown to have opposite effects of estrogens in the cardiovascular system. Studies in several experimental models support the argument of that androgens increase blood pressure and boost the development of arterial hypertension (38, 46). The role of testosterone on cardiovascular risk has gained much attention with the suggestion that androgens play a role in modulating the renin-angiotensin system and contribute to the development of endothelial dysfunction (38, 42). The higher testosterone levels in males, rather than estrogen in females, are considered key players for the sex-associated differences in the prevalence of cardiovascular diseases (39). The hypothesis is strengthened by data from animal models showing that cardiovascular diseases are more common in male at an earlier age when testosterone levels are elevated and that gonadectomy prevents/decreases

http://www.ajpheart.org 0363-6135/15 Copyright © 2015 the American Physiological Society H723
cardiovascular diseases-associated organ damage (30). Few data from observational studies have established a positive correlation of endogenous testosterone levels and increased cardiovascular risk in women. Nonetheless, the role of exogenous administration of testosterone in the control of vascular function of females is largely unknown.

Even though appropriated amounts of testosterone may improve women’s metabolic, psychological, and sexual function (2), the risks it may pose to the cardiovascular function are not well established and should be considered, especially in women with an existing risk. In the present study we sought to determine whether testosterone interferes with the protective effects of conjugated equine estrogen (CEE), which is most often used in postmenopausal women, although the cardiovascular risk in women. Nonetheless, the role of exogenous administration of testosterone in the control of vascular function of females is largely unknown.

**MATERIALS AND METHODS**

Female SHR rats were obtained from the breeding stock of the Institute of Biomedical Sciences of the University of São Paulo. Rats were housed in a temperature-controlled room on a 12-h:12-h light-dark cycle with 60% humidity and with standard rat chow and water ad libitum. All the procedures used in this study were approved and performed in accordance with the guidelines of the Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo (protocol 145, page 95, book 2. 06.12.2010), following the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). At 12 wk of age, female SHR rats were OVX as previously described (12). Thirty days after ovariectomy, a group of OVX-SHRs was treated during 15 days with a solution of CEEs (Premarin, 9.6 μg·kg⁻¹·day⁻¹) by gavage (OVX + CEE group). This dose was chosen as an equivalent dose of 0.625 mg/day, used in postmenopausal women (15), and calculated to this animal model (6). Another OVX-SHR group received CEE (9.6 μg·kg⁻¹·day⁻¹) associated with testosterone cypionate (2.85 mg·kg⁻¹·wk⁻¹ im; OVX + CEE + T group) as previously described (37). The selected dose of testosterone (2.85 mg·kg⁻¹·wk⁻¹) was compatible with that used in clinical studies for hypoactive sexual dysfunction in postmenopausal women (16). On the day of euthanasia, blood samples were collected from the abdominal aorta of anesthetized animals and then centrifuged to separate the serum. Levels of estrogen and testosterone were determined by radioimmunoassay (Siemens Healthcare Diagnostic, Tarrytown, NY).

**Arterial blood pressure measurement.** Rats were anesthetized with a mixture of ketamine (113 mg/kg) and xylazine (7.4 mg/kg), and the right carotid artery was cannulated with a heparinized polyethylene catheter that was exteriorized in the midscapular region. After 24 h, mean arterial pressure was measured in conscious animals by a pressure transducer (PE-399) and recorded using an interface and software for computer data acquisition (PowerLab, ADInstruments, Melbourne, Australia).

**Vascular reactivity.** Intact segments (4 mm) of dissected thoracic aorta were set up in tissue baths for measurement of isometric contractile force, as previously described (5). After a 60-min equilibration period, contractile responses to angiotensin II (ANG II; 0.1 nM to 10 μM) were performed in rings with (E²) and without (E’) endothelium. In another series of experiments, endothelium-dependent and endothelium-independent relaxations to acetylcholine (ACh, 0.1 nM to 10 μM) and sodium nitroprusside (SNP, 0.1 nM to 1 μM) were performed in the absence of either vehicle 1 μM losartan (ANG-II type-1 receptor (AT1R) antagonist) or 10 μM apocynin (a putative NADPH-oxidase inhibitor). Each drug was added separately to the Krebs-Henseleit solution during the equilibration period and was maintained throughout the experiment. Endothelial integrity was assessed by testing the relaxant effect of ACh (100 nM) in vessels precontracted with 1 μM norepinephrine.

**Detection of reactive oxygen species generation in aortic sections.** Vascular reactive oxygen species (ROS) production was determined in situ in aortic sections by dihydroethidium, following the method previously described (36). Aortic segments (10 μm) were incubated in a light-protected and humidified chamber (37°C, 30 min) with 5-μM dihydroethidium solution. Fluorescence was detected with a 585–590-nm long-pass filter under a microscope ( Axioskop, Zeiss) with a ×40 objective lens coupled to a digital camera. Fluorescent images were recorded and analyzed by measuring the mean optical density of the fluorescence in a computer system (KS-300 Software, Zeiss). Fluorescence in each image was evaluated at least in three locations and normalized by the area. The roles of AT1R and NADPH oxidase on ROS generation were evaluated by a 30-min preincubation of sections with 1 μM losartan or 10 μM apocynin, respectively.

**Quantitative real-time PCR for detection of NADPH-oxidase subunits.** Total RNA was isolated from aortas using TRIzol reagent according to the manufacturer’s instructions. mRNAs encoding AT1R and ANG-II type-2 receptor (AT2R), as well as the subunits of NADPH oxidase (NOX1, NOX2, NOX4, p47phox, p22phox) and eNOS, were quantified by quantitative real-time PCR (qPCR) based on SYBR Green fluorescence (Applied Biosystems, Carlsbad, CA). β-Actin was used as an internal control. Primer sequences used are described on Table 1. qPCR reactions were performed, recorded, and analyzed using the Corbett Research system (Corbett Life Sciences, Sydney, Australia). The conditions for qPCR were as follows: 95°C for 2 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Cycle threshold (Ct) values obtained for each gene were referenced to β-actin (ΔCt) and converted to the linear form using the term 2⁻ΔΔCt as a value directly proportional to the copy number of complementary DNA and initial quantity of mRNA.

**Immunoblot analysis of NADPH-oxidase phosphorylation.** Frozen aortas were homogenized in lysis buffer, consisting of (in mmol/l) 50 M tris(hydroxymethyl)aminomethane (Tris), 150 M NaCl, 5 M β-mercaptoethanol, and 0.1% Triton X-100. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes. The membranes were probed overnight with primary antibodies in appropriate dilutions. The following primary antibodies were used in this study: anti-phosphorylated p47phox (Abcam, Cambridge, MA, USA), anti-nonphosphorylated p47phox (Abcam, Cambridge, MA, USA), anti-phosphorylated p22phox (Abcam, Cambridge, MA, USA), anti-nonphosphorylated p22phox (Abcam, Cambridge, MA, USA), and anti-AT1R (Abcam, Cambridge, MA, USA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA, USA). Immunoreactive bands were stained with diaminobenzidine and visualized with ultrahigh-speed X-ray film (Konica Minolta, Minolta, Tokyo, Japan). The density of the bands was quantified by densitometry using the ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

### Table 1. Primers designed for qPCR experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1R (NM_030985.4)</td>
<td>GACCTTCTCTGAGTGTCTGTA</td>
<td>CAGCGAAGAAGCGCTAGACAC</td>
</tr>
<tr>
<td>AT2R (NM_007429.5)</td>
<td>CTCGCTGGATGCCTATAAGA</td>
<td>AGCAGAGATTTTTTGTCTGACG</td>
</tr>
<tr>
<td>eNOS (NM_021838.2)</td>
<td>TCTCGGAGAGAGCGATTACCCAT</td>
<td>AAAGCGAGAGAGCATTTCGAAAA</td>
</tr>
<tr>
<td>NOX2 (NM_053683.1)</td>
<td>TTTCCTGAGAAGAAAGAGAGG</td>
<td>GCAGTGATGGAAGCTCTGAAAA</td>
</tr>
<tr>
<td>NOXI (NM_053683.1)</td>
<td>GAGATTGCTACAGAAGAGACCGA</td>
<td>ACCAGAGTCCTTCCTGAAA</td>
</tr>
<tr>
<td>NOX4 (NM_055204.1)</td>
<td>GTATGGGCGCTGCTGCGGAG</td>
<td>TCTCTGGCCAGCACAATCTGCTG</td>
</tr>
<tr>
<td>p22phox (NM_007806.3)</td>
<td>GGGUTCTACCTCTCACCAGCCT</td>
<td>TACGGCAATGGGAGGCGCTGCAA</td>
</tr>
</tbody>
</table>

qPCR, quantitative PCR; AT1R, ANG-II type-1 receptor; AT2R, ANG-II type-2 receptor; eNOS, endothelial nitric oxide synthase; NOX, NADPH oxidase.
OVX, OVX

Basic parameters (uterus weight, plasma estrogen and testosterone levels, and blood pressure) measured in Sham, OVX, OVX+CEE, and OVX+CEE+T rats (Fig. 1, A and B). Nevertheless, differences in ANG-II responses were seen only in endothelium-intact aortas (E\(^+\)) (Fig. 1A). In E\(^+\) aortic rings, ANG II evoked a higher contractile response in OVX than in Sham, as evidenced by the increased AUC (Fig. 1C) and maximum response (MaxR) (Fig. 1D). CEE treatment restored the vascular responses to ANG II in OVX to similar levels observed in Sham. The protective effects of CEE were abolished when testosterone was added to the treatment (Fig. 1). Contractions to ANG II were equivalent in endothelium-denuded (E\(^-\)) vessels from all groups (Fig. 1B), establishing a key role for the endothelial cells in the regulation of ANG-II responses by ovariectomy and estrogens.

As expected in aorta isolated from Sham SHRs, ACh evoked a biphasic response (relaxation at lower concentrations and contraction at higher concentrations) (Fig. 2A). Although ACh induces similar levels of MaxR in all groups (Table 3), there was significant change in the sensitivity and pattern of the curve when EC\(_{50}\) and AUC values were compared. Despite the absence of differences in EC\(_{50}\) values, OVX markedly decreased AUC in response to ACh, probably because of an increase in the contractile component of the biphasic curve. The contractile phase of ACh responses started at lower concentration and reached higher levels in OVX than in Sham. At ACh maximum concentration (10 \(\mu M\)), there was significant reduction in the relaxation induced by this agonist in aortas from OVX compared with Sham (Fig. 2C). CEE treatment brought the responses to ACh to similar levels observed in Sham. On the other hand, OVX+CEE+T worsened the responses observed in OVX. Even though testosterone did not interfere with the benefits of CEE to diminish the contractile component of the curve, it considerably decreased the sensitivity of aortic to ACh (Table 3, Fig. 2B). The endothelium-independent relaxation to SNP was similar among the groups studied, demonstrating that the different changes of sex hormone levels in female SHRs do not interfere with the aortic smooth muscle cells sensitivity to NO (Table 3).

To evaluate the contribution of ANG II via AT\(_1\)R and NADPH oxidase on the impaired endothelial function in OVX and OVX+CEE+T groups, curves to ACh were performed in E\(^-\) aortic rings incubated with losartan (1 \(\mu M\)) or apocynin (10 \(\mu M\)). Both losartan (Fig. 3) and apocynin (Fig. 4) improved ACh-induced relaxation of aortas from OVX (Figs. 3B and 4B) and OVX+CEE+T (Figs. 3D and 4D) but did not interfere with ACh responses in rings from Sham (Figs. 3A and 4A) or OVX-CEE (Figs. 3C and 4C) rats. Whereas in OVX aortas the inhibition of AT\(_1\)R (Fig. 3, E and F) and NADPH oxidase (Fig. 4, E and F) diminished the contractile component of the curves.

### RESULTS

Uterine weight and plasma levels of estrogen and testosterone one were reduced in OVX rats when compared with those in Sham rats (Table 2). Treatment with CEE restored uterine weight and increased estrogen levels when compared with Sham and OVX groups. The association of testosterone with estrogen levels. The mean arterial pressure of freely moving female SHRs was neither modified by OVX nor by the treatment with CEE. However, an addition of testosterone markedly increased blood pressure levels in CEE-treated OVX-SHRs (Table 2).

ANG II induced concentration-dependent contractions in aortic rings with and without endothelium isolated from Sham, OVX, OVX+CEE, and OVX+CEE+T SHRs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>OVX</th>
<th>OVX+CEE</th>
<th>OVX+CEE+T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterus weight, mg/cm tibia</td>
<td>2.6 ± 0.1</td>
<td>0.7 ± 0.1*</td>
<td>2.3 ± 0.1*#</td>
<td>2.2 ± 0.1#</td>
</tr>
<tr>
<td>Plasma estrogen, pg/ml</td>
<td>31.9 ± 1.2</td>
<td>17.9 ± 0.3*</td>
<td>114.4 ± 13.7*#</td>
<td>80.7 ± 4.1*#</td>
</tr>
<tr>
<td>Plasma testosterone, ng/ml</td>
<td>4.8 ± 0.6</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>29.4 ± 3.8*#+</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
<td>145.7 ± 1.2</td>
<td>145.6 ± 2.6</td>
<td>147.0 ± 1.7</td>
<td>158.5 ± 2.6*#</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5–10 animals. Groups consist of sham-operated (Sham), ovariectomized (OVX), OVX treated with conjugated equine estrogens (OVX+CEE), and OVX treated with CEE associated to testosterone (OVX+CEE+T) spontaneously hypertensive rats (SHRs). *P < 0.05, compared with Sham; #P < 0.05, compared with OVX; +P < 0.05, compared with OVX+CEE.

AJP-Heart Circ Physiol • doi:10.1152/ajpheart.00681.2014 • www.ajpheart.org

---

Tris-HCl (pH 7.4), 5 EGTA, 2 EDTA, 0.1 PMSF, 1 pepstatin A, 1 leupeptin, and 1 aproetin. Equal amounts of protein (50 \(\mug\)) from each aorta were resolved by SDS-PAGE on 10% gels and electroblotted onto nitrocellulose membrane. After blockade for 1-h blocking with 5% nonfat milk in Tris-buffered saline with 0.1% (vol/vol) Tween 20 (TBST), membranes were incubated overnight at 4°C with 1:1,000 dilution of polyclonal phospho-Ser345 p47\(_{phox}\) antibody (Biorbyt, Cambridge, UK). After five washes with TBST, membranes were incubated for 1 h with a 1:2,000 dilution of horseradish peroxidase-labeled goat anti-rabbit secondary antibody (Thermo Scientific, Waltham, MA). After five additional washes in TBST, chemiluminescent signal was visualized by LAS4000 imaging system (GE Healthcare, Cleveland, OH). Blots were then placed in stripping solution (Restore, Thermo Scientific) for removal of primary and secondary antibodies. After being stripped, all membranes were reblotted using a monoclonal antibody anti-p47\(_{phox}\) (1:500; Santa Cruz Biotechnology) as a loading control. Densitometry analyses of Western blots were performed using a Mac Biophotonic ImageJ Software. Data are expressed as the ratio of phospho-p47\(_{phox}\) densities to the corresponding values of total p47\(_{phox}\) densitometry.

Statistical analysis. Data are expressed as means ± SE. Contractions to ANG II are shown as absolute tension (g). Relaxation was expressed as the percentage to the precontraction in response to norepinephrine (1 \(\mumol/l\)). Area under the concentration-response curve (AUC) was used as an overall measure of cumulative response induced by ANG II, ACh, and SNP. EC\(_{50}\) was determined from individual concentration-response curves by nonlinear regression analysis. AUC was calculated from each individual contractile or relaxing and was expressed as arbitrary units. Differences between groups [sham-operated (Sham), OVX, OVX+CEE, and OVX+CEE+T] were analyzed by one-way ANOVA, and their interaction with experimental treatment groups (losartan and apocynin) were analyzed by two-way ANOVA with Bonferroni’s post-test to compare replicate means. Statistical significance was accepted at \(P < 0.05\). The statistical analysis was carried out using the Prism 5 software (GraphPad Software, San Diego, CA).

### Table 2. Basic parameters (uterus weight, plasma estrogen and testosterone levels, and blood pressure) measured in Sham, OVX, OVX+CEE, and OVX+CEE+T SHRs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>OVX</th>
<th>OVX+CEE</th>
<th>OVX+CEE+T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterus weight, mg/cm tibia</td>
<td>2.6 ± 0.1</td>
<td>0.7 ± 0.1*</td>
<td>2.3 ± 0.1*#</td>
<td>2.2 ± 0.1#</td>
</tr>
<tr>
<td>Plasma estrogen, pg/ml</td>
<td>31.9 ± 1.2</td>
<td>17.9 ± 0.3*</td>
<td>114.4 ± 13.7*#</td>
<td>80.7 ± 4.1*#</td>
</tr>
<tr>
<td>Plasma testosterone, ng/ml</td>
<td>4.8 ± 0.6</td>
<td>1.4 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>29.4 ± 3.8*#+</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
<td>145.7 ± 1.2</td>
<td>145.6 ± 2.6</td>
<td>147.0 ± 1.7</td>
<td>158.5 ± 2.6*#</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5–10 animals. Groups consist of sham-operated (Sham), ovariectomized (OVX), OVX treated with conjugated equine estrogens (OVX+CEE), and OVX treated with CEE associated to testosterone (OVX+CEE+T) spontaneously hypertensive rats (SHRs). *P < 0.05, compared with Sham; #P < 0.05, compared with OVX; +P < 0.05, compared with OVX+CEE.
to ACh, in aortic rings from CEE+T losartan and apocynin improved the sensitivity (lower EC_{50}) and MaxR to ACh (Table 3, Figs. 3 and 4).

ROS generation was observed in aortic cross sections from all groups (Fig. 5A). In isolated aorta from OVX and OVX/CEE/T, detected levels of ROS were higher when compared with Sham and OVX-CEE aortas, suggesting that testosterone abrogates the antioxidant effects of CEE (Fig. 5B). The incubation with losartan (Fig. 5C) and apocynin (Fig. 5D) reduced ROS generation in aortas from OVX and OVX+CEE+T but did not modify ROS generation in aortas from CEE or Sham rats. As previously described, OVX significantly decreased eNOS expression (Fig. 6A). CEE treatment restored eNOS mRNA expression, an effect that was not

Fig. 1. Angiotensin II (ANG II)-induced contractions. Shown are concentration-effect curves determined in endothelium-intact (E^+; A) or endothelium-denuded (E^-; B) aortas from female spontaneously hypertensive rats (SHRs). Vascular responses are expressed as the area under the curve (AUC; C) and maximum (Max) contraction (D) to ANG II. Curves were obtained in vessels from sham-operated (Sham), ovariectomized (OVX), OVX treated with conjugated equine estrogens (OVX+CEE), and OVX treated with CEE associated to testosterone (OVX+CEE+T) rats. Each point represents mean ± SE from 6–10 independent experiment. *P < 0.05.

Fig. 2. Acetylcholine (ACh)-induced vasodilatations. Shown are concentration-effect curves determined in endothelium-intact aortas from female SHRs (A). Differences in endothelium-dependent relaxations are expressed as AUC (B) and relaxation at the highest concentration of ACh (10^{-5} M; C). Curves were obtained in vessels from Sham, OVX, OVX+CEE, and OVX+CEE+T rats. Each point represents the mean ± SE from 6–10 independent experiments. *P < 0.05.
modified by the association with testosterone (Fig. 6A). mRNA levels of membrane-bound NADPH-oxidase subunits p22\textsubscript{phox} and NOX2 were greater in aortas from OVX compared with Sham (Fig. 6, B and E). Both CEE and CEE+T treatments restored NOX2 and p22\textsubscript{phox} levels to those observed in aortas from Sham. Although OVX did not modify NOX1 expression, a marked decrease on NOX1 mRNA was observed after both CEE and CEE+T treatments (Fig. 6D). The expression of p47\textsubscript{phox} and NOX4 mRNA was similar among the groups (Fig. 6, C and F). The expression of AT1R and AT2R was not detected (data not shown). Nonetheless, CEE+H11001T treatment increased p47\textsubscript{phox} phosphorylation at serine residues, which has been associated with increased NADPH-oxidase activity (Fig. 6G).

Table 3. MaxR and EC\textsubscript{50} to \textit{ACh} and SNP in aortic rings from Sham, OVX, OVX+CEE, and OVX+CEE+T SHRs

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>OVX</th>
<th>OVX+CEE</th>
<th>OVX+CEE+T</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh MaxR %</td>
<td>73.9±3.8</td>
<td>67.2±3.7</td>
<td>80.6±6.2</td>
<td>66.5±3.9</td>
</tr>
<tr>
<td>EC\textsubscript{50} nM</td>
<td>19.2 (13.1–28.2)</td>
<td>7.9 (3.1–20.2)</td>
<td>9.8 (5.0–19.0)</td>
<td>174.9 (123.7–247.2)*#+</td>
</tr>
<tr>
<td>SNP MaxR %</td>
<td>99.6±0.5</td>
<td>95.4±2.4</td>
<td>95.0±4.9</td>
<td>100±0.1</td>
</tr>
<tr>
<td>EC\textsubscript{50} nM</td>
<td>337.7 (292–392)</td>
<td>497.7 (371–662)</td>
<td>33.9 (22.8–52.0)¥</td>
<td>438.1 (256–747)</td>
</tr>
</tbody>
</table>

Values are means ± SE for maximum response (MaxR) and means with 95% confidence interval for EC\textsubscript{50} in 5–10 animals. Statistical significance calculated by 2-way ANOVA for acetylcholine (ACh) and 1-way ANOVA for sodium nitroprusside (SNP). *P<0.05, compared with Sham; #P<0.05, compared with OVX; ¥P<0.05, compared with matched controls.
DISCUSSION

This is the first study aimed to determine systematically the effects of testosterone supplementation on the vascular effects induced by estrogens. Observational epidemiological studies described and discussed consequences of the combination of estrogens with testosterone; however, the mechanisms involved in this interaction are largely unknown. We found that the addition of testosterone to CEE therapy increases ROS generation throughout the activation of cytosolic NADPH-oxidase subunit p47\(^{phox}\), reversing the protective effects of estrogen on ANG-II contraction and endothelial function.

We used hypertensive female OVX-SHRs as an experimental model to represent menopause in women (22). The rational of the model is based on the observation that a high number of postmenopausal women display a certain degree of endothelial dysfunction and increased blood pressure compared with young premenopausal women (1). There is evidence that changes in vascular function after menopause, either by aging or estrogen withdrawn, influences the actions of hormones and may interfere with the outcomes of HT. In those conditions, women may get the best benefits, or the highest risks, from HT (with or without testosterone). Therefore, the use of a health normotensive and young female rat may not be representative of what actually happens in menopausal women.

Experimental studies in animal models and vascular cells as well as early observational studies have suggested protective vascular effects by estrogens and, more specifically, by estradiol (E\(_2\)). However, this theory has been questioned by clinical trials, such as the Women’s Health Initiative (41). The negative results from the Women’s Health Initiative have raised concerns over the use of estrogens and, more specifically, the CEE not only in cardiovascular disease prevention but also to relieve menopausal symptoms. CEE represents the most common form of HT, although their specific effects on cardiovascular function are not well established and remain rather controversial. Few basic studies have described similarities between the CEE mixture and E\(_2\) in exerting potentially beneficial effects on cardiovascular function (9). In contrast, others have de-
scribed differences in E2 efficacy compared with CEE in modulating NO production in porcine and human endothelial cells (34, 35). Among the criticism for the use of CEE is the fact that CEE consists of a complex mixture lacking the main physiological estrogen in women (1, 19).

In the present study, we found a protective effect by CEE in OVX females with endothelial dysfunction, characterized by a biphasic (relaxation-contraction) response to ACh (31). Treatment of OVX-SHRs with CEE at equivalent doses of HT in women decreases the contractile responses to ANG II in endothelium-intact aortic rings, establishing that the protective effects of estrogen mostly take place in the endothelium. In fact, we also found that CEE treatment markedly diminishes the contractile component of the endothelium-dependent curve to ACh in OVX-SHRs.

The best-characterized mechanism for long-term estrogen modulation of vascular function involves estrogen receptor (ER) binding to recognition sites of the genome and modulation of transcription, which results in increased or decreased gene expression in endothelial cells (44). The vascular protection conferred by estrogen has been largely associated to its use (48). Similar to estrogens, testosterone exerts wide-ranging and concentration-specific effects via interaction with specific receptors found throughout the body, including the vascular wall (30). The dose of testosterone used for hormonal treatments in postmenopausal women has been widely discussed. Studies have described that low doses (150 μg/day) of testosterone can contribute to the cardioprotection in OVX rats by E2 (29). Another group has described that 300 μg of testosterone twice/week improves insulin sensitivity and muscle strength in elderly women with chronic heart failure (25). Although they clearly have an important role in sexual desire and arousal, the exact effect of androgens in other systems in female remains poorly understood.

Experimental studies suggest that androgens contribute to increased cardiovascular risk in males. Adult male SHRs have exacerbated hypertension than females, and castration of male SHRs is associated with a reduction in blood pressure to levels found in females. In addition, treatment of OVX-SHRs with testosterone increases blood pressure to levels seen in males.
Testosterone treatment has also been associated with increased vasoconstriction in descending coronary artery of female pig (21). How testosterone may induce vascular dysfunction remains unknown, although a modulatory role on ANG-II signaling pathway has been proposed. Treatment of males with androgens has been described to increase pressor responses to ANG II in several animal models and vascular territories (42). Testosterone can also cause endothelial dysfunction by increasing ROS generation. Increased ROS by androgens was reported in vascular smooth muscle cells of SHRs and Wistar male rats (4). Accordingly, the addition of testosterone to HT may modify the protective actions of estrogen or have direct detrimental effects in the vasculature. Although several studies have explored the independent or isolated effects of estrogen or testosterone in the cardiovascular system, the effects of their association in the vasculature are largely unknown. The present study demonstrates a major role of testosterone in modulating oxidative stress, which aggravates endothelial dysfunction and reverses the beneficial effects produced by CEE in O VX-SHRs. Although testosterone did not modify the effects of CEE in the contractile phase of ACh curve, it significantly reduced the sensitivity of aortas to

![Graphs showing mRNA expression and phosphorylation](image)

**Fig. 6. NADPH-oxidase expression and phosphorylation in aortas of female SHRs.** Shown are mRNA expression of endothelial nitric oxide synthase (eNOS; A) and NADPH-oxidase subunits p22phox (B) and p47phox (C), NOX1 (D), NOX2 (E), and NOX4 (F), normalized to the expression of β-actin. G: results of immunoblots analyzed in single aortas and probed with antibodies against phosphorylated (p)-serine-345-p47phox or total (t)-p47phox, as indicated, and plotted as the ratio of p-p47phox to t-p47phox. Analysis was performed in aortas from Sham, OVX, OVX+CEE, and OVX+CEE+T rats. Data represent the mean ± SE from 6–8 independent experiments. *P < 0.05.

(40). Testosterone treatment has also been associated with increased vasoconstriction in descending coronary artery of female pig (21). How testosterone may induce vascular dysfunction remains unknown, although a modulatory role on ANG-II signaling pathway has been proposed. Treatment of males with androgens has been described to increase pressor responses to ANG II in several animal models and vascular territories (42). Testosterone can also cause endothelial dysfunction by increasing ROS generation. Increased ROS by androgens was reported in vascular smooth muscle cells of SHRs and Wistar male rats (4). Accordingly, the addition of testosterone to HT may modify the protective actions of estrogen or have direct detrimental effects in the vasculature. Although several studies have explored the independent or isolated effects of estrogen or testosterone in the cardiovascular system, the effects of their association in the vasculature are largely unknown. The present study demonstrates a major role of testosterone in modulating oxidative stress, which aggravates endothelial dysfunction and reverses the beneficial effects produced by CEE in O VX-SHRs. Although testosterone did not modify the effects of CEE in the contractile phase of ACh curve, it significantly reduced the sensitivity of aortas to
ACh (as evidenced by both EC50 and AUC). Testosterone also abrogated the protective effect of CEE in the contractile responses to ANG II. The degree of ANG-II contraction in vessels from OVX+CEE+T was similar to that observed in OVX aorta. In addition, treatment with losartan and apocynin improved endothelial function in OVX+CEE+T to levels observed in Sham and OVX+CEE groups. Together, these data establish that the effects of estrogen on endothelial function are lessened by testosterone via mechanisms that might involve both a direct action of testosterone in the ANG-II system and an indirect action, inhibiting estrogen actions. Nevertheless, in our studies neither CEE nor CEE+T treatments modified mRNA expression of the receptors for estrogen (ER-α, ER-β), androgen, or ANG II (AT1R or AT2R) (data not shown).

The activation of NADPH oxidase has a fundamental role in the vascular effects of ANG II and is the major source of ROS in the vessel wall (27). Previous studies have shown that estrogen downregulates the expression of NADPH-oxidase subunits and receptors for ANG II (11, 13, 47). Corroborating these data, we have found a reduced vascular expression of NADPH-oxidase subunits in OVX-SHRs treated with CEEs. In the present study, we have shown that the increase in ROS generation observed in OVX+CEE+T rats was prevented by losartan and apocynin, suggesting a contribution of AT1R-mediated activation of NADPH oxidase induced by testosterone. Curiously, even though the addition of testosterone to CEE treatment increases ROS generation and impairs vascular function, testosterone did not modify the effects of estrogen on NADPH-oxidase subunits mRNA expression.

NADPH oxidases are membrane-associated proteins that transfer electrons across biological membranes using NADPH as an electron donor and as such generate ROS. The prototype NADPH oxidase possesses cytosolic (p47phox, p67phox or homologues) and membrane-bound [p22phox and gp91phox or homologues (NOX)] subunits (26). This study provides evidence that CEE+T treatment does not interfere with the NOX homologues (NOX1, NOX2, and NOX4), p22phox, and p47phox mRNA expression but augments phosphorylation of the respiratory burst of the oxidase, the p47phox subunit. Phosphorylation of the p47phox subunit induces its translocation from the cytosolic compartment to the cellular membrane, where it combines with other subunits to assemble the fully activated oxidase and to increase ROS generation (20). Results from mutation of phosphorylated serine sites demonstrated that p47phox phosphorylation is absolutely required for NADPH-oxidase activation. p47phox is phosphorylated on selective serine sites by different type of protein kinases, such as PKC, PKA, MAPK ERK1/2, and p38-MAPK (20). A recent study has shown that testosterone stimulates short-term, nongenomic ROS production by a unique mechanism that involves c-Src tyrosine kinase activation only in vascular smooth muscle cells from SHR (8). Although the same study showed that testosterone also upregulates p47phox expression via genomic mechanism, we believe that this effect may not overcome the inhibitory effect induced by estrogen. The tyrosine kinase c-Src is an upstream molecule of the p38-MAPK cascade, which is known to phosphorylate p47phox at Ser345 site and promote NADPH-oxidase assembly (10). In our study, we found increased phosphorylation of p47phox at this specific site, suggesting an increased activation of NADPH oxidase by testosterone in OVX-SHRs.

Taken together, our results suggest that the association of testosterone with CEE treatment impairs the benefits of CEE on endothelial cells function in hypertensive OVX. The detrimental effects of testosterone are mediated by mechanisms that involve AT1R activation and ROS generation. Testosterone does not interfere with the ability of CEE to upregulate eNOS or on the downregulation of membrane-bound NADPH-oxidase subunits induced by CEE, but it acts by an independent mechanism, which increases p47phox phosphorylation and stimulates NADPH oxidase. Cardiovascular diseases are the leading cause of death for menopausal women in Western countries, and the risk and benefits of HT to the cardiovascular system are still theme of a heated discussion. Although the association of estrogen and testosterone still needs to be better addressed in both experimental and clinical studies, our data demonstrate that this androgen has complex biological effects and may negatively influence the risk of cardiovascular events and other outcomes when associated to HT.

ACKNOWLEDGMENTS

We are grateful to Sônia Maria Rodrigues Leite, Marta Rodrigues da Silva, and Antônio Garcia Soares, Jr., for excellent technical support.

GRANTS

We thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/BDU 269/12). T. J. Costa was supported by a fellowship from FAPESP (2010/03608-1). Ana Paula Dantas is supported by grant from Ministerio de Economia y Competitividad, Instituto de Salud Carlos III (RIC RD12/00042/0006) and Programa Hispano-Brasileño de Cooperación Interuniversitaria (HBB-2011-0054 PC).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.H.C.C. conception and design of research; M.H.C.C. approved final version of manuscript; T.J.C., G.S.C., and M.H.C.C. prepared figures; T.J.C., G.S.C., A.P.D., and M.H.C.C. interpreted results of experiments; T.J.C., G.S.C., A.P.D., and M.H.C.C. analyzed data; T.J.C., G.S.C., R.C.T., E.H.A., Z.B.F., A.P.D., and M.H.C.C. contributed to writing of the manuscript; T.J.C., G.S.C., R.C.T., E.H.A., Z.B.F., A.P.D., and M.H.C.C. reviewed and edited the manuscript; T.J.C., G.S.C., A.P.D., and M.H.C.C. approved final manuscript.

REFERENCES


