Cellular Physiology and Biochemistry

Cell Physiol Biochem 2017;44:1796-1809
DOI: 10.1159/000485817
Published online: November 07, 2017
Accepted: October 23, 2017

Original Paper

Sildenafil (Viagra®) Prevents Cox-1/TXA2 Pathway-Mediated Vascular Hypercontractility in ApoE$^{-/-}$ Mice

Marcos A.S. Leal[a] Ananda T. Dias[a] Marcella L. Porto[b] Bruna F. Brun[a] Agata L. Gava[a] Silvana S. Meyrelles[a] José Gil-Longo[c] Manuel Campos-Toimil[c] Thiago M.C. Pereira[b,d] Elisardo C. Vasquez[a,d]

[a]Laboratory of Translational Physiology, Federal University of Espirito Santo, Vitoria, ES, Brazil, [b]Federal Institute of Education, Science and Technology (IFES), Vila Velha, ES, Brazil; [c]Farmacología de las Enfermedades Crónicas (CDPHARMA), Centro de Investigación en Medicina Molecular y Enfermedades Crónicas (CIMUS), Universidade de Santiago de Compostela, Santiago de Compostela, Spain: [d]Laboratory of Translational Physiology & Pharmacology, Pharmaceutical Sciences Graduate Program, Vila Velha University, Vila Velha, ES, Brazil

Key Words
Atherosclerosis • Endothelial dysfunction • Oxidative stress • TXA2 • ET-1 • Cyclooxygenase

Abstract
Background/Aims: The atherosclerotic apolipoprotein E-deficient (apoE$^{-/-}$) mouse exhibits impaired vasodilation and enhanced vasoconstriction responsiveness. The objectives of this study were: a) to determine the relative contribution of cyclooxygenases (Cox-1 and Cox-2), thromboxane A2 (TXA2) and endothelin-1 (ET-1) to enhancing vascular hyperresponsiveness in this model of atherosclerosis and b) to investigate the beneficial effects of the phosphodiesterase 5 inhibitor sildenafil on this endothelial dysfunction. ***Methods:*** Adult male apoE$^{-/-}$ mice were treated with sildenafil (40 mg/kg/day, for 3 weeks) and compared with non-treated ApoE$^{-/-}$ and wild-type mice. The beneficial effects of sildenafil on vascular contractile response to phenylephrine (PE) in aortic rings were evaluated before and after incubation with Cox-1 (SC-560) or Cox-2 (NS-398) inhibitors or the TP antagonist SQ-29548, and on contractile responsiveness to ET-1. ***Results:*** ApoE$^{-/-}$ mice exhibited enhanced vasoconstriction to PE (R_{max} ~35%, $p<0.01$), which was prevented by treatment with sildenafil. The enhanced PE-induced contractions were abolished by both Cox-1 inhibition and TP antagonist, but were not modified by Cox-2 inhibition. Aortic rings from ApoE$^{-/-}$ mice also exhibited enhanced contractions to ET-1 (R_{max} ~30%, $p<0.01$), which were attenuated in sildenafil-treated ApoE$^{-/-}$ mice. In addition, we observed augmented levels of vascular proinflammatory cytokines in ApoE$^{-/-}$ mice, which were partially corrected by treatment with sildenafil (IL-6, IL-10/IL-6 ratio and MCP-1). ***Conclusion:*** The present data show that the Cox-1/TXA2 pathway prevails over the Cox-2 isoform in the mediation of vascular hypercontractility observed in apoE$^{-/-}$ mice. The results also show a beneficial effect of sildenafil on this endothelial dysfunction and on the proinflammatory cytokines in atherosclerotic animals, opening new perspectives for the treatment of other endothelium-related cardiovascular abnormalities.

Elisardo C Vasquez
and Thiago MC Pereira

Pharmaceutical Sciences Graduate Program, Vila Velha University, ES (Brazil)
E-Mail evasquez@terra.com.br/pereiratmc@gmail.com

Introduction

Atherosclerosis (AT) is a complex vascular inflammatory disease of conductance arteries and its subclinical condition is an important trigger of future unfavorable cardiovascular outcomes, such as cerebrovascular events or acute myocardial infarction [1-4]. It is initiated and sustained by a combination of endothelial dysfunction and chronic exposure to modifiable risk factors (e.g. inadequate lifestyle) and non-modifiable risk factors (e.g. aging and male gender), which have been widely demonstrated by experimental [2, 5-8] and clinical studies [9-12].

In the last three decades, genetically modified mice have been extensively used as models for understanding the causes and mechanisms involved in human diseases and have contributed to the consolidation of translational research in cardiovascular and metabolic disorders [5, 7, 8, 13, 14]. Among the available models, the apoE$^{-/-}$ mouse was the first and most widely used for this purpose. Loss of apoE gene expression in this animal compromises the hepatic clearance of VLDL and LDL, leading to spontaneous hypercholesterolemia [1, 3, 7]. Moreover, apoE$^{-/-}$ mice exhibit impaired vasodilation and enhanced vasoconstriction responsiveness, which are hallmarks of endothelial dysfunction in this experimental model and in atherosclerotic humans [4, 5, 7]. Previous studies have contributed to understanding of the mechanisms of impaired relaxation of both resistance and conductance vasodilation in the apoE$^{-/-}$ mouse [7, 15]. However, the mechanisms underlying vascular hypercontractility, and approaches to correct this disturbance in this model, have not yet been completely elucidated. Thus, there is a need for research into putative therapies to treat and prevent the vascular endothelium-dependent hypercontractility that is commonly observed in chronic cardiovascular diseases, including AT.

More recently, our laboratory and others have investigated some mechanisms for the progression of AT, and alternative strategies to improve endothelial function using the apoE$^{-/-}$ model [5, 7, 15-17]. In this context, we and others have previously shown that the decreased endothelium-dependent vasodilation response to acetylcholine observed in this atherosclerotic model is mainly due to decreased bioavailability of nitric oxide, increased production of reactive oxygen species (ROS) and chronic inflammation [7, 15, 16, 18, 19]. In addition, we demonstrated an exacerbated vasoconstrictor response to sympathetic agonists in the same experimental model, with relative contribution of eicosanoids and ROS using nonselective cyclooxygenase (Cox-1/Cox-2) and NADPH oxidase inhibitors, respectively [18, 20]. In parallel, our laboratories [15, 18, 21-23] and others [24, 25] have published several data supporting the idea that chronic use of sildenafil, a phosphodiesterase 5 (PDE5) inhibitor, improves endothelial function in the apoE$^{-/-}$ mouse. However, the beneficial effects of this drug on the prostanoid vasoconstrictors and on inflammatory cytokines have not yet been evaluated in this suitable animal model of AT [26, 27].

The present study was therefore designed to determine whether the exaggerated contractile response observed in conductance vessels from apoE$^{-/-}$ mice could be mediated by: 1) eicosanoids derived from COX-1 or COX-2) pathways, 2) enhanced responsiveness to endothelin 1 (ET-1) and 3) aortic ROS overproduction. The hypotheses that inflammatory cytokines play a pivotal role, and that sildenafil is able to attenuate or abolish those endothelial dysfunctions, were also tested.

Materials and Methods

Animals

The experiments were conducted in adult (18-week-old) male apoE$^{-/-}$ mice that had a C57BL/6J genetic background, and in wild-type (WT) mice. The animals were bred and maintained in the animal care facility at the Laboratory of Translational Physiology of the Health Sciences Center of the Federal University of Espírito Santo, Brazil. The mice were housed in individual acclimatized plastic cages with temperature control (22-23°C) and humidity (60%) and were exposed to a 12/12-h light–dark cycle. All experimental procedures were performed in accordance with the guidelines for the care and handling of laboratory

animals as recommended by the National Institutes of Health (NIH), and the study protocols were approved by the Institutional Animal Care Committee (CEUA).

To accelerate the spontaneous hyperlipidemia and the progression of endothelial dysfunction and AT, the apoE$^{-/-}$ animals were fed a Western-type diet for 10 weeks, beginning at 8 weeks of age. After 7 weeks (15-week-old), the apoE$^{-/-}$ mice were divided into the following groups: a) apoE$^{-/-}$ mice treated with the PDE5 inhibitor, sildenafil (Viagra®, 40 mg/kg/day, for 3 weeks, by oral gavage), and b) apoE$^{-/-}$ mice treated with the vehicle. Wild-type mice were used as negative controls.

Plasma lipids profile

Blood samples were obtained by puncturing the animals' right ventricles, after euthanization with an overdose of sodium thiopental (Cristalia, Sao Paulo, Brazil, 200 mg/kg, i.p.). The blood was immediately transferred to a tube containing heparin and centrifuged at 956 g for 10 min. Plasma was separated immediately and kept at −20 °C until assayed. The levels of total plasma cholesterol, high density lipoproteins (HDL) and triglycerides were measured using commercial colorimetric assay kits (Bioclin, Belo Horizonte, Brazil). The levels of non-HDL were estimated by subtracting HDL from total serum cholesterol.

Vascular function

At the end of the treatment, animals were euthanized as previously described (see above) and the descending thoracic aorta was carefully excised by midline abdominal incision, and cleaned of fat and connective tissue, taking care to do not stretch the vessel excessively to ensure the integrity of the endothelium. Next, the aorta was then cut into small rings (~ 3 mm length) and placed in cold Krebs–Henseleit buffer with the following composition (in mM): NaCl 115, KCl 4.7, $CaCl_2 \bullet 2H_2O$, 2.5, $MgSO_4 \bullet 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, EDTA 0.01 and glucose 11.1.

Aortic rings were then mounted on stainless steel triangles, suspended in vertical organ baths containing 5 mL Krebs solution, maintained at a resting tension of 0.5 g at 37°C, pH 7.4 and continuously gassed with a mixture of 95% O_2 and 5% CO_2. The rings were connected to force transducers to measure isometric tension using a computerized acquisition system (MP100, Biopac Systems Inc., Santa Barbara, USA).

After a 60-min equilibration period, aortic rings were exposed to KCl (125 mM) to assess their maximal tension. After washout (30 min), aortic rings pre-contracted with the α-agonist phenylephrine (PE, 10 μM) were challenged with the vasodilator acetylcholine (10 μM). Relaxing responses greater than 60% indicated the presence of intact endothelial cells [18]. After washout with fresh buffer solution (30 min) and an equilibration period for 30 minutes, the contractile responsiveness of aortic rings was tested through cumulative concentrations of PE (from 100 pM to 30 mM).

To evaluate the relative contribution of endothelium-derived vasoactive factors to hypercontractile responsiveness, we initiated contractile response analyses. Thus, after another washout, the contractile responses of aortic rings to PE were repeated, after previous incubation for 20 min with one of the following agents: a) selective Cox-1 inhibitor (SC-560, 1 μM), b) selective Cox-2 inhibitor (NS-398, 1 μM) or c) thromboxane-prostanoid receptor (TP) antagonist (SQ 29548, 1 μM).

The curve-concentration responses were analyzed based on the calculation of the a) area under the curve (AUC) when testing the responsiveness to PE or endothelin 1, or the difference in the area under the curve (Δ AUC), when comparing concentration-response curves to PE before and after an inhibitor, expressed in arbitrary units (a.u.), b) the sensitivity of aortic rings to PE or endothelin 1, which was estimated using the pEC_{50} ($-\log EC_{50}$) and c) the efficacy estimated using the maximum response (R_{max}) to PE or endothelin 1 in both conditions.

Contribution of oxidative stress to enhanced contractile vascular responsiveness

At the end of the treatment, the lucigenin-derived chemiluminescence assay was used to determine superoxide anion ($\bullet O_2$-) levels in the aorta. Isolated vascular ring segments (3 mm), obtained as described in the previous section were acutely incubated in saline phosphate buffer. Luminescence was quantified in a luminometer (Orion II luminometer, Berthold Detection Systems, Pforzheim, Germany) and the results are expressed as percentage of control group (arbitrary units).

Determination of cytokines in aortic tissue using cytometric bead array

The levels of inflammatory interleukin 6 (IL-6) chemokine (MCP-1) and tumor necrosis factor alpha (TNF-α) cytokines, and the anti-inflammatory IL-10 cytokine were measured in the aortic tissue by

flow cytometry using a cytometric bead array, according to the manufacturer's instructions described in a mouse inflammation Kit, (CBA - BD Biosciences, San Diego, CA, USA). The BD CBA system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure different soluble analytes in a particle-based immunoassay in a single sample. Based on the manufacturer's recommended procedure, a typical forward and side scatter gate was set to exclude aggregates; a total of 5, 000 events in the gate were analyzed using FACSCanto II and FACSDiva Software (BD). Samples were quantified by comparing them with standard curves of recombinant cytokines using FCAP Array software (BD). The results are expressed as pg/mL.

Statistical analysis

The values are expressed as means±SEM. The Gaussian distribution of the variables was previously analyzed using the D'Agostino-Pearson omnibus normality test. Fitting concentration-response curves were constructed and analyzed using nonlinear regression analysis. Statistical comparisons between the different groups were performed by one- or two-way analysis of variance (ANOVA), followed by Bonferroni's *post hoc* test. Differences between means with a value of $p<0.05$ were considered statistically significant. Statistical software: Prism 7.0, GraphPad Software Inc.

Results

Plasma lipid profiles

The plasma lipid profiles of the three groups of animals are summarized in Table 1. ApoE$^{-/-}$ mice showed a significant increase in triglycerides (~3-fold, $p<0.001$), total plasma cholesterol (~14-fold, $p<0.001$) and non-HDL (~28-fold, $p<0.001$), and the treatment with sildenafil did not modify these differences in relation to the wild-type group. As expected, HDL was significantly reduced (~2- fold, $p<0.001$) in both the non-treated and the sildenafil-treated apoE$^{-/-}$ groups, compared with wild-type mice.

Contractile response to α1-adrenoceptor stimulation

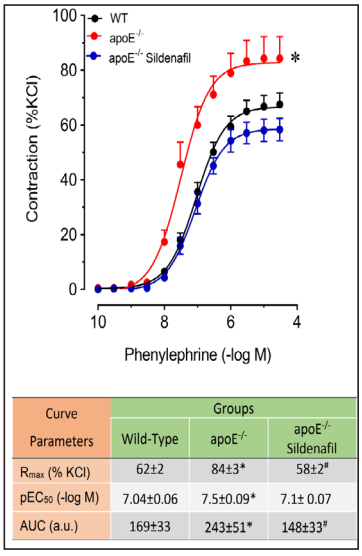
Fig. 1 summarizes the results of the evaluation of PE-induced contractions in aortic rings from the apoE$^{-/-}$ and wild-type groups. As shown in the line-graph, the apoE$^{-/-}$ group exhibited a left- and upward-shift in the concentration-response curve to PE when compared with the wild-type animals ($p<0.05$), indicating an enhanced contractile responsiveness in those atherosclerotic animals. In contrast, the curve of apoE$^{-/-}$ mice treated with sildenafil was right- and downward-shifted, close to the concentration-response curve of wild-type mice. Accordingly, apoE$^{-/-}$ mice exhibited a significant increase in efficacy (R_{max} ~35%, $p<0.01$), sensitivity (~7%, $p<0.01$) and AUC (~44%, $p<0.01$) when compared with wild-type mice, and the treatment with sildenafil completely supressed these parameters (R_{max}: 31%, sensitivity: 7% and AUC: 39%, $p<0.05$ vs apoE$^{-/-}$ mice; $p>0.05$ vs. wild-type mice) as demonstrated in the table at the bottom of Fig. 1.

Contribution of the cyclooxygenase-2 pathway to enhanced contractility

Following the demonstration of an exacerbated vasoconstriction to PE in apoE$^{-/-}$ mice, we focused our analysis on the contribution of the prostanoid molecular pathway in this abnormal response. The average data of concentration–response curves to PE and the respective parameters are shown in the line-graphs and in the bottom table in Fig. 2, comparing the PE-induced values before and after the specific Cox-2 blockade with NS-398 in the three groups of animals. No significant differences were observed in the concentration-response curves (line-graphs) or in the parameters of the curves (bottom table) obtained before and after incubation of the aortic rings with NS-398 in the wild-type group. Similarly, pre-incubation of aortic rings with the specific of Cox-2 inhibitor caused only a trend towards an

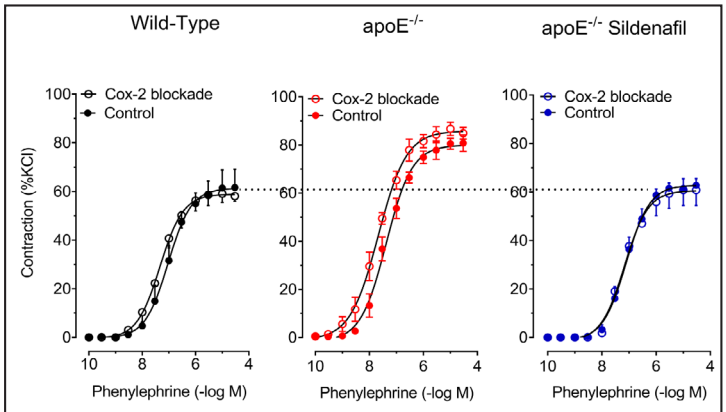
Table 1. Serum lipid profiles of sildenafil-treated apoE$^{-/-}$ mice compared with non-treated apoE$^{-/-}$ mice and wild-type control mice. Values are means ± SEM. *$p<0.05$ vs. WT (one-way ANOVA)

Lipid profile (mg/dL)	Wild-Type	apoE$^{-/-}$	
		Vehicle	Sildenafil
Triglycerides	70±8	229±19*	242±18*
Total plasma cholesterol	88±5	1,278±63*	367±172
High-density lipoprotein (HDL)	42±4	20±1*	21±3*
Non-HDL lipoprotein	46±2	1,258±16	346±173



Curve Parameters	Groups		
	Wild-Type	apoE-/-	apoE-/- Sildenafil
R_{max} (% KCl)	62±2	84±3*	58±2#
pEC_{50} (-log M)	7.04±0.06	7.5±0.09*	7.1± 0.07
AUC (a.u.)	169±33	243±51*	148±33#

Fig. 1. The effect of chronic treatment with sildenafil on phenylephrine-induced contraction in aortic rings from apoE-/- mice. R_{max} (maximum PE-induced contractions), AUC (area under the curve) and pEC_{50} (sensitivity). Values are means±SEM. *p<0.05 vs. Wild-Type mice; #p<0.05 vs. apoE-/- mice. Two-way ANOVA for repeated measurements (pEC_{50} and R_{max}), and one-way ANOVA (ΔAUC).



Curve Parameters	Groups					
	Wild-Type		apoE-/-		apoE-/- Sildenafil	
	Control	NS-398	Control	NS-398	Control	NS-398
R_{max} (% KCl)	62±4	59±2	79±2*	84±3*	62±3#	60±2#
pEC_{50} (-log M)	7.0±0.1	7.3±0.1	7.4±0.06	7.6±0.07	7.0±0.1	7.2±0.07
Delta AUC (a.u.)	11±7		41±17*		1±13#	

Fig. 2. The contribution of the prostanoids derived from Cox-2 to PE-induced vasoconstriction in aortic rings from apoE-/- mice compared to nontreated apoE-/- and wild-type mice. NS-398 is a selective Cox-2 inhibitor. The table shows the maximum vasoconstrictor response (R_{max}) to KCl, sensitivity (pEC_{50}) to PE and the difference between areas under the curve before and after the Cox-2 inhibitor in the same group. The values are means ± SEM. *p<0.05 vs. respective wild-type column; #p<0.05 vs non-treated apoE-/- group . Two-way ANOVA for repeated measurements (pEC_{50} and R_{max}), and one-way ANOVA (ΔAUC).

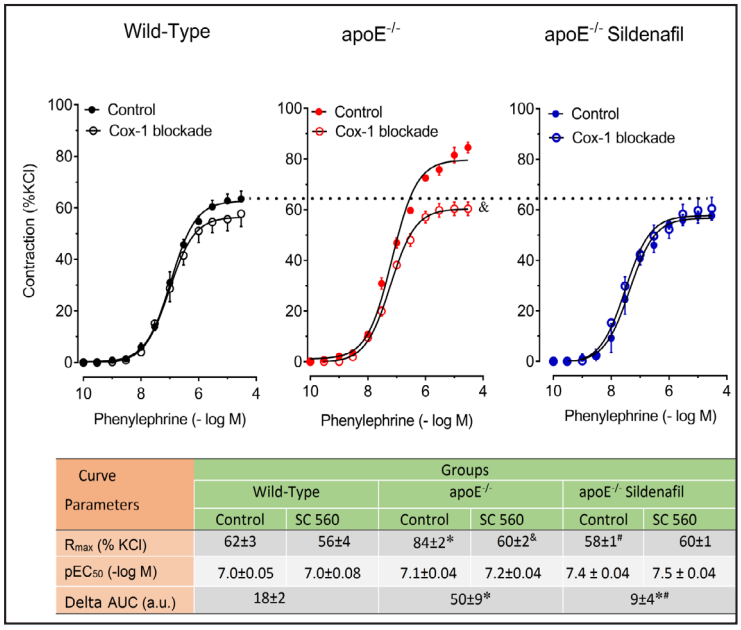
upward shift (p>0.05) in the curves, indicating a worsening of contractile hyperresponsiveness to PE in the apoE-/- group. In the group apoE-/- mice, in which hypercontractility to PE had been normalized by the treatment with sildenafil, pre-incubation with the NS-398 inhibitor did not cause additional significant changes in the concentration-response curves when compared with the data obtained without the Cox-2 blockade. These data suggest that the Cox-2-derived pathway does not contribute to the vascular hyperresponsiveness exhibited by apoE-/- mice.

Contribution of the cyclooxygenase-1 pathway to hypercontractility responsiveness

Next, we evaluated the relative contribution of Cox-1 to this process in a different group of animals, using SC-560, a specific inhibitor of this pathway. As indicated in the upper panel graphs and in the respective parameters in the bottom table (Fig. 3), pre-incubation of aortic rings with the Cox-1 inhibitor did not modify the curve parameters in the wild-type group. However, this blockade caused a right- and downward shift in the concentration-response curve to PE in aortic rings from apoE-/- mice (line-graph of Fig. 3). Accordingly, the maximum response (R_{max}) was significantly reduced (39%, p<0.05) and consequently the difference in AUC was significantly augmented (2.7-fold, p<0.05) when compared with the wild-type group. In contrast, pre-incubation of aortic rings with the Cox-1 inhibitor did not cause significant effects on the parameters of the concentration-response curves of apoE-/- mice treated with sildenafil, in which treatment with sildenafil had already normalized the concentration-response curve to PE.

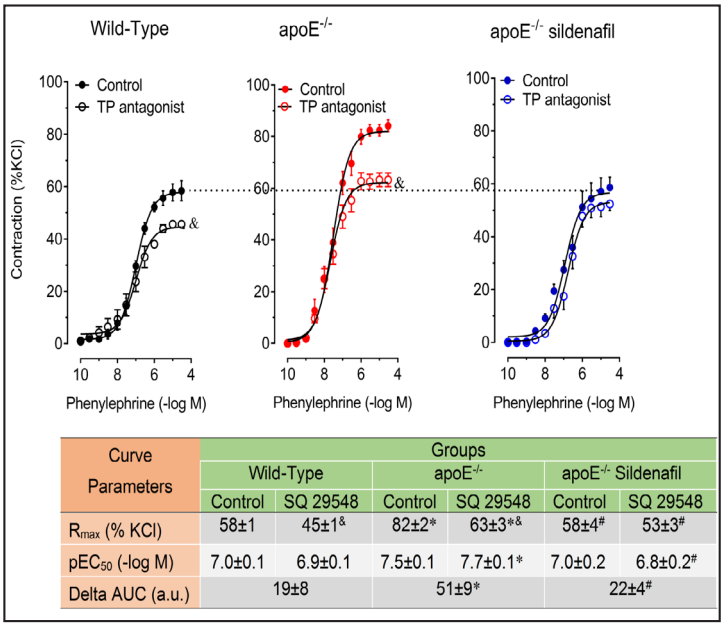
Contribution of TXA_2 to enhanced contractile responsiveness

Next, we evaluated the contribution of TXA2 to hyperresponsiveness in the vasoconstrictor PE in aortic rings from apoE-/- mice. Fig. 4, shows the average data of



Curve Parameters	Groups					
	Wild-Type		apoE$^{-/-}$		apoE$^{-/-}$ Sildenafil	
	Control	SC 560	Control	SC 560	Control	SC 560
R_{max} (% KCl)	62±3	56±4	84±2*	60±2$^{\&}$	58±1$^{\#}$	60±1
pEC_{50} (-log M)	7.0±0.05	7.0±0.08	7.1±0.04	7.2±0.04	7.4 ± 0.04	7.5 ± 0.04
Delta AUC (a.u.)	18±2		50±9*		9±4*$^{\#}$	

Fig. 3. The contribution of the prostanoids derived from Cox-1 to the PE-induced vasoconstriction in aortic rings from apoE$^{-/-}$ mice compared to non-treated apoE$^{-/-}$ and wild-type mice using the selective Cox-1 inhibitor SC-560. The table shows the maximum vasoconstrictor response (Rmax) to KCl, sensitivity (pEC_{50}) to PE and the difference between areas under the curve (ΔAUC) before and after the Cox-1 inhibitor in the same group. The values are means±SEM. *p<0.05 vs. respective wild-type column; $^{\&}$p<0.05 vs control group; $^{\#}$p<0.05 vs non-treated apoE$^{-/-}$ group . Two-way ANOVA for repeated measurements (pEC_{50} and R_{max}), and one-way ANOVA (ΔAUC).



Curve Parameters	Groups					
	Wild-Type		apoE$^{-/-}$		apoE$^{-/-}$ Sildenafil	
	Control	SQ 29548	Control	SQ 29548	Control	SQ 29548
R_{max} (% KCl)	58±1	45±1$^{\&}$	82±2*	63±3*$^{\&}$	58±4$^{\#}$	53±3$^{\#}$
pEC_{50} (-log M)	7.0±0.1	6.9±0.1	7.5±0.1	7.7±0.1*	7.0±0.2	6.8±0.2$^{\#}$
Delta AUC (a.u.)	19±8		51±9*		22±4$^{\#}$	

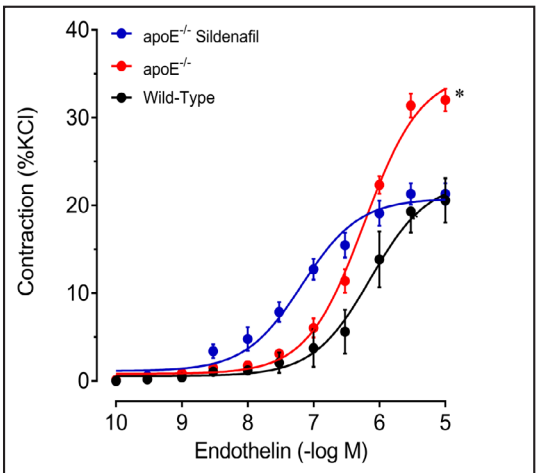
Fig. 4. The contribution of thromboxane A2 (TXA2) to the PE-induced vasoconstriction in aortic rings from apoE$^{-/-}$mice compared to nontreated apoE$^{-/-}$ and wild-type mice. SQ-29548 is a selective thromboxane receptor antagonist. The table shows the maximum vasoconstrictor response (R_{max}) to KCl, sensitivity (pEC_{50}) to PE and the difference between areas under the curve (ΔAUC) before and after the TP antagonist in the same group. The values are means±SEM. *p<0.05 vs. respective wild-type column; $^{\&}$p<0.05 vs control group; $^{\#}$p<0.05 vs non-treated apoE$^{-/-}$ group. Two-way ANOVA for repeated measurements (pEC_{50} and R_{max}), and one-way ANOVA (ΔAUC).

concentration–response curves to PE under the effect of the TP antagonist SQ-29.548 (1 μM) in the three groups of animals. As indicated in the upper panel graphs and in the respective parameters in the bottom table, pre-incubation of aortic rings with the TP antagonist did not modify the sensitivity in the previous response to PE in the three groups of animals (p>0.05). However, in terms of R_{max} and AUC, a reduction in the concentration-response curve to PE was observed. For instance, when we compared the AUC in the presence and absence of blockade as demonstrated in Fig. 4, a minor reduction was noted in the wild-type group (-17% p<0.05), but this reduction was significantly greater in the apoE$^{-/-}$ mice (-26 % p<0.05). Interestingly, treatment with sildenafil normalized this parameter (-18 % p<0.05). On the other hand, the R_{max} to PE under the TP blockade was significantly decreased in apoE$^{-/-}$ mice (- 23%, p <0.05), but it was not significant either in wild-type (-14% p>0.05) or

in apoE$^{-/-}$ mice treated with sildenafil (-8%, p>0.05).

Contractile vascular responsiveness to endothelin-1

Having established that both Cox-1 and the TXA2 pathways have an important relative contribution to the vascular hypercontractility to PE in apoE$^{-/-}$ mice, we decided to evaluate the role of the endogenous vasoconstrictor endothelin-1. Fig. 5 summarizes the results of the evaluation of endothelin-induced contractions in aortic rings from all groups. As expected, aortic rings of apoE$^{-/-}$ animals exhibited an enhanced contractile response to endothelin-1 when compared with wild-type animals, as indicated by the three main concentration-response curve parameters (bottom of Fig. 5). The data showed a significant increase in efficacy (~68%), sensitivity (~7%) and AUC (~2-fold) in apoE$^{-/-}$ mice when compared with wild-type mice (p<0.05). Looking at figure 5, we see that among the three parameters of the curve response to endothelin-1, the one most affected by sildenafil treatment was R_{max} (-32%p<0.05), with small changes in all other parameters.



Curve Parameters	Groups		
	Wild-Type	apoE$^{-/-}$	apoE$^{-/-}$ Sildenafil
R_{max} (% KCl)	19±2	32±2*	21±1$^{\#}$
pEC_{50} (-log M)	5.6±0.3	6.0±0.1	7.2±0.1*$^{\#}$
AUC (a.u.)	15±5	31±2*	37±3*

Fig. 5. Dose-response curves showing the aortic responsiveness to the vasoconstrictor endothelin-1 in apoE$^{-/-}$ mice and the beneficial effect of sildenafil. The table shows the area under the curve (AUC), the maximum vasoconstrictor response (R_{max}) and the sensitivity (pEC_{50}) in each group. The values are means±SEM. *p<0.05 vs. wild-type and $^{\#}$p<0.05 vs. non-treated apoE$^{-/-}$ group. Two-way ANOVA for repeated measurements (pEC_{50} and R_{max}), and one-way ANOVA (ΔAUC).

Contribution of oxidative stress to enhanced contractile vascular responsiveness

We also evaluated the oxidative stress by measuring $\bullet O_2^-$, the most important ROS. Fig. 6 shows the production of $\bullet O_2^-$ generated in the three groups of animals, which was significantly augmented in aortas from apoE$^{-/-}$ (2.6-fold, p<0.05) when compared with wild-type control animals. In the group of apoE$^{-/-}$ treated with sildenafil, the levels of $\bullet O_2^-$ were attenuated 1.7-fold (p<0.05).

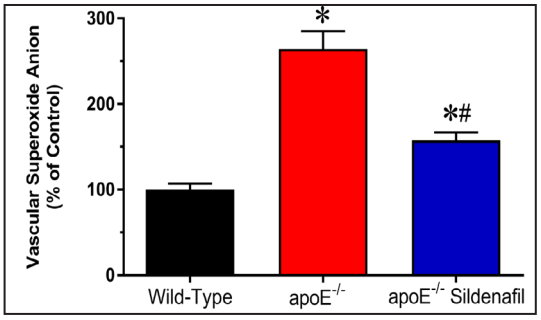


Fig. 6. The effect of sildenafil treatment on superoxide anion levels in aortic tissue in apoE$^{-/-}$ mice. The values are means±SEM (n= 6 to 8 animals per group. *p<0.05 vs. Wild-Type mice and $^{\#}$p<0.05 vs. non-treated apoE$^{-/-}$ group (1-way ANOVA). Two-way ANOVA for repeated measurements (pEC_{50} and R_{max}), and one-way ANOVA (ΔAUC).

Aortic inflammatory cytokines

In a separated group of animals, we evaluated the levels of proinflammatory IL-6, MCP-1 and TNF-α cytokines, and the anti-inflammatory IL-10 cytokine in aortic tissue. As expected and as shown in Fig. 7, aortas from apoE$^{-/-}$ mice showed augmented levels of the proinflammatory cytokine IL-6 (3-fold, p<0.05) when compared with wild-type mice, and this difference was significantly attenuated by chronic treatment with sildenafil (1.6-fold, p<0.05). Although no significant changes in the anti-inflammatory IL-10 were observed when compared with the wild-type group, the apoE$^{-/-}$ mice exhibited a significant reduction in the IL-10/IL-6 ratio (3.3-fold, p<0.05) when compared with wild-type mice. The

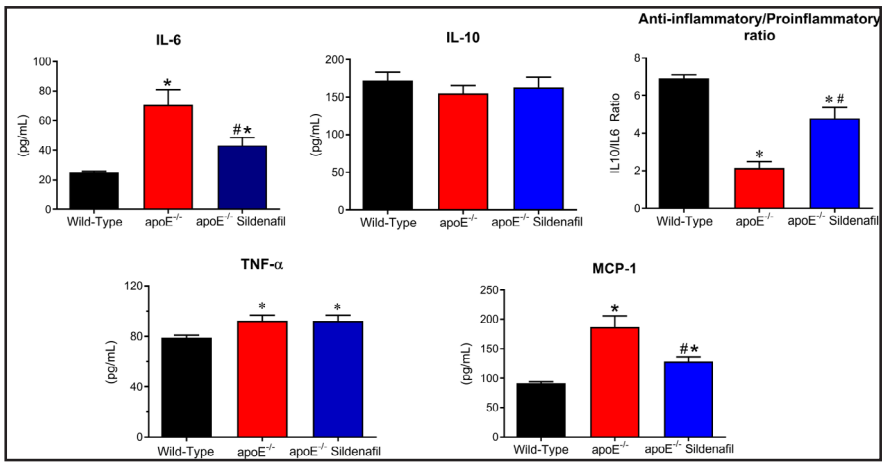


Fig. 7. The effect of sildenafil treatment on the proinflammatory interleukin (IL-6), the anti-inflammatory IL-10 and the IL-10/IL-6 ratio. The proinflammatory cytokines monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor alpha (TNF-α) are also shown. Values are means±SEM (n= 6 to 8 animals per group. *p<0.05 vs. Wild-type mice and #p<0.05 vs. non-treated apoE$^{-/-}$ group (One-way ANOVA).

aortic chemokine TNF-α was also significantly augmented in apoE$^{-/-}$ mice (1.2-fold, p<0.05), and the treatment with sildenafil did not have significant effects. The level of tissue cytokine MCP-1 was also significantly augmented (2-fold, p<0.05) and this difference was attenuated with chronic treatment with sildenafil (1.6-fold, p<0.05).

Discussion

This study was designed to elucidate the mechanisms involved in the vascular hypercontractility of aortic rings from apoE$^{-/-}$ mice and to test the beneficial effects of the PDE 5 inhibitor sildenafil on this abnormal response. Our data show a hyperactivation of COX-1/TXA2 pathway and an exacerbated contractile response to ET-1 beyond the increased levels of proinflammatory cytokines and $\bullet O_2^-$. Interesting that the exacerbation of these four different but complementary pathways was successfully attenuated by sildenafil, reinforcing the vascular protective effects exhibited by this PDE5 inhibitor.

In the present study, we observed a hypercontractility in aortic rings from apoE$^{-/-}$, which is in agreement with previous data from our laboratory [18] and with other studies that have observed enhanced vasoconstrictions even in apoE$^{+/-}$, a model of mild dyslipidemia [28]. Data from previous studies suggest that the exacerbated vascular contractile response to α1-adrenoceptor agonists, which was observed in the apoE$^{-/-}$ murine model, could be mediated by altered vascular responsiveness to endogenous vasoconstrictors and an imbalance of the vasodilatory/vasoconstriction prostanoid pathways [18, 20]. It has also been suggested that this hypercontractile response to phenylephrine (R_{max}) is due to higher levels of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in the smooth muscle cells of apoE$^{-/-}$ mice [29]. Accordingly, it has been established that Cox-derived pro-inflammatory prostanoids play a pivotal role in the generation of endothelial dysfunction and AT [26, 30-32]. Cox-1 and Cox-2 convert arachidonic acid to final vasoactive prostanoids, including balanced actions of PGI_2 and TXA2 to maintain the regulation of vascular function [33]. In general, Cox-1 is constitutively expressed in most tissues, whereas Cox-2 is an inducible isoform by various physiological stimuli such as inflammation and shear stress [34-38]. However, few studies have been conducted to evaluate the role of these endothelial enzymes in the apoE$^{-/-}$ murine model. Therefore, the novelty of the present study includes the elucidation of the relative role of Cox-1 and Cox-2 mediating the hypercontractility induced by phenylephrine, and the pleiotropic effects of the PDE-5 inhibitor sildenafil on the Cox-1 and Cox-2 pathways.

The activation of Cox, the diffusion of endothelium-dependent contracting factors towards the smooth muscle cells, and the subsequent stimulation of their TP are important contributors to endothelial dysfunction [39]. In apoE$^{-/-}$ mice, in which elevated production of TXA2 [34] has been observed, interruption of TXA2 signaling by deletion of its receptor

(TP) limits atherogenesis [40]. By using the specific inhibitors SC-560 for Cox-1 and NS-398 inhibitor for Cox-2 (Fig. 3), we observed that Cox-1 prevails over Cox-2 in the mediation of the endothelium-dependent vasoconstrictor response to PE apoE$^{-/-}$ mice. This is in agreement with the finding that aortic endothelial cells preferentially express Cox-1 versus Cox-2 [31, 41], and that as Cox-1 expression is increased, the major source of vasoconstrictor prostanoids is converted into the endothelial cells, which diffuse to contract the underlying vascular smooth muscle in arteries of mouse models of cardiovascular diseases [31, 42]. Importantly, chronic treatment with sildenafil normalized the vascular hypercontractility (R_{max}) in this model of AT. Interestingly, pre-incubation of the aortic rings with the TXA_2 inhibitor SQ-29548 (data in Fig. 4) reduced hypercontractility to PE to a similar extent to that observed when the aortic rings were pre-incubated with a specific Cox-1 inhibitor. Therefore, using different protocols, our data indicate that Cox-1, but not Cox-2 is an important contributor to the endothelium-dependent hypercontractility observed in the apoE$^{-/-}$ mouse model of AT, which is in agreement with other studies [40]. The present data is also corroborated by other studies that show that activation of TP is of high relevance for vasoconstriction [26, 43-46], and that the pharmacological antagonism of TXA_2 can delay the progression of AT [47, 48]. This could be explained by the fact that the activation of TP lead to the increase in $[Ca^{+2}]_c$, a critical step for vasoconstriction and the atherosclerotic process [18, 49, 50]. In addition, other studies have shown that TXA2 up-regulates NADPH oxidase [42, 51, 52], increasing the levels of superoxide anion, which reacts with NO, compromising its bioavailability and its vasodilator activity. This hypothesis is supported by the present data showing a significant increase (more than 2-fold) of superoxide anions in aortic tissue of apoE$^{-/-}$ mice (Fig. 6). The novelty of the present study is the demonstration that sildenafil treatment was able to correct the Cox-1/TXA2 pathway disturbance. However, one cannot rule out the participation of other endothelium-derived vasocontractile agents.

We also considered a possible contribution of ET-1 to the hyperreactivity in this atherosclerotic model. This peptide is a potent vasoconstrictor that is continuously secreted by vascular endothelial cells and acts on the underlying smooth muscle cells through ET_A receptors that increase $[Ca^{+2}]_c$, [53-56], potentiating the TXA_2 pathway. It has been associated with many forms of cardiovascular disease [57, 58]. Based on this concept, and considering the evidence that conductance vessels from atherosclerotic apoE$^{-/-}$ mice exhibit an overproduction of ET-1 and ETA hypersensitivity [58-60], we evaluated the responsiveness in aortic rings from apoE$^{-/-}$ to ET-1. Our data show an exacerbated vascular contractile response to ET-1 in apoE$^{-/-}$ mice, which could also contribute (via NADPH oxidase) to the overproduction of $\bullet O_2^-$ and to the increased degree of endothelial dysfunction [53, 61] that we observed in the present study. Moreover, it has been shown that activation of Cox is a source of $\bullet O_2^-$because of its ability to co-oxidize substances such as NADPH [36], which corroborates the present study. Therefore, our cumulative findings show an overproduction of vascular ROS (Fig. 6), an exacerbated contraction force of aortic rings to TXA2 (Fig. 4) and enhanced response to ET-1 (Fig. 5) in apoE$^{-/-}$ mice. This led us to determine whether sildenafil could minimize these synergic mechanisms in the progression of endothelial dysfunction.

Recent data have demonstrated that $\bullet O_2^-$ may upregulate PDE4 and PDE5, which rapidly inactivates the second messengers involved in vasodilation such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) [15, 18, 62]. Additionally, superoxide anions appear to stimulate the production of TXA2 and 8-isoprostane, an equipotent agonist of TP [52, 62] and ET-1 [63, 64]. Thus, pharmacological strategies such as pleiotropic drugs to interrupt this vicious cycle cascade seem to be relevant against AT. In fact, in the present study we observed that treatment of apoE$^{-/-}$ animals with this PDE5 inhibitor reduced the vascular hyperactivity probably by decreasing the TP/ET-1/NADPH oxidase activity. Previous reports from our laboratory and other studies have revealed that sildenafil, besides having vasodilatory effect (by preserving cGMP signaling and consequently reducing $[Ca^{+2}]_c$) presents additional anti-atherosclerotic effects, such as: exhibiting indirect antioxidative and antigenotoxic activity [15, 21, 23], increasing the number and function of endothelial progenitor cells [21], and reducing significantly lipid deposition in the

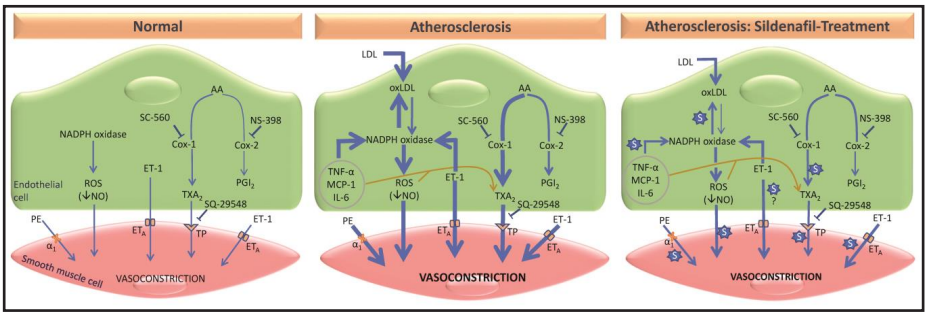


Fig. 8. Schematic summary of the main contributors to the enhanced vasoconstriction in the apoE$^{-/-}$ mouse model of AT and the beneficial effects of sildenafil based on the present data. AA: arachidonic acid, α1: α1-adrenoceptor, Cox: Cyclooxygenase, ET-1: Endothelin-1, ET_A: Endothelin-$_A$ receptor, IL-6: Interleukin-6, MCP-1: Monocyte chemoattractant protein-1, oxLDL: oxidized low-density lipoprotein, PE: Phenylephrine, PGI_2: Prostacyclin, ROS: Reactive oxygen species, S: possible actions of sildenafil, TNF-α: tumor necrosis factor alpha cytokine, TP: thromboxane-prostanoid receptor antagonist, TXA2: thromboxane A2.

conductance arteries, even under dyslipidemia [15, 18]. Confirming our previous observations, the new finding in this issue is that treatment with sildenafil shifted the exacerbated contractile response to TP and ET-1 close to that exhibited by normal wild-type animals. These responses can be explained by three main factors: 1) normalization of $[Ca^{+2}]_c$ in smooth vascular muscle cells by increasing cGMP, correcting this abnormal pathway; 2) reduction of levels $\bullet O_2$-, restoring the bioavailability of NO and lastly (and most surprisingly) the reduction of proinflammatory IL-6, TNF-α and MCP-1 (important stimulators of ROS) [8, 19, 65-67]. In addition to the data relating to changes in vascular inflammatory cytokines, we are demonstrating, for the first time, evidence of an anti-inflammatory effect of sildenafil in atherosclerotic apoE$^{-/-}$ mice (Fig. 7).

AT is characterized by chronic inflammation and enrichment of inflammatory cells in the vessel wall [39]. The crucial role of pro-inflammatory cytokines (e.g., MCP-1, TNF-α and IL-6) in the initiation and progression of AT is well documented [8, 28, 65-67]. However, there are many challenges when studying the immune system in mice aortic tissues, due to the size and the number of cells obtained from the aorta. As a solution, techniques such as polymerase chain reaction and immunohistochemistry can be useful, although they also have some limitations, such as poor correlation between mRNA and protein and semi-quantitative data, respectively [68-70]. In this study, we demonstrated, for the first time, an increase of IL-6, TNF-α and MCP-1 proteins in aortas of apoE$^{-/-}$ mice using the cytometry assay (Fig. 7). The observation that the ratio anti-inflammatory/proinflammatory cytokines was markedly reduced in the atherosclerotic animals and significantly attenuated by the treatment with sildenafil was also important. Previous studies have shown that MCP-1 plays an important role in the recruitment and trans-endothelial migration of monocytes in early atherogenesis [1, 71, 72]. At the same time, IL-6 is an important biomolecule that increases the oxidative stress, promotes fatty streaks and induces leukocytes recruitment into the sub-endothelial space [65, 73]. This led us to propose that the reduction of pro-inflammatory mediators by sildenafil might be justified by an increase in cGMP. This possibility is supported by previous findings in other experimental models demonstrating that intracellular cGMP diminishes cytokine production and oxidative stress, thereby modulating the inflammatory response [74-76].Therefore, it seems reasonable to consider that the correction of the enhanced contractile responsiveness promoted by sildenafil in aortic rings from apoE$^{-/-}$ mice could act, at least in part, via its antioxidant properties and indirect interaction with pro-inflammatory mediators, highlighting the benefits of the pleiotropic effects of sildenafil, as observed in other experimental models [4, 22].

Conclusion

A schematic summary of the present data is shown in Fig. 8. Our data show that the enhanced vasoconstriction of aortic rings from atherosclerotic mice is mediated by Cox-1 (but not Cox-2) via thromboxane A2 and ET-1, which can be repaired by sildenafil,

independently of the preserved hypercholesterolemia. Additionally, the antioxidative and immunomodulatory properties of sildenafil (i.e., reduction of $\bullet O_2$-, IL-6 and MCP-1 and an increase in IL-10/IL-6 ratio) might explain, at least in part, the vascular protective effects exhibited by this PDE5 inhibitor. The present data provide new insights into the mechanisms involved in endothelium-dependent hypercontractility in this murine model of AT, and shows that the beneficial effects of chronic treatment with sildenafil on endothelial dysfunction in this model open new perspectives for treatment of other endothelium-related cardiovascular diseases.

Abbreviations

AA (arachidonic acid); apoE$^{-/-}$ Apolipoprotein (E-deficient mouse); AT (Atherosclerosis); AUC (Area under the curve); Cox (Cyclooxygenases); $[Ca^{2+}]_c$ (Cytosolic Ca^{2+} concentration); ET (Endothelin); HDL (High density lipoprotein); IL (Interleukin); LDL (Low density lipoprotein); MCP-1 (Monocyte chemoattractant protein-1); oxLDL (Oxidized low-density lipoprotein); PGI_2 (Prostacyclin); PDE (Phosphodiesterase); PE (Phenylephrine); R_{max} (Maximum response (efficacy)); ROS (Reactive oxygen species); TNF-α (Tumor necrosis factor alpha); TP (Thromboxane-prostanoid receptor); TXA2 (Thromboxane A2); VLDL (Very low density lipoprotein).

Acknowledgements

This research was supported by National Council for the Development of Science and Technology (CNPq) (Ref. Grants #303001/2015-1, #307584/2015-1, #445736/2014-3 and # 445080/2014-0) and the State Foundation for Science and Technology (Fapes) (Ref. Grant Universal 2014 Proc #67597483/15). The funding bodies had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Disclosure Statement

The authors declare that they have no competing interests.

References

1 Zhang T, Tian F, Wang J, Jing J, Zhou SS, Chen YD: Endothelial Cell Autophagy in Atherosclerosis is Regulated by miR-30-Mediated Translational Control of ATG6.Cell Physiol Biochem 2015;37:1369-1378.
2 Liu X, Guo CY, Ma XJ, Wu CF, Zhang Y, Sun MY, Pan YT, Yin HJ: Anti-inflammatory effects of tanshinone IIA on atherosclerostic vessels of ovariectomized ApoE mice are mediated by estrogen receptor activation and through the ERK signaling pathway. Cell Physiol Biochem 2015;35:1744-1755.
3 Zhang T, Tian F, Wang J, Jing J, Zhou SS, Chen YD: Atherosclerosis-Associated Endothelial Cell Apoptosis by MiR-429-Mediated Down Regulation of Bcl-2. Cell Physiol Biochem 2015;37:1421-1430.
4 Vasquez EC, Gava AL, Graceli JB, Balarini CM, Campagnaro BP, Pereira TM, Meyrelles SS: Novel therapeutic targets for phosphodiesterase 5 inhibitors: Current state-of-the-art on systemic arterial hypertension and atherosclerosis. Curr Pharm Biotechnol 2016;17:347-364.
5 Pereira TM, Nogueira BV, Lima LC, Porto ML, Arruda JA, Vasquez EC, Meyrelles SS: Cardiac and vascular changes in elderly atherosclerotic mice: the influence of gender. Lipids Health Dis 2010;9:87.
6 Cesar L, Suarez SV, Adi J, Adi N, Vazquez-Padron R, Yu H, Ma Q, Goldschmidt-Clermont PJ, Agatston A, Kurlansky P, Webster KA: An essential role for diet in exercise-mediated protection against dyslipidemia, inflammation and atherosclerosis in ApoE$^{-/-}$ mice. PLoS One 2011;6:e17263.
7 Vasquez EC, Peotta VA, Gava AL, Pereira TM, Meyrelles SS: Cardiac and vascular phenotypes in the apolipoprotein E-deficient mouse. J Biomed Sci 2012;19:22.
8 Santanna AF, Filete PF, Lima EM, Porto ML, Meyrelles SS, Vasquez EC, Endringer DC, Lenz D, Abdalla D, Pereira TM, Andrade TU: Chronic administration of the soluble non-bacterial fraction of kefir attenuates lipid deposition in LDLr$^{-/-}$ mice. Nutrition 2017;35:100-105.
9 Ijzelenberg W, Hellemans IM, van Tulder MW, Heymans MW, Rauwerda JA, van Rossum AC, Seidell JC: The effect of a comprehensive lifestyle intervention on cardiovascular risk factors in pharmacologically treated

patients with stable cardiovascular disease compared to usual care: a randomised controlled trial. BMC Cardiovasc Disord 2012;12:71.
10 Szostak J, Laurant P: The forgotten face of regular physical exercise: a 'natural' anti-atherogenic activity. Clin Sci (Lond) 2011;121:91-106.
11 Tobaldini E, Costantino G, Solbiati M, Cogliati C, Kara T, Nobili L, Montano N: Sleep, sleep deprivation, autonomic nervous system and cardiovascular diseases. Neurosci Biobehav Rev 2017;74:321-329.
12 Kreatsoulas C, Anand SS: The impact of social determinants on cardiovascular disease. Can J Cardiol 2010;26:8C-13C.
13 Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM, Maeda N: Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc Natl Acad Sci USA 1992;89:4471-4475.
14 Lima LC, Porto ML, Campagnaro BP, Tonini CL, Nogueira BV, Pereira TM, Vasquez EC, Meyrelles SS: Mononuclear cell therapy reverts cuff-induced thrombosis in apolipoprotein E-deficient mice. Lipids Health Dis 2012;11:96.
15 Balarini CM, Leal MA, Gomes IB, Pereira TM, Gava AL, Meyrelles SS, Vasquez EC: Sildenafil restores endothelial function in the apolipoprotein E knockout mouse. J Transl Med. 2013;11:3.
16 Meyrelles SS, Peotta VA, Pereira TM, Vasquez EC: Endothelial dysfunction in the apolipoprotein E-deficient mouse: insights into the influence of diet, gender and aging. Lipids Health Dis 2011;10:211.
17 Liu W, Wang B, Wang T, Liu X, He X, Liu Y, Li Z, Zeng H: Ursodeoxycholic Acid Attenuates Acute Aortic Dissection Formation in Angiotensin II-Infused Apolipoprotein E-Deficient Mice Associated with Reduced ROS and Increased Nrf2 Levels. Cell Physiol Biochem 2016;38:1391-1405.
18 Leal MA, Balarini CM, Dias AT, Porto ML, Gava AL, Pereira TM, Meyrelles SS, Vasquez EC: Mechanisms of enhanced vasoconstriction in the mouse model of atherosclerosis: the beneficial effects of sildenafil. Curr Pharm Biotechnol 2015;16:517-530.
19 Shao Y, Cheng Z, Li X, Chernaya V, Wang H, Yang XF: Immunosuppressive/anti-inflammatory cytokines directly and indirectly inhibit endothelial dysfunction: a novel mechanism for maintaining vascular function: J Hematol Oncol 2014;7:80.
20 Arruda RM, Peotta VA, Meyrelles SS, Vasquez EC: Evaluation of vascular function in apolipoprotein E knockout mice with angiotensin-dependent renovascular hypertension. Hypertension 2005;46:932-936.
21 Rodrigues BP, Campagnaro BP, Balarini CM, Pereira TM, Meyrelles SS, Vasquez EC: Sildenafil ameliorates biomarkers of genotoxicity in an experimental model of spontaneous atherosclerosis. Lipids Health Dis 2013;12:128.
22 Pereira TM, Pimenta FS, Porto ML, Baldo MP, Campagnaro BP, Gava AL, Meyrelles SS, Vasquez EC: Coadjuvants in the diabetic complications: Nutraceuticals and drugs with pleiotropic effects. Int J Mol Sci. 2016;17:E1273.
23 Bernardes FP, Batista AT, Porto ML, Vasquez EC, Campagnaro BP, Meyrelles SS: Protective effect of sildenafil on the genotoxicity and cytotoxicity in apolipoprotein E-deficient mice bone marrow cells. Lipids Health Dis 2016;15:100.
24 Valatsou A, Briasoulis A, Vogiatzi G, Pantopoulou A, Oikonomou E, Miliou A, Perrea D, Tousoulis D: Beneficial effects of sildenafil on tissue perfusion and inflammation in a murine model of limb ischemia and atherosclerosis. Curr Vasc Pharmacol 2017;15:282-287.
25 Dussault S, Maingrette F, Ménard C, Michaud SE, Haddad P, Groleau J, TurgeonJ, Perez G, Rivard A: Sildenafil increases endothelial progenitor cell functionand improves ischemia-induced neovascularization in hypercholesterolemicapolipoprotein E-deficient mice. Hypertension 2009;54:1043-1049.
26 Vanhoutte PM, Shimokawa H, Feletou M, Tang EH: Endothelial dysfunction and vascular disease - a 30th anniversary update. Acta Physiol (Oxf) 2017;219:22-96.
27 Le NT, Sandhu UG, Quintana-Quezada RA, Hoang NM, Fujiwara K, Abe JI: Flow signaling and atherosclerosis. Cell Mol Life Sci 2017;10:1835-1858.
28 Cavieres V, Valdes K, Moreno B, Moore-Carrasco R, Gonzalez DR: Vascular hypercontractility and endothelial dysfunction before development of atherosclerosis in moderate dyslipidemia: role for nitric oxide and interleukin-6 Am J Cardiovasc Dis 2014;4:114-122.
29 Van AT, Fransen P, Guns PJ, Herman AG, Bult H: Altered Ca2+ handling of smooth muscle cells in aorta of apolipoprotein E-deficient mice before development of atherosclerotic lesions. Cell Calcium 2007;41:295–302.
30 Félétou M, Huang Y, Vanhoutte PM: Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. Br J Pharmacol 2011;164:894-912.

31 Li S, Liu B, Luo W, Zhang Y, Li H, Huang D, Zhou Y: Role of cyclooxygenase-1 and -2 in endothelium-dependent contraction of atherosclerotic mouse abdominal aortas. Clin Exp Pharmacol Physiol 2016;43:67-74.
32 Zhou Y, Luo W, Zhang Y, Li H, Huang D, Liu B: Cyclo-oxygenase-1 or -2-mediated metabolism of arachidonic acid in endothelium-dependent contraction of mouse arteries. Exp Physiol 2013;98:1225-1234.
33 Zhao WG, Richardson JS: Prostacyclin, thromboxane A2, and hypertension. Clin Invest Med 1990;13:343-352.
34 Schenning M, van Tiel CM, Van Manen D, Stam JC, Gadella BM, Wirtz KW, Snoek GT: Phosphatidylinositol transfer protein alpha regulates growth and apoptosis of NIH3T3 cells: involvement of a cannabinoid 1-like receptor. J Lipid Res 2004;45:1555-1564.
35 Funk CD, FitzGerald GA: COX-2 inhibitors and cardiovascular risk. J Cardiovasc Pharmacol 2007;50:470-479.
36 Félétou M, Huang Y, Vanhoutte PM: Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. Br J Pharmacol 2011;164:894-912.
37 Linton MF, Fazio S: Cyclooxygenase products and atherosclerosis. Drug Discov Today Ther Strateg 2008;5:25-36.
38 Kraus S, Naumov I, Shapira S, Kazanov D, Aroch I, Afek A, Eisenberg O, George J, Arber N, Finkelstein A: Aspirin but not meloxicam attenuates early atherosclerosis in apolipoprotein E knockout mice. Isr Med Assoc J 2014;16:233-238.
39 Félétou M, Huang Y, Vanhoutte PM: Vasoconstrictor prostanoids. Pflugers Arch 2010;459:941-950.
40 Kobayashi T, Tahara Y, Matsumoto M, Iguchi M, Sano H, Murayama T, Arai H, Oida H, Yurugi-Kobayashi T, Yamashita JK, Katagiri H, Majima M, Yokode M, Kita T, Narumiya S: Roles of thromboxane A(2) and prostacyclin in the development of atherosclerosis in apoE-deficient mice. J Clin Invest 2004;114:784-794.
41 Vanhoutte PM, Tang EH: Endothelium-dependent contractions: when a good guy turns bad! J Physiol 2008;586:5295-5304.
42 Tang EH, Leung FP, Huang Y, Feletou M, So KF, Man RY, Vanhoutte PM: Calcium and reactive oxygen species increase in endothelial cells in response to releasers of endothelium-derived contracting factor. Br J Pharmacol 2007;151:15-23.
43 Pfister SL: Aortic thromboxane receptor deficiency alters vascular reactivity in cholesterol-fed rabbits. Atherosclerosis 2006;189:358-363.
44 Zhou Z, Sun C, Tilley SL, Mustafa SJ: Mechanisms underlying uridine adenosine tetraphosphate-induced vascular contraction in mouse aorta: Role of thromboxane and purinergic receptors. Vascul Pharmacol 2015;73:78-85.
45 Pettersen AÅ, Arnesen H, Opstad TB, Bratseth V, Seljeflot I: Markers of endothelial and platelet activation are associated with high on-aspirin platelet reactivity in patients with stable coronary artery disease. Thromb Res 2012;130:424-428.
46 Cavka A, Cosic A, Jukic I, Jelakovic B, Lombard JH, Phillips SA, Seric V,Mihaljevic I, Drenjancevic I: The role of cyclo-oxygenase-1 in high-salt diet-induced microvascular dysfunction in humans. J Physiol 2015;593:5313-5324.
47 Cherdon C, Rolin S, Hanson J, Ooms A, de Leval L, Drion P, Michiels C, Pirotte B, Masereel B, Sakalihassan N, Defraigne JO, Dogné JM: BM-573 inhibits the development of early atherosclerotic lesions in Apo E deficient mice by blocking TP receptors and thromboxane synthase. Prostaglandins Other Lipid Mediat 2011;94:124-132.
48 Bots ML, Ford I, Lloyd SM, Laurent S, Touboul PJ, Hennerici MG: Prevention of cerebrovascular and Cardiovascular events of ischemic origin with terutroban in patients with a history of ischemic stroke or transient ischemic attack vascular ultrasound study investigators. Thromboxane prostaglandin receptor antagonist and carotid atherosclerosis progression in patients with cerebrovascular disease of ischemic origin: a randomized controlled trial. Stroke 2014;45:2348-2353.
49 Suzuki K, Saito SY, Ishikawa T: Involvement of phosphatidylcholine-specific phospholipase C in thromboxane A_2 receptor-mediated extracellular Ca^{2+} influx in rat aorta. Eur J Pharmacol 2012;677:123-130.
50 Luo W, Liu B, Zhou Y: The endothelial cyclooxygenase pathway: Insights from mouse arteries. Eur J Pharmacol 2016;780:148-158.
51 Cohen RA, Tong X: Vascular oxidative stress: the common link in hypertensive and diabetic vascular disease. J Cardiovasc Pharmacol 2010;55:308-316.
52 Ellinsworth DC, Shukla N, Fleming I, Jeremy JY: Interactions between thromboxane A_2, thromboxane/prostaglandin (TP) receptors, and endothelium-derived hyperpolarization. Cardiovasc Res 2014;102:9-16.

53 Kowalczyk A, Kleniewska P, Kolodziejczyk M, Skibska B, Goraca A: The role ofendothelin-1 and endothelin receptor antagonists in inflammatory response and sepsis. Arch Immunol Ther Exp (Warsz) 2015;63:41-52.
54 Nasser SA, El-Mas MM: Endothelin ETA receptor antagonism in cardiovascular disease. Eur J Pharmacol 2014;737:210-213.
55 Ohkita M, Tawa M, Kitada K, Matsumura Y: Pathophysiological roles of endothelin receptors in cardiovascular diseases. J Pharmacol Sci 2012;119:302-313.
56 Nishiyama SK, Zhao J, Wray DW, Richardson RS: Vascular function and Endothelin-1: Tipping the Balance between Vasodilation and Vasoconstriction. J Appl Physiol (1985) 2017;122:354-360.
57 Sánchez A, Martínez P, Muñoz M, Benedito S, García-Sacristán A, Hernández M, Prieto D: Endothelin-1 contributes to endothelial dysfunction and enhanced vasoconstriction through augmented superoxide production in penile arteries from insulin-resistant obese rats: role of ET(A) and ET(B) receptors. Br J Pharmacol 2014;171:5682-5695.
58 Yamashiro K, Milsom AB, Duchene J, Panayiotou C, Urabe T, Hattori N, Ahluwalia A: Alterations in nitric oxide and endothelin-1 bioactivity underlie cerebrovascular dysfunction in ApoE-deficient mice. J Cereb Blood Flow Metab 2010;30:1494-1503.
59 Barton M, Haudenschild CC, d'Uscio LV, Shaw S, Münter K, Lüscher TF: Endothelin ETA receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. Proc Natl Acad Sci U S A. 1998;95:14367-14372.
60 Fan J, Unoki H, Iwasa S, Watanabe T: Role of endothelin-1 in atherosclerosis. Ann N Y Acad Sci 2000;902:84-93
61 Callera GE, Touyz RM, Teixeira SA, Muscara MN, Carvalho MH, Fortes ZB, Nigro D, Schiffrin EL, Tostes RC: ETA receptor blockade decreases vascular superoxide generation in DOCA-salt hypertension. Hypertension 2003;42:811-817.
62 Muzaffar S, Jeremy JY, Angelini GD, Shukla N: NADPH oxidase 4 mediates upregulation of type 4 phosphodiesterases in human endothelial cells. J Cell Physiol 2012; 227:1941-1950.
63 Saleh MA, De Miguel C, Stevens DI, Carmines PK, Pollock DM, Pollock JS: Free radical scavenging decreases endothelin-1 excretion and glomerular albumin permeability during type 1 diabetes. Physiol Rep 2016;4.
64 Idris-Khodja N, Ouerd S, Mian MO, Gornitsky J, Barhoumi T, Paradis P, Schiffrin EL: Endothelin-1 overexpression exaggerates diabetes-induced endothelial dysfunction by altering oxidative stress. Am J Hypertens 2016;26.
65 Fatkhullina AR, Peshkova IO, Koltsova EK: The role of cytokines in the development of atherosclerosis. Biochemistry (Mosc) 2016;81:1358-1370.
66 Huber SA, Sakkinen P, Conze D, Hardin N, Tracy R: Interleukin-6 exacerbates early atherosclerosis in mice. Arterioscler Thromb Vasc Biol 1999;19:2364-2367.
67 Zhang H, Wu LM, Wu J: Cross-talk between apolipoprotein E and cytokines. Mediators Inflamm 2011;2011:949072.
68 Sullivan KE, Cutilli J, Piliero LM, Ghavimi-Alagha D, Starr SE, Campbell DE, Douglas SD: Measurement of cytokine secretion, intracellular protein expression, and mRNA in resting and stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2000;7:920-924.
69 Gjurich BN, Taghavie-Moghadam PL, Galkina EV: Flow cytometric analysis of immune cells within murine aorta. Methods Mol Biol 2015;1339:161-175.
70 Maier T, Güell M, Serrano L: Correlation of mRNA and protein in complex biological samples. FEBS Lett 2009;583:3966-3973.
71 Harrington JR: The role of MCP-1 in atherosclerosis. Stem Cells 2000;18:65-66.
72 Antunes BM, Cayres SU, Lira FS, Fernandes RA: Arterial thickness and immunometabolism: The mediating role of chronic exercise. Curr Cardiol Rev 2016;12:47-51.
73 Wassmann S, Stumpf M, Strehlow K, Schmid A, Schieffer B, Böhm M, Nickenig G: Interleukin-6 induces oxidative stress and endothelial dysfunction by overexpression of the angiotensin II type 1 receptor. Circ Res 2004;94:534-541.
74 Khoshakhlagh P, Bahrololoumi-Shapourabadi M, Mohammadirad A, Ashtaral-Nakhai L, Minaie B, Abdollahi M: Beneficial effect of phosphodiesterase-5 inhibitor in experimental inflammatory bowel disease; molecular evidence for involvement of oxidative stress. Toxicol Mech Methods 2007;17:281-288.
75 Nunes AK, Rapôso C, Luna RL, Cruz-Höfling MA, Peixoto CA: Sildenafil (Viagra®)down regulates cytokines and prevents demyelination in a cuprizone-induced MS mouse model. Cytokine 2012;60:540-551.
76 Dias AT, Rodrigues BP, Porto ML, Gava AL, Balarini CM, Freitas FP, Palomino Z, Casarini DE, Campagnaro BP, Pereira TM, Meyrelles SS, Vasquez EC: Sildenafil ameliorates oxidative stress and DNA damage in the stenotic kidneys in mice with renovascular hypertension. J Transl Med 2014;12:35.