ORIGINAL RESEARCH—BASIC SCIENCE

Diminazene Protects Corpus Cavernosum Against Hypercholesterolemia-Induced Injury

Rodrigo A. Fraga-Silva, PhD,* Fabiana P. Costa-Fraga, BSc,* Fabrizio Montecucco, MD,†‡ Mikael Sturny, BSc,* Younoss Faye, BSc,* François Mach, MD,† Graziano Pelli, PhD,† Vinayak Shenoy, PhD,§ Rafaela F. da Silva, PhD,¶ Mohan K. Raizada, PhD,§ Robson A.S. Santos, MD, PhD,¶ and Nikolaos Stergiopulos, PhD*

*Laboratory of Hemodynamics and Cardiovascular Technology, Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland; †Faculty of Medicine, Division of Cardiology, Foundation for Medical Researches, Geneva University Hospitals, Geneva, Switzerland; ‡First Clinic of Internal Medicine, Department of Internal Medicine, University of Genoa, Genoa, Italy; §Physiology and Functional Genomics, College of Medicine, University of Florida, Gainesville, FL, USA; ¶Department of Physiology and Biophysics, Federal University of Minas Gerais, Belo Horizonte, Brazil

DOI: 10.1111/jsm.12757

ABSTRACT

Introduction. Angiotensin-converting enzyme 2 (ACE2) is a key enzyme of the renin angiotensin system, which breaks down angiotensin II and forms angiotensin-(1–7). In erectile tissues, it has been documented that angiotensin II contributes to the development of erectile dysfunction (ED), while treatment with angiotensin-(1–7) improves penile erection. However, the expression and function of ACE2 in erectile tissues have never been investigated.
Aim. Here, we examined the expression of ACE2 in erectile tissues and its actions against hypercholesterolemia-induced corpus cavernosum (CC) injury.
Methods. Hypercholesterolemic apolipoprotein E knockout ($ApoE^{-/-}$) mice, a well-known model of ED, were treated with diminazene aceturate (DIZE), an ACE2 activator compound, or vehicle for 3 weeks. Reactive oxygen species (ROS), collagen content, and protein expression of ACE2, neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) subunits were evaluated in the penis of DIZE-treated and untreated $ApoE^{-/-}$ mice. Functional studies were performed in CC strips.
Main Outcome Measures. ACE2 expression and its role in modulating nitric oxide (NO)/ROS production and fibrosis within the CC of hypercholesterolemic mice were the main outcome measures.
Results. ACE2 was expressed in smooth muscle and endothelial cells of mouse CC. Interestingly, ACE2 was downregulated in penis of hypercholesterolemic mice with ED, suggesting a protective role of ACE2 on the CC homeostasis. In accordance with that, pharmacological ACE2 activation by DIZE treatment reduced ROS production and NADPH oxidase expression, and elevated nNOS and eNOS expression and NO bioavailability in the penis of $ApoE^{-/-}$ mice. Additionally, DIZE decreased collagen content within the CC. These beneficial actions of DIZE on the CC were not accompanied by improvements in atherosclerotic plaque size or serum lipid profile.
Conclusion. ACE2 is expressed in erectile tissue and its reduction is associated with hypercholesterolemia-induced ED. Additionally, treatment with DIZE improved hypercholesterolemia-induced CC injury, suggesting ACE2 as a potential target for treating ED. **Fraga-Silva RA, Costa-Fraga FP, Montecucco F, Sturny M, Faye Y, Mach F, Pelli G, Shenoy V, da Silva RF, Raizada MK, Santos RAS, and Stergiopulos N. Diminazene protects corpus cavernosum against hypercholesterolemia-induced injury. J Sex Med 2015;12:289–302.**

Key Words. Erectile Dysfunction; Hypercholesterolemia; Angiotensin-Converting Enzyme 2; Angiotensin II; Angiotensin-(1–7)

Introduction

The renin angiotensin system (RAS) is a key modulator of cardiovascular homeostasis [1–3]. Also, evidences indicate that RAS also plays an essential role in erectile function [4–10]. Besides endocrinal actions, the RAS is articulated within the cavernosal tissue and acts in a paracrine manner, modulating corpus cavernosum (CC) smooth muscle cell activity [4,8,10]. In fact, the physiological amount of angiotensin II (Ang II), the main RAS effector, produced in erectile tissues is significantly higher than those found in the systemic circulation [4], indicating an intense modulation of RAS in the erectile tissues. Within the CC, Ang II produces smooth muscle cell contraction and oxidative stress and decreases nitric oxide (NO) bioavailability [8,11,12]. Moreover, it was reported that Ang II levels in penile plasma were elevated in patients with erectile dysfunction (ED) [4], suggesting that the hyperactivity of this peptide is closely associated with the pathogenesis of ED.

Conversely, Ang-(1–7), known as a pivotal contraregulator of Ang II, appears to mediate penile erection [6,7,9]. Ang-(1–7) produces CC relaxation and NO release and potentiates erection by acting through Mas receptor [7]. This peptide is mainly formed by angiotensin-converting enzyme 2 (ACE2), which cleaves the C-terminal phenylalanine of Ang II, leading to Ang-(1–7) formation [13–15]. Thus, ACE2 is a key enzyme of the RAS that reduces Ang II and increases Ang-(1–7) bioavailability. However, the expression and function of this enzyme have never been investigated in erectile tissues.

Recently, a novel pharmacological strategy has been developed to target ACE2. Based on the crystal 3D structure of ACE2 and using a virtual screening strategy, about 140,000 small molecules (from a chemical library) were molecularly docked into structural pockets present in structures of ACE2 in different conformations [16]. Small molecules able to interact with a specific pocket of this enzyme were able to maintain a favorable conformation for optimal activity, consequently activating the enzyme [16]. It was documented that two compounds are able to activate ACE2, a xanthenone called XNT [16–19] and diminazene aceturate (DIZE) [20–22]. It has been shown that ACE2 activator compounds produce several beneficial cardiovascular effects [16–22]. For instance, DIZE attenuates pulmonary hypertension [20], reduces damages in cardiac [21] and stroke ischemia [22], and has other beneficial outcomes [23,24]; however, its action on erectile tissues has never been addressed.

In the present study, we aim to evaluate the expression of ACE2 in erectile tissue. Moreover, we sought to investigate if treatment with the novel ACE2 activator compound, DIZE, would protect the CC against injury induced by hypercholesterolemia.

Materials and Methods

Experimental Design

A well-established animal model of hypercholesterolemia-induced ED was used [25–27]. This ED model was chosen foremost because hypercholesterolemia is one of the most prevalent risk factors for the development of ED [28,29]. Moreover, the pathophysiological mechanism of hypercholesterolemic ED condition involves molecular pathways related to actions of the RAS on systemic vasculature and CC, such as modulation of oxidative stress, NO bioavailability, and matrix collagen deposition [28,30–33]. Apolipoprotein E knockout ($ApoE^{-/-}$) mice in a C57BL/6J background (N = 40) were obtained from Charles River Laboratories (Les Oncins, France). Animals at 15–20 weeks of age were randomly assigned in two groups: (i) treated with the ACE2 activator DIZE [20] and (ii) treated with vehicle (saline 0.9%). During the 11-week experimental period, the animals were fed with Western-type diet consisting of 15% (wt/wt) cocoa butter and 0.25% (wt/wt) cholesterol (Diet W, cat# 4021.06, abDiets, Woerden, Netherlands). The mice were administered with DIZE (15 mg/kg per day, provided by Prof. Raizada) or vehicle subcutaneously during the last 3 weeks. After experimental period, the animals were anesthetized (ketamine 100 mg/kg, xylazine 10 mg/kg) and blood samples were collected by cardiac puncture for serum extraction. Immediately following cardiac puncture, the penis was removed and snap-frozen in liquid nitrogen and stored at –80°C for protein measurements or frozen in cryoembedding medium for histological analysis. Some penises were excised and mounted in the isolated organ bath as described below. Age-matched C57BL/6J wild type mice (n = 20) fed with standard diet were used as additional controls. All animal study was approved by local ethics committee and Swiss authorities and is in accordance with the United States National Institutes of Health (NIH) guidelines.

Dosage of Serum Lipid Profile

Serum lipid profile was routinely measured on total blood and expressed as millimole per liter.

Briefly, blood samples were collected by cardiac puncture, incubated at room temperature for 15 minutes (for clotting), and the serum was sequentially obtained by centrifugation (4,500 rpm for 10 minutes). The lipids were measured by photometric enzymatic reaction using commercial available kits (total cholesterol, cat. number: #12016630; low-density lipoprotein cholesterol [LDL-c], cat. number: #03038661; high-density lipoprotein cholesterol [HLDL-c] from Roche Diagnostics GmbH, Mannheim, Germany) using the chemistry analyzer Roche Hitachi 902 (Roche Diagnostics GmbH). The assay was performed as described in the instruction manual.

Immunostaining in Mouse Penis

The expression and localization of ACE2 in mouse penis were assessed by co-immunostaining of ACE2 with CD31 and α-actin. Six-micrometer cryosections of wild type and ApoE$^{-/-}$ mice penises were fixed in acetone at room temperature and immunostained overnight with the following primary antibodies: anti-ACE2 (1:100, cat# sc-20998, Santa Cruz Biotechnology, Inc., Dallas, TX, USA); anti-α-actin (1:100, cat# sc-32251, Santa Cruz Biotechnology, Inc.); and anti-CD31 (1:100, cat# sc-1506, Santa Cruz Biotechnology, Inc.). Sequentially, the following second antibodies were used: anti-rabbit immunoglobulin G (IgG) conjugated with Alexa Fluor-555 secondary antibody (1:400, cat# A31572, Invitrogen, Inc., Carlsbad, CA, USA); anti-mouse IgG conjugated with Alexa Fluor-647 secondary antibody (1:400, cat# A31571, Invitrogen, Inc.); and anti-goat IgG conjugated with Alexa Fluor-488 secondary antibody (1:400, cat# A21467, Invitrogen, Inc.). The sections were finally closed with coverslip and mounting medium containing DAPI (Vectashield®, cat# H-1200, Vector Laboratories, Inc., Burlingame, CA, USA) and examined on a confocal microscope equipped with a digital imaging system (Carl Zeiss LSM 700, Carl Zeiss LSM, Zeiss, Oberkochen, Baden-Württemberg, Germany).

Western Blotting of ApoE$^{-/-}$ Mouse Penis

The protein expressions of ACE2, neuronal (nNOS) and endothelial (eNOS) nitric oxide synthases, and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) subunits p67-phox (p67) and p22-phox (p22) were evaluated by western blotting. Forty micrograms of protein extracted from mice penises was run on a 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel and the proteins were transferred onto a polyvinylidene fluoride membrane. After blocking for 1 hour (Li-Cor Odyssey Blocking Buffer, cat# 927-40000, Li-Cor Biosciences, Lincoln, NE, USA), the membranes were probed with one of the following primary antibodies: anti-ACE2 (1:100, cat# sc-20998, Santa Cruz Biotechnology, Inc.); anti-eNOS (dilution 1:200, cat# sc-654, Santa Cruz Biotechnology, Inc.); anti-nNOS (dilution 1:200, cat# sc-8309, Santa Cruz Biotechnology, Inc.); anti-p22 (dilution 1:200, cat# sc-11712, Santa Cruz Biotechnology, Inc.); anti-p67 (dilution 1:200, cat# sc-7663, Santa Cruz Biotechnology, Inc.); and anti-α-actin (dilution 1:600, cat# 32251, Santa Cruz Biotechnology, Inc.). Membranes were washed three times for 10 minutes in tris-buffered saline-Tween and incubated with anti-mouse IgG conjugated with IRDye 680 secondary antibody (dilution 1:3,000, cat# 926-68072, Li-Cor Biosciences) or anti-goat IgG conjugated with IRDye 800 secondary antibody (dilution 1:3,000, cat# 926-32214, LiCor, Inc.) or anti-rabbit IgG conjugated with IRDye 800 secondary antibody (dilution 1:3,000, cat# 926-32213, LiCor, Inc.) for 2–3 hours at room temperature. After a series of final washes (phosphate-buffered solution [PBS]), the blots were detected using a fluorescence detector (Odyssey Imaging System, Li-Cor Biosciences).

Detection of Reactive Oxygen Species in CC

Reactive oxygen species (ROS) detection in mice CC was performed by dihydroethidium (DHE; Sigma-Aldrich, St. Louis, MO, USA, cat# 37291) staining. Cryosections of 6 μm were allowed to thaw at room temperature and sequentially washed with PBS. Later, the sections were stained with DHE at 2 μmol/L in PBS for 20 minutes at 37°C in dark. The slices were washed with PBS, closed with coverslip and mounting medium containing DAPI, and examined on a confocal microscope (Carl Zeiss LSM 700). DHE fluorescence intensity of acquired digital images was quantified by Image J software (NIH, Bethesda, MD, USA).

Total Nitrite and Nitrate Assay in Serum

Nitrite (NO_2^-) and nitrate (NO_3^-) were measured as an indirect measurement of NO content in mouse serum using a commercially available kit (Griess assay; R&D Systems, Minneapolis, MN, USA, cat# KGE001). Prior to conducting the assay, serum was filtered using a 10,000 molecular weight cutoff filters (cat# UFC501096, Millipore, Billerica, MA, USA). The assay was performed as

described previously [31] and according to the manual instructions. Each sample was run as a technical triplicate.

Functional Studies in Cavernosal Tissue

After euthanasia, penises were excised and dissected in Krebs–Henseleit buffer (mmol/L: NaCl 110.8, KCl 5.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.07, $CaCl_2$ 2.49, NaH_2PO_4 2.33, and glucose 11.51). The tunica albuginea was carefully removed and the strip preparations were obtained. Cavernosal strips were mounted in isolated organ chamber system containing Krebs–Henseleit buffer at 37°C and continuously aerated with a mixture of 95% O_2 and 5% CO_2. The mechanical activity was recorded isometrically by a force transducer (ADInstruments, Colorado Springs, CO, USA). The tissue was stretched to a passive force of 3.0 mN and allowed to equilibrate for 60 minutes, and the solutions were replaced every 15 minutes [31]. Changes in isomeric force were recorded using a PowerLab/8SP data acquisition system (Chart software, version 5.0; ADInstruments).

To evaluate cavernosal endothelial function, the endothelial-dependent relaxation was induced by acetylcholine (ACh, 10^{-9} mol/L to 10^{-6} mol/L) in strips pre-constricted with phenylephrine (10^{-5} mol/L). To correct the differences in vascular smooth muscle reactivity, the response to ACh was normalized by the sodium nitroprusside (SNP) response (10^{-4} mol/L).

Sirius Red Staining for Collagen Content in Mouse Penis

Sirius red staining was performed in penis cryosections as described previously [27,31]. Briefly, mouse penis cryosections (6 μm) were rinsed with water; nuclei were stained with Weigert's hematoxylin for 10 minutes, washed in tap water, and incubated with 0.1% Sirius red (Sigma Chemical Co, St Louis, MO, USA) in saturated picric acid for 60 minutes. Sections were rinsed twice with 5% acetic acid in water for 10 seconds, then immersed in absolute ethanol three times before clearing in xylene twice and coverslipping. The sections were photographed with identical exposure settings under ordinary light microscopy. Quantifications were performed with Image J software.

Atherosclerotic Lesion Analysis

For quantification of atherosclerotic lesions, en face with Oil-red-O was performed in aortic tree of $ApoE^{-/-}$ mice. After sacrifice, arterial trees were perfused with PBS and dissected from the heart, extending 2–3mm after the bifurcation of the iliac arteries. After fixation in 4% paraformaldehyde for 24 hours, the aortas were opened longitudinally to expose the area of definable lesion covering the luminal en face surface. Oil-red-O staining was carried out as previously described [34]. Images were captured and the quantification of stained area was determined with Image J software.

Data Analysis

All results are expressed as mean ± standard error of the mean. Statistical analyses for western blot, Sirius red, and DHE staining were performed using one-way analysis of variance (ANOVA) followed by Bonferroni posttest. Statistical analyses for Griess assay, serum lipid, and Oil-Red-O staining red were done using Student *t*-test. Finally, the statistical analyses of the corpus cavernosal tissue bath experiments were performed using two-way ANOVA followed by Bonferroni posttest. A value of $P < 0.05$ was considered significant.

Results

ACE2 Is Expressed in Mouse Penis and It Is Reduced in Hypercholesterolemic Mice

ACE2 protein expression was shown in CC of wild type and $ApoE^{-/-}$ mice by immunostaining (Figure 1). This enzyme expression was co-localized with α-actin and CD31, markers for smooth muscle and endothelial cells, respectively (Figure 1A). The expression of ACE2 in the mouse penis was confirmed by western blotting (Figure 1B). Interestingly, ACE2 protein expression was reduced in $ApoE^{-/-}$ mice when compared with wild type (Figure 1C). Additionally, 3 weeks of treatment with DIZE increased its protein expression (Figure 1B, C) at similar levels than wild type. These data revealed that ACE2 is present in erectile tissues and its regulation may differ under pathological conditions.

ACE2 Activation Decreases ROS Production in $ApoE^{-/-}$ Mouse Penis

Based on the data showing the expression of ACE2 on the CC, we sought to evaluate if pharmacological increase of this enzyme would produce beneficial effects against injures induced by hypercholesterolemia. As previously observed [28,35], we found that ROS production was augmented in $ApoE^{-/-}$ mouse penis (Figure 2), which was associated to an increased protein expression of NADPH oxidase (Figure 3). Remarkably, the treatment with DIZE decreases

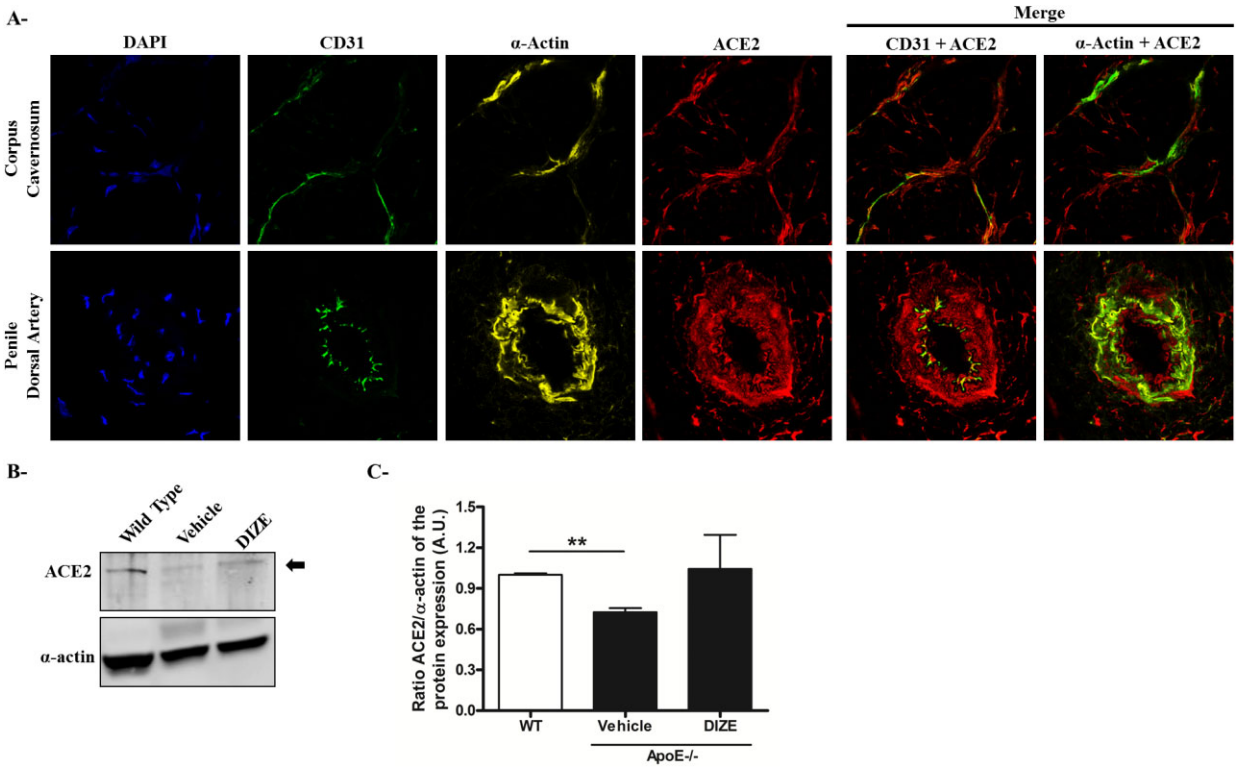

Figure 1 ACE2 is expressed in mouse penis. Immunostaining and western blotting analysis were used to detect angiotensin-converting enzyme 2 (ACE2) on penis from wild type and apolipoprotein E knockout (ApoE$^{-/-}$) mouse penis. (A) ACE2 was strongly co-localized with α-actin (smooth muscle cells marker) as well as CD31 (endothelial cell marker) in the corpus cavernosum and dorsal artery of wild type and ApoE$^{-/-}$ mice. The graphs show representative images obtained from eight different animals. (B) ACE2 expression in the mouse penis was confirmed by western blotting, which revealed a specific single band at the expected molecular weight. Additionally, ACE2 expression was reduced in ApoE$^{-/-}$ mouse penis when compared with wild type, while 3 weeks of treatment with diminazene aceturate (DIZE) normalized this alteration. The graph show representative gel of western blotting from three independent experiments. (C) Quantification of ACE2 protein expression by western blotting. Data were normalized using α-actin. $^{**}P < 0.01$ (One-way ANOVA followed by Bonferroni post-test). A.U. = arbitrary unity; WT = wild type.

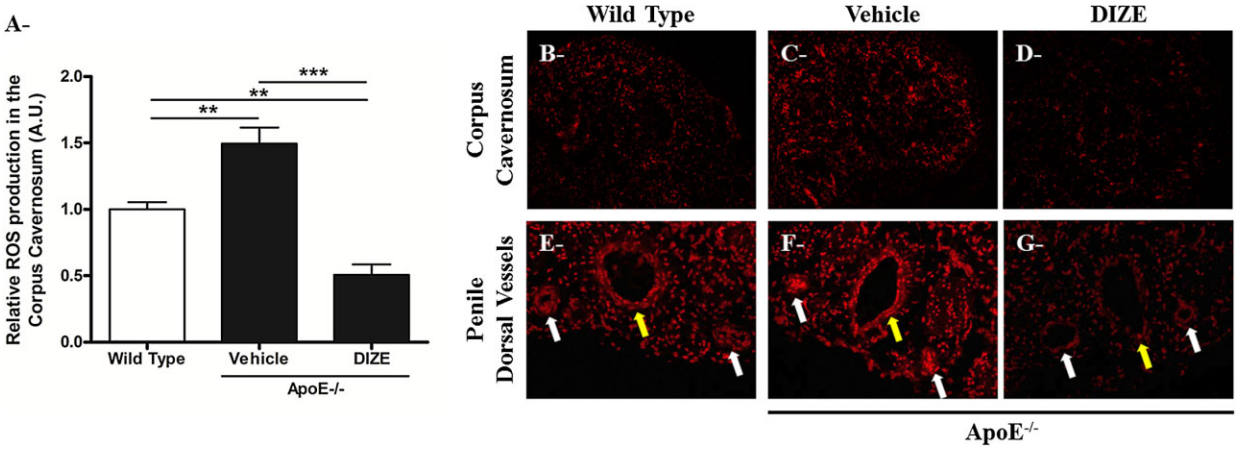

Figure 2 Angiotensin-converting enzyme 2 (ACE2) activation reduces reactive oxygen species (ROS) production induced by hypercholesterolemia. Diminazene aceturate (DIZE) treatment decreases ROS content into corpus cavernosum of ApoE$^{-/-}$ mice. (A) Quantification of ROS content. (B–G) Representative photomicrographs of penis sections showing the ROS production using dihydroethidium (DHE) staining. Figure parts B–D show the corpus cavernosum and E–F show the dorsal vessels area. $^{**}P < 0.01$ and $^{***}P < 0.001$ (one-way analysis of variance [ANOVA] followed by Bonferroni posttest). Each column represents the mean ± standard error of the mean (SEM) (n = 12) of relative fluorescence in arbitrary unity (A.U.).
ApoE$^{-/-}$ = apolipoprotein E knockout.

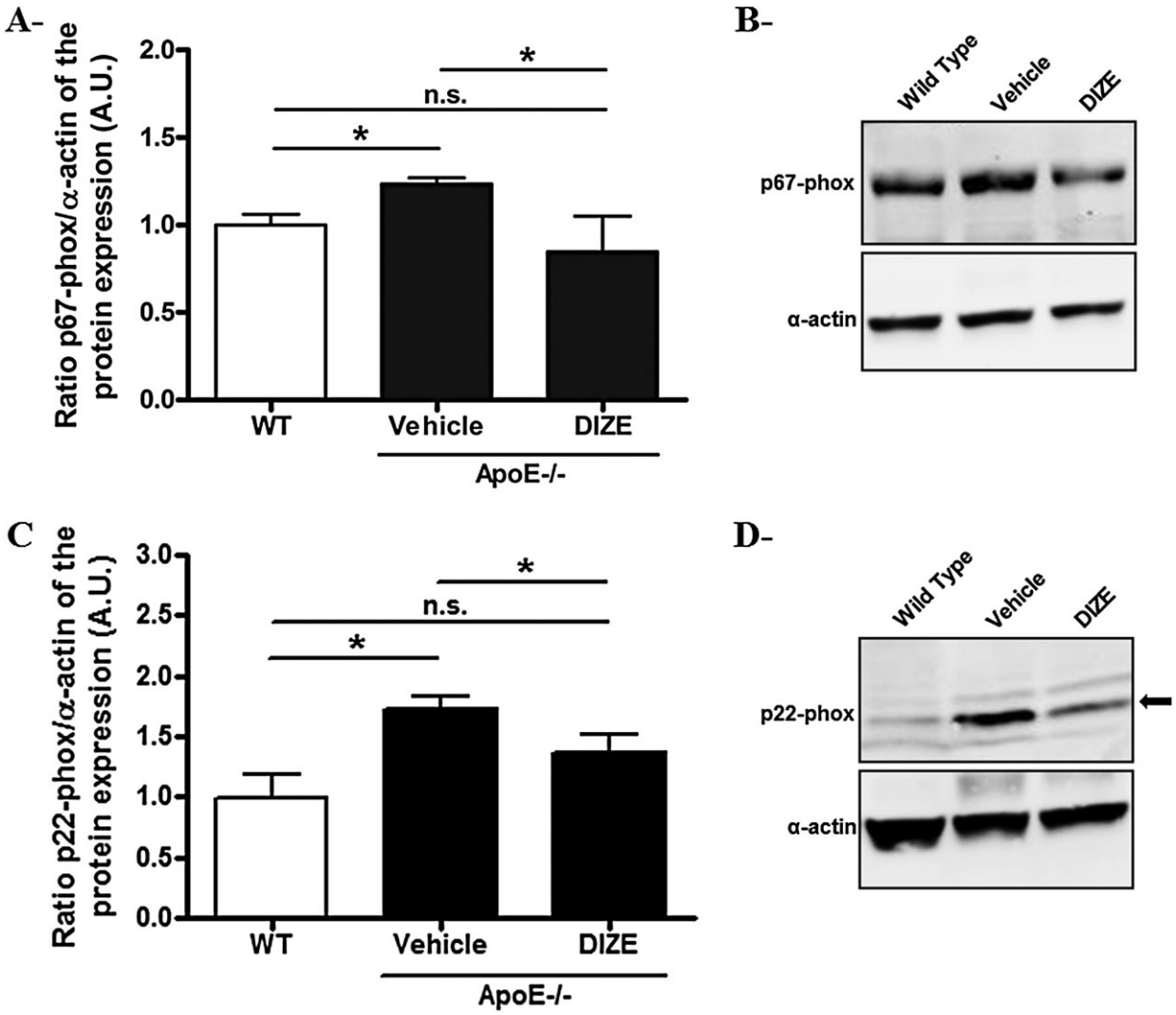

Figure 3 Treatment with diminazene aceturate (DIZE) reduces nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase expression in penis from apolipoprotein E knockout ($ApoE^{-/-}$) mice. (A and C) Quantification of NADPH oxidase protein expression, subunits p67-phox (A) and p22-phox (C), was performed using western blotting in protein extracted from penis of wild type, untreated, and DIZE-treated $ApoE^{-/-}$ mice. (B and D) Representative western blotting gels of p67-phox (C) and p22-phox (D). Data were normalized using α-actin. $^*P < 0.05$ (one-way analysis of variance [ANOVA] followed by Bonferroni posttest). Each column represents the mean ± standard error of the mean (SEM) (n = 6 to 7) of relative protein expression in arbitrary unit (A.U.) from three independent experiments. n.s. = nonsignificant; WT = wild type.

ROS production in the CC of $ApoE^{-/-}$ mice to a comparable level of wild type mice (Figure 2A–D). Similarly, DIZE treatment also decreased ROS production in the dorsal vessels of $ApoE^{-/-}$ mice (Figure 2E–G). Accordingly, DIZE treatment reduced the protein expression of NADPH oxidase subunits p67 (Figure 3A, B) and p22 (Figure 3C, D) in $ApoE^{-/-}$ mouse penis as compared with vehicle-treated mice.

DIZE Increases NO Bioavailability in $ApoE^{-/-}$ Mouse Penis

NO stable metabolites (NO_2^- and NO_3^-) in serum samples were increased in DIZE-treated $ApoE^{-/-}$ mice compared with untreated $ApoE^{-/-}$ mice (Figure 5C). Because this result only indicates a systemic vascular increase in NO production, we decided to evaluate the local protein levels of nNOS and eNOS. The eNOS and nNOS protein

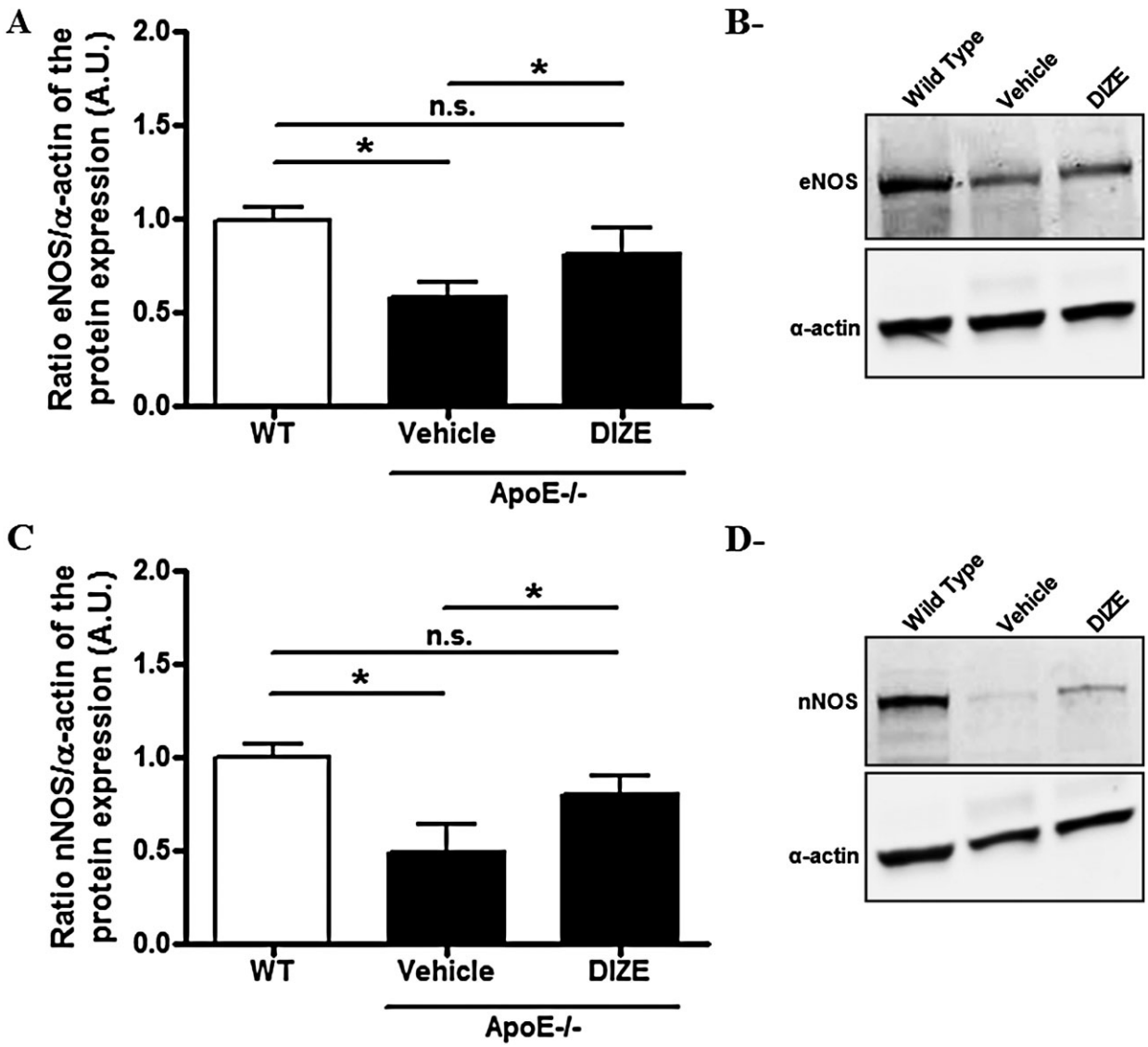

Figure 4 Treatment with diminazene aceturate (DIZE) increases endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase expressions in penis from ApoE$^{-/-}$ mice. (A and C) Quantification of eNOS (A) and nNOS (C) protein expressions was performed using western blotting in protein extracted from penis of wild type, untreated, and DIZE-treated apolipoprotein E knockout (ApoE$^{-/-}$). Representative western blotting gels of eNOS (B) and nNOS (D). Data were normalized using α-actin. *$P < 0.05$ (one-way analysis of variance [ANOVA] followed by Bonferroni posttest). Each column represents the mean ± standard error of the mean (SEM) (n = 7 to 8) of relative protein expression in arbitrary unit (A.U.) from three independent experiments. n.s. = nonsignificant; WT = wild type.

expressions were reduced in ApoE$^{-/-}$ compared with wild type mice. Interestingly, treatment with DIZE increased both eNOS (Figure 4A, B) and nNOS (Figure 4C, D) protein expressions, suggesting an increase in NO bioavailability in the CC. Similar results were obtained by immunostaining. We observed that the eNOS expression was reduced in penile endothelial cells identified by co-immunostaining with CD31 (Figure 5A, B).

In order to confirm the increased NO production within erectile tissues by treatment with DIZE, functional studies in the CC strip were performed. As expected, the NO-dependent relaxation induced by ACh was deeply reduced in ApoE$^{-/-}$ when compared with wild type mice (Figure 6A). Interestingly, the cavernosal relaxation induced by ACh was significantly increased by treatment with DIZE in ApoE$^{-/-}$ mice

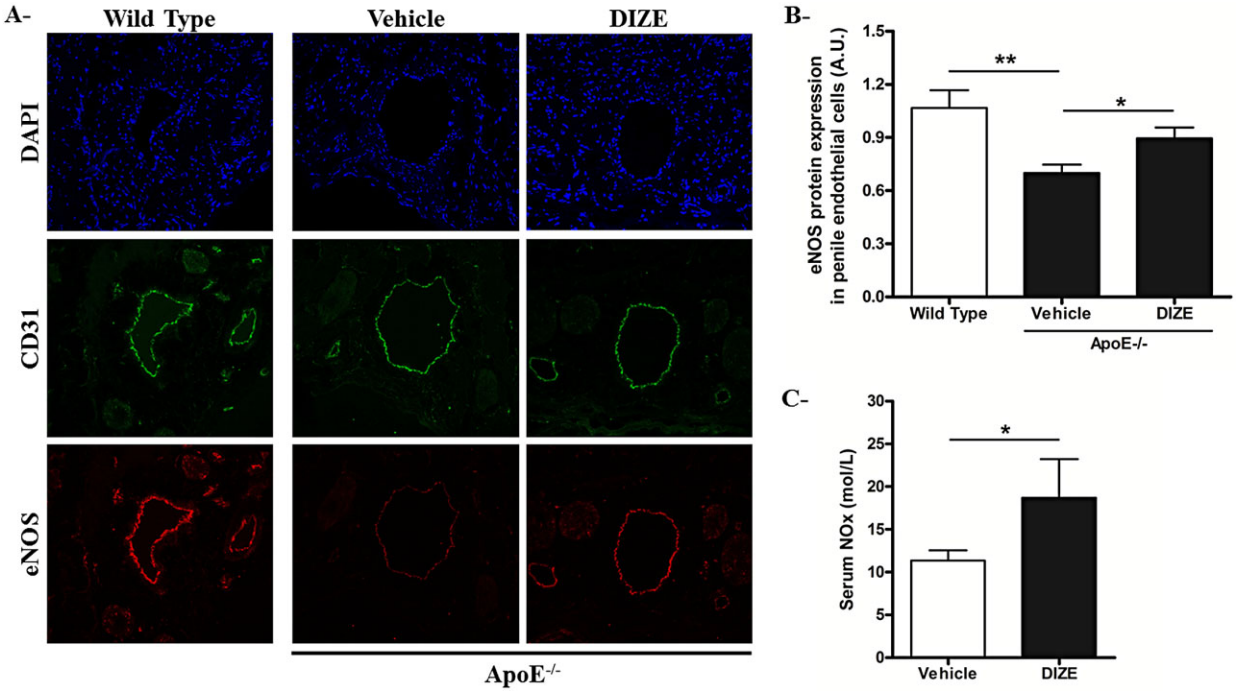

Figure 5 Treatment with diminazene aceturate (DIZE) increases endothelial nitric oxide synthase (eNOS) in vascular penile endothelium and increases systemic nitric oxide (NO) bioavailability. (A–B) Immunostaining analysis was used to detect eNOS expression in endothelial cells of dorsal penile vessels (arteries and vein). (C) Griess assay was used to determine the NO metabolites, nitrite, and nitrate in mice serum. (A) The graphs show representative images. (B) Quantification of eNOS expression. $^{*}P < 0.05$ and $^{**}P < 0.01$ (one-way analysis of variance [ANOVA] followed by Bonferroni posttest). Each column represents the mean ± standard error of the mean (SEM) (n = 8) of relative protein expression in arbitrary unit (A.U.). (C) Graphic showing the levels of nitrite and nitrate in serum from untreated and DIZE-treated apolipoprotein E knockout ($ApoE^{-/-}$) mice. $^{*}P < 0.05$ (unpaired Student's *t*-test). Each column represents the mean ± SEM (n = 10 to 12) of nitrite and nitrate concentration (μmol/L).

when compared with vehicle group (Figure 6A). Normalization of the ACh response with the SNP (NO donor) evidenced even more the beneficial effect of ACE2 activation by DIZE on NO bioavailability within the CC (Figure 6B).

Treatment with DIZE Reduces Collagen Deposition

It has been reported that $ApoE^{-/-}$ mice have a significant increase in penile collagen content [36,37]. Many studies have reported that ACE2 actions protect against pathological collagen deposition in diverse organs [19,38]. Therefore, we evaluated the effects of DIZE in penile fibrosis of $ApoE^{-/-}$ mice. Penile collagen content of $ApoE^{-/-}$ mice was significantly increased when compared with wild type mice. The treatment with DIZE produced a robust decrease in the collagen content within the CC of $ApoE^{-/-}$ mice, returning it to similar levels observed in the wild type mice (Figure 7).

Beneficial Action of Treatment with DIZE on the CC Is Not Accompanied by Reduction in Atherosclerotic Plaque Size or Lipid Profile Changes

It has been reported that ACE2 has atheroprotective effects [39]. However, the treatment with DIZE did not change atherosclerotic plaque size (Figure 8A, B) or total cholesterol, LDL, and HDL serum levels in $ApoE^{-/-}$ mice (Figure 8C–E). These data indicate that the beneficial effects of ACE2 activation by DIZE in the CC were not related to a decreased plaque deposition of lipid profile changes.

Discussion

The RAS is critically involved in the cardiovascular homeostasis, being an important target to manage cardiovascular disorders [40,41]. This system is also considerably involved in ED pathophysiology [5,7,11]. Evidences indicate that RAS acts in a paracrine manner in the erectile tissues

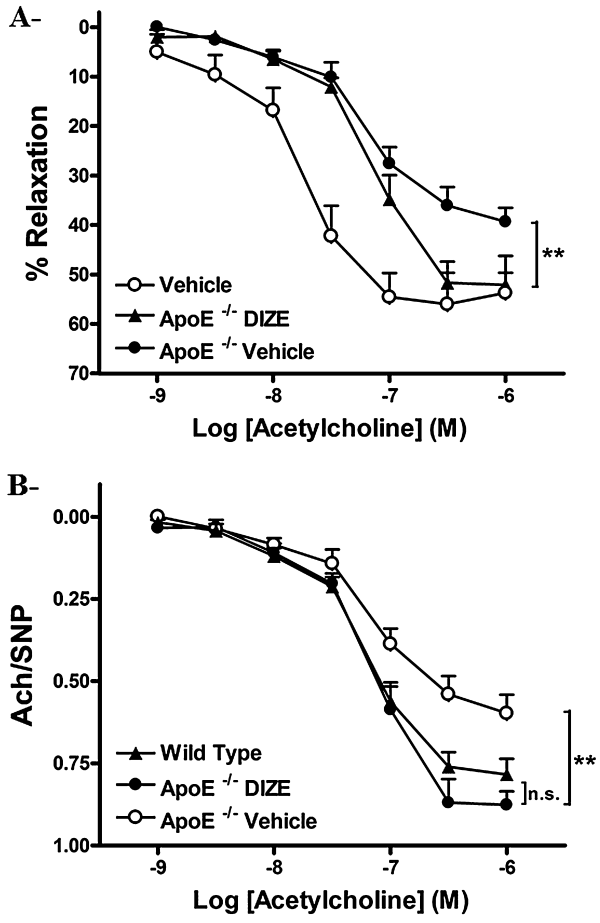

Figure 6 Angiotensin-converting enzyme 2 (ACE2) activation increases nitric oxide (NO) production within the corpus cavernosum from apolipoprotein E knockout ($ApoE^{-/-}$) mice. Relaxation is produced by increasing cumulative concentrations of acetylcholine (ACh) (A) in cavernosal strip from wild type, untreated, and DIZE-treated $ApoE^{-/-}$ mice. (B) Normalized curve response of ACh by SNP response at 10^{-4} mol/L. $^{**}P < 0.01$ and n.s. = nonsignificant (two-way analysis of variance [ANOVA] followed by the Bonferroni's multiple comparison test). Each point represents the mean ± standard error of the mean (SEM) (n = 8). DIZE = diminazene aceturate; SNP = sodium nitroprusside.

[4,7,8]. For instance, Becker et al. have shown that Ang II levels in human CC are higher than in the systemic plasma [4], which indicates an intense modulation of the RAS within penis. Supporting these data, it was observed that ACE activity were 30-fold higher in canine CC than carotid artery [42]. Additionally, da Costa Goncalves et al. observed that intracavernosal injection of A-779, a Mas receptor antagonist, reduced the erectile response in rats, indicating an endogenous role of Ang-(1–7) in penile physiological erection [7]. Many components of the RAS such as AT1 receptor, ACE, Ang I, Ang II, Ang III, Mas receptor, and Ang-(1–7) were detected within the CC [7,8]; however, no study has addressed the expression and activity of ACE2 in the penis. ACE2 is a key enzyme of the RAS that breaks down the octapeptide Ang II and forms the heptapeptide Ang-(1–7) [3,14]. Generally, the RAS is modulated by two opposite branches, a constrictive and proliferative axis, composed of Ang II/AT1 receptor, and a vasodilatory and anti-proliferative axis, composed of Ang-(1–7)/Mas receptor [43–45]. Therefore, ACE2 modulates the balance of the two major effectors of RAS, promoting beneficial effects due to an increased Ang-(1–7) production with concomitant degradation of Ang II [3,46]. In erectile tissues, Ang II produces penile detumescence and contributes to the development of ED [4], while Ang-(1–7) mediates penile erection [7]. Thus, it leads to the postulation that ACE2 would produce beneficial and pro-erectile actions in the penis. In the present study, we found that ACE2 is present within the mouse penis and that its reduction may contribute to the erectile deficiency in this hypercholesterolemic-induced ED. Moreover, ACE2 activation protects the CC against hypercholesterolemic-induced injuries.

The activation of ACE2 was achieved by pharmacological treatment with DIZE. The concept of ACE2 activators was recently developed by the group of Prof. Raizada, which documented two bioactive compounds, XNT [16] and DIZE [20]. These compounds are able to interact with specific pockets in the structure of the enzyme (non-catalytic sites) and favor the maintenance of the optimal activity conformation [16]. Therefore, they act by modulating ACE2 activity and, consequently, shifting the levels of Ang II and Ang-(1–7) [47]. Indeed, it has been shown that the beneficial cardiovascular actions of DIZE was abolished by C-16, an ACE2 inhibitor [20,21], and attenuated by co-treatment with A-779, an Ang-(1–7)-Mas receptor antagonist [22,23]. Beyond activating intrinsic ACE2, it has been shown that ACE2 activators, when given chronically, may also increase the ACE2 expression [19,20]. The relevance of each mechanism, intrinsic activation, or increase in enzyme amount in a chronic scenario is still unknown; however, both mechanisms imply the increase of ACE2 activity. In the present study, the levels of Ang II and Ang-(1–7) within the penis or ACE2 activity were not evaluated, and the mechanism by which DIZE protects erectile tissues against hypercholesterolemic-induced injuries should be carefully taken into account. Despite this limitation, we observed that DIZE treatment

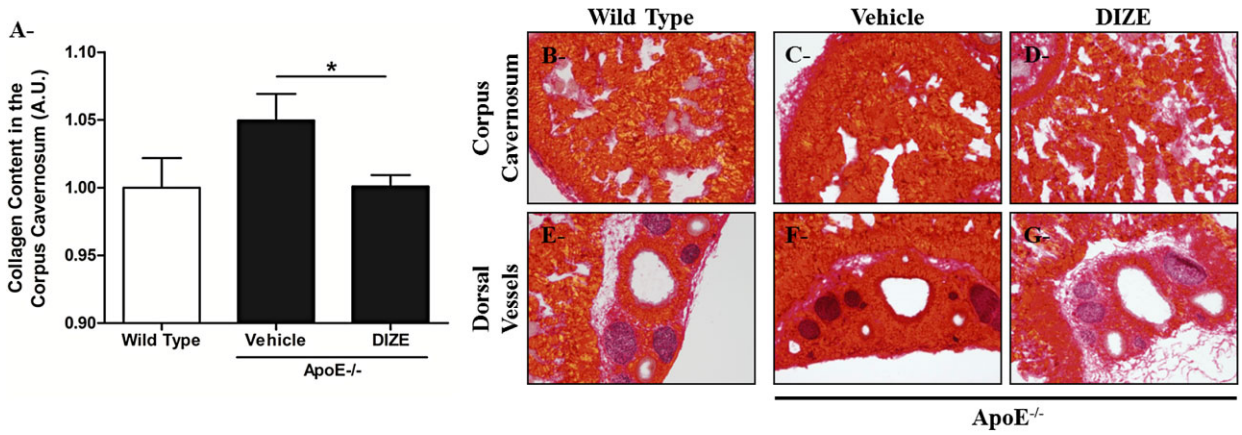

Figure 7 Treatment with diminazene aceturate (DIZE) reduces penile fibrosis induced by hypercholesterolemia. Collagen content was evaluated by Sirius red staining in penis from wild type and apolipoprotein E knockout ($ApoE^{-/-}$) mice treated or not with DIZE. (A) Quantification of collagen content into the corpus cavernosum. (B–G) Representative photomicrographs of penis sections showing the collagen content (red staining). Figure parts B–D show the corpus cavernosum and E–F show the dorsal vessels area. $^*P < 0.05$ (one-way analysis of variance [ANOVA] followed by Bonferroni posttest). Each column represents the mean ± standard error of the mean (SEM) (n = 10) of relative collagen content in arbitrary unit (A.U.).

increased ACE2 protein expression in the penis, suggesting that this compound modulates, somehow, ACE2 activity within the CC.

Many studies have pointed out that an augmented oxidative stress is a critical pathway involved in the impairment of cavernosal function observed in ED [48]. An increased ROS production may cause vascular impairment by decreasing the ability of arteries to respond to vasodilation factors [49,50]. ACE2 decreases vascular oxidative stress by dual action, decreasing Ang II and increasing Ang-(1–7) actions [44]. Ang II is a classic pro-oxidant peptide that increases ROS production through the activation of NADPH oxidases [51]. On the other hand, Ang-(1–7) is an antioxidant agent [52]. Acting thought Mas receptor, this peptide reduces ROS production with consequent improvement of endothelial function [53]. In keeping with that, our study has shown that the DIZE treatment normalized the augmented ROS production observed in $ApoE^{-/-}$ mouse penis, possibly by modulating NADPH oxidase expression.

In addition to its role of decreasing ROS, DIZE treatment also increased NO bioavailability in the penis of $ApoE^{-/-}$ mice. NO is the main effector in the erectile response and an impaired NO bioactivity is a major pathogenic mechanism of ED [54]. In the present study, we found that treatment with DIZE increased the stable NO metabolites $NO2^-$/$NO3^-$ in serum samples from $ApoE^{-/-}$ mice. Moreover, the expressions of eNOS and nNOS in the penis of $ApoE^{-/-}$ mice were restored by treatment with DIZE, suggesting that this compound improves NO bioavailability within the penis. Supporting these data, the functional study in cavernosal strip revealed a marked increase in the relaxation produced by ACh, which indicates an enhanced production of NO in the DIZE-treated group. Possibly, the beneficial effect of DIZE treatment on NO generation is mediated by an increase in local Ang-(1–7) levels. In fact, several studies have reported that Ang-(1–7) improves endothelial function by increasing NO bioavailability [55,56].

In our experimental protocol, DIZE was given in the last 3 weeks, after 8 weeks of high-fat diet. Previous reports have shown that after 6 weeks, these animals have already developed atherosclerosis in the aortic arc and carotid artery [57]; therefore, it is expected that cavernosal alterations had already take place in the beginning of the treatment. However, the direct evaluation of the cavernosal condition at 8 weeks of high-fat diet was not assessed, and the interpretation of DIZE actions as reversing or preventive should be carefully taken.

It has been shown that ACE2 has anti-atherosclerotic activity [39,58]. This enzyme inhibits the development of atherosclerotic lesions, reduces intraplaque inflammation, and increases plaque stability [39,58,59]. Thus, the beneficial effects produced by ACE2 activation in the $ApoE^{-/-}$ mouse penis could be due, in part, to a decrease in atherosclerosis development or a lipid profile changes. However, in the present study, no significant difference was observed in the lesion area when

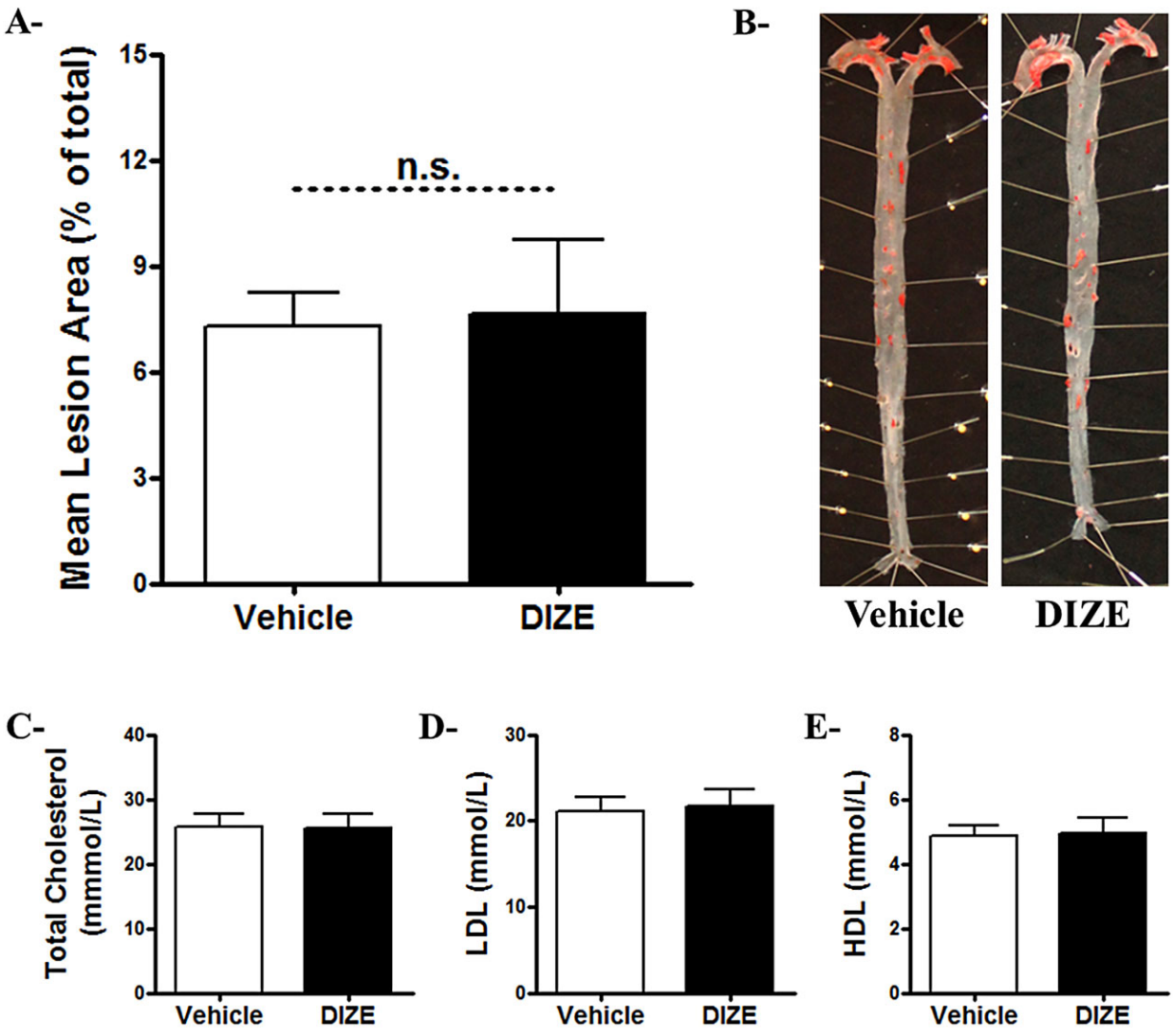

Figure 8 Treatment with diminazene aceturate (DIZE) is not accompanied by improvements in atherosclerotic plaque and serum lipid profile. (A) Quantification of lipid deposition in the aorta measured by Oil Red O (n = 7 to 8). (B) Representative photographs of entire aorta (lipid deposition in red). (C–D) Serum lipid profile in treated and untreated apolipoprotein E knockout ($ApoE^{-/-}$) mice. (C) Total cholesterol (mmol/L); (D) low-density lipoprotein (LDL) (mmol/L); (E) high-density lipoprotein (HDL) (mmol/L). n.s. = nonsignificant (unpaired Student's *t*-test). Each column represents the mean ± standard error of the mean (SEM) (n = 12).

comparing DIZE-treated with untreated $ApoE^{-/-}$ mice. Possibly, the duration of 3 weeks treatment with DIZE was not sufficient to establish/detect changes on the development of atherosclerotic plaques. Additionally, no modification in lipid profile was observed.

In summary, our findings demonstrate that ACE2 is expressed in the CC and its downregulation was associated with hypercholesterolemia-induced ED. Furthermore, the treatment with DIZE, a known ACE2 activator compound, attenuated ROS production and increased NO bioavailability. Importantly, these effects were accompanied by a reduction in penile collagen content. These results suggest that ACE2 might have significant therapeutic benefits for the treatment of ED.

Acknowledgments

This research was funded by the Ecole Polytechnique Fédérale de Lausanne (grant to Dr. N. Stergiopulos), Novartis Consumer Health Foundation (grant to Dr. R.A. Fraga-Silva), the Swiss National Science Foundation (grant #310030-118245 to Dr. F. Mach and grant #32003B-134963/1 to Dr. F. Montecucco), Novartis

Foundation (grant to Dr. F. Montecucco), Foundation "Gustave and Simone Prévot" (grant to Dr. F. Montecucco), and the University of Genoa (grant to Dr. F. Montecucco). We thank the histology core facility of the EPFL Faculty of Life Science for helping with the histological sectioning and Sirius red staining.

Corresponding Author: Rodrigo A. Fraga da Silva, PhD, Laboratory of Hemodynamics and Cardiovascular Technology, Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne, Station 17, BM 5115, CH-1015, Lausanne, Switzerland. Tel: (+41) 021-693-1119; Fax: (+41) 21-693-1120; E-mail: rodrigo.fragasilva@epfl.ch

Conflict of Interest: The authors report no conflicts of interest.

Statement of Authorship

Category 1

(a) Conception and Design
Rodrigo A. Fraga-Silva; Fabrizio Montecucco; François Mach; Rafaela F. da Silva; Mohan K. Raizada; Robson A.S. Santos; Nikolaos Stergiopulos

(b) Acquisition of Data
Rodrigo A. Fraga-Silva; Fabiana P. Costa-Fraga; Mikael Sturny; Younoss Faye; Graziano Pelli; Vinayak Shenoy

(c) Analysis and Interpretation of Data
Rodrigo A. Fraga-Silva; Fabiana P. Costa-Fraga; Mikael Sturny; Younoss Faye; Graziano Pelli; Vinayak Shenoy

Category 2

(a) Drafting the Article
Rodrigo A. Fraga-Silva; Fabrizio Montecucco; Nikolaos Stergiopulos; Robson A.S. Santos

(b) Revising It for Intellectual Content
Rodrigo A. Fraga-Silva; Fabrizio Montecucco; Robson A.S. Santos; Nikolaos Stergiopulos

Category 3

(a) Final Approval of the Completed Article
Rodrigo A. Fraga-Silva; Fabiana P. Costa-Fraga; Fabrizio Montecucco; Mikael Sturny; Younoss Faye; François Mach; Graziano Pelli; Vinayak Shenoy; Rafaela F. da Silva; Mohan K. Raizada; Robson A.S. Santos; Nikolaos Stergiopulos

References

1 Ma TK, Kam KK, Yan BP, Lam YY. Renin-angiotensin-aldosterone system blockade for cardiovascular diseases: Current status. Br J Pharmacol 2010;160:1273–92.
2 Schiffrin EL. Vascular and cardiac benefits of angiotensin receptor blockers. Am J Med 2002;113:409–18.
3 Fraga-Silva RA, Ferreira AJ, Dos Santos RA. Opportunities for targeting the Angiotensin-converting enzyme 2/angiotensin-(1–7)/mas receptor pathway in hypertension. Curr Hypertens Rep 2013;15:31–8.
4 Becker AJ, Uckert S, Stief CG, Scheller F, Knapp WH, Hartmann U, Jonas U. Plasma levels of angiotensin II during different penile conditions in the cavernous and systemic blood of healthy men and patients with erectile dysfunction. Urology 2001;58:805–10.
5 Becker AJ, Uckert S, Stief CG, Truss MC, Machtens S, Scheller F, Knapp WH, Hartmann U, Jonas U. Possible role of bradykinin and angiotensin II in the regulation of penile erection and detumescence. Urology 2001;57:193–8.
6 da Costa Goncalves AC, Fraga-Silva RA, Leite R, Santos RA. AVE 0991, a non-peptide Mas-receptor agonist, facilitates penile erection. Exp Physiol 2013;98:850–5.
7 da Costa Goncalves AC, Leite R, Fraga-Silva RA, Pinheiro SV, Reis AB, Reis FM, Touyz RM, Webb RC, Alenina N, Bader M, Santos RA. Evidence that the vasodilator angiotensin-(1–7)-Mas axis plays an important role in erectile function. Am J Physiol Heart Circ Physiol 2007;293:H2588–96.
8 Kifor I, Williams GH, Vickers MA, Sullivan MP, Jodbert P, Dluhy RG. Tissue angiotensin II as a modulator of erectile function. I. Angiotensin peptide content, secretion and effects in the corpus cavernosum. J Urol 1997;157:1920–5.
9 Yousif MH, Kehinde EO, Benter IF. Different responses to angiotensin-(1–7) in young, aged and diabetic rabbit corpus cavernosum. Pharmacol Res 2007;56:209–16.
10 Fraga-Silva RA, Montecucco F, Mach F, Santos RA, Stergiopulos N. Pathophysiological role of the renin-angiotensin system on erectile dysfunction. Eur J Clin Invest 2013;43:978–85.
11 Park JK, Kim SZ, Kim SH, Park YK, Cho KW. Renin angiotensin system in rabbit corpus cavernosum: Functional characterization of angiotensin II receptors. J Urol 1997;158:653–8.
12 Jin L, Lagoda G, Leite R, Webb RC, Burnett AL. NADPH oxidase activation: A mechanism of hypertension-associated erectile dysfunction. J Sex Med 2008;5:544–51.
13 Fraga-Silva RA, Da Silva DG, Montecucco F, Mach F, Stergiopulos N, da Silva RF, Santos RA. The angiotensin-converting enzyme 2/angiotensin-(1–7)/Mas receptor axis: A potential target for treating thrombotic diseases. Thromb Haemost 2012;108:1089–96.
14 Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A, Tummino P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J Biol Chem 2002;277:14838–43.
15 Tipnis SR, Hooper NM, Hyde R, Karran E, Christie G, Turner AJ. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. J Biol Chem 2000;275:33238–43.
16 Hernandez Prada JA, Ferreira AJ, Katovich MJ, Shenoy V, Qi Y, Santos RA, Castellano RK, Lampkins AJ, Gubala V, Ostrov DA, Raizada MK. Structure-based identification of small-molecule angiotensin-converting enzyme 2 activators as novel antihypertensive agents. Hypertension 2008;51:1312–7.
17 Fraga-Silva RA, Costa-Fraga FP, Murca TM, Moraes PL, Martins Lima A, Lautner RQ, Castro CH, Soares CM, Borges CL, Nadu AP, Oliveira ML, Shenoy V, Katovich MJ, Santos RA, Raizada MK, Ferreira AJ. Angiotensin-converting enzyme 2 activation improves endothelial function. Hypertension 2013;61:1233–8.
18 Fraga-Silva RA, Sorg BS, Wankhede M, Dedeugd C, Jun JY, Baker MB, Li Y, Castellano RK, Katovich MJ, Raizada MK, Ferreira AJ. ACE2 activation promotes antithrombotic activity. Mol Med 2010;16:210–5.

19 Ferreira AJ, Shenoy V, Qi Y, Fraga-Silva RA, Santos RA, Katovich MJ, Raizada MK. Angiotensin-converting enzyme 2 activation protects against hypertension-induced cardiac fibrosis involving extracellular signal-regulated kinases. Exp Physiol 2011;96:287–94.
20 Shenoy V, Gjymishka A, Jarajapu YP, Qi Y, Afzal A, Rigatto K, Ferreira AJ, Fraga-Silva RA, Kearns P, Douglas JY, Agarwal D, Mubarak KK, Bradford C, Kennedy WR, Jun JY, Rathinasabapathy A, Bruce E, Gupta D, Cardounel AJ, Mocco J, Patel JM, Francis J, Grant MB, Katovich MJ, Raizada MK. Diminazene attenuates pulmonary hypertension and improves angiogenic progenitor cell functions in experimental models. Am J Respir Crit Care Med 2013;187:648–57.
21 Qi Y, Zhang J, Cole-Jeffrey CT, Shenoy V, Espejo A, Hanna M, Song C, Pepine CJ, Katovich MJ, Raizada MK. Diminazene aceturate enhances angiotensin-converting enzyme 2 activity and attenuates ischemia-induced cardiac pathophysiology. Hypertension 2013;62:746–52.
22 Mecca AP, Regenhardt RW, O'Connor TE, Joseph JP, Raizada MK, Katovich MJ, Sumners C. Cerebroprotection by angiotensin-(1–7) in endothelin-1-induced ischaemic stroke. Exp Physiol 2011;96:1084–96.
23 Foureaux G, Nogueira JC, Nogueira BS, Fulgencio GO, Menezes GB, Fernandes SO, Cardoso VN, Fernandes RS, Oliveira GP, Franca JR, Faraco AA, Raizada MK, Ferreira AJ. Antiglaucomatous effects of the activation of intrinsic Angiotensin-converting enzyme 2. Invest Ophthalmol Vis Sci 2013;54:4296–306.
24 Rigatto K, Casali KR, Shenoy V, Katovich MJ, Raizada MK. Diminazene aceturate improves autonomic modulation in pulmonary hypertension. Eur J Pharmacol 2013;713:89–93.
25 Xie D, Odronic SI, Wu F, Pippen AM, Donatucci CF, Annex BH. A mouse model of hypercholesterolemia-induced erectile dysfunction. J Sex Med 2007;4(4 Pt 1):898–907.
26 Schlimmer N, Kratz M, Bohm M, Baumhakel M. Telmisartan, ramipril and their combination improve endothelial function in different tissues in a murine model of cholesterol-induced atherosclerosis. Br J Pharmacol 2011;163:804–14.
27 Fraga-Silva RA, Costa-Fraga FP, Montecucco F, Faye Y, Savergnini SQ, Lenglet S, Mach F, Steffens S, Stergiopulos N, dos Santos RA, da Silva RF. Treatment with CB2 agonist JWH-133 reduces histological features associated with erectile dysfunction in hypercholesterolemic mice. Clin Dev Immunol 2013;2013:263846.
28 Musicki B, Liu T, Lagoda GA, Strong TD, Sezen SF, Johnson JM, Burnett AL. Hypercholesterolemia-induced erectile dysfunction: Endothelial nitric oxide synthase (eNOS) uncoupling in the mouse penis by NAD(P)H oxidase. J Sex Med 2010;7:3023–32.
29 McKinlay JB. The worldwide prevalence and epidemiology of erectile dysfunction. Int J Impot Res 2000;12(suppl 4):S6–11.
30 Chung E, De Young L, Brock GB. Investigative models in erectile dysfunction: A state-of-the-art review of current animal models. J Sex Med 2011;8:3291–305.
31 Fraga-Silva RA, Costa-Fraga FP, Savergnini SQ, De Sousa FB, Montecucco F, da Silva D, Sinisterra RD, Mach F, Stergiopulos N, da Silva RF, Santos RA. An oral formulation of angiotensin-(1–7) reverses corpus cavernosum damages induced by hypercholesterolemia. J Sex Med 2013;10:2430–42.
32 Yesilli C, Yaman O, Anafarta K. Effect of experimental hypercholesterolemia on cavernosal structures. Urology 2001;57: 1184–8.
33 Fraga-Silva RA, Costa-Fraga FP, Faye Y, Sturny M, Santos RA, da Silva RF, Stergiopulos N. An increased arginase activity is associated with corpus cavernosum impairment induced by hypercholesterolemia. J Sex Med 2014;11:1173–81.
34 Maganto-Garcia E, Tarrio M, Lichtman AH. Mouse models of atherosclerosis. In: Coligan JE, Bierer BE, Margulies DH, Shevach EM and Strober W, eds. Current protocols in immunology. John Wiley & Sons, New York, NY; 2012:11–23. Chapter 15:Unit 15 24.
35 Schlimmer N, Kratz M, Bohm M, Baumhakel M. Telmisartan, ramipril and their combination improve endothelial function in different tissues in a murine model of cholesterol-induced atherosclerosis. Br J Pharmacol 2011;163:804–14.
36 Fraga-Silva R, Costa-Fraga F, Savergnini S, De Sousa F, Montecucco F, da Silva D, Sinisterra R, Mach F, Stergiopulos N, da Silva R, Santos RA. An oral formulation of angiotensin-(1–7) protects against hypercholesterolemic-induced corpus cavernosum damages. J Sex Med 2012;9(suppl 4):231–2. In: World Meeting on Sexual Medicine 2012: 2012; Chicago, USA.
37 Baumhakel M, Custodis F, Schlimmer N, Laufs U, Bohm M. Improvement of endothelial function of the corpus cavernosum in apolipoprotein E knockout mice treated with irbesartan. J Pharmacol Exp Ther 2008;327:692–8.
38 Shenoy V, Ferreira AJ, Qi Y, Fraga-Silva RA, Diez-Freire C, Dooies A, Jun JY, Sriramula S, Mariappan N, Pourang D, Venugopal CS, Francis J, Reudelhuber T, Santos RA, Patel JM, Raizada MK, Katovich MJ. The angiotensin-converting enzyme 2/angiogenesis-(1–7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension. Am J Respir Crit Care Med 2010;182:1065–72.
39 Wang Y, Tikellis C, Thomas MC, Golledge J. Angiotensin converting enzyme 2 and atherosclerosis. Atherosclerosis 2013;226:3–8.
40 Unger T. The role of the renin-angiotensin system in the development of cardiovascular disease. Am J Cardiol 2002;89(2A):3A–9A, discussion 10A.
41 Nicholls MG, Richards AM, Agarwal M. The importance of the renin-angiotensin system in cardiovascular disease. J Hum Hypertens 1998;12:295–9.
42 Iwamoto Y, Song K, Takai S, Yamada M, Jin D, Sakaguchi M, Ueda H, Katsuoka Y, Miyazaki M. Multiple pathways of angiotensin I conversion and their functional role in the canine penile corpus cavernosum. J Pharmacol Exp Ther 2001;298:43–8.
43 Ferreira AJ, Santos RA. Cardiovascular actions of angiotensin-(1–7). Braz J Med Biol Res 2005;38:499–507.
44 Ferreira AJ, Santos RA, Bradford CN, Mecca AP, Sumners C, Katovich MJ, Raizada MK. Therapeutic implications of the vasoprotective axis of the renin-angiotensin system in cardiovascular diseases. Hypertension 2010;55:207–13.
45 Bader M, Ganten D. Update on tissue renin-angiotensin systems. J Mol Med 2008;86:615–21.
46 Ferreira AJ, Murca TM, Fraga-Silva RA, Castro CH, Raizada MK, Santos RA. New cardiovascular and pulmonary therapeutic strategies based on the Angiotensin-converting enzyme 2/angiotensin-(1–7)/mas receptor axis. Int J Hypertens 2012; 2012:147825.
47 Fraga-Silva RA, Ferreira AJ, Dos Santos RA. Opportunities for targeting the angiotensin-converting enzyme 2/angiotensin-(1–7)/mas receptor pathway in hypertension. Curr Hypertens Rep 2013;15:31–8.
48 Bivalacqua TJ, Usta MF, Champion HC, Kadowitz PJ, Hellstrom WJ. Endothelial dysfunction in erectile dysfunction: Role of the endothelium in erectile physiology and disease. J Androl 2003;24(6 suppl):S17–37.
49 Kim SC, Kim IK, Seo KK, Baek KJ, Lee MY. Involvement of superoxide radical in the impaired endothelium-dependent relaxation of cavernous smooth muscle in hypercholesterolemic rabbits. Urol Res 1997;25:341–6.
50 Azadzoi KM, Saenz de Tejada I. Hypercholesterolemia impairs endothelium-dependent relaxation of rabbit corpus cavernosum smooth muscle. J Urol 1991;146:238–40.
51 Touyz RM, Berry C. Recent advances in angiotensin II signaling. Braz J Med Biol Res 2002;35:1001–15.

52 Rabelo LA, Alenina N, Bader M. ACE2-angiotensin-(1–7)-Mas axis and oxidative stress in cardiovascular disease. Hypertens Res 2011;34:154–60.
53 Xu P, Costa-Goncalves AC, Todiras M, Rabelo LA, Sampaio WO, Moura MM, Santos SS, Luft FC, Bader M, Gross V, Alenina N, Santos RA. Endothelial dysfunction and elevated blood pressure in MAS gene-deleted mice. Hypertension 2008;51:574–80.
54 Burnett AL, Lowenstein CJ, Bredt DS, Chang TS, Snyder SH. Nitric oxide: A physiologic mediator of penile erection. Science 1992;257:401–3.
55 Beyer AM, Guo DF, Rahmouni K. Prolonged treatment with angiotensin 1–7 improves endothelial function in diet-induced obesity. J Hypertens 2013;31:730–8.
56 Faria-Silva R, Duarte FV, Santos RA. Short-term angiotensin(1–7) receptor MAS stimulation improves endothelial function in normotensive rats. Hypertension 2005;46:948–52.
57 Cheng C, Tempel D, van Haperen R, van der Baan A, Grosveld F, Daemen MJ, Krams R, de Crom R. Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress. Circulation 2006;113:2744–53.
58 Zhang C, Zhao YX, Zhang YH, Zhu L, Deng BP, Zhou ZL, Li SY, Lu XT, Song LL, Lei XM, Tang WB, Wang N, Pan CM, Song HD, Liu CX, Dong B, Zhang Y, Cao Y. Angiotensin-converting enzyme 2 attenuates atherosclerotic lesions by targeting vascular cells. Proc Natl Acad Sci U S A 2010;107:15886–91.
59 Dong B, Zhang C, Feng JB, Zhao YX, Li SY, Yang YP, Dong QL, Deng BP, Zhu L, Yu QT, Liu CX, Liu B, Pan CM, Song HD, Zhang MX, Zhang Y. Overexpression of ACE2 enhances plaque stability in a rabbit model of atherosclerosis. Arterioscler Thromb Vasc Biol 2008;28:1270–6.