UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE BIOCIÊNCIAS
DEPARTAMENTO DE FISIOLOGIA E FARMACOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA E FISIOLOGIA

JOSÉ JAIRO TEIXEIRA DA SILVA

ROLE OF PHOSPHODIESTERASE 5 IN CARDIOVASCULAR PATHOPHYSIOLOGY IN SPONTANEOUSLY HYPERTENSIVE RATS

Recife

2019

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Tese em cotutela apresentada ao Programa de Pós-Graduação em Bioquímica e Fisiologia, Centro de Biociências da Universidade Federal de Pernambuco (UFPE), e apresentada ao Dottorato di Ricerca in Morfogenesi e Ingegneria Tissutale - (Sapienza) - Ciclo XXXII, Dipartimento di Scienze Anatomiche, Istologiche, Medico-legali e dell 'Apparato Locomotore da Università di Roma, "Sapienza", como parte dos requisitos parciais para obtenção do título de doutor em Bioquímica e Fisiologia pela UFPE e doutor em Morfogenesi e Ingegneria Tissutale pela Università di Roma.

Área de concentração: Bioquímica e Fisiologia.

Orientadora: Profª. Drª. Glória Isolina Boente Pinto Duarte

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Recife

2019

Catalogação na fonte:
Bibliotecária Claudina Queiroz, CRB4/1752

Silva, José Jairo Teixeira da
Role of phosphodiesterase 5 in cardiovascular pathophysiology in spontaneously hypertensive rats / José Jairo Teixeira da Silva - 2019.
120 folhas: il., fig., tab.

Orientadores: Glória Isolina Boente Pinto Duarte, Fabio Naro
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-Graduação em Bioquímica e fisiologia. Recife, 2019.

Inclui referências e apêndices.

1. Hipertensão 2. Fosfodiesterase 5 3. Sildenafil
I. Duarte, Glória Isolina Boente Pinto (Orientadora) II. Naro, Fabio (Orientador) III. Título

573.1 CDD (22.ed.) UFPE/CB-2020-008

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This thesis is dedicated to my mother, Elza Teixeira.

ACKONWLEDGE

I would like to express my deep gratitude to Professor Glória Isolina Boente Pinto Duarte and Professor Fabio Naro, my research supervisors, for their valuable and constructive suggestions during the planning and development of this research work.

I would like to thank the Professors Fabiano Elias Xavier, Cristina de Oliveira Silva, Valéria Nunes and Saad Lahlou for their essential contribution and technical assistance.

I would like to thank Professor Vera Lúcia de Menezes Lima, coordinator of the "Programa de Doutorado em Bioquímica e Fisiologia – UFPE", as well as Professor Antonio Mussaró, coordinator of the "Dottorato di Ricerca in Morfogenesi ed Ingegneria Tissutale, Curriculum in Scienze Morfogenetiche e Citologiche, Ciclo XXXII – Sapienza", for the opportunity.

I would like to express my very great appreciation to all components of the "Departamento de Bioquímica e Fisiologia, UFPE", as well as all components of the "Dipartimento di Scienze Anatomiche, Istologiche, Medico-legali e dell 'Apparato Locomotore – Università di Roma, La Sapienza".

I wish to acknowledge the contribution of all members of the "Laboratório de Fisiologia and Farmacologia Cardiovascular", as well as all the members of the "Laboratório de Reatividade Vascular" - UFPE – Brazil. I would like to express my very great appreciation in special to Hicla Moreira, Geórgia Leal, Thayane Rebeca, Marcelo Rocha, Brydja Denko and Leonardo Gomes.

I also would like to thank all components of the "Laboratorio Meccanismi Regolativi della Morfogenisi Normale e Patologica and Locomotor" - University of Rome, 'La Sapienza' - Italy. I would like to express my very great appreciation in special to Andrea Isidori, Andrea Lenzi, Mary Venneri, Manuela Pellegrini and Salvatore Aguanno.

I wish to thank all laboratory animals used in this study for making this thesis possible. I expressed my respect and sincere gratitude.

I would like to express my deep gratitude to all funding agencies, specially, to the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior", Brazil (CAPES, Finance code 001, Grant number: 88881.068093/214-01); the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq/Brazil); and FIRB Grant (grant number: FIRB2010RBAP109BLT_003).

Finally, I need to thank all my Brazilians and Italians friends,

Valquíria Medeiros and Ricardo Lima [...] **muito obrigado.**

Rita Assenza, Erica Leonetti, Ernestina Capuano, Claudia Tito, Thays Carvalho, Marisa Cornacchione, “Zia” Federica Barbagallo Sergio Forcelloni, Anna Benedetti, Cesar Augusto, Sara Di Persio, “Don” Carlo Lamanna, Anna Maria Lustri, Valentina Mularoni, Ciro Francesco, Luigi Fattore, Sara Di Siena, Silvia Carderelli, Ramtim Bidares [...] **grazie mille, siete indimenticabili.**

“One must still have chaos in oneself to give birth to a dancing star” (NIETZSCHE, 1891).

ABSTRACT

The aim of this study was to investigate the impact of chronic phosphodiesterase type 5A inhibition (PDE5Ai) by sildenafil in different stages of development in a model of essential hypertension. For this purpose, male spontaneously hypertensive rats (SHRs) were chronically treated with PDE5A inhibitor sildenafil (45mg/kg/60days) during the advance of hypertension in young SHRs (pre-hypertensive protocol, 4 until 12 weeks-old rats). The impact of sildenafil treatment on established hypertension in adult SHRs (hypertensive protocol, 16 until 24 weeks-old rats) was also evaluated. In the pre-hypertensive protocol, chronic PDE5Ai promotes a significant increase in acetylcholine-induced relaxation without altering the relaxation evoked by sodium nitroprusside in conductance arteries. By contrast, in the same preparations, it promotes a reduction in contractile response induced by phenylephrine in conductance arteries from SHRs. Sildenafil improvement of vascular function during the development of hypertension since it decreased oxidative stress, a mechanism dependent of cyclooxygenase type 2 (COX-2) and mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK1/2) in aortic tissues. Resting blood pressure, vascular remodeling and the reactivity of resistance arteries were not modified after sildenafil treatment. In hypertensive protocol, chronic inhibition of PDE5A counteracts sympathetic signaling in hearts from SHRs treated with sildenafil, probably by blunting systolic β-adrenergic receptor (β-AR) stimulation in hypertensive hearts. Still, at 6 months of age analysis of cross-sectional area of cardiac myocytes demonstrated a significant reduction of myocytes area in SHR-Sild group compared to the area of cardiomyocytes from untreated rats, suggesting an antihypertrophic effect related to PDE5Ai. Furthermore, chronic sildenafil administration not only contributes with the reduction of left ventricle cardiac reactive oxygen species (ROS) production but also significantly increased of superoxide dismutase activity (SOD), without significant effects on catalase (CAT) activity. In SHRs, sildenafil treatment resulted in downregulation of beta-myosin heavy chain (β-MHC), transforming factor GATA binding protein 4 (GATA4) and nuclear factor of activated T-cells (NFATc3) protein expression which are markers of cardiac hypertrophy, and promotes downregulation of and transforming growth factor β (TGF-β) protein expression, a marker of fibrosis. On the other hand, atrial natriuretic peptide (ANP) protein expression was upregulated in SHR-Sild group suggesting a mechanism related with cardioprotection. Thus, in hypertensive protocol we reported the first direct evidence that sildenafil blunts cardiac contractility and heart rate (HR) after β-adrenergic stimulation in SHR-Sild group, as well as attenuates cardiac oxidative

stress and cardiac hypertrophy secondary to hypertension. Both, vascular function improvement and antihypertrophic effect in our model, are involved with the decrease of oxidative stress after sildenafil administration. Taken together, these finds demonstrates a potential role of PDE5Ai as an adjunct therapy in hypertension.

Keywords: Hypertension. Phosphodiesterase 5. Sildenafil.

RESUMO

O objetivo desse estudo foi investigar os efeitos da inibição crônica da fosfodiesterase 5 (PDE5Ai) na hipertensão essencial. Para isto, foram utilizados ratos espontaneamente hipertensos (SHRs) cronicamente tratados com sildenafil (45mg/kg/60dias), um inibidor da PDE5A, durante o desenvolvimento da hipertensão em SHRs jovens (protocolo pré-hipertensivo, 4 semanas até 12 semanas de idade). O impacto da inibição crônica da PDE5A também foi avaliado em SHRs adultos, momento no qual a pressão arterial se encontrava bem estabelecida nesse modelo (protocolo hipertensivo, 16 semanas até 22 semanas de idade). Aos 3 meses, o tratamento com sildenafil promoveu um aumento significativo do relaxamento vascular induzido pela acetilcolina, sem alterações significativas no relaxamento vascular induzido pelo nitroprussiato de sódio. Ademais, a PDE5Ai induziu uma redução da resposta contrátil à fenilefrina na aorta do grupo SHR-Sild. O tratamento crônico com sildenafil resultou em melhora da função vascular durante o desenvolvimento da hipertensão, uma vez que reduziu o estresse oxidativo tecidual, por modular a via da ciclooxigenase do tipo 2 e modular a ativação de proteínas quinases reguladas por mitógeno (MAP)/quinases reguladas por sinal extracelular (ERK1/2) na aorta. Pressão arterial, frequência cardíaca, remodelamento vascular e reatividade de vasos de resistência não foram modificados pelo tratamento com o sildenafil. Aos 6 meses de idade, no protocolo hipertensivo, a PDE5Ai atenuou a resposta à estimulação simpática no coração de animais tratados com sildenafil quando comparados com os animais não tratados. A área de secção transversa dos cardiomiócitos dos animais do grupo SHR-Sild foi reduzida quando comparada aos animais tratados com o veículo, sugerindo um efeito anti-hipertrófico associado a inibição da PDE5A. O tratamento não apenas atenuou a hipertrofia ventricular esquerda, mas também contribuiu com a redução da produção cardíaca de espécies reativas de oxigênio, e promoveu aumento significativo da atividade da superóxido dismutase, sem alterações na atividade da catalase. Neste protocolo experimental, a PDE5Ai resultou em menor expressão proteica da miosina de cadeia pesada (β-MHC), do fator de transcrição GATA4 e do fator nuclear de células T ativadas (NFATc3), marcadores clássicos de hipertrofia cardíaca. Além disso, promoveu diminuição da expressão proteica do fator transformador de crescimento beta (TGF-β), o qual é diretamente relacionado a fibrose tecidual. Foi observado um aumento significativo da expressão proteica do peptídeo natriurético atrial (ANP) no tecido cardíaco, o qual foi associado a cardioproteção. Em ambos os protocolos experimentais, a melhora da função vascular e os efeitos anti-hipertróficos associados a inibição da PDE5A pelo sildenafil estão relacionados com a diminuição do

estresse oxidativo tecidual, o que demonstra uma nova possibilidade terapêutica na utilização do sildenafil no tratamento da hipertensão arterial.

Palavras-chave: Hipertensão. Fosfodiesterase 5. Sildenafil.

LIST OF FIGURES

Figure 1 –	Regulatory network interconnecting cardiac phosphodiesterase.	29
Figure 2 –	Signaling pathway underlying PDE5A inhibitory effects of acute β-adrenergic stimulation.	32
Figure 3 –	Effects of chronic PDE5A inhibition on endothelium-dependent and endothelium-independent relaxation response to acetylcholine (A) and sodium nitroprusside (B), respectively and on the vasoconstriction response to phenylephrine (C) in aortic rings from animals treated with sildenafil (SHR-Sild) and untreated SHR.	41
Figure 4 –	Effects of endothelium removal (A), pretreatment with L-NAME (B) or ODQ (C) preincubation on contractile response induced by phenylephrine in aortic rings from treated (SHR-Sild) and untreated SHR.	42
Figure 5 –	Left panel: Representative blots of phospho-eNOS Ser1177, total-eNOS, phospho-Akt 1/2/3, total-Akt 1/2/3, PKG and caveolin and α-actin in aorta from sildenafil-treated (SHR-Sild) and untreated SHR.	43
Figure 6 –	Effect of COX inhibition by indomethacin on relaxation response to acetylcholine (A) and contractile response to phenylephrine (B) in aortic rings from sildenafil-treated (SHR-Sild) and untreated SHR.	44
Figure 7 –	Left top panel: Representative blots of COX-1, COX-2 and α-actin in aorta from sildenafil-treated (SHR-Sild) and untreated SHR.	45
Figure 8 –	Western blot analysis of protein expression of total-ERK (A) and phospho-ERK (B) in aorta from sildenafil-treated (SHR-Sild) and untreated SHR.	46
Figure 9 –	Top panel: Representative blots of cardiac phospho-ERK, total-ERK and GAPDH expression in sildenafil-treated (SHR-Sild) and untreated SHR groups. Wet heart weight/body weight (BW) ratio (A) and western blot analysis of left ventricular cardiac protein expression.	47

Figure 10 – Relationship between increased intraluminal pressure and vessel diameter of mesenteric arteries under active (+Ca^{2+}) and passive (0 Ca^{2+}) conditions in untreated SHR (panel A) and SHR treated with sildenafil (panel B). Comparison of such relationship in either active (panel C) or passive (panel D) between untreated and sildenafil-treated SHR. 49

Figure 11 – Relationship between increased intraluminal pressure and lumen diameter of mesenteric arteries under active (+Ca^{2+}) and passive (0 Ca^{2+}) conditions in untreated SHR (panel A) and SHR treated with sildenafil (B). Comparison of such relationship in either active (panel C) or passive (panel D) between untreated and Sild-treated SHR. 50

Figure 12 – Comparison of the relationship of increased intraluminal pressure and wall thickness of mesenteric arteries between untreated SHR and SHR treated with sildenafil in either active (panel A) or passive (panel B) conditions. 51

Figure 13 – Comparison of the relationship of increased intraluminal pressure and media/lumen ratio of mesenteric arteries between untreated SHR and SHR treated with sildenafil (SHR-Sild) in either active (panel A) or passive (panel B) conditions. 52

Figure 14 – Comparison of the relationship of increased intraluminal pressure and cross-sectional area of mesenteric arteries between untreated SHR and SHR-Sild in either active (panel A) or passive (panel B) conditions. 53

Figure 15 – Comparison of the relationship of increased intraluminal pressure and incremental distensibility of mesenteric arteries between untreated SHR and SHR-Sild under active conditions (+ Ca^{2+}). 54

Figure 16 – Comparison of the relationship of increased intraluminal pressure and intrinsic tone (panel A), myogenic index (panel B) or myogenic contraction (panel C) of mesenteric arteries between SHR untreated and treated with sildenafil (SHR-Sild). 55

Figure 17 – Effect of chronic PDE5A inhibition on endothelium-dependent response to acetylcholine (A) and norepinephrine (B) in mesenteric rings from sildenafil-treated (SHR-Sild) and untreated SHR. 56

Figure 18 – Effect of pretreatment with INDO (A), L-NAME (B), INDO+L-NAME (C) and ODQ (D) on vasodilatation response induced by acetylcholine in mesenteric resistance arteries rings from sildenafil-treated (SHR-Sild) and untreated SHR. 57

Figure 19 – Effect of pretreatment with INDO (A), L-NAME (B), INDO+L-NAME (C) and ODQ (D) on contraction response induced by norepinephrine in mesenteric resistance arteries rings from sildenafil-treated (SHR-Sild) and untreated SHR. 58

Figure 20 – Modifications of cardiac growth in hypertension. (A) Representative Masson Thricome staining of adult heart sections. 64

Figure 21 – Maximum variations in left ventricular systolic pressure (ΔLVSP) (panel A) and maximum variation in HR (ΔHR, panel B) during infusion of increasing concentrations of ISO in isolated perfused hearts from sildenafil (Sild) treated and untreated SHR. 65

Figure 22 – Comparison of the maximal rate of pressure development (Max dP/dt) and the minimum relaxation rate (Min dP/dt) after stimulation of α_1-adrenoceptors by ISO in isolated hearts from sildenafil-treated SHR and untreated SHR. 66

Figure 23 – Comparison of left ventricular ROS production (panel A), t-SOD: total superoxide dismutase activity (panel B), and CAT: catalase activity (panel C) in SHR treated with sildenafil (SHR-Sild) and untreated SHR. 67

Figure 24 – Cardiac hypertrophic markers. Above panel: Representative blots of β-MHC, GATA4, TGFβ, NFATc3, ANP and PDE5A in left ventricular cardiac tissue from treated animals (SHR-Sild) and untreated SHR. 69

Figure 25 – Cardiac hypertrophic signaling pathways. Above panel: Representative blots of phospho-ERK1/2, total-ERK2, phospho- 70

AKT and total-AKT in left ventricular cardiac tissue from treated animals (SHR-Sild) and untreated SHR.

Figure 26 – Schematic experimental design 77

Figure 27 – Representative image of pressurized arteries 79

Figure 28 – Representative images of mesenteric arteries used to vascular reactivity study 80

Figure 29 – Demonstrative isolated perfused heart preparation 81

LIST OF TABLES

Table 1 – Baseline blood pressure and heart rate values in sildenafil-treated (SHR-Sild) and untreated SHR – pre-hypertensive protocol 40

Table 2 – Baseline blood pressure and heart rate values in sildenafil-treated (SHR-Sild) and untreated SHR – hypertensive protocol 62

LIST OF ABREVIATURES

AC	adenylyl cyclase
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
BNP	brain natriuretic peptide
Ca^{2+}	calcium
CaM	calmodulin
cAMP	3',5'- cyclic adenosine monophosphate
CAT	catalase
CNP	C-type natriuretic peptide
COX-2	cyclooxygenase type 2
CSA	cross-sectional area
DBP	diastolic arterial pressure
D_e	external diameter
D_i	internal diameter
eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular signal-regulated kinase
GATA4	GATA binding protein 4
GC	guanylyl cyclase
GTP	guanosine triphosphate
H_2DCF-DA	2',7'-dichlorofluorescein diacetate
HR	heart rate
IL-6	interleukin type 6
iNOS	inducible nitric oxide synthase
ISO	isoproterenol

L-NAME	N(ω)-nitro-L-arginine methyl ester
LTCC	L-type calcium channel
LVEDP	left ventricle end-diastolic pressure
LVSP	left ventricular systolic pressure
MAP	mean arterial pressure
max dP/dt	maximum rate of pressure development
MCP-1	monocyte chemoattractant protein-1
min dP/dT	maximum rate of relaxation
MCP-1	monocyte chemoattractant protein-1
min dP/dT	maximum rate of relaxation
NADPH	nicotinamide-adenine-dinucleotide-phosphate
NFATc3	nuclear factor of activated T-cells
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthases
NPR	natriuretic peptides-membrane associated-guanylyl cyclase receptors
NPs	natriuretic peptides
O^{2-}	superoxide anions
ODQ	oxadiazolo- [4,3-a]quinoxalin-1-one
PDE5A	phosphodiesterase type 5A
PE	polyethylene
pGC	particulate guanylyl cyclase
PI3K	phosphoinositide-3 kinase
PKA	protein kinase A
PKG	protein kinase G
PLB	phospholamban

RGS2	regulator of G-coupled signaling 2
ROS	reactive oxygen species
SBP	systolic arterial pressure
sGC	soluble guanylyl cyclase
SHRs	spontaneously hypertensive rats
SOD	superoxide dismutase activity
SPB	sodium pentobarbital
TAC	transverse aortic constriction
TEMs	TIE2-expressing monocytes
TGF-β	transforming growth factor β
TnI	troponin I
VCAM-1	vascular cell adhesion molecule 1
VSMCs	vascular smooth muscle cells
WGA	Wheat Germ Agglutin
WKYs	Wistar Kyoto rats
WT	wall thickness
β-AR	β-adrenergic receptor
β-MHC	beta-myosin heavy chain
ΔHR	maximum variation in heart rate
ΔLVSP	Maximum variations in left ventricular systolic pressure

SUMMARY

1	**INTRODUCTION**	**22**
1.1	HYPERTENSION	22
1.1.1	**Hypertension: definition and classification**	**22**
1.1.2	**Structural changes in hypertension**	**23**
1.1.3	**Experimental models for the study of hypertension in humans**	**25**
1.2	CYCLIC NUCLEOTIDES AND PHOSPHODIESTERASE IN THE CARDIOVASCULAR SYSTEM	27
1.2.1	**Cyclic nucleotides as regulators in the heart and vessels**	**27**
1.2.2	**Activation and regulation of cGMP/PKG/PDE5A signaling pathway in heart**	**29**
1.2.3	**Regulation of PDE5A function in cardiovascular system**	**33**
1.3	CGMP/PKG/PDE5A IN PHATOPHYSIOLOGY OF CARDIOVASCULAR DISEASES	34
2	**AIM**	**37**
3	**RESULTS**	**38**
3.1	PRE-HYPERTENSIVE PROTOCOL	38
3.1.1	**Chronic PDE5Ai improves endothelial function by decreasing COX-2/MAPK expression and oxidative stress in aorta from SHRs**	**38**
3.1.2	**Structural and mechanical study of mesenteric resistance arteries on the pressure myograph**	**48**
3.1.3	**Vascular effects of sildenafil treatment on mesenteric resistance arteries**	**48**
3.2	HYPERTENSIVE PROTOCOL	59
3.2.1	**Effects of chronic PDE5Ai by sildenafil on biometric parameters, blood pressure and heart rate**	**59**
3.2.2	**Inhibition of PDE5A by sildenafil counteracts sympathetic signaling in the heart**	**59**
3.2.3	**Impact of chronic PDE5Ai and oxidative stress in hypertension**	**60**
3.2.4	**PDE5Ai and cardiac hypertrophy in hypertension**	**60**
4	**DISCUSSION**	**71**
5	**MATERIAL AND METHODS**	**71**
5.1	ANIMALS	77

5.2	EXPERIMENTAL DESIGN	77
5.2.1	**Pre-hypertensive protocol (4-week-old until 12-week-old rats, Figure 26A)**	**77**
5.2.2	**Hypertensive protocol (16-week-old until 20-week-old rats, Figure 26B)**	**78**
5.3	STRUCTURAL AND MECHANICAL STUDY OF MESENTERIC RESISTANCE ARTERIES UNDER ACTIVE (+0 CA2+) AND PASSIVE CONDITIONS (0CA2+) ON THE PRESSURE MYOGRAPH	78
5.3.1	**Pressurized arteries (pressure myograph)**	**78**
5.3.2	**Equations for structural analysis**	**79**
5.4	VASCULAR REACTIVITY STUDY	79
5.4.1	**Experimental protocols**	**80**
5.5	ARTERIAL BLOOD PRESSURE MEASUREMENT	80
5.6	ISOLATED PERFUSED HEART STUDY (LANGENDORFF)	81
5.6.1	**Experimental protocol of isolated perfused heart study**	**82**
5.7	HISTOLOGICAL ANALYSIS	82
5.8	IMMUNOFLUORESCENCE ANALYSIS	82
5.9	CARDIAC OXIDATIVE STRESS ANALYSES	83
5.9.1	**Cardiac reactive oxygen species quantification**	**83**
5.9.2	**Total superoxide dismutase activity**	**83**
5.9.3	**Cardiac catalase activity**	**83**
5.10	**WESTERN BLOT ANALYSIS**	**84**
5.11	**STATISTICAL ANALYSIS**	**85**
	REFERENCES	**86**
	APPENDICE A: FUNDINGS	**109**
	APPENDICE B: ARTICLE PUBLISHED IN LIFE SCIENCES	**110**

1 INTRODUCTION

1.1 HYPERTENSION

1.1.1 Hypertension: definition and classification

Hypertension is the leading cause of death and disability worldwide. High blood pressure (systolic blood pressure (SBP) values ≥ 140 mmHg and/or diastolic blood pressure (DBP) values ≥ 90 mmHg) is one of the most important risk factors for cardiovascular, cerebrovascular and renal diseases **[Mills, 2016; NCD-Risk 2017; Williams, *Guidelines 2018]**. Long-term high blood pressure particularly contributes to highest risk for coronary artery disease, stroke, heart failure, peripheral vascular disorders, arrythmias, dementia and chronic kidney disease **[Mendis et al., 2011; Lau et al., 2017; Hernandorena et al., 2017].**

The global occurrence of hypertension was estimated in 1.13 billion people in 2015 (31% of all adults). It is projected that this number will increase by 20-25% in 2025, reaching close to 1.56 billion people **[Kearney et al., 2005; WHO, 2013]**. Usually, hypertension prevalence is higher in low- and middle-income countries than in high-income countries **[NCD-Risk 2017, Mills et al., 2017]**. Prevalence of high blood pressure varies according to population and method of evaluation **[Malachias et al., 2016]**. Picon and colleagues, showed a decrease of the number of patients with hypertension in Brazil in the last three decades, from 36.1% to 31.0% **[Picon et al., 2012]**. Nowadays, the prevalence of self-reported high blood pressure in Brazil, among individual with 18 years or more is estimated in 24.3% **[Vigitel, 2018]**. In Italy, 26% of adult outpatients had hypertension **[Tocci et al., 2016a]**. Additionally, overall hypertension prevalence in Italy is higher in North (36.8 %) compared to Center (29.3 %) and South (33.8 %) **[Tocci et al., 2016b].**

High blood pressure is also classified as primary or secondary hypertension. Essential hypertension, also called primary or idiopathic is the form of hypertension that has no identifiable cause. Its prevalence accounts for 90-95% of all cases of hypertension **[Oscar and Oparil, 2000; Williams, *Guidelines 2018]**. Essential hypertension is considered a multifactorial disease, mostly in young and middle-aged adults, and is defined as high blood pressure in which secondary causes of arterial hypertension are not present **[Oscar and Oparil, 2000; Wise et al., 2016]**. Essential hypertension tends to cluster in families and results from a complex interaction between genetically based diseases and environmental factors **[Oscar and Oparil, 2000]**. However, it is still not fully understood what genes are involved in the genesis of hypertension.

In its turn, secondary hypertension is characterized by an identifiable underlying primary cause and accounts for only a small fraction (5-10%) of the overall prevalence of hypertension. The most common causes of secondary hypertension according to the prevalence are primary aldosteronism, obstructive sleep apnea, renal parenchymal disease and thyroid disease. But also, there are others causes (<1%), such as fibromuscular dysplasia, pheochromocytoma, Cushing's syndrome, hyperparathyroidism and coarctation of the aorta **[Williams, *Guidelines 2018].**

Many pathophysiological factors have been well recognized and implicated in the genesis of essential hypertension such as – genotypic influence, aging, exposure to persistent physical and social stress and increased sympathetic nervous system activity **[Mancia and Grassi, 2014; Grassi et al., 2016]**; unhealthy diet, lack of physical activity, obesity, diabetes, insulin resistance **[Cheung et al., 2012]**; long-term high salt intake **[Stolarz-Skrzypek et al., 2013]**; alcohol intake **[Roereck et al., 2018]**; smoking **[Virdis et al., 2010]**; inadequate dietary intake of potassium **[Stolarz-Skrzypek et al., 2013]**, calcium and magnesium deficiency **[Houston et al., 2008]**; vitamin deficiency **[Chen et al., 2015]**; alterations in expression of the kallikrein–kinin **[Rhaleb et al., 2011]**; increased renin release, increased production of angiotensin II and aldosterone, oxidative stress, endothelial dysfunction, vascular remodeling **[Riet et al., 2015; Siti et al., 2015; Briones and Touyz, 2018]** as well as alterations in inotropic and chronotropic properties of the heart **[Mayet and Hughes 2003].**

1.1.2 Structural changes in hypertension

Arterial hypertension has been related with several outcomes, such as myocardial remodeling and cardiac hypertrophy **[González et al., 2018]**, as well as vascular abnormalities **[Intengan and Schiffrin, 2001; Schiffrin, 2012]**. Hypertensive heart disease is defined as the response of the heart to pressure overload imposed on the cardiac left ventricle by the sustained high blood pressure and total peripheral resistance produced by vascular disease. It is related with the presence of left ventricular hypertrophy or left ventricular systolic and diastolic dysfunction and their clinical manifestations in response to an increased preload and afterload **[Frohlich et al., 1992; González, 2018]**. Left ventricular hypertrophy prevalence ranged from 36% to 41% in all hypertensive patients **[Cuspidi et al., 2012]**.

Cardiac hypertrophy is an adaptative response to cardiac stress, and could be classified as physiological and pathological, which differs in terms of the molecular mechanisms, cardiac phenotype and prognosis **[Grossman et al., 1975; Nakamura and Sadoshima, 2018]**. The

physiological type is a result of a transient hemodynamic changes, such as those observed in postnatal and gestational periods, as well as in endurance training. However, the pathological hypertrophy, results from persistent hemodynamic overload, as a result of a hypertensive condition, valvular diseases, myocardial infarction, metabolic syndrome and cardiomyopathies **[Levy et al., 1990; Nakamura and Sadoshima, 2018; González et al., 2018]**.

Pathological hypertrophy secondary to hypertension is initially identified by a reduced in ventricular chamber dimension with increased cardiomyocytes wall thickness, resulting in concentric hypertrophy. Persistent cardiac workload and cardiac hypertrophy leads to ventricular chamber dilatation (congestive heart failure) with impaired contractile function, also named as eccentric hypertrophy **[Bernardo et al., 2010; Nakamura and Sadoshima, 2018]**. The maladaptive changes results in cardiomyocytes hypertrophy and death; interstitial inflammation and fibrosis; arteriolar wall thickening; as well as reduced capillarization. Furthermore, sustained high blood pressure and cardiac hypertrophy configure an independent risk factor for cardiac morbidity and mortality **[González et al., 2018].** Pathological hypertrophy stimuli usually are associated with maladaptive gene expression, arrythmias, contractile dysfunction, fibrosis, mitochondrial dysfunctions and apoptosis **[Levy et al., 1990; Nakamura and Sadoshima, 2018]**.

Hypertension is also linked with structural and functional vascular abnormalities. These include enhanced vasoconstrictor response, endothelial dysfunction and vascular remodeling **[Touyz et al., 2018]**. The endothelium plays a crucial role in acute regulation of vascular tone and in the long-term vascular structural adaptations. In physiological conditions, endothelial cells synthetize and release several vasodilator factors such as [nitric oxide (NO), endothelium-derived hyperpolarizing factor and prostacyclin] and some vasoconstrictor factors [thromboxane A2, endotelin-1, prostaglandin H2 and radical superoxide, O^{2-}] **[Furchgott et al., 1980; Yanagisawa et al., 1988; Palmer et al., 1987; Palmer et al., 1988; Lüscher et al., 1992; Garland et al., 2011; Touyz et al., 2018]**.

The balance between vasodilator and vasoconstrictor mediators is essential in maintenance of vascular tone and regulation of blood pressure **[Deanfield et al., 2007; Siti et al., 2015]**. In essential hypertension, increased media to lumen ratio which characterizes vascular remodeling, can result in eutrophic or hypertrophic remodeling. This is characterized by a reduced outer diameter by an increase in wall thickness. Vascular eutrophic remodeling is the

most prevalent found in patients with essential hypertension **[Rizzoni et al., 2000; Rizzoni et al., 2001; Bruno et al., 2017].**

Increase of oxidative stress **[Takimoto, 2007a; Siti, 2015]** and increase of sympathetic nerve activity **[Schlaich, 2003; Bruno, 2012]** are important risk factors for, cardiac hypertrophy, as well as vascular remodeling and endothelial dysfunction. Oxidative stress is defined as a disturbance between ROS and antioxidants systems in favor of oxidants **[Birden et al., 2012]**. Basal oxidative species generation is a component of diverse physiological signaling pathways. However, increase of ROS production is strongly associated with a broad variety of hypertrophy signaling kinases and transcription factors in cardiovascular system **[Takimoto et al., 2007a; Siti et al., 2015].** In humans, biomarkers of systemic oxidative stress are elevated in hypertension. Treatment with SOD mimetics or antioxidants improves vascular function, regress vascular remodeling and reduces blood pressure. **[Redon, 2003; Rodriguez-Iturbe, 2003; Touyz et al., 2018].** NO breakdown by ROS is the principal cause of reduced NO availability and endothelial dysfunction, both in physiological aging and in many pathological conditions, including arterial hypertension **[Bruno et al., 2012; Bruno et al., 2017]**.

Increased cardiac norepinephrine release is related to the development of left ventricular hypertrophy **[Schlaich et al., 2003]**. Intensification of sympathetic nerve activity not only is associated with cardiac hypertrophy, but also induces vascular dysfunction. Sympathetic activity is associated with sustained high blood pressure through several mechanisms, such as increasing of ROS production, decreasing of antioxidant systems activity, enhancing peripheral vasoconstriction, potentiating cardiac contraction, affecting renal function and water excretion, as well as inducing baroreflex dysfunction **[Krieger, 1970; Irigoyen and Krieger, 1998; Takimoto et al., 2007a; 2007b; Bruno et al., 2012].**

1.1.3 Experimental models for the study of hypertension in humans

Animals are appreciated tools for understanding the pathophysiology and in developing therapeutic interventions for hypertension **[Lerman et al., 2019]**. Animals for study of high blood pressure can be categorized according to etiology of hypertension, as models of primary or secondary hypertension **[Lin et al., 2016]**. Usually, experimental hypertension can be induced by pharmacological induction, exposition to environmental adverse conditions, as well as genetic modifications. Indeed, Lerman and colleagues (2005) categorized animals for study of hypertension as genetic (phenotype or genotype-driven models) and non-genetic

models. The phenotype-driven models comprise animals that has a natural variation among inbreed strains, such as SHRs. The most commonly used animal model of essential hypertension is the SHRs with the Wistar Kyoto rats (WKYs) as the normotensive control **[Pinto et al., 1998]**. SHR strain was produced by Okamoto and Aoki (1963) by selective inbreeding of WKYs with high blood pressure. However, WKYs are not inbreed, the biological variability of this strain may be greater than that of SHRs **[Kurtz et al., 1987].**

The causes of increased blood pressure in the SHRs are undoubtedly multifactorial and include changes in vascular, renal, cardiac and sympathetic systems. Functional and structural alterations that results in increased of blood pressure in this strain begin at 4-5 weeks of age, and steadily increases to reach systolic arterial pressure to (~180 – 200 mmHg). In male WKYs animals, blood pressure remains at 131-136 mmHg after 10 weeks of age **[Okamoto and Aoki, 1963; Lerman et al., 2005; Lin et al., 2016]**. Increased cardiac output with unaltered total vascular resistance were observed in the early stages of hypertension in SHRs. However, with the progress of establishment of hypertension in this strain, cardiac output returns to basal levels and now hypertrophic blood vessels produce a sustained increase in total peripheral resistance **[Doggrell et al., 1998]**.

Hypertension and cardiac hypertrophy are associated with increase of basal sympathetic hyperactivity and hyperexcitability in SHRs. This strain develops as a classic phenotype characteristic, cardiac hypertrophy (~30% of increasing when compared to WKYs hearts), endothelial dysfunction and heart failure. At 16-weeks of age, there is evidence of left ventricular hypertrophy, as well as sustained elevated arterial pressure and HR become evident in SHRs **[Herring et al., 2011]**.

Pressure overload in SHRs not only induces concentric hypertrophy in cardiomyocytes, but also modifies changes in mechanical and electrical activities of left ventricular cells. Such changes depend on transmural localization of these cells which differ across the left ventricular wall. **[McCrossan et al., 2004]**. Besides, SHRs only develop heart failure at 18-24 months of age, characterized by impairment of left ventricular function, dilation of cardiac chambers, increasing of collagen deposition and significant reduction of cardiac ejection fraction without an additional increase in left ventricular mass **[Boluyt et al., 1995]**. Brooksby and colleagues (1993) demonstrated that compared to cardyomyocytes from normotensive rats, hypertrophied cardiomyocytes from SHRs display an increase in calcium (Ca^{2+}) transient which is responsible for their increased contraction. Besides that, prolonged

action potential in SHRs myocytes leads to a greater sarcoplasmic reticular Ca^{2+} release and an increased contraction in hypertrophied SHRs myocytes**.**

SHRs also exhibit impaired endothelial vasodilation and enhanced vasoconstriction. Endothelial dysfunction in SHRs is characterized by decrease in endothelium-dependent relaxation to acetylcholine, where the maximum relaxation response is reduced to ~ 40% in comparison with normotensive WKYs (80-100% of ACh-inducing relaxation) **[Konishi et al., 1983]**. Hypertension is commonly associated with structural and functional vascular abnormalities **[Touyz et al., 2018]**. Eutrophic vascular remodeling is a hallmark in SHRs **[Schiffrin et al., 2012]**.

Rizzoni and colleagues (1994) also demonstrated through a time-course analysis, that the development of vascular and cardiac hypertrophy in SHRs are variables age-dependent. At 4-week-old SHRs, an enhanced contractile response to norepinephrine related to vascular hypertrophy was detected whereas endothelial dysfunction was not evident. Endothelial dysfunction was observed only in 12-week-old SHRs, after the development of hypertension and cardiac hypertrophy. Until this date, it is quite debatable whether endothelial dysfunction during hypertension is a cause or a consequence of increased blood pressure in SHRs **[Bernatova et al., 2014]**. As described in the literature, impaired NO signaling is associated with hypertension and its complications **[Schiffrin et al., 2012; Su et al., 2015].** NO plays an important role in basal endothelial function and cardiac contractility in both humans and experimental models **[Massion et al., 2003; Brandes et al., 2014]**, although until this data there are some contradictory results about the NO production and its availability in cardiovascular in SHRs **[Chou et al., 1998; Vazari et al., 2000; Kristek, 2007; Pùzserovà et al., 2007].**

1.2 CYCLIC NUCLEOTIDES AND PHOSPHODIESTERASE IN THE CARDIOVASCULAR SYSTEM

1.2.1 Cyclic nucleotides as regulators in the heart and vessels

3',5'- cyclic adenosine monophosphate (cAMP) and cGMP are nucleotides that act as regulators in several process, such as those involved in vasodilation and vasoconstriction, neuronal function, immune response, metabolic response, cellular growth, differentiation and proliferation, apoptosis and cardiac contractility **[Zaccolo and Movsesian, 2007; Bork and Nikolaev, 2018].**

Synthetized by adenylyl cyclase (AC) from ATP, cAMP regulates cardiac function, as well as increasing inotropic and chronotropic responses in the heart. The main downstream effector of cAMP is the protein kinase A (PKA). In its turn, cGMP, which is generated by guanylyl cyclase (GC) in response to NO and natriuretic peptides (NPs) modulates cardiac contractility via the activation of protein kinase G (PKG). In the cardiovascular system, PKG activation is associated with vasorelaxation and cardiac negative inotropic effects **[Zaccolo and Movsesian, 2007].**

cAMP/PKA and cGMP/PKG signaling pathways usually exert opposing effects on heart function, because of the differing effects of PKA- and PKG-activation and phosphorylation on different target proteins in the heart **[Wegener et al., 2002; Zaccolo and Movsesian, 2007; Stangherlin and Zaccolo, 2012].** These two signaling pathways can be modulated by PDEs. These cellular components comprising a superfamily of enzymes, encoded by 21 different genes, categorized into 11 families (PDE1 to PDE11), which catalyze the breakdown of both cAMP and cGMP into their linear inactive forms, 5'- AMP and 5'- GMP **[Bender and Beavo, 2006; Lukowski et al., 2014; Azevedo et al., 2014].**

Cyclic nucleotides balance between synthesis and degradation is modulated by PDEs. PDEs are found in all tissues, but their distribution varies among different tissues and cellular compartments. In the heart, 10 PDEs families have been described – PDE1, PDE2, PDE3, PDE4, PDE5, PDE7, PDE8, PDE9, PDE10 and PDE11. It has been reported that PDE1, PDE2, PDE3, PDE10 and PDE11 are dual-specificity enzymes that can hydrolyze both cAMP and cGMP; PDE4, PDE7 and PDE8 hydrolyze only cAMP; PDE5A and PDE9 are selective for cGMP **[Bender and Beavo, 2006; Azevedo et al., 2014; Maurice et al., 2014].**

PDEs differ in their structure, intracellular localization, cellular expression, regulation and kinetic properties **[Bender and Beavo, 2006; Lukowski et al., 2014].** cGMP selectively modulates cardiac PDE2 and PDE5A activity. Whereas PDE2 hydrolyzes both cAMP and cGMP; PDE5A selectively hydrolyzes cGMP (Figure 1). In addition, PDE1, PDE3 and PDE9 are relevant in the cardiac function **[Bender and Beavo, 2006].**

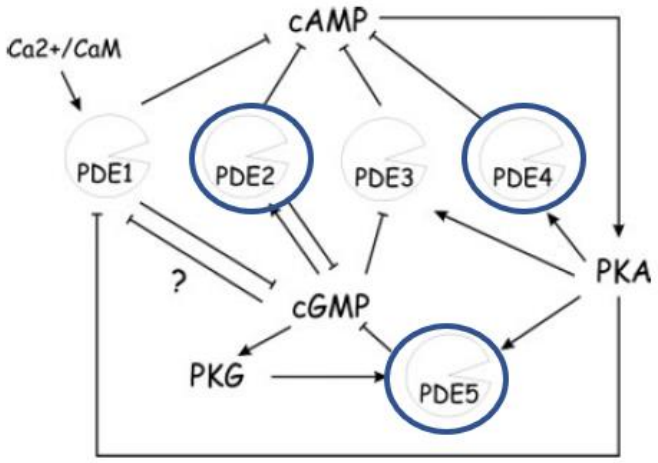


Figure 1. Regulatory network interconnecting cardiac phosphodiesterase. cAMP – 3',5'-cyclic adenosine monophosphate, cGMP – 3',5'- cyclic guanosine monophosphate, PDE – phosphodiesterase, PKG – protein kinase G, PKA – protein kinase A, CaM – calmodulin. Arrows indicate activation while blunt ends indicate inhibition or cyclic nucleotide hydrolysis. Adapted from **Zaccolo and Movsesian, 2007**.

1.2.2 Activation and regulation of cGMP/PKG/PDE5A signaling pathway in heart

In the cardiovascular system, cGMP is crucial to numerous cell types, as cardiomyocytes, cardiac fibroblasts, vascular smooth muscle cells (VSMCs) and endothelial cells **[Bork and Nikolaev, 2018].** cGMP mediates an extensive field of physiologic processes by activating PKG, cyclic nucleotide-gated ion channels and PDEs **[Zaccolo and Movsesian, 2007; Azevedo, 2014].** cGMP is synthetized from guanosine triphosphate (GTP) by different isoforms of GC, one which is soluble (sGC) and acts as a biological target for NO and a particulate isoform (pGC) that acts as a membrane receptor to NPs **[Tsai and Kass, 2009; Kotz et al., 2009].**

sGC which is activated by NO, resides in the cytosol and is a heterodimer composed by $\alpha_{1,2}$ subunit and $\beta_{1,2}$ subunit. The $\alpha_1\beta_1$ heterodimer is the most prevalent sGC isoform. NO diffuses directly across the plasma membrane and activates sGC by binding to both heme and non-heme sites of sGC which activates catalytic sites of the enzyme to rise intracellular cGMP levels **[Tsai and Kass, 2009; Montfort et al., 2016]**.

NO is produced by a group of enzymes called NO synthases (NOS), whose main representatives are neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) isoforms. All three isoforms are expressed in the heart, but only, nNOS and eNOS are constitutively

expressed. NO/sGC/cGMP/PKG pathway modulates myocardial contractility and HR, as well as exerts cardiac anti-remodeling effects. Contractile function is modulated by the amount of NO; which is enhanced by low concentrations and depressed by high concentrations **[Mohan et al., 1996; Massion et al., 2003; Rastaldo et al., 2007]**. Both, cardiac hypertrophy **[Brede, 2003]** and endothelial dysfunction **[Atochin and Huang, 2010]** are associated with decreased eNOS expression and its activity.

NPs mediates biosynthesis of cGMP by binding to pGC. NPs are classified as ANP, brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Briefly, ANP is secreted by atria and ventricles in response to myocardial wall stretch, while BNP and CNP is secreted by the brain, vascular endothelium, macrophages and cardiac fibroblasts **[Nishikimi et al., 2006].** NPs production and release can also be influenced by neurohumoral factors, such as catecholamines, antidiuretic hormone (also called vasopressin), glucocorticoids, angiotensin II and endothelin **[Nishikimi et al., 2006; Cerra and Pelegrino, 2007; Volpe et al., 2014; Song et al., 2015].** pGC is also called as NP-membrane associated-GC receptors (NPR). There are at least seven pGCs (NPR-A to NPR-G) **[Bork and Nikolaev, 2018]**. Both ANP and BNP bind preferentially to NPR-A, whereas CNP preferentially binds to NPR-B. NPR-A and NPR-B are responsible for the common physiological effects promoted by NPs, such as regulation of blood pressure, venous capacitance, arterial resistance and cardiac contractility **[Nishikimi et al., 2006; Cerra and Pelegrino, 2007; Volpe et al., 2014].**

NPs not only regulate arterial blood pressure and volume, but also, exert local antihypertrophic and antifibrotic effects **[Nishikimi et al., 2006; Song et al., 2015]**. Overexpression of NPR-A in the heart did not alter blood pressure but reduced cardiac myocyte size in both wild type and NPR-$A^{-/-}$ mice **[Kishimoto et al., 2001]**. Pressure overload induced by transverse aortic constriction (TAC) in NPR-$A^{-/-}$ mice resulted in significant cardiac hypertrophy and increased in mRNA expression of cardiac hypertrophic markers when compared to NPR-$A^{+/+}$ **[Holtwick et al., 2003]**. Calderone and colleagues demonstrated in culture of neonatal cardiomyocytes that NO and ANP can attenuate the effects of norepinephrine on the growth of cardiac myocytes and fibroblasts by a cGMP-mediated inhibition of norepinephrine-stimulated calcium Ca^{2+} influx **[Calderone et al., 1998]**. Interestingly, in SHR, there is a strong evidence that natriuretic peptide precursor A gene/variant modifications predisposing to spontaneous hypertension and cardiac hypertrophy **[Ye and West, 2003]**. NPs/NPR-A/cGMP/PKG signaling negatively regulates calcineurin-

NFAT signaling pathway **[Fiedler et al., 2002]** and MAPK/ERK in hypertensive eNOS$^{-/-}$ mice **[Bubikat et al., 2005].**

NO/sGC/cGMP and NPs/pGC/cGMP signaling converges to PKG activation. There are at least two PGK genes, PKG type I (PKG-I) and type II (PKG-II) **[Bork and Nikolaev, 2018]**. cGMP dependent PKG-I represents the best characterized cGMP downstream signaling effector in cardiovascular system. PKG-I comprised two different isoforms, PKG-Iα and PKG-Iβ, which are splice variants of the same gene and differ basically on the sequence of their N-terminal domain. PKG-I phosphorylation regulates major components of the cardiac excitation–contraction coupling **[Zaccolo and Movsesian, 2007]**.

Activation of PKG-I and phosphorylation of target proteins is involved with cardiac negative inotropic response **[Lee et al., 2010]**, cardiac antihypertrophic effects **[Takimoto et al., 2005b]**, protection against ischemia/reperfusion-related injury **[Fisher et al., 2005; Salloum et al., 2008; Salloum et al., 2009]** and promotes cardioprotective effects against apoptosis **[Costa et al., 2005; Salloum et al., 2007]**.

cGMP pool production and distribution differ depending of which type of GC is activated. PKG-I activation limits the accumulation of cGMP induced by NO via PDE5A stimulation; in its turn PKG-I activation increases the accumulation of cGMP induced by NPs (a positive feed-back); which suggests that cGMP is not homogeneously distributed in the cytosol of adult cardiomyocytes **[Castro et al., 2010]**. cGMP pool production induced by pGC activation is controlled primarily by the action of the PDE2, while PDE5A action limits the cytosolic cGMP pool induced by NO-sGC activation **[Castro et al., 2006; Fischmeister et al., 2006; Francis et al., 2010; Castro et al., 2010]**.

cGMP signaling regulates cardiac contractility **[Rastaldo et al., 2007]**. Several studies have been investigated the effects of both NO/sGC/cGMP and NPs/pGC/cGMP-PKG-I activation on cardiomyocytes contractility. In the heart, cGMP/PKG/PDE5A exerts modest effects on rest function, however, has the capacity to counteract to acute and chronic stress responses, in both physiological and pathological conditions **[Zhang et al., 2008; Zhang et al., 2011]**.

Stimulation of cardiac β-AR and activation of G-protein-coupled receptors leads to the generation of cAMP by stimulation of adenylyl cyclase and subsequent activation of PKA which results in enhancement of cardiac contractility and Ca^{2+} transient **[de Lucia et al., 2018]**. Stimulation of β-AR not only rises cAMP levels, but also stimulates cGMP production by NO-sGC-PKG pathway, which can modulate cAMP signaling in numerous ways

[Castellano and Böhm, 1997; de Lucia et al., 2018]. First, cGMP/PKG can phosphorylates troponin I (TnI) **[Layland et al., 2002]** and L-type calcium channel (LTCC) **[Yang et al., 2007]**, resulting in myofilament responsiveness to Ca^{2+} and reduction of Ca^{2+} current, respectively. Second, cGMP triggers PDE2 enhancing cAMP catabolism **[Mongillo et al., 2006; Stangherlin and Zaccolo, 2012]**. Third, cGMP acts as a competitive inhibitor of PDE3, which result in a positive inotropic effect of low levels of NO/cGMP **[Mohan et al., 1996]**. Furthermore, cGMP not only acts as a regulator of the activity of cAMP hydrolyzing PDEs, but also influences the intracellular concentration of cAMP **[Zaccolo and Movsesian, 2007]**. cGMP also increases the activity of RGS2 (regulator of G-coupled signaling 2) and in that way interferes in Gαq/11 signaling, which results in blunted β-adrenergic response in the heart **[Takimoto et al., 2009]**. Takimoto and coworkers showed that regulation of β-AR by cGMP is linked to NO-synthesis / PDE5A-hydrolyzing cGMP **[Takimoto et al., 2007]**.

Interestingly data from Lee and coworkers demonstrated that acute PDE5Ai modulates myocyte β-AR stimulation by isoproterenol (ISO) through suppressing sarcomere shortening without altering Ca^{2+} transient. The authors suggest that this mechanism requires modulation of β3 signaling coupled to PKG-mediated TnI phosphorylation. This modulation was unaltered in the presence of both PDE2 and PDE3 inhibitors **[Lee et al., 2010]** (Figure 2).

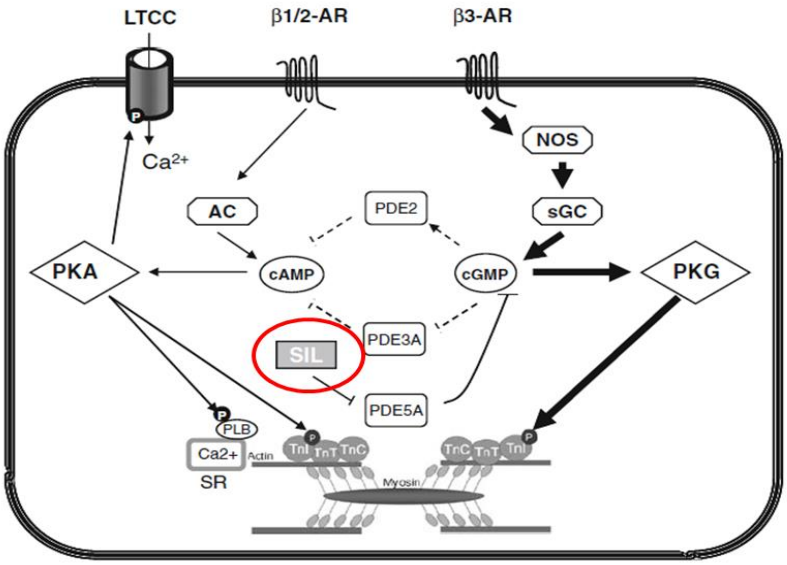


Figure 2. Signaling pathway underlying PDE5A inhibitory effects of acute β-adrenergic stimulation. LTCC – L-type calcium channel, PKA – protein kinase A, β1,2,3-AR – β-adrenergic receptor, AC – adenylyl cyclase, cAMP – 3',5'- cyclic adenosine monophosphate, PLB – phospholamban, NOS – nitric oxide synthase, sGC – soluble

guanylyl cyclase, cGMP – 3',5'- cyclic guanosine monophosphate PKG – protein kinase B, SIL – sildenafil. Adapted from Lee et al., 2010.

Recently, Isidori and colleagues (2015) demonstrated a potential role of sildenafil, on β-adrenergic signaling contraction frequency, as well as a receptor-specific interplay between cGMP and cAMP, through different PDE activities. Their results indicate that PDE5Ai has a minimal impact on the maximum response induced by ISO while significantly affecting the sustained phase of β-adrenergic response in culture of cardiomyocytes, besides that, PDE5Ai selectively depressed contraction rate stimulated by β2-, but not β1-AR activation.

Some authors have also demonstrated that activation β1/β2-adrenoceptors involves mechanism related with β3-adrenergic receptors counteracting cAMP generation in the heart [**Mongillo et al., 2006].** Borlaug and colleagues showed that PDE5Ai by sildenafil blunts systolic response to β-adrenergic dobutamine-stimulation, which corroborates the idea that PDE5A in the human heart modulates stimulated cardiac function **[Borlaug et al., 2005]**. However, the effects of PDE5Ai in cardiac β-AR signaling remain under investigation.

1.2.3 Regulation of PDE5A function in cardiovascular system

The most widely studied cGMP-specific-PDE is PDE5A **[Zhang and Kass, 2011]**. Pharmacologic inhibition targeting PDE5A to promote raise of cGMP concentration has evidenced to be a worthwhile treatment strategy for several conditions, such as erectile dysfunction, pulmonary arterial hypertension, heart failure, thrombosis and for the treatment of male lower urinary tract symptoms **[Corbin et al., 2002; Kaplan et al., 2007; Andersson, 2018].** Sildenafil (Viagra®; Pfizer In., US), avanalafil (Stendra®; Vivus Inc., US), vardenafil (Levitra®; Glaxo Smith Kline, UK) and tadalafil (Cialis®; Eli Lilly, US) are the commonly used, Food and Drug Administration-approved PDE5Ai. It is noteworthy that sildenafil was initially developed as an antihypertensive and antianginal agent **[Ribaudo et al., 2016; Andersson et al., 2018]**.These selective inhibitors have also been useful as research tools to study the physiological role of PDE5A in various pathological conditions, such as hypertension **[Bender and Beavo, 2006; Keravis and Lugnier, 2012]**.

PDE5A is encoded by a single PDE5A gene, localized in chromosome 4 (4q26) **[Yanaka et al., 1998]**. PDE5A contain a catalytic domain that hydrolyzes cGMP and a regulatory (R) domain that contain two highly homologous GAF domains – GAF-A and GAF-B. GAF-A is responsible for allosteric binding to cGMP, which promotes phosphorylation of human

PDE5A at Ser102 **[Zoraghi et al., 2005]**. In bovine and rodents, cGMP promotes phosphorylation of PDE5A at Ser92 **[Corbin et al., 2000; Puzzo et al., 2008]**. PDE5A catalytic activity not only is further enhanced by PKG, but also can be phosphorylated by PKA, which provides a positive feedback mechanism that can be activated by cGMP synthesis; hence PDE5A catalytic activity can be prolonged. These mechanisms of phosphorylation increase cGMP-biding affinity, as well as increase enzymatic catalytic activity. In the heart, three N-terminal variants of PDE5A mRNA and protein have been identified in humans and rodents (PDE5A1, PDE5A2, and PDE5A3). These isoforms differ only in the initial portion of exon-1 in the N-terminal. Both, PDE5A1 and PDE5A2 are widely expressed and distributed; PDE5A3 has been suggested to be the isoform mostly expressed in cardiomyocytes and VSMCs. It is important to highlight that, all three N-terminal variants have similar activity among them **[Bender and Beavo, 2006; Azevedo et al., 2014; Maurice et al., 2014; Campolo et al., 2017]**.

PDE5A is described as a cytosolic protein **[Bender and Beavo, 2006]**, firstly described as a dimer **[Fink et al., 1999]**, although recently was reported that dimers and tetramers of PDE5A coexist *in vivo* **[Keravis and Lugnier, 2012; Cardarelli et al., 2018]**. PDE5A was initially identified and isolated from platelets **[Coquil et al., 1980]** and later, from the lung **[Francis et al., 1980]**. Nowadays, PDE5A has also been described in smooth muscle cells, vascular myocytes, diseased cardiac myocytes, brain, cerebellum, liver, kidney, gastrointestinal tissues, pancreas and corpus cavernosum **[Bender and Beavo, 2006; Conti and Beavo, 2007; Francis et al., 2010; Keravis and Lugnier, 2012]**. In normal and failing mice hearts, PDE5A contributed ~22 and 43% of the cytosolic cGMP-hydrolytic activity, respectively **[Vandeput et al., 2009]**. PDE5A is up-regulated 2- to 6-fold in mice and humans heart disease **[Takimoto et al., 2005a; Nagendran et al., 2007; Pokreisz et al., 2009; Vandeput et al., 2009].** In cardiomyocytes, PDE5A is particularly expressed in z-bands and its sub-cellular organization depends of NOS/NO/cGMP/PKG signaling **[Takimoto et al., 2005; Kass et al., 2007a; Kass et al., 2007b].** Interestingly, both, mice genetically lacking eNOS and/or after chronic inhibition of eNOS by N(ω)-nitro-L-arginine methyl ester (L-NAME), PDE5A distribution and/or localization becomes diffuse **[Senzaki et al., 2001; Takimoto et al., 2005b].**

1.3 CGMP/PKG/PDE5A IN PHATOPHYSIOLOGY OF CARDIOVASCULAR DISEASES

Recently there has been great interest of treating cardiovascular diseases based on the use of drugs that act on cGMP regulatory cascade **[Lukowski et al., 2014]**. PDE5A expression and

activity is enhanced in several conditions, such as endothelial dysfunction **[Korkmaz et al., 2009]**, arterial hypertension **[Mergia et al., 2016]** and cardiac hypertrophy **[Nagendran et al., 2007]**. There are several evidences reported in animal models and clinical trials regarding the cardiovascular protective effects of PDE5Ai **[Rodrigues et al., 2013; Lukowski et al., 2014; Das et al., 2015 Leal et al., 2017]**.

Several clinical and experimental studies suggest that PDE5Ai does not modified or only exerts modest effects on blood pressure and HR **[Zhao et al., 2001; Gardiner et al., 2004; Lewis et al., 2007; Guazzi et al., 2011; Sasser and Baylis, 2010]**. Conversely, antihypertensive effects of PDE5Ai not only were observed in SHR treated chronically with sildenafil, but also in humans **[Oliver et al., 2006; Toblli et al., 2007; Oliver et al., 2010; Yaguas et al., 2010]**.

Regarding to sildenafil anti-hypertrophic effects, Takimoto and colleagues (2005) demonstrated that PDE5Ai by sildenafil not only prevents and reverses cardiac hypertrophy induced by sustained cardiac pressure overload, but also deactivates calcineurin/NFAT, phosphoinositide-3 kinase (PI3K)/Akt, and ERK1/2 signaling pathways. Similarly, chronic sildenafil administration prevents progressive chamber dilatation and dysfunction; development of fibrosis as well cardiac molecular remodeling by increasing PKG activity in mice subjected to TAC compared to non-treated TAC-hearts **[Nagayama et al., 2009].**

Likewise, PDE5Ai reverts the left ventricular hypertrophy and fibrosis induced by NOS blockers such as L-NAME **[Ferreira-Melo et al., 2006; Rossoni et al., 2007]**, protects against ischemia/reperfusion-related injury **[Salloum et al., 2003; Salloum et al., 2008; Salloum et al., 2009]**, as well as protects against doxorubicin-induced cardiomyocytes apoptosis **[Costa et al., 2005; Fisher et al., 2005]**. Interestingly, the cardioprotective effect of sildenafil against ischemia-reperfusion injury depends of NO synthase isoforms (eNOS and iNOS) inducing delayed preconditioning **[Salloum et al., 2003]**.

Ockaili and colleagues demonstrated that sildenafil induced acute and delayed protective effects against ischemia-reperfusion injury, probably by opening of mitochondrial K_{ATP} channels **[Ockaili et al., 2002]**. Treatment with tadalafil, the long-acting PDE5A inhibitor, improved cardiac performance and prevented cardiomyocytes apoptosis in doxorubicin-induced cardiomyopathy. This effect seems to be mediated through enhancing cGMP and PKG activity probably via up-regulation of mitochondrial Mn SOD without interfering with chemotherapeutic anti-cancer effect **[Koka et al., 2010]**. Yaguas and colleagues (2010) showed that chronic sildenafil administration reversed endothelial dysfunction, reduced renal

oxidative stress and renal macrophage accumulation in SHR which may suggest its use as an adjunct therapy in essential hypertension. Data regarding the effects of chronic PDE5Ai in SHR are scarce and remain under investigation.

Gianneta and colleagues also demonstrated that chronic inhibition of PDE5A improves cardiac diabetic cardiomyopathy in humans, exerting an anti-remodeling effect, improving cardiac and circulating markers **[Gianneta et al., 2012].** Similarly, PDE5Ai in diabetic mice expanded cardiac anti-inflammatory TIE2-expressing monocytes (TEMs) which promotes tissue repair, and reduces vascular inflammatory proteins, such as COX2 and vascular cell adhesion molecule 1 (VCAM-1), promoting tissue protection **[Venneri et al., 2015]**. At vascular level, chronic PDE5Ai promotes increasing of NO/cGMP/PKG pathway activation and improvement of vascular function, which results in vasodilatation, as well as anti-inflammatory and anti-genotoxic effects **[Balarini et al., 2013; Rodrigues et al., 2013]**. Leal and colleagues (2017) also reported a reduction of vascular proinflammatory cytokines, such as interleukin type 6 (IL-6), IL-10/IL-6 ratio and monocyte chemoattractant protein-1 (MCP-1) in $ApoE^{-/-}$ mice after treatment with sildenafil. These authors also demonstrated that chronic PDE5Ai decreases O^{2-} and COX-derived thromboxane. The anti-inflammatory mechanisms after sildenafil administration comprise interference of MAPK activation and NF-kB transactivation **[Zhao, 2011]**. Interestingly, PDE5Ai also increases the number and function of endothelial progenitor cells **[Rodrigues et al., 2013]** and decrease lipid deposition in conductance arteries. These reported findings reinforce the potential role of sildenafil in the vascular protection.

Oxidative stress and inflammation are key regulators of the initiation, regulation and clinical implications of vascular dysfunction secondary to hypertension **[Siti, 2005]**. Regarding the effects of sildenafil, its anti-inflammatory effects are mediated through the inhibition of PDE5A which in turn potentiates the inhibitory action of NO-sGC-cGMP pathway on nicotinamide-adenine-dinucleotide-phosphate (NADPH) oxidase expression and activity **[Koupparis et al., 2005]**. Furthermore, O^{2-} upregulates PDE5A protein expression and impairment of NO-mediated vasodilation **[Muzaffar et al., 2008]**.

2 AIM

The aim of this thesis was to investigate the impact of chronic PDE5Ai in different stages of development of essential hypertension. To accomplish this aim, two different experimental protocols were performed.

The impact of PDE5Ai sildenafil was investigated on development of hypertension in young SHRs (pre-hypertensive protocol). To assess how chronic PDEA5i affects the resting blood pressure and the functional vascular properties in young SHRs, sildenafil administration was started in the pre-hypertensive period (4 weeks-old), until 12 weeks of age when the establishment of hypertension occurs.

The effects of the chronic treatment with sildenafil were investigated on sustained high blood pressure in adult SHRs hearts. To explore how chronic PDE5Ai affects cardiac contractility, sildenafil administration was started 16 weeks-old, until 24 weeks of age. Following treatment, we examined the impact of sildenafil administration on cardiac performance under β-adrenergic stimulation, as well as its influence on cardiac oxidative stress and cardiac hypertrophy.

3 RESULTS

3.1 PRE-HYPERTENSIVE PROTOCOL

To investigate how chronic PDE5Ai affects the resting blood pressure and the functional vascular properties in young SHRs. Sildenafil administration was started in the pre-hypertensive period (4 weeks-old), until 12 weeks of age when the establishment of hypertension occurs in this strain. Functional and structural mechanisms that result from increased blood pressure in this strain begin at 5 weeks of age. Since SHRs have a pre-hypertensive state, this strain is used in studies of cause and development of hypertension. Vascular reactivity of aorta and mesenteric arteries were investigated in this experimental protocol.

3.1.1 Chronic PDE5Ai improves endothelial function by decreasing COX-2/MAPK expression and oxidative stress in aorta from SHRs

In the pre-hypertensive protocol, sildenafil treatment for 60 days did not alter the measured SBP, DBP, mean arterial pressure (MAP) and HR when compared to the same parameters of untreated SHRs (Table 1). Until this date, it is quite debatable whether endothelial dysfunction during hypertension is a cause or a consequence of increased blood pressure in this strain. To further investigate that, vascular reactivity of aorta was examined in this model after chronic sildenafil administration. Even in the absence of significant effects on blood pressure and HR, the PDE5Ai sildenafil, promoted a significant increase in acetylcholine-induced relaxation without altering the relaxation evoked by sodium nitroprusside; as well as promotes a reduction in contractile response induced by the α1-adrenoceptor agonist phenylephrine in isolated aortic rings (Figure 3). In aortic preparation from both SHR and SHR-Sild group the contractile response to phenylephrine was significantly enhanced in the presence of L-NAME or the specific inhibitor of sGC, ODQ. However, this enhanced effect was greater in preparations from SHR-Sild than those from untreated SHR group. This suggest an increasing of NO bioavailability after PDE5Ai (Figure 4). As demonstrated in Figure 5, protein expression of eNOS, phosphorylated eNOS at Ser1177, AKT 1/2/3, phosphorylated-AKT 1/2/3, PKG, and caveolin were unchanged by Sild treatment.

To evaluate the contribution of COX-derived prostaglandins to the acetylcholine and phenylephrine responses, arteries were preincubated with the COX inhibitor indomethacin.

COX inhibition increased relaxation response to acetylcholine and decreased contraction response to phenylephrine in aortic rings from treated and untreated animals. However, these effects were lowest in arteries isolated from sildenafil treated SHR, suggesting an anti-inflammatory effect associated with chronic PDE5Ai (Figure 6). Sildenafil administration potentiates the NO-mediated effects exerting antioxidative and anti-inflammatory actions. In this study was demonstrated that this protective effect of sildenafil could be attributed to reduced superoxide anions (O^{2-}) generation, reduced of COX-2 protein expression (Figure 7) and reduced of ERK1/2 protein phosphorylation (Figure 8); as well as increased NO bioavailability. Interestingly, the effects of chronic PDE5Ai not only promotes improvement of endothelial function, but also exerts interesting effects in the heart. At 12 weeks-old rats, chronic PDE5Ai by sildenafil prevented cardiac hypertrophy expressed as a reduction of wet heart weight/body weight ratio (mg/g) in SHR-Sild group compared to untreated group. As observed in aorta, ERK1/2 protein expression levels were reduced in SHR-Sild hearts (Figure 9).

The above-mentioned data were published in Life Sciences (2019), as an article entitled “Chronic administration of sildenafil improves endothelial function in spontaneously hypertensive rats by decreasing COX-2 expression and oxidative stress”, as a part of the global aim thesis. For the first time, it was demonstrated that PDE5Ai has a potential role in the improvement of vascular function during the development of hypertension in SHR. These mechanisms are associated with reduction of oxidative stress, a mechanism dependent of cyclooxygenase type 2 (COX-2) and ERK1/2 in aorta (see appendices B of this thesis, page 110).

Table 1. Baseline blood pressure and heart rate values in sildenafil-treated (SHR-Sild) and untreated SHR – pre hypertensive protocol

	SHR	SHR-Sild	P value
SAP (mm Hg)	180 ± 10	190 ± 14	0.84
DAP (mm Hg)	129 ± 6	134 ± 6	0.60
MAP (mm Hg)	150 ± 8	153 ± 11	0.81
HR (bpm)	327 ± 6	325 ± 13	0.90

Values are means ± SEM (n=5 rats per group). SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MAP, mean arterial pressure; HR, heart rate. Data were analyzed by Student's t-test.

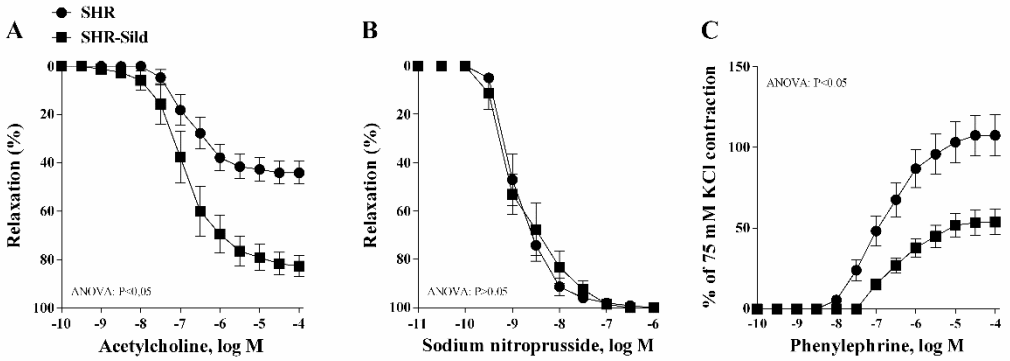


Figure 3. Pre-hypertensive protocol. Effects of chronic PDE5Ai on endothelium-dependent and endothelium-independent relaxation response to acetylcholine (A) and sodium nitroprusside (B), respectively and on the vasoconstriction response to phenylephrine (C) in aortic rings from animals treated with sildenafil (SHR-Sild) and untreated SHR. Results are expressed as mean ± SEM (n = 8 rats per group).

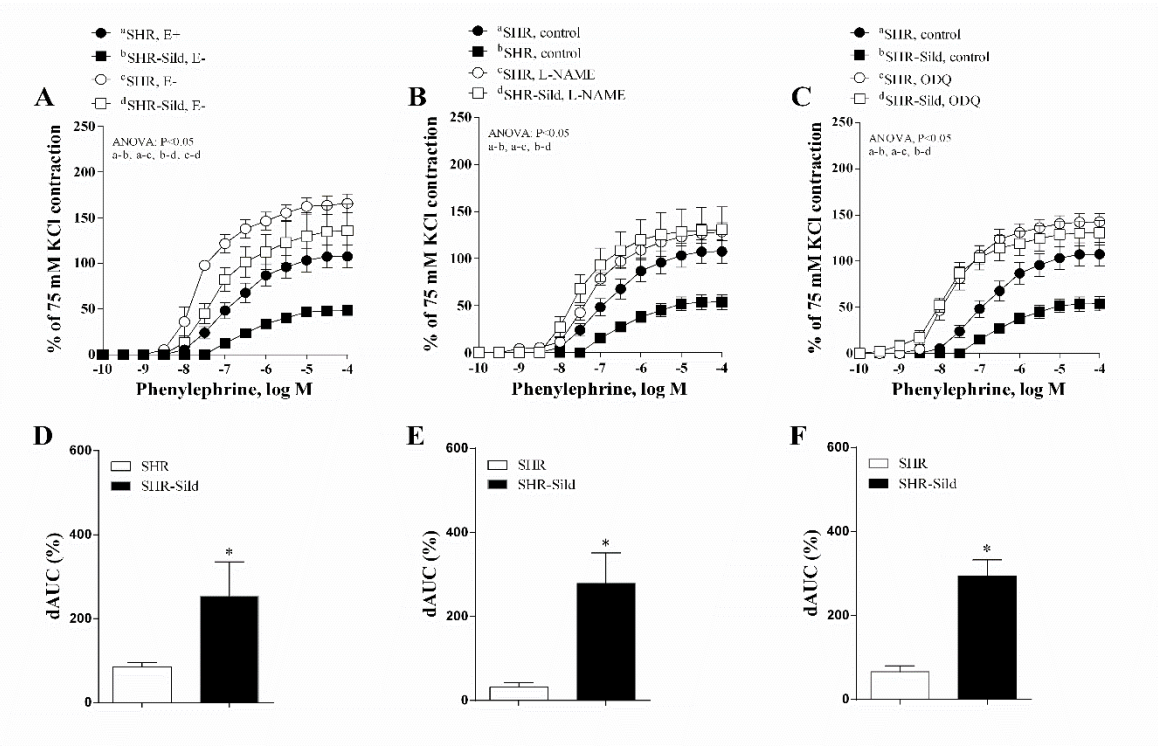


Figure 4. Pre-hypertensive protocol. Effects of endothelium removal (A), pretreatment with L-NAME (B) or ODQ (C) preincubation on contractile response induced by phenylephrine in aortic rings from treated (SHR-Sild) and untreated SHR. Differences in area under the concentration-response curve (dAUC) to phenylephrine in segments with (E+) and without (E−) endothelium, in the absence and presence of L-NAME or ODQ are shown in figures D, E and F, respectively. Results are expressed as mean ± SEM (n = 5–8 rats per group). *P < 0.05 vs. SHR by Student's t-test.

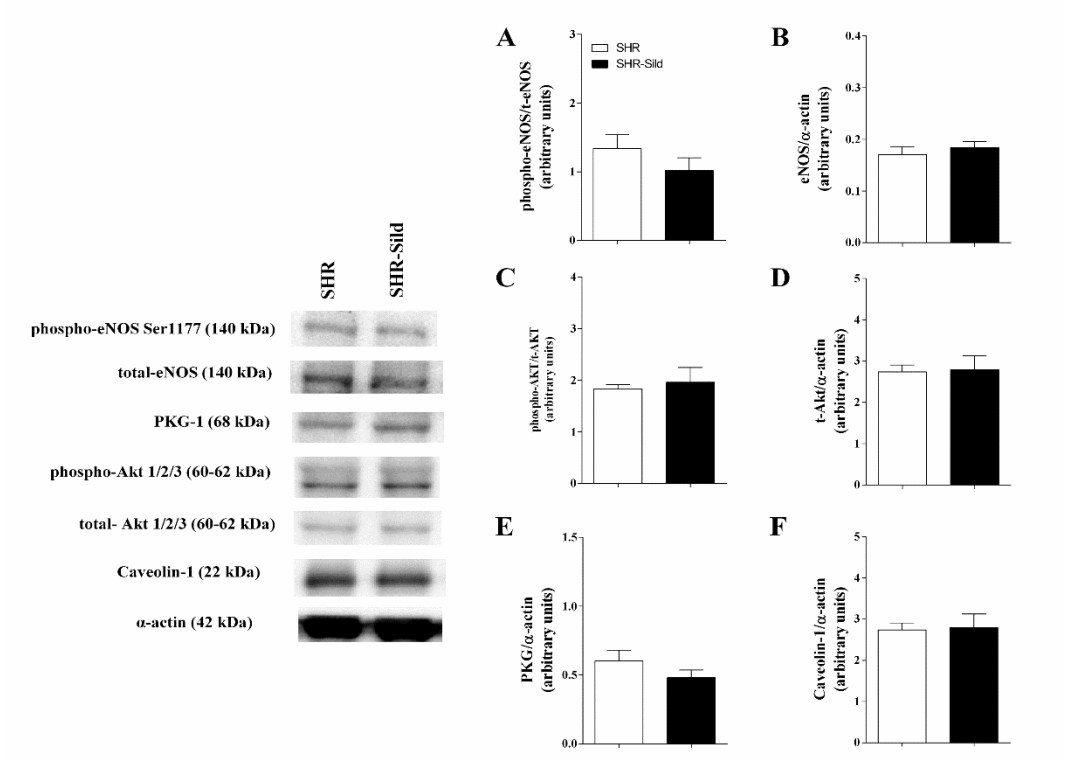


Figure 5. Pre-hypertensive protocol. Left panel – Representative blots of phospho-eNOS Ser1177, total-eNOS, phospho-Akt 1/2/3, total-Akt 1/2/3, PKG and caveolin and α-actin in aorta from sildenafil-treated (SHR-Sild) and untreated SHR. Right panel (A–F) – Averaged densitometric data for phospho-eNOS Ser1177 (A), total-eNOS (B), phospho-Akt 1/2/3 (C), total-Akt 1/2/3 (D), PKG (E) and caveolin (F) expression in aortic tissues from SHR and SHR-Sild groups. Densitometry of proteins were normalized by α-actin levels. Results were expressed as mean ± SEM (n = 6 rats per group).

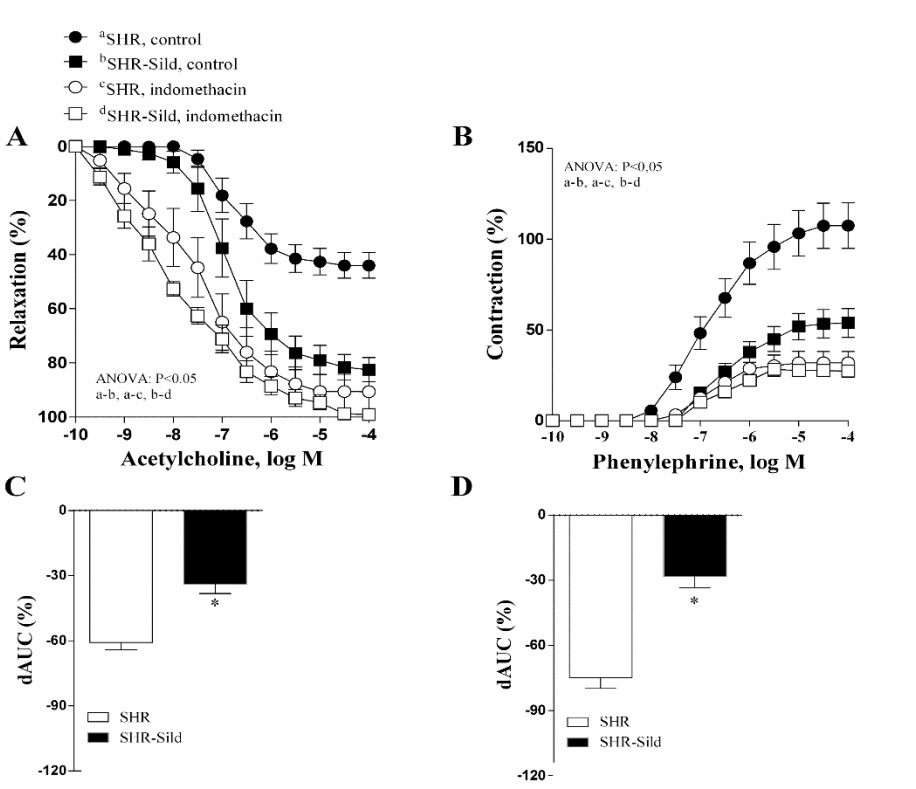


Figure 6. Pre-hypertensive protocol. Effect of COX inhibition by indomethacin on relaxation response to acetylcholine (A) and contractile response to phenylephrine (B) in aortic rings from sildenafil-treated (SHR-Sild) and untreated SHR. Differences in area under the concentration-response curve (dAUC) to acetylcholine (C) and phenylephrine (D) in segments in the absence and in the presence of indomethacin. Results are expressed as mean ± SEM (n = 5 – 8 rats per group). *P < 0.05 vs. SHR by Student's t-test.

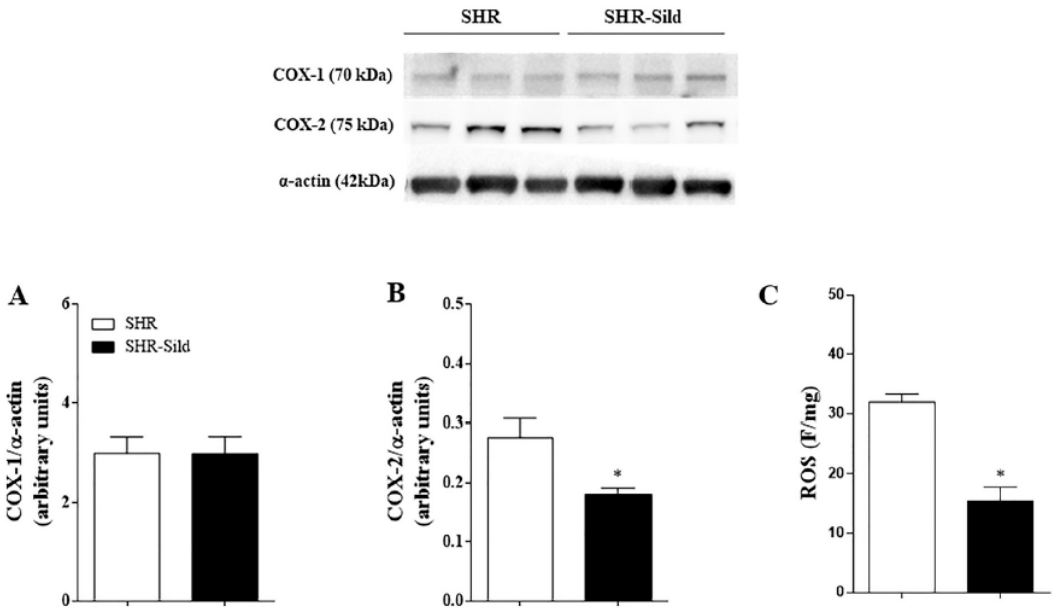


Figure 7. Pre-hypertensive protocol. Left top panel – Representative blots of COX-1, COX-2 and α-actin in aorta from sildenafil-treated (SHR-Sild) and untreated SHR. Averaged densitometric data for COX-1 (A) and COX-2 (B) expression in aortic tissues from SHR and SHR-Sild groups. Densitometry of proteins were normalized by α-actin levels. Aortic relative reactive oxygen species levels (C) in SHR and SHR-Sild groups. Results are expressed as mean ± SEM (n = 6–8 rats per group). *P < 0.05 vs. SHR by Student's test.

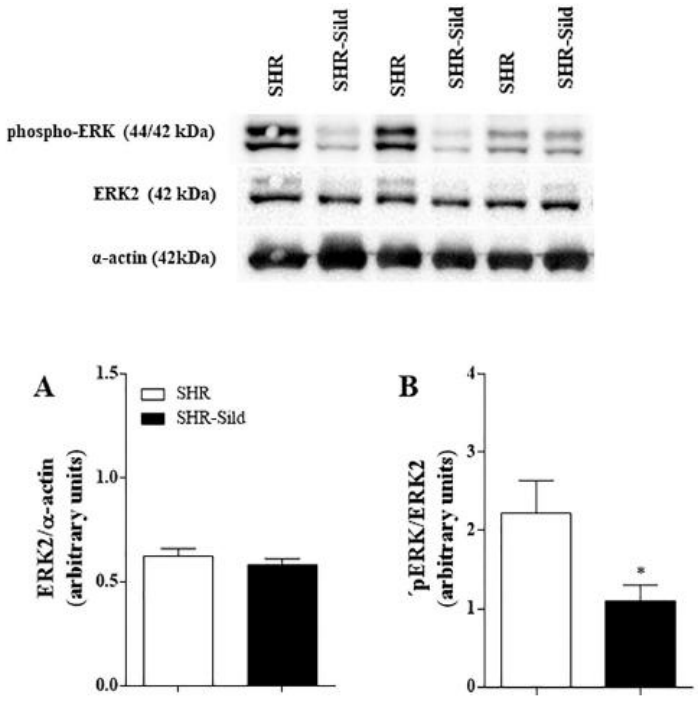


Figure 8. Pre-hypertensive protocol. Western blot analysis of protein expression of total-ERK (A) and phospho-ERK (B) in aorta from sildenafil-treated (SHR-Sild) and untreated SHR. Densitometry of proteins was normalized by α-actin levels. Results are expressed as mean ± SEM (n = 6 rats per group). *P < 0.05 vs. SHR by Student's t-test.

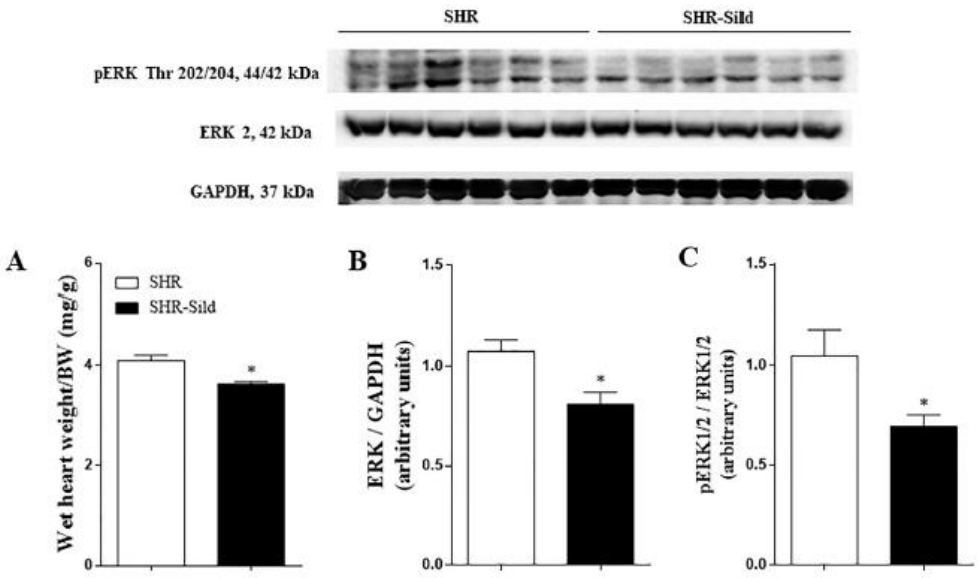


Figure 9. Pre-hypertensive protocol. Top panel – Representative blots of cardiac phospho-ERK, total-ERK and GAPDH expression in sildenafil-treated (SHR-Sild) and untreated SHR groups. Wet heart weight/body weight (BW) ratio (A) and western blot analysis of left ventricular cardiac protein expression of phospho-ERK (B) and total-ERK (C) from sildenafil-treated (SHR-Sild) and untreated SHR. Densitometry of proteins was normalized by GAPDH levels. Results are expressed as mean ± SEM (n = 6 rats per group). *P < 0.05 vs. SHR by Student's t-test.

***All above-mentioned data were published in Life sciences by JJ Teixeira-da-Silva and colleagues., (2019), 15;225 – 29-38. doi – 10.1016/j.lfs.2019.03.07.**

3.1.2 Structural and mechanical study of mesenteric resistance arteries on the pressure myograph

Regarding vascular remodeling, the impact of chronic PDE5Ai was investigated on mesenteric resistance arteries of SHR during the development of hypertension. In passive (0 Ca^{2+}) and active (+ Ca^{2+}) stimuli under isobaric conditions, chronic PDE5Ai did not alter the following parameters – vessel diameter (Figure 10), lumen diameter (Figure 11), wall thickness (Figure 12), media/lumen ratio (Figure 13) and cross-sectional area (Figure 14). Subsequently, were analyzed as mechanical parameters – incremental distensibility (Figure 15), intrinsic tone (Figure 16A), myogenic index (Figure 16B) and myogenic contraction (Figure 16C). In 12-weeks-old, sildenafil administration did not prevent the vascular remodeling during the advance of hypertension.

3.1.3 Vascular effects of sildenafil treatment on mesenteric resistance arteries

In endothelium-intact mesenteric arteries rings from SHRs, sildenafil treatment did not affect acetylcholine-induced relaxation (Figure 17A) and the contractile response induced by alfa-1 adrenoceptor agonist norepinephrine (Figure 17B). To investigate the involvement of NO/sGC/PKG pathway on vascular effects of sildenafil in SHRs, pretreatment with indomethacin, L-NAME, L-NAME+ODQ and ODQ were performed in endothelium intact mesenteric arteries rings. Any of these pre-treatments modulated vasodilation response to acetylcholine (Figure 18) and contractile response (Figure 19) to norepinephrine in sildenafil-treated (SHR-Sild) and untreated rats.

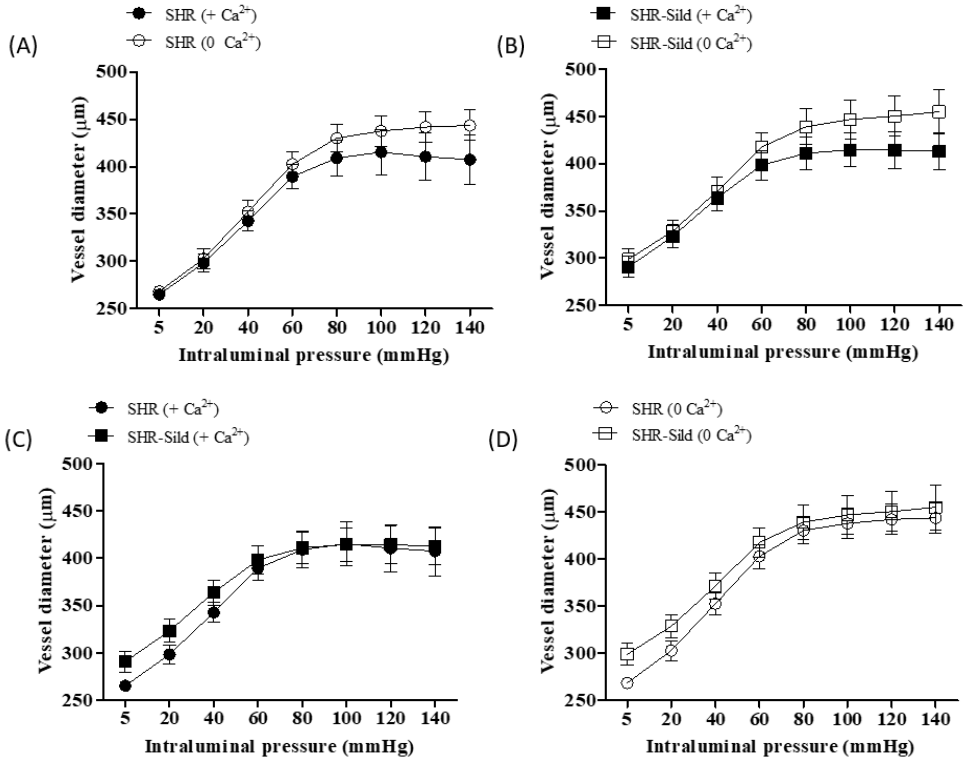


Figure 10. Relationship between increased intraluminal pressure and vessel diameter of mesenteric arteries under active (+Ca^{2+}) and passive (0 Ca^{2+}) conditions in untreated SHR (panel A) and SHR treated with sildenafil (panel B). Comparison of such relationship in either active (panel C) or passive (panel D) between untreated and sildenafil-treated SHR. Results were expressed as ± SEM (n = 8 rats per group).

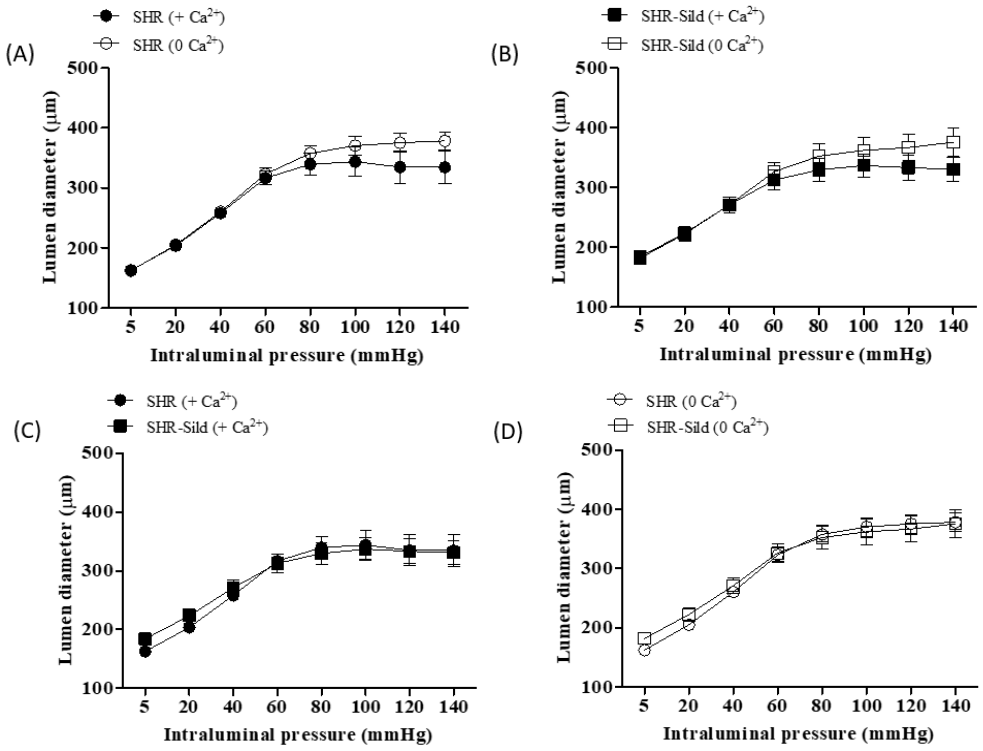


Figure 11. Relationship between increased intraluminal pressure and lumen diameter of mesenteric arteries under active (+Ca^{2+}) and passive (0 Ca^{2+}) conditions in untreated SHR (panel A) and SHR treated with sildenafil (B). Comparison of such relationship in either active (panel C) or passive (panel D) between untreated and Sild-treated SHR. Results were expressed as ± SEM (n = 8 rats per group).

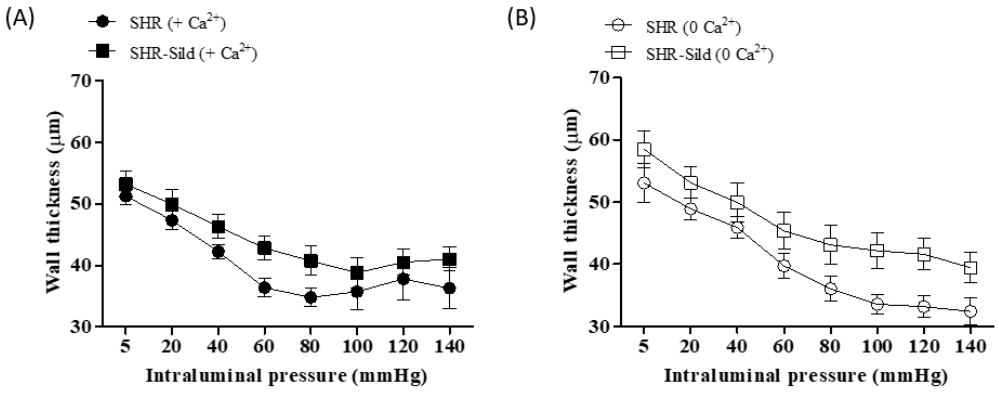


Figure 12. Comparison of the relationship of increased intraluminal pressure and wall thickness of mesenteric arteries between untreated SHR and SHR treated with sildenafil in either active (panel A) or passive (panel B) conditions. Results were expressed as ± SEM (n = 8 rats per group).

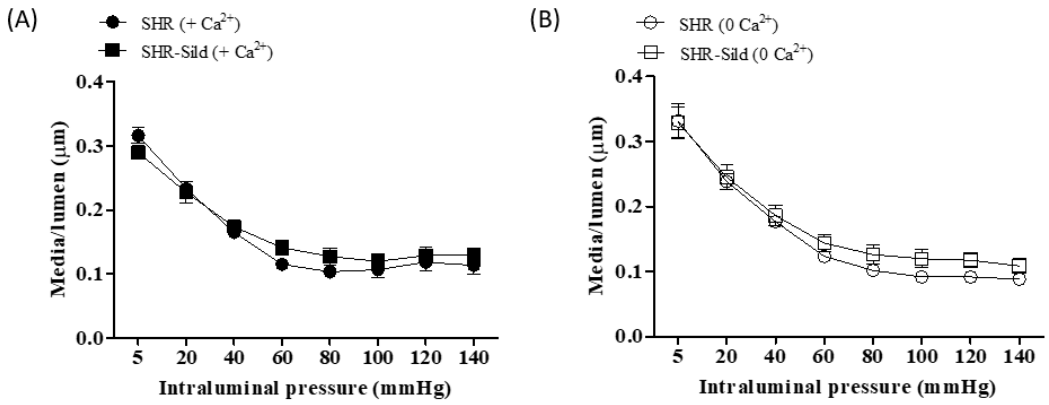


Figure 13. Comparison of the relationship of increased intraluminal pressure and media/lumen ratio of mesenteric arteries between untreated SHR and SHR treated with sildenafil (SHR-Sild) in either active (panel A) or passive (panel B) conditions. Results were expressed as ± SEM (n = 8 rats per group).

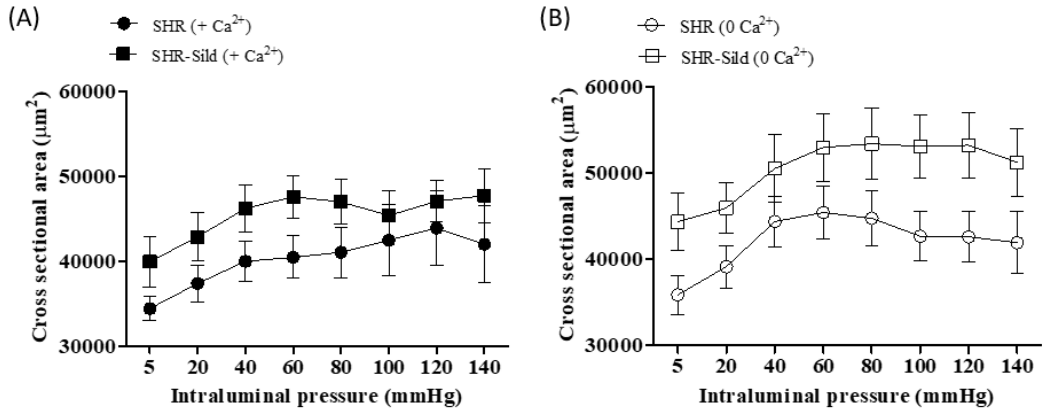


Figure 14. Comparison of the relationship of increased intraluminal pressure and cross-sectional area of mesenteric arteries between untreated SHR and SHR-Sild in either active (panel A) or passive (panel B) conditions. Results were expressed as ± SEM (n = 8 rats per group).

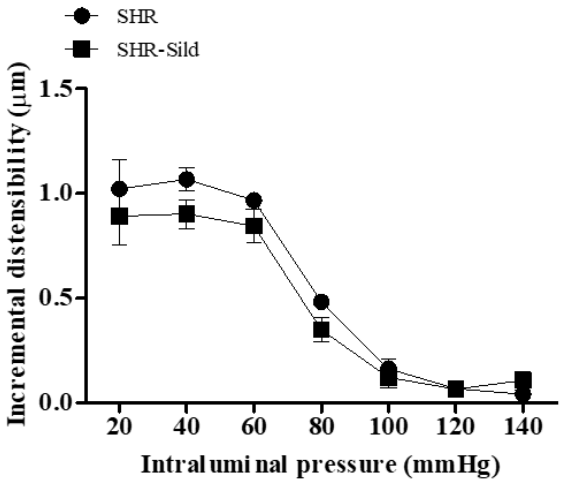


Figure 15. Comparison of the relationship of increased intraluminal pressure and incremental distensibility of mesenteric arteries between untreated SHR and SHR-Sild under active conditions (+ Ca^{2+}). Results were expressed as ± SEM (n = 8 rats per group).

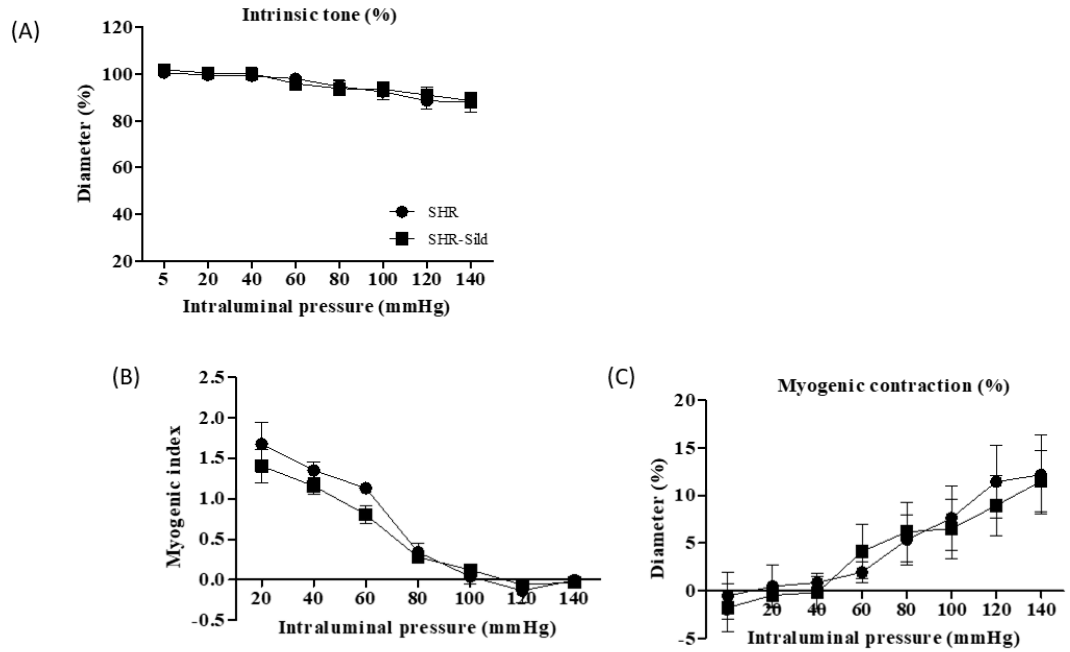


Figure 16. Comparison of the relationship of increased intraluminal pressure and intrinsic tone (panel A), myogenic index (panel B) or myogenic contraction (panel C) of mesenteric arteries between SHR untreated and treated with sildenafil (SHR-Sild). Results were expressed as ± SEM (n = 8 rats per group).

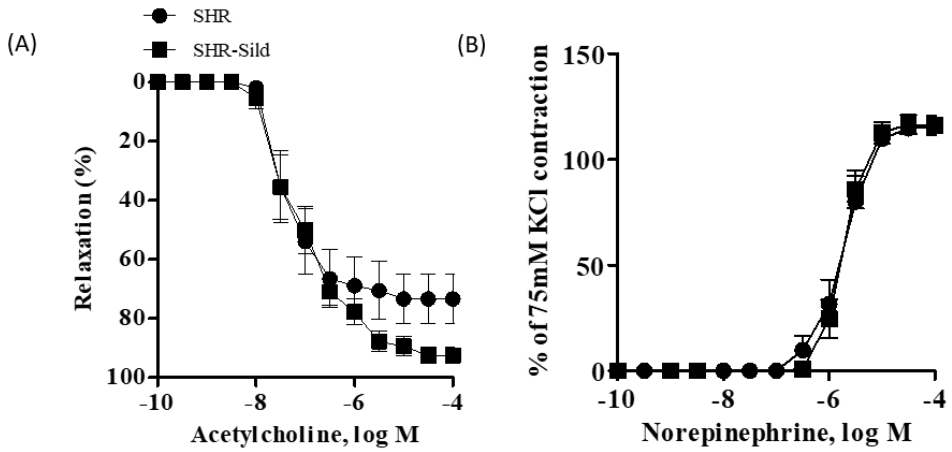


Figure 17. Effect of chronic PDE5Ai on endothelium-dependent response to acetylcholine (A) and norepinephrine (B) in mesenteric rings from sildenafil-treated (SHR-Sild) and untreated SHR. Results were expressed as ± SEM (n = 8 rats per group).

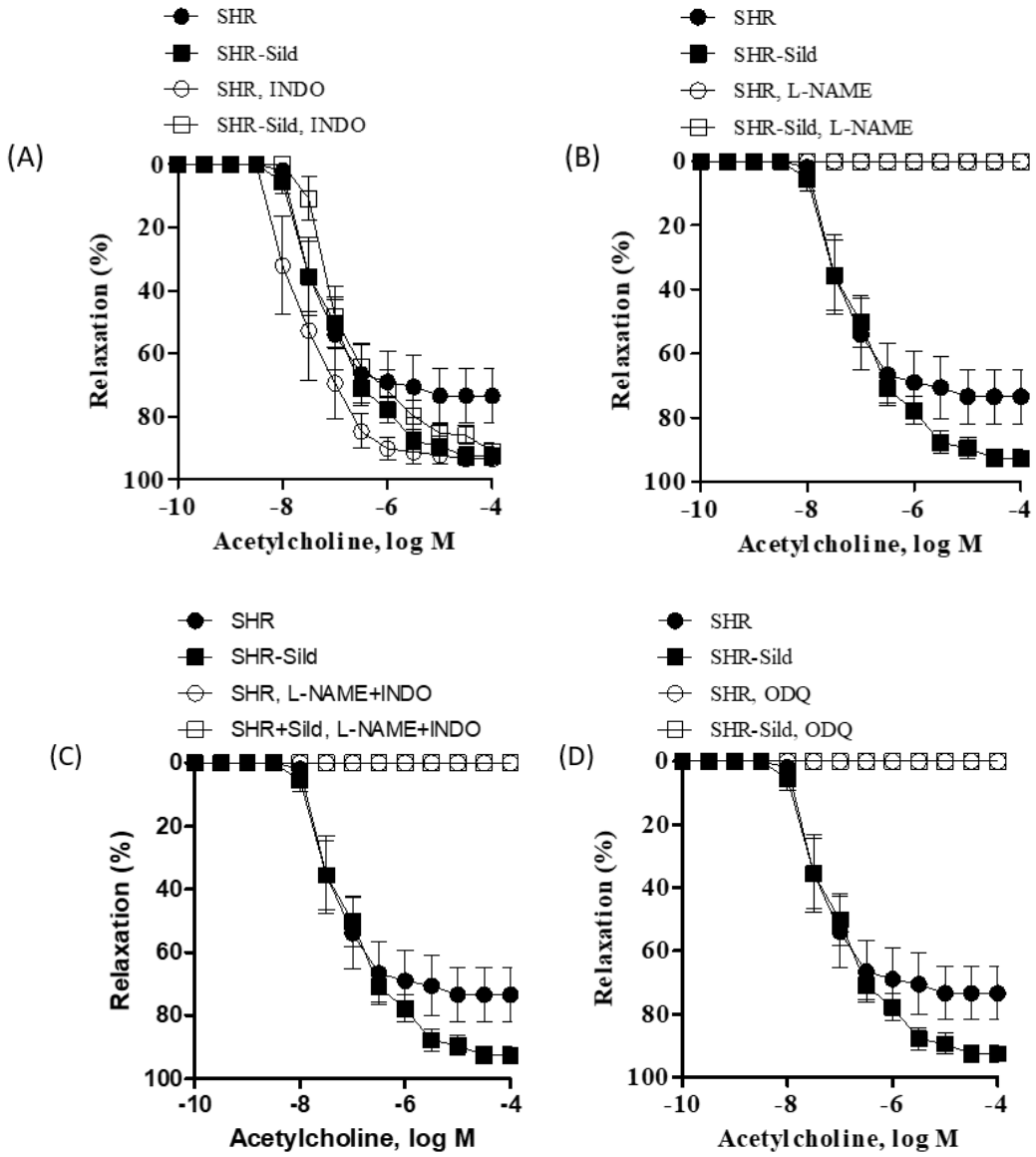


Figure 18. Effect of pretreatment with INDO (A), L-NAME (B), INDO+L-NAME (C) and ODQ (D) on vasodilatation response induced by acetylcholine in mesenteric resistance arteries rings from sildenafil-treated (SHR-Sild) and untreated SHR. Results were expressed as ± SEM (n = 8 rats per group).

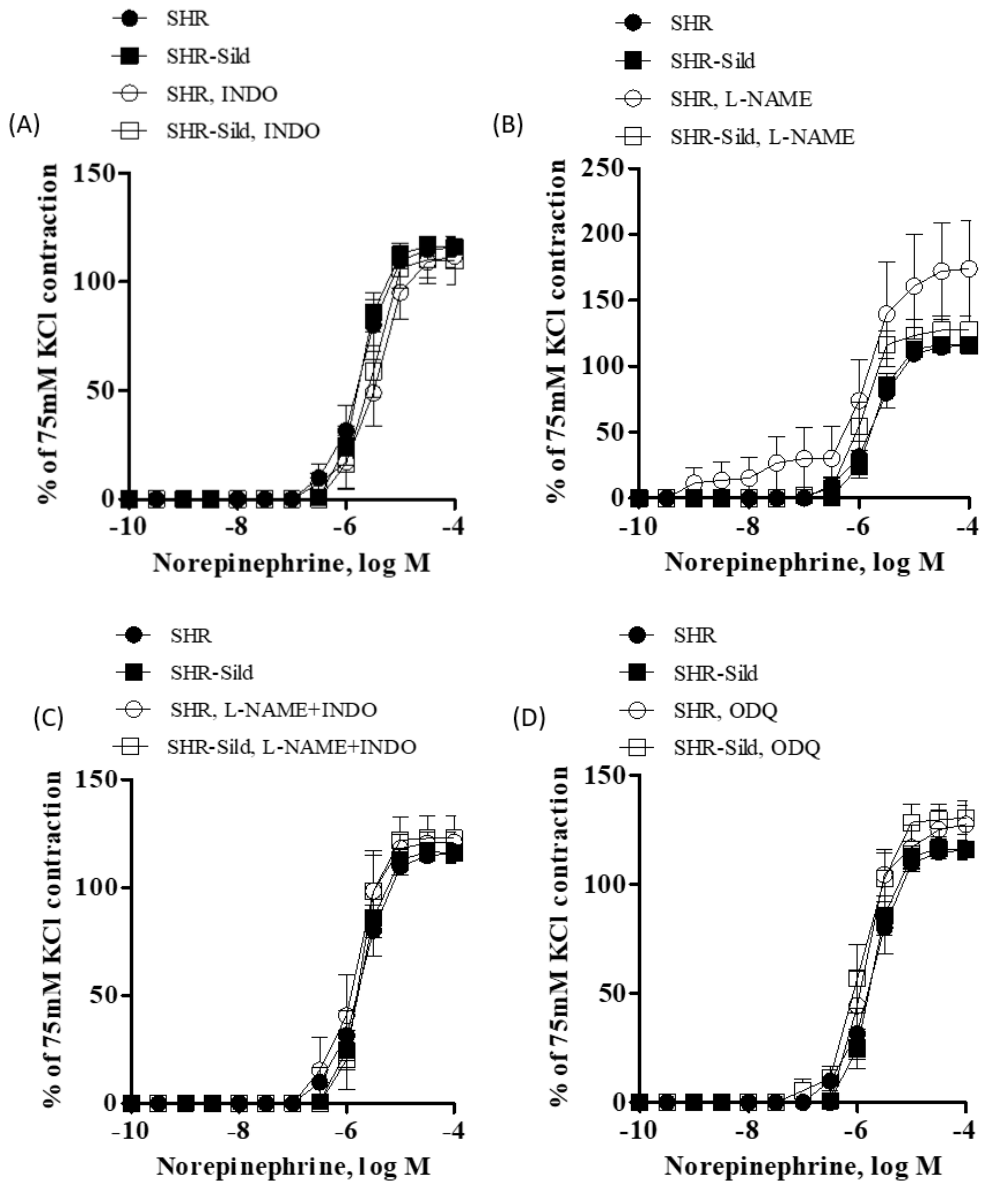


Figure 19. Effect of pretreatment with INDO (A), L-NAME (B), INDO+L-NAME (C) and ODQ (D) on contraction response induced by norepinephrine in mesenteric resistance arteries rings from sildenafil-treated (SHR-Sild) and untreated SHR. Results were expressed as ± SEM (n = 8 rats per group).

3.2 HYPERTENSIVE PROTOCOL

To investigate how chronic PDE5Ai affects cardiac dysfunction secondary to hypertension, sildenafil administration was started at 16-weeks-old rats. After 60 days of treatment, was investigate the impact of sildenafil treatment on cardiac performance under β-adrenergic stimulation, as well as how its influence cardiac oxidative stress and cardiac hypertrophy.

3.2.1 Effects of chronic PDE5Ai by sildenafil on biometric parameters, blood pressure and heart rate

At 24-weeks, body weight was not different between treated and untreated animals (SHR-Sild – 310 ± 11 *vs*. SHR – 290 ± 10 g, $P > 0.05$). As demonstrated in the pre-hypertensive protocol, no significant differences were observed in SBP, DBP, MAP and HR between untreated SHR and chronic sildenafil-treated SHR (Table 2). In 24-week-old rats, even in the absence of significant effects on resting blood pressure and HR, PDE5Ai with sildenafil significantly attenuated cardiac hypertrophy, as evidenced by the significant reduction of the wet heart weight/final body weight ratio (mg/g) (SHR-Sild – 3.5 ± 0.05 *vs*. SHR – 3.9 ± 0.07; $P < 0.01$; Figure 20C).

Masson's Trichome Staining analysis showed that cardiac structure as well as the collagen deposition were not modified in SHR-Sild group compared to untreated SHR (Figure 20A). The analysis of cross sectional area (Figure 20B and 20D) showed a significant reduction of myocytes area in the hearts from animals treated with a PDE5A inhibitor sildenafil (SHR-Sild – 205 ± 4.2 *vs*. SHR – 301 ± 7.2 μm^2), which suggests an antihypertrophic effect related to chronic PDE5Ai against sustained high blood pressure. As shown in Figure 20E, the total number of myocytes nuclei were not modified by chronic sildenafil treatment (SHR-Sild – 226 ± 9 *vs.* SHR – 266 ± 14 mm^2).

3.2.2 Inhibition of PDE5A by sildenafil counteracts sympathetic signaling in the heart

Left ventricular function from sildenafil- treated and untreated animals were evaluated in Langendorff-perfused hearts beating spontaneously under a constant pressure of 80 mmHg. After 30-min of equilibration period, there were no significant differences in basal left ventricular systolic pressure (LVSP) levels between animals treated and untreated with sildenafil (SHR-Sild – 94 ± 5.9 *vs*. SHR – 100 ± 5.9 mmHg, $P > 0.05$). The same result was obtained for basal levels of HR (SHR-Sild – 170 ± 3.4 *vs*. SHR – 180 ± 8.6 bpm, $P > 0.05$).

Values at the end of equilibration period were set to 100%. Cardiac functionality was verified through the positive inotropic and chronotropic responses to increasing concentrations of ISO (10^{-13} to 10^{-3} M) (Figure 21). As expected, ISO infusion evoked an increase, in a concentration-dependent manner, not only in the left ventricular contractility response, but also in HR in both studied groups.

A two-way ANOVA revealed that positive inotropic responses to increasing concentrations (10^{-11}-10^{-5} M) of the β-nonselective adrenergic agonist ISO were significantly ($P < 0.001$) reduced in hearts from sild-treated rats compared to the values recorded in untreated rats (Figure 21A). Likewise, positive chronotropic responses to ISO were significantly ($P < 0.01$) reduced in hearts from sildenafil-treated rats compared to untreated ones (Figure 21B). Taken together these results suggest that PDE5Ai blunts systolic β-adrenergic stimulation in hypertensive hearts. To analyze the impact of sildenafil on cardiac performance, we also investigated the derivatives of pressure over time. The maximal rate of pressure development (Max dP/dt, mmHg/s) and the minimum relaxation rate (Min dP/dt, mmHg/s) after ISO stimulation were not modified by chronic sildenafil treatment (Figure 22A and 22B, respectively). Max dP/dt and Min dP/dt afford a biphasic signal in the heart analysis which can be used to evaluate changes of contractility or relaxation of the ventricles, respectively.

3.2.3 Impact of chronic PDE5Ai and oxidative stress in hypertension

The reduction of the cross-sectional area of cardiomyocytes in SHRs treated with sildenafil could be associated to decreased ROS production and increased antioxidant systems activity. After 60 days of PDE5Ai, SHR-Sild group showed a significant decrease in the intensity of DCF fluorescence in the left ventricular cardiac mass when compared to intensity fluorescence measured in samples obtained from untreated animals. This may suggest a significant reduction of ROS production in the left ventricular myocardial tissue of animals chronically treated with sildenafil (Figure 23A). Chronic PDE5Ai resulted in reduction of cardiac ROS levels and increased cardiac total t-SOD in SHR-Sild group (Figure 23B), suggesting an improvement of antioxidant defense after PDE5Ai. CAT activity was unchanged between the studied groups (Figure 23C).

3.2.4 PDE5Ai and cardiac hypertrophy in hypertension

In SHRs, sildenafil treatment resulted in protection against cardiac pathological remodeling, demonstrated by downregulation of β-MHC, GATA4 and NFATc3 protein expression which

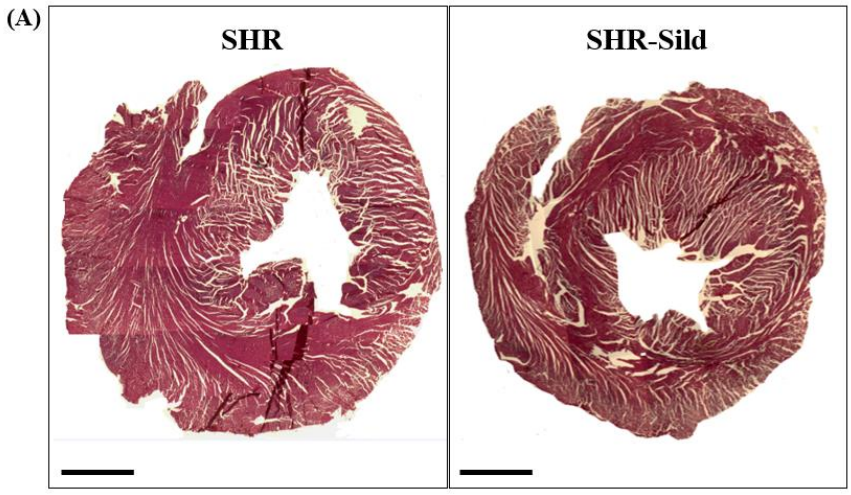
are markers of cardiac hypertrophy (Figure 24A, B, D, respectively). As shown in Figure 24C, TGFβ protein levels, a negative autocrine growth factor related with fibrosis, were downregulated in sildenafil treated SHRs compared to untreated SHRs. Furthermore, cardiac left ventricular ANP protein expression was upregulated in SHR-Sild group compared with untreated group (Figure 24E), suggesting a mechanism related with cardioprotection. PDE5A expression was not modified in treated and untreated rats (Figure 24F).

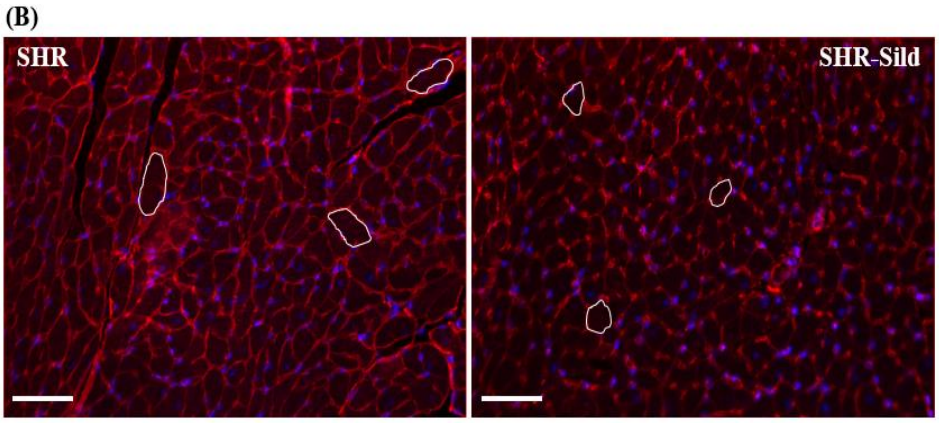
Basal levels of p42/p44 ERK protein expression is usually augmented in hypertension and it is related with increase cardiac oxidative stress [Roberts et al., 2012]. Total ERK protein expression (Figure 25A) did not change in the left ventricle of SHR treated with sildenafil. However, a significantly ($P < 0.01$) reduction of ERK1/2 phosphorylation was observed in the homogenate of myocardium obtained from sildenafil-treated animals compared to the levels measured in the homogenate of ventricles of untreated animals (Figure 25B). Besides that, increase of AKT kinase phosphorylation leads to activation of transcription and protein synthesis resulting in cardiac hypertrophy [Abeyrathna, 2015]. Densitometry analysis from hearts of SHR-Sild group showed a significant ($P < 0.01$) reduction of AKT1 (Ser 473) phosphorylation as compared to SHR group (Figure 25D). However, total AKT1 levels remained unchanged irrespective of whether SHR were treated or not with Sild (Figure 25C).

Table 2. Baseline blood pressure and heart rate values in sildenafil-treated (SHR-Sild) and untreated SHR – hypertensive protocol

Parameters	SHR	SHR-Sild
SBP	190 ± 3.2	200 ± 10
DBP	140 ± 1.0	150 ± 4.5
MAP	160 ± 2.0	180 ± 9.0
HR	340 ± 17	358 ± 12

Values are means ± SEM (n = 4 rats per group). SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate.


(A)
SHR
SHR-Sild


(B)
SHR
SHR-Sild

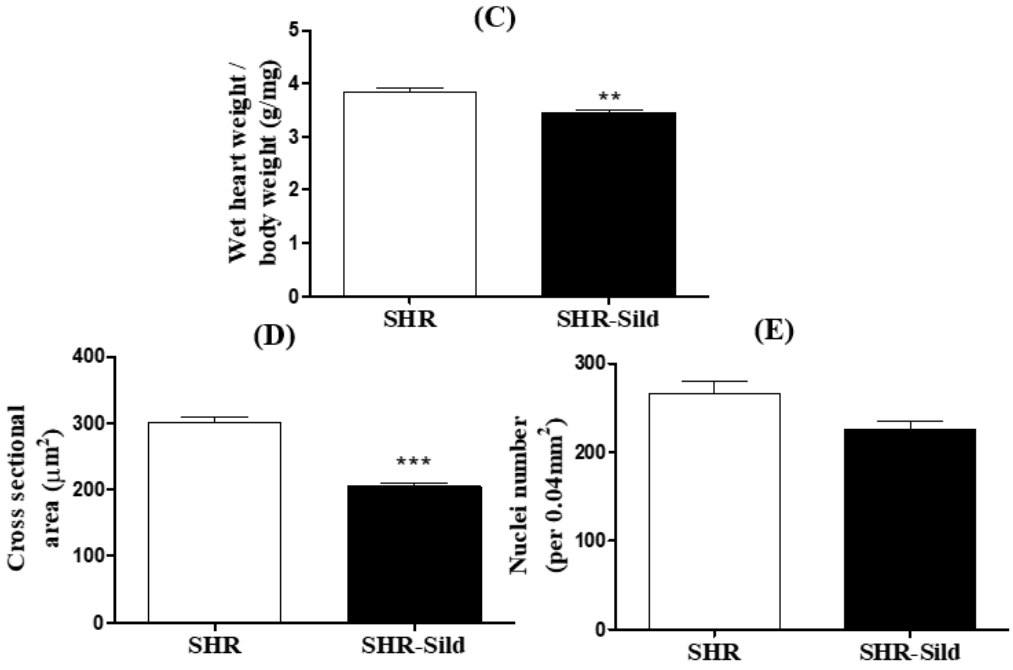


Figure 20. Modifications of cardiac growth in hypertension. (A) Representative Masson Thricome staining of adult heart sections. Scale bar – 100 μm. (B) Representative images of immunofluorescence for Wheat Germ Agglutin (WGA). The white circle highlighted the different cell size. Scale bar – 50 μm. (C) The graph represents the wet heart weight/body weight ratio (g/mg) of SHR-Sild and SHR (n = 8 rats per group). (D) Cross-sectional area of cardiomyocytes and (E) number of nuclei from sildenafil treated and untreated. Data were analyzed by Student's t-test, ** $P < 0.01$; *** $P < 0.001$.

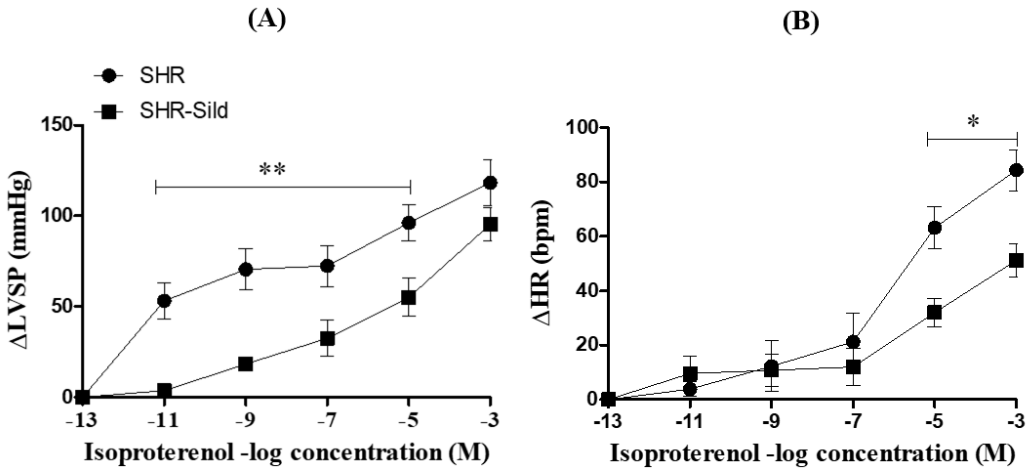


Figure 21. Maximum variations in left ventricular systolic pressure (ΔLVSP) (panel A) and maximum variation in HR (ΔHR, panel B) during infusion of increasing concentrations of ISO in isolated perfused hearts from sildenafil (Sild) treated and untreated SHR. Results were expressed as ± SEM (n = 8 rats per group). Data were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparison test, *P < 0.05. bpm = beats per minute.

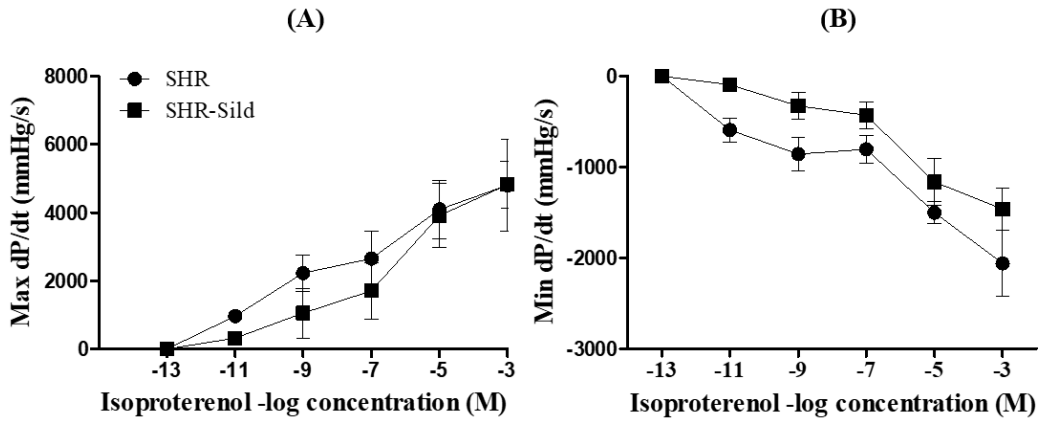


Figure 22. Comparison of the maximal rate of pressure development (Max dP/dt) and the minimum relaxation rate (Min dP/dt) after stimulation of α_1-adrenoceptors by ISO in isolated hearts from sildenafil-treated SHR and untreated SHR. Results were expressed as ± SEM (n = 8 rats per group).

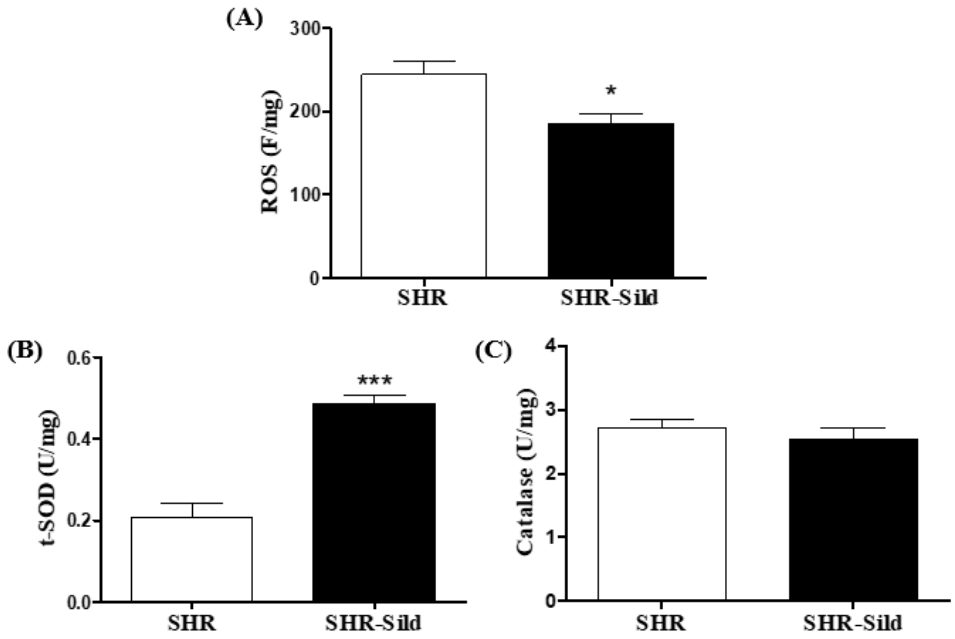
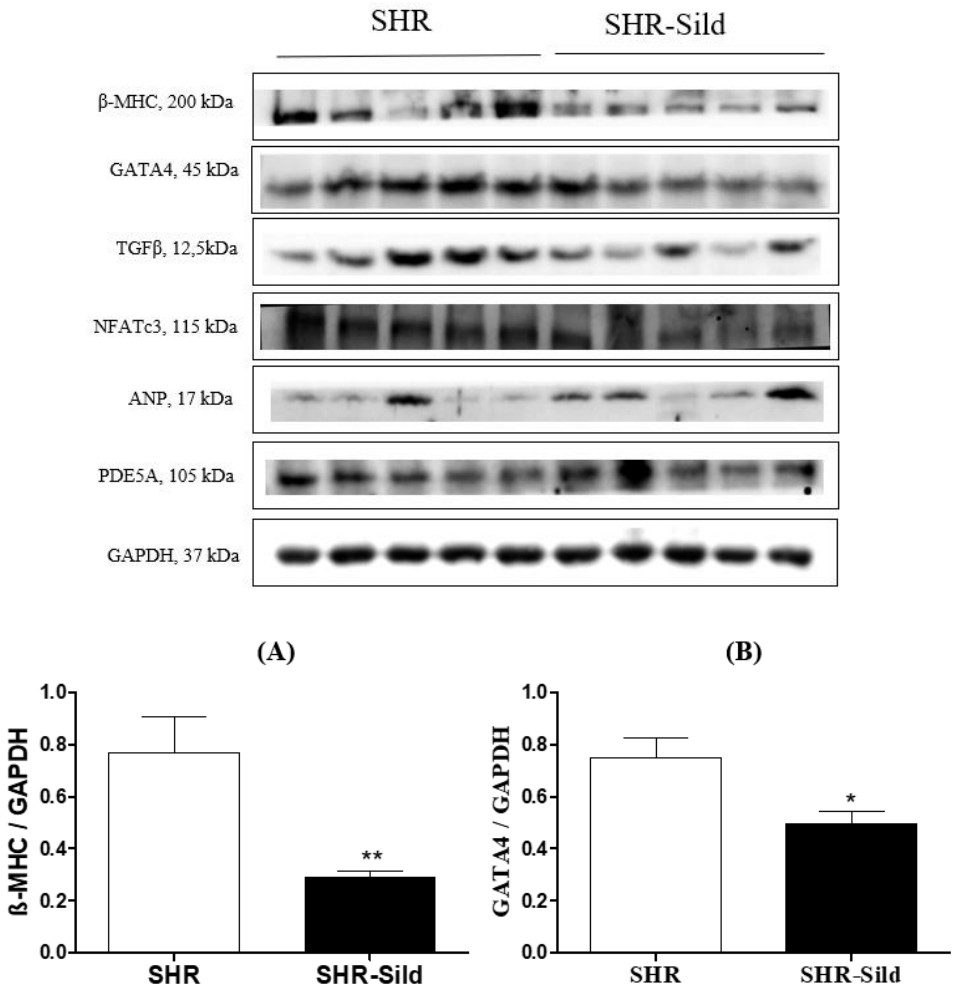


Figure 23. Comparison of left ventricular ROS production (panel A), t-SOD – total superoxide dismutase activity (panel B), and CAT – catalase activity (panel C) in SHR treated with sildenafil (SHR-Sild) and untreated SHR. Results were expressed as mean ± SEM (n = 8 rats per group). Data were analyzed by Student's *t*-test, * $P < 0.05$; *** $P < 0.001$.


SHR
SHR-Sild
β-MHC, 200 kDa
GATA4, 45 kDa
TGFβ, 12,5kDa
NFATc3, 115 kDa
ANP, 17 kDa
PDE5A, 105 kDa
GAPDH, 37 kDa
(A)
(B)
ß-MHC / GAPDH
GATA4 / GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
**
*
SHR
SHR-Sild

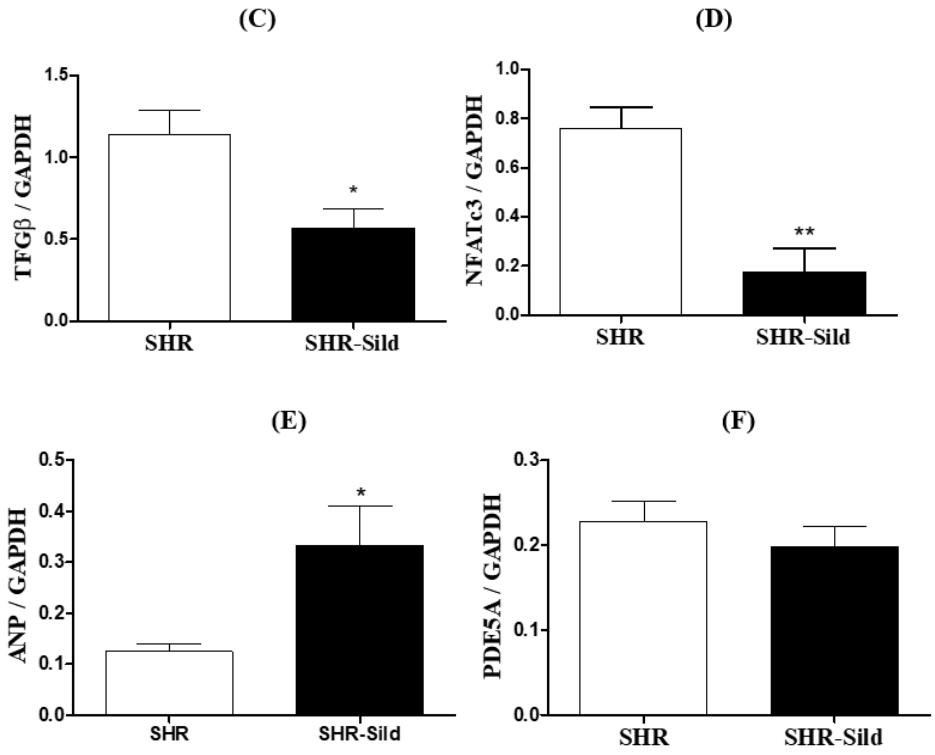


Figure 24. Cardiac hypertrophic markers. Above panel – Representative blots of β-MHC, GATA4, TGFβ, NFATc3, ANP and PDE5A in left ventricular cardiac tissue from treated animals (SHR-Sild) and untreated SHR. Below panel (A-F) – average densitometric data for β-MHC (A), GATA4 (B), TGFβ (C), NFATc3 (D), ANP (E), PDE5A (F) and expression in hearts from both experimental groups. Densitometry of proteins were normalized by GAPDH levels. Results were expressed as mean ± SEM (n = 5 rats per group). Data were analyzed by Student's *t*-test, * P < 0,05; ** P < 0.01.

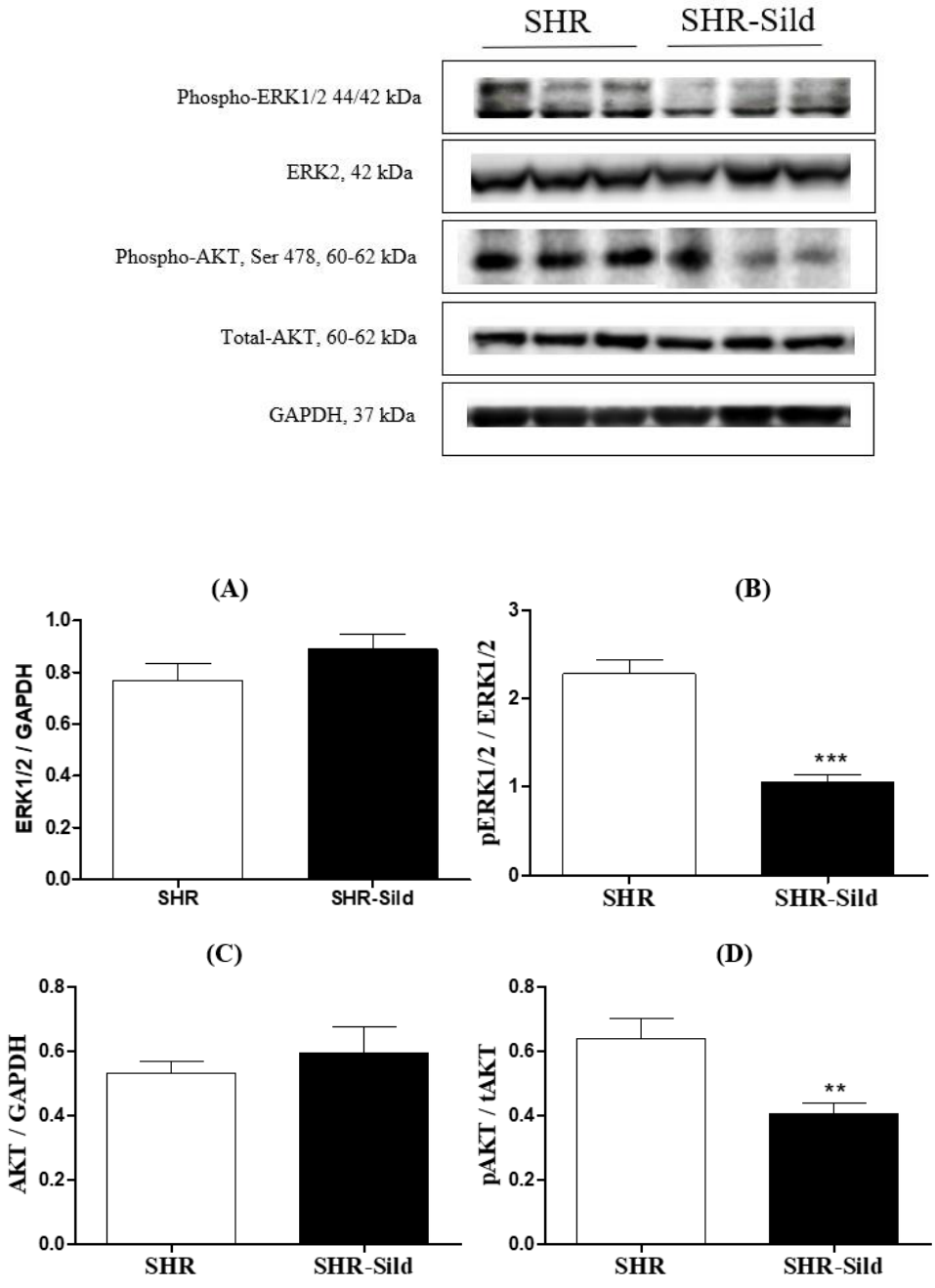


Figure 25. Cardiac hypertrophic signaling pathways. Above panel – Representative blots of phospho-ERK1/2, total-ERK2, phospho-AKT and total-AKT in left ventricular cardiac tissue from treated animals (SHR-Sild) and untreated SHR. Below panel (A-D) – average densitometric data for total-ERK2 (A), phospho-ERK1/2 (B), total-AKT (C) and phospho-AKT (D) expression in hearts from both experimental groups. Densitometry of proteins were normalized by GAPDH levels. Results were expressed as mean ± SEM (n = 5 rats per group). Data were analyzed by Student's *t*-test, ** $P < 0.01$; *** $P < 0.001$.

4 DISCUSSION

Hypertension and its cardiovascular repercussion are the major causes of death and disability worldwide **[Hernandorena et al., 2017]**. Increased activity of cGMP hydrolyzing PDE5A is associated with high blood pressure **[Taddei et al., 2006; Oliver et al., 2006; Mergia et al., 2016;]**, vascular dysfunction **[Rodrigues et al., 2013; Leal et al., 2017;]**, cardiac remodeling and heart failure **[Nagendran et al., 2007; Pokreisz et al., 2009]**. The treatment of these conditions based on the use of drugs that act on cGMP regulatory cascade, represents an important alternative of the management of hypertension and its complications. In this study, the effects of chronic PDE5Ai on cardiovascular system of SHRs was examined.

First, we investigated the effects of treatment with sildenafil during the advance of hypertension in young SHRs. This treatment did not alter resting blood pressure and HR. Nevertheless, it improved the vascular function, as evidenced by the enhanced vasorelaxation response to acetylcholine as well as the reduced contractile response to phenylephrine in aortic rings from pre-hypertensive SHRs. The absence of modulation of blood pressure and HR could be explained once that sildenafil administration did not modify vascular reactivity, mechanical and structural parameters in resistance vessels, the kind of vessels that directly contributes to the creation of the resistance to flow or impedance to ventricular ejection and regulation of blood pressure. In the pre-hypertensive protocol, the improvement of vascular function could be associated to the decreasing oxidative stress, a mechanism dependent of COX-2 and ERK1/2 in aorta from young SHR (see appendices B, page 110).

Second, the effects of chronic PDE5Ai were investigated on cardiac performance, cardiac oxidative stress, as well as cardiac hypertrophy in sildenafil-treated and untreated rats at 6 months of age. Our results reveal that treatment with sildenafil blunted cardiac β-adrenergic stimulation by ISO in SHRs, promoted cardioprotection against sustained high blood pressure by decreasing ROS production and increasing SOD activity. As was observed in pre-hypertensive protocol, treatment with PDE5Ai did not alter significantly baseline SBP, DBP, MAP and HR as compared to untreated SHR. Kristek and colleagues (2007), demonstrated through indirectly measurement of blood pressure by plethysmography, that PDE5Ai by sildenafil did not evoke significant changes on SBP in SHRs **[Kristek et al., 2007]**. To date, there is not a consensus regarding the antihypertensive effects of PDE5Ai in humans **[Oliver et al., 2006; Lewis et al., 2007; Oliver et al., 2010; Giannetta et al., 2012]** and experimental models **[Gardiner et al., 2004; Yaguas et al., 2010].**

Even in the absence of significant effects on blood pressure and HR, our data demonstrated that potentiation of cGMP/PKG pathway by sildenafil decreases cardiac oxidative stress, prevents progression of cardiac pathological hypertrophy induced by pressure overload by modulating cardiac systolic β-adrenergic signaling in SHRs. Analysis of biometric parameters in 24-week-old rats treated by sildenafil shows a significant reduction of wet heart weight/body weight ratio, as well as downregulation of cardiac hypertrophic markers, suggesting an anti-hypertrophic effect. Increase cGMP/PKG signaling pathway activation was described as a negative regulator of cardiac hypertrophy **[Zhang et al., 2011]**. In the present study, analysis of cross-sectional area showed a significant reduction of 33% of myocytes area from SHR-Sild compared to untreated rats, suggesting an antihypertrophic effect related to chronic PDE5Ai. Several authors demonstrated that PDE5Ai by sildenafil not only prevents and reverses cardiac hypertrophy, but prevents progressive chamber dilatation, as well as prevents increase of collagen deposition in the heart secondary to hypertension **[Takimoto et al., 2005; Ferreira-Melo et al., 2006; Rossoni et al., 2007; Nagayama et al., 2009].**

Increase of sympathetic nerve activity is considered an important risk factor for cardiac hypertrophy secondary to high blood pressure **[Schlaich et al., 2003; Bruno et al., 2012].** The hypertensive protocol reports the first evidence that sildenafil blunts β-adrenergic signaling in adult heart from adult SHRs. Our data demonstrated that chronic PDE5Ai counteracts systolic β-adrenergic stimulation in isolated perfused Langendorff hearts, evidenced by the significant decreased of the positive inotropic response to a β-nonselective adrenergic agonist. Positive chronotropic response to ISO was significantly attenuated in heart from SHR-Sild compared to those from untreated rats. Borlaug and coworkers reported the first evidence that sildenafil modulates cardiac contractility in health humans, by suppressing β-adrenergic systolic function induced by dobutamine. Their observations might suggest a beneficial role of PDE5Ai in disorders associated with an increase of adrenergic neurohormonal stimulation, such as hypertension and cardiac hypertrophy **[Borlaug et al., 2005]**.

Lee and colleagues demonstrated that acute PDE5Ai modulates myocyte β-adrenergic stimulation to ISO by suppressing sarcomere shortening. cGMP acts as a regulator of the activity of cAMP hydrolyzing PDEs as well as influences the intracellular concentration of cAMP, crucial in cardiac contractility response **[Lee et al., 2010]**. Augmented cGMP levels modulate RGS2/ Gαq/11 signaling which results in blunted β-adrenergic response in the heart

[Takimoto et al., 2009]. Isidori and colleagues (2015) demonstrated a receptor-specific interplay between cGMP and cAMP, via PDE5A–PDE2-mediated crosstalk in cardiomyocytes. On the contrary, their results indicate that cGMP/PKG pathway has a slight influence on the peak of response to ISO while significantly affects the constant phase of β-adrenergic response in cultured cardiomyocytes. These authors suggest that PDE5Ai sildenafil selectively depressed contraction rate stimulated by β2-, but not β1-adrenergic activation**.** These evidences reinforce the participation of PDE5A as a modulator of β-adrenergic signaling in adult heart from SHRs.

Increase of oxidative stress is associated with pathological cardiac remodeling secondary to hypertension **[Takimoto et al., 2007]**. This phenomenon could be considered a cause, a consequence, or more often a potentiating factor for hypertension, contractile dysfunction and pathological cardiac remodeling **[Takimoto et al., 2007; Maulik et al., 2012]**. In the current study, chronic sildenafil administration not only reduced left ventricular cardiac ROS production but also significantly increased SOD activity, without inducing significant effects on CAT activity. To the best of our knowledge, our data represents the first demonstration that chronic sildenafil modulates SOD activity in adult hearts from SHRs. The pre-hypertensive protocol showed that chronic PDE5Ai by sildenafil (60 days of treatment) attenuates the development of endothelial dysfunction, by modulation of vascular inflammatory activation pathways, such as COX-2 and p42/44 MAPK. Several authors showed that PDE5A expression and activity impact on oxidative stress [**Takimoto et al., 2007; Maulik et al., 2012]**. Scheweita and coworkers showed that both sildenafil and vardenafil treatments increased SOD and CAT activities in the liver of male rats **[Sheweita et al., 2015]**. PDE5Ai decreases O^{2-} generation by acting as a SOD-mimetic **[Fernandes et al., 2008]**. SOD3 gene deletion is associated with increased myocardial PDE5A expression and exacerbated pathological cardiac remodeling induced by TAC in mice **[Lu et al., 2010]**. Koka and colleagues (2010) demonstrated that chronic PDE5Ai by tadalafil, the long-acting PDE5A inhibitor, improved cardiac performance and prevented cardiomyocyte apoptosis in doxorubicin-induced cardiomyopathy by enhancing cGMP and PKG activity through up-regulation of mitochondrial MnSOD. Itani and colleagues (2017) using chick embryo hearts exposed to hypoxic conditions showed that oxidative stress results in a significant reduction of SOD protein expression, increased expression of PDE5A protein levels, reduction of NO bioavailability and dysfunction of peripheral endothelial cells. These authors reported that sildenafil treatment decreases oxidative stress but had no effects on the expression of SOD

protein levels. These observations reinforce the hypothesis of a beneficial role of P PDE5Ai as an anti-inflammatory component in cardiac dysfunction secondary to hypertension.

At the end of hypertensive protocol, β-MHC, GATA4 and NFATc3 protein levels, standard markers of hypertrophic cardiomyopathy, were reduced in the heart of sildenafil-treated rats compared to that of untreated rats. TGFβ protein levels, a negative autocrine growth factor related with fibrosis, were also downregulated in sildenafil treated SHRs compared to untreated animals. These findings may support the potential use of sildenafil as an anti-hypertrophic agent in the treatment of hypertensive hearts. Cardiac antihypertrophic effects were demonstrated through ERK1/2 pathway inhibition **[Touyz et al., 2002].** ERK protein overexpression is involved in both vasoconstriction and VSMCs proliferation, as well as cardiac hypertrophy **[Mutlak et al., 2015]** and hypertension **[Roberts et al., 2012]**. Basal levels of p42/p44 ERK are augmented in several hypertensive models of hypertension such as Dahl Salt rats **[Kim, 1997]**, DOCA-salt rats **[Kim et al., 2005]** and SHRs **[Toyz et al., 2002; Tabet et al., 2005]**. In the pre-hypertensive protocol, we showed that antihypertrophic effect by sildenafil is associated with decrease of phosphorylation of p42/p44 ERK in both aorta and left ventricular cardiac mass of young SHR. In the hypertensive protocol, chronic PDE5Ai also downregulate ERK pathway, which is activated in cardiac hypertrophy.

GATA4, a classic cardiac hypertrophic marker, once activated induces cardiac hypertrophy by phosphorylation at Serine 105; this activity could be directly modulated by ERK1/2 and p38 in cardiomyocytes **[Liang et al., 2001]**. In the present study, both ERK1/2 and GATA4 protein expression levels were downregulated after sildenafil administration, suggesting an antihypertrophic effect in the hearts from SHR treated compared to untreated rats. Furthermore, the serine/threonine protein kinase AKT and PI3-kinase are important modulators of cardiac cell proliferation, growth and survival. The long-term activation of AKT promotes pathological hypertrophy and heart failure **[Chaanine et al., 2011]**. Left ventricular cardiac tissue from adult sildenafil-treated SHR showed a significant reduction of AKT1 (Ser473) phosphorylation when compared to untreated rats. Taken together, these results (ERK1/2, GATA4 and AKT downregulation) suggest that chronic PDE5Ai by sildenafil, exerts an important antihypertrophic effect in the heart against pressure overload in hypertension.

At 6 months of age, ANP protein levels were increased in left ventricular mass from sildenafil-treated rats compared to untreated group. As described, NPs usually bind to their receptors and subsequently activates pGC to generate cGMP, thereby activating PKG and

subsequently promoting antihypertrophic effects **[Nakamura et al., 2018]**. Until this data there is not a consensus about the role of natriuretic peptides in cardiac contractility. Calderone and colleagues (1998) showed that ANP and NO attenuate the effects of adrenergic stimulation by norepinephrine on the growth of cardiac myocytes and fibroblasts via a cGMP-mediated inhibition norepinephrine-stimulated Ca^{2+} influx. Exogenous administration of both ANP and ANP antagonist (HS-142–1) confirms the idea that ANP has antihypertrophic, antifibrotic effect, as well as attenuate the growth response to adrenergic phenylephrine-stimulation **[Horio et al., 2000].** Knowles and coworkers **[Knowles et al, 2001]** demonstrated that mice lacking NPR-type A exposed to a pressure overload induced by TAC have marked cardiac hypertrophy when compared to NPR-type $A^{+/+}$ mice. Interestingly, this effect is not directly dependent of regulation of blood pressure by ANP. These studies support the idea that increased ANP protein expression after chronic Sild administration could be associated with cardioprotection in our model.

Overexpression of myocardial ANP protein levels has been reported, in congestive hypertrophic failure hearts of humans and experimental models **[Lu et al., 2010]**. It is noteworthy that heart failure does not occurs at 6 months of age in SHRs but only at 18-24 months of age **[Boluyt et al., 1995]**. This observation suggests that overexpression of cardiac ventricular ANP protein levels might be associated with cardioprotection in SHRs who received chronic sildenafil treatment. Therefore, ANP/pGC/cGMP/PKG signaling may be related to the attenuation of a non-selective β-adrenergic agonist stimulation as well as an antihypertrophic effect in the heart from SHR-Sild group compared to untreated rats.

The present work has some limitations. The first one was the non-quantification of cardiac tissue cGMP levels after PDE5Ai sildenafil administration. Second, ROS, CAT and SOD assays were performed using whole left ventricular cardiac tissue, which make their correlation with cardiomyocyte remodeling of some weakness. In this case, further experiments are needed to reinforce this correlation in isolated cardiac cells. To provide more insight into the mechanisms by which sildenafil induces cardiac antihypertrophic effects, by decreasing oxidative stress, measurements of expression and/or activity of ROS-generating enzymes would be necessary (e.g. NAPDH oxidase system). However, additional experiments must to be performed to understand if this phenomenon is associated with desensitization of cardiac a β-adrenoreceptors, differences in β1/β2-adrenoreceptor density and selectivity as well as the impact of PDE5Ai on Ca^{2+} signaling (e.g. calcineurin-NFAT pathway) in this strain.

This doctoral thesis reports the first direct evidence that chronic PDE5Ai improves vascular function during the development of hypertension in SHR, as well as blunts cardiac β-adrenergic stimulation induced by ISO in adult SHR, which results in antihypertrophic effects against cardiac pressure overload secondary to hypertension. Both, vascular function improvement and cardioprotection in our model, are involved with the decrease of oxidative stress after sildenafil administration. Additional experiments must to be carry out to give more insights about how sildenafil decreases oxidative stress and sympathetic neural activity signaling in models of essential hypertension.

5 MATERIAL AND METHODS

5.1 ANIMALS

Male SHR were obtained from the animal facility of the Universidade Federal de Pernambuco (UFPE). Animals were housed in individually ventilated cages (425 mm x 266 mm x 185 mm; Tecniplast, Buguggiate, Italy), three animals were hosted per cage. Rats were maintained in a climate-controlled room within the range of 22-24°C, 60% of humidity and the room had a 12h-12h light-dark cycle. All experimental protocols were performed in accordance with the Ethical Principles in Animal Research set forth by US National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications, 2011), and approved by the Institutional Animal Care and Use Committee (reference number – CEUA, nº 0046/2016).

5.2 EXPERIMENTAL DESIGN

The impact of pharmacological PDE5Ai by sildenafil was investigated in a model with essential hypertension during the advance of hypertension in young SHR (pre-hypertensive protocol). Furthermore, the impact of sildenafil treatment on established hypertension (hypertensive protocol) was also investigated.

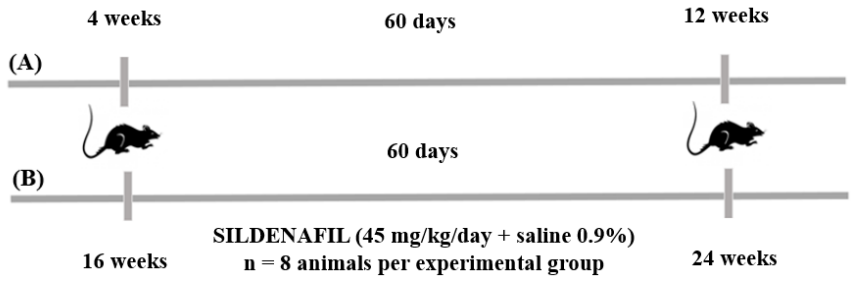


Figure 26. Schematic experimental design

5.2.1 Pre-hypertensive protocol (4-week-old until 12-week-old rats, Figure 26A)

Male SHR (n = 8 per group) were chronic treated with sildenafil (45 mg/kg/day) **[Walker, 1999]**. Chronic PDE5Ai was started in the pre-hypertensive period (4 weeks-old), until 12 weeks of age when the establishment of hypertension occurs in this strain. According to the treatment, rats were divided into two experimental groups –

i. SHR, which were treated with vehicle (NaCl, 0.9%);
ii. SHR-Sild, which were chronic treated with sildenafil.

Specifically, in this protocol was investigated the impact of PDE5Ai on vascular reactivity of resistance arteries, as well as structural properties of these vessels after sildenafil administration. Data and methods from vascular reactivity of aorta are present in the final section of this thesis (see appendices B, pag. 110).

5.2.2 Hypertensive protocol (16-week-old until 20-week-old rats, Figure 26B)

16-Week-old male SHR (n=8 per group) were orally treated by gavage with sildenafil, a PDE5Ai, sildenafil (45 mg/kg/day). Untreated animals received only vehicle (saline, 0.9%). According to the treatment rats were divided into two experimental groups –

i. SHR, which were treated with vehicle;
ii. SHR-Sild, which were chronic treated with sildenafil.

After 8 weeks of treatment, animals were intraperitoneally (i.p.) anesthetized by sodium pentobarbital (SPB, 50 mg/kg) and euthanized. The organs of interest were surgically removed, cleaned of connective tissues, dried and accurately weighed. Wet tissue weights were measured and normalized to the body weight. After 60 days of treatment, was investigated the impact of PDE5Ai was investigated in adult SHR hearts.

5.3 STRUCTURAL AND MECHANICAL STUDY OF MESENTERIC RESISTANCE ARTERIES UNDER ACTIVE (+0 CA^{2+}) AND PASSIVE CONDITIONS (0CA^{2+}) ON THE PRESSURE MYOGRAPH

5.3.1 Pressurized arteries (pressure myograph)

Pressure myograph was used to study '*in vitro*', both structural and mechanical parameters of vascular segments under isobaric conditions, as described by Duling and colleagues (1981). In this protocol, the third-order branch of superior mesenteric arteries arcade were dissected and cut into segments (~2 mm) and mounted on a pressure myograph (Danish Myo Tech, Model P100, J.P.Trading I / S, Aarhus, Denmark). The two extremities of the arteries were cannulated with glass micropipettes and tied with nylon suture wire (Figure 2). Arterial length was adjusted by increasing of intraluminal pressure to approximately 140 mmHg. In this condition the vascular walls were disposed parallelly and without stretching. Subsequently, the pressure of the system was adjusted to 70 mmHg and the arteries were maintained in Krebs-Henseleit Solution (KHS), continuously bubbled with a mixture of O_2 (95%) and CO_2 (5%), at 37ºC, for 60 minutes (min). After the stabilization period, the intraluminal pressure

was reduced to 3 mmHg and was started a curve of pressure, from 3 to 140 mmHg, with increments of 20 mmHg every 5 min. All protocols were performed in the presence (Ca^{2+}) and absence of calcium ($0Ca^{2+}$). The KHS-$0Ca^{2+}$ solution was prepared by omitting $CaCl_2$ and adding 10 mM of ethylene glycol-bis(2-aminoethylether)-N, N, N', N'-tetra acetic acid (EGTA). For each intraluminal pressure value, at the end of the 5 min, the internal diameter (D_i) and external diameter (D_e) were measured. Both, D_i and D_e measurements, were used to determinate the structural and parameters of mesenteric arteries under passive conditions, i.e. KHS without Ca^{2+} (KHS-$0Ca^{2+}$).

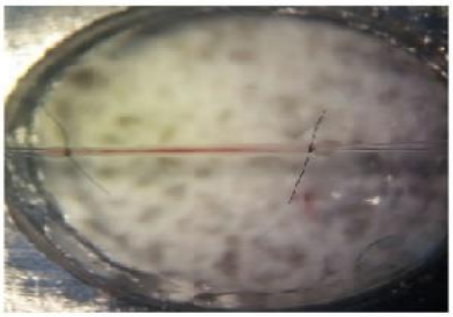


Figure 27. Representative image of pressurized arteries.

5.3.2 Equations for structural analysis

The following equations describe how was calculated vascular structural parameters and how was obtained information about arterial vascular remodeling –

$$\mathrm{WT} = \frac{(D_e - D_i)}{2} \qquad \mathrm{CSA} = \frac{\pi}{4} \cdot \left(D_e^2 - D_i^2\right) \qquad \mathrm{W:L} = \frac{(D_e - D_i)}{2 \cdot D_i}$$

Legend – WT – wall thickness; CSA – cross-sectional area; (W – L) – wall-lumen ratio; D_e – external diameter; D_i – internal diameter.

5.4 VASCULAR REACTIVITY STUDY

At 3 months of age, animals were anesthetized with SPB and euthanized by exsanguination. The descending third-order branch of the mesenteric arteries arcade was carefully excised and cleaned of fat and connective tissues, dissected and cut into segments (~3 mm length each one) and placed in KHS of the following composition (in mM) – [115 NaCl, 2.5 $CaCl_2$, 4.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4.7H_2O$, 25 $NaHCO_3$, 11 glucose and 0.03 EDTA (Sigma, St. Louis, MO, USA)]. Segments of mesenteric resistance arteries were mounted in myographs

chambers (Danish Myo Technology A/S, Aarhus, Denmark) to measure isometric tension as described by Mulvany and Halpern (1977).

Figure 28. Representative images of mesenteric arteries used to vascular reactivity study.

5.4.1 Experimental protocols

After 60-min of equilibration period, mesenteric arteries rings were exposed to 120 mmol/L KCl, to check their functional integrity and to assess the maximum contractility. After a washout period of 30 min, cumulative concentration-response curves to acetylcholine (00.1 nM to 100 µM, Sigma, St. Louis, MO, USA) were performed in vessels pre-contracted with norepinephrine, at a concentration that produced approximately 50-70% of the contraction induced by KCl. After a washout period of 60 min, the contractile responses to norepinephrine (10 nM to 30 µM) were evaluated. Furthermore, mesenteric vascular responsivity to norepinephrine was analyzed in the presence of a nonselective NO synthase inhibitor L-NAME (100 µM, Sigma, St. Louis, MO, USA), a specific inhibitor of soluble guanylate cyclase (sGC), [1H- [1,2,4] oxadiazolo- [4,3-a]quinoxalin-1-one] (ODQ, 0.1 µM, Tocris®, USA), and a non-selective COXs inhibitor, indomethacin (1 µM, Sigma, St. Louis, MO, USA). All drugs were added 30 min before performing the concentration-response curve.

5.5 ARTERIAL BLOOD PRESSURE MEASUREMENT

SHR were anesthetized with SPB (50 mg/kg, i.p.) and submitted to catheterization procedure [Alves-Santos, 2016]. A polyethylene (PE) catheter [PE10 coupled to PE50, filled with heparin (125 UI/mL)] was inserted into the abdominal aorta via the left femoral artery (Figure 5). Postoperatively, animals were kept in individual cages and treated with Penicillin (1.200.000 UI). After 24 hours, SBP, DBP, MAP and HR were measured for 60 min in conscious, freely moving rats. Subsequently, arterial catheter was connected to a blood pressure transducer (MLT0380; ADInstruments Pty Ltd, Castle Hill, New South Wales,

Australia) connected to a signal amplifier (Bridge Amp, ML224; ADInstruments, Australia) and an acquisition system (PowerLab 4/30, ML866; ADInstruments, Australia).

5.6 ISOLATED PERFUSED HEART STUDY (LANGENDORFF)

The method for isolating the beating heart '*ex vivo*' was firstly described by Langendorff [Langendorff, 1895]. Animals were anesthetized with SPB (50 mg/kg, i.p.) and the hearts were rapidly excised and submerged in ice-cold modified KHS before being fixed through the aorta in Langendorff apparatus, under a constant pressure of 80 mmHg. Perfusion was carried out using KHS, of the following composition (in mM) – [NaCl, 129, $NaHCO_3$, 21, KH_2PO_4, 1.2, KCl, 5.6, $MgCl_2$.6 H_2O, 1.25, $CaCl_2.2H_2O$, 1.25]. Glucose (10 mM) as substrate and sodium pyruvate (2 mM) as co-substrate were added to preserve the myocardium performance. Perfusing solution was gassed with a mixture of 95% O_2 + 5% CO_2, pH 7.4, the temperature was maintained at 37 °C. A collapsed latex balloon was introduced in the left ventricular cavity via an incision in the left atrium.

The balloon was filled with distilled water, and its volume was adjusted to obtain an left ventricle end-diastolic pressure (LVEDP) between 5- and 10-mm Hg. This value was kept constant thought the experiment. The balloon was connected to a transducer coupled to a signal amplifier. Data were transmitted to a data acquisition program (Power Lab model ML870 8/30, ADInstruments Pty Ltd, Castle Hill, New South Wales, Australia).

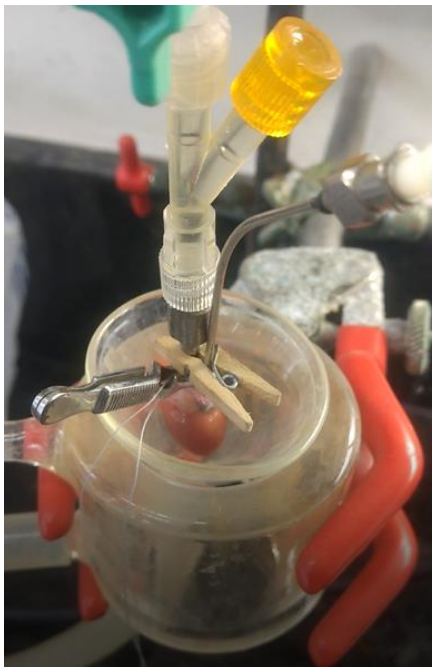


Figure 29. Demonstrative isolated perfused heart preparation

5.6.1 Experimental protocol of isolated perfused heart study

After 30-min of equilibration period, basal left ventricular systolic pressure and HR were measured. In order to evaluate the impact of sildenafil treatment on cardiac function, inotropic and chronotropic responses were evaluated by adding increasing concentrations of ISO (10^{-13} to 10^{-3}M). The system allowed to record and analyze multiple cardiac parameters such as – developed pressure – maximal systolic pressure and minimal diastolic pressure (mmHg); ii) HR (beats/min); iii) maximum rate of pressure development, max dP/dt (mmHg/s) and iv) maximum rate of relaxation, min dP/dT (mmHg/s).

5.7 HISTOLOGICAL ANALYSIS

Once removed from rats, the hearts were transversely sectioned into equal parts. The apex was fixed with 10% neutral buffered formalin for 24 hours. Subsequently, the samples were dehydrated in ethyl alcohol at increasing concentrations, diaphanized with xylene, and embedded in paraffin (Sigma® 327212). For each tissue sample, were made sections of 5 μm and then processed for Masson Trichrome Staining (Thermo Scientific 879019). To start, the slides were placed in a heater, 56° C for 15 min. Paraffin was dissolved in xylene and rehydrated through a graded series of alcohol and placed in distilled water. For Masson Trichrome Staining, slides were placed in a fixative Boiun's solution for 1 hour at 56ºC and rinsed in running tap water for 5 min until yellow color is removed. Subsequently, the slides were rinsed in a nuclear staining, Weigert's solution, and bathed in running tap water for 2 min. Slides were rinsed in Biebrich Scarlet/Acid Fuchsin Solution 10 min and rinsed in distilled water for 1 minute. After that, the slides were rinsed in Phosphomolybdic / Phosphotungstic Acid Solution for 10 min, followed by Aniline Blue Solution for 5 min and quickly in distilled water. Finally, samples were rinsed in Acetic Acid Solution (1%) for 3 min, after which the sections were dehydrated very quickly in increasing ethanol solutions to xylene and they mounted in Eukitt medium. Sections were randomly selected and analyzed in blind. Collagen levels were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

5.8 IMMUNOFLUORESCENCE ANALYSIS

For analysis of myocyte cross sectional area was used the protocol of WGA staining. To start, adult hearts sections (5 μm) were deparaffinized, rehydrated, and heated in 10 mM Antigen Retrieval Sodium Citrate Solution, pH = 6.0 for 10 min. After 3 washes in phosphatase-buffered saline (PBS), for 5 min, samples were incubated with 5 μg/mL of WGA Alexa Fluor 594 Conjugate (Thermo Fisher W11262) in PBS for 10 min at room temperature, protected

from light. Following extensive washes in PBS, the sections were counterstained with Hoechst 33258 (Thermo Fisher H3569) and mounted with PBS - 50% Glycerol v/v. Immunofluorescence images were acquired by Nikon Eclipse Ti-S microscope. Sections were randomly selected and analyzed in blind. myocyte cross sectional area analysis was performed on WGA positive cells using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

5.9 CARDIAC OXIDATIVE STRESS ANALYSES

Left ventricular cardiac tissues were homogenized using a Tris-base (50 mM) containing EDTA (1 mM) buffer, with protease inhibitors (PMSF and OTV, 1 mg/mL). Samples were centrifuged at 1000 g, 10 min at 4°C.

5.9.1 Cardiac reactive oxygen species quantification

The relative levels of ROS were measured through a fluorometric reaction between left ventricular cardiac supernatant samples and the 2',7'-dichlorofluorescein diacetate (H_2DCF-DA) reagent (50 µL) in black polystyrene 96-well plate incubated for 45 min at 37ºC. The final product of this reaction is a highly fluorescent compost DCF in presence of ROS [Nijmeh, 2010]. Blank readings were subtracted from loaded sample readings and fluorescence was measured at λ-excitation = 504 nm and λ-emission = 522 nm. Values were reported as fluorescence units per milligram of cardiac tissue (FU/mg).

5.9.2 Total superoxide dismutase activity

t-SOD was performed according to the methodology proposed by Misra and Fridovich **[Misra & Fridovich, 1972]**. Triplicates of left ventricular cardiac supernatants from treated and untreated animals were incubated at 37 ºC, followed by addition of sodium carbonate buffer (0.05%, pH=10.2) in 0,1 mM EDTA. The reaction was started by adding epinephrine (150 mM) diluted in acetic acid (0.05%). Absorbance changes were evaluated at 240nm every 15 seconds for 3 min. t-SOD values were expressed as units per mg of protein (U/mg).

5.9.3 Cardiac catalase activity

CAT activity was performed according to Aebi **[Aebi, 1984]**. CAT activity was measured through the reaction between left ventricular cardiac supernatants from treated and untreated

animals, sodium phosphate buffer (pH = 7.0) and hydrogen peroxide solution (300 mM). Absorbance were measured at 240 nm in defined intervals of 10 s for 3 min. CAT levels were expressed as units per mg of protein (U/mg).

5.10 WESTERN BLOT ANALYSIS

Left ventricle cardiac mass samples were homogenized using an extraction buffer (pH = 7.4) composed by KCl (3 mmol/L), HEPES (1 mmol/L, Sigma, St. Louis, MO, USA), $MgCl_2$ (1 mmol/L, Sigma, St. Louis, MO, USA), EDTA (0.5 mmol/L, Sigma, St. Louis, MO, USA), 1,4-dithiothreitol (DTT, 1 mmol/L, Bio-Rad, Hercules, CA, USA), glycerol 10% (Sigma, St. Louis, MO, USA) and sodium dodecyl sulfate (SDS, 10%, Sigma, St. Louis, MO, USA), in the presence of protease inhibitors such as – sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), pepstatin, aprotinin and leupeptin, all at 1 mg/mL (Sigma, St. Louis, MO, USA). Cardiac samples were centrifuged for 10 min at 10000 g, at 0°C. Protein amount was measured according to the method described by Bradford **[Bradford, 1976]**.

Cardiac supernatant samples (30 μg of protein) were run on SDS-PAGE (SDS - Polyacrylamide Gel Electrophoresis), transferred to polyvinylidene fluoride membranes (GE HealthCare, Little Chalfont-Buckinghamshire, UK) using the Mini Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). Membranes were previously blocked with of nonfat dry milk (5%, Sigma, St. Louis, MO, USA) dissolved in Tris-buffered saline Tween 20 (TBST, 0.1%) (0.05 M Tris, 0.15M NaCl, pH 7.5 and 1% Tween-20). Subsequently the membranes were incubated overnight in 4ºC on a shaker, with anti β-myosin (1 – 200, Sigma, St. Louis, MO, USA), anti-transcription factor GATA4 (1 – 500, GTX113194, GeneTex), anti-atrial natriuretic peptide (ANP, 1 – 200, Santa Cruz Biotechnology, Santa Cruz, CA), anti-transforming growth factor beta (TGFβ, 1 – 500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-nuclear factor activated T-cells (1 – 500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphodiesterase 5A, 1 – 1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-Akt 1/2/3 Ser 473-R (1 – 500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Akt 1/2/3 (1 – 1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-ERK p44/42 (1 – 1000, Cell Signaling Technology, Danvers, MA), anti-ERK2 (1 – 1000, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1 – 1000, Santa Cruz Biotechnology, Santa Cruz, CA) as a housekeeping. Membranes were washed three times with TBST 0.1% and incubated with specifics peroxidase-conjugated secondary antibodies (GE HealthCare, Little Chalfont-Buckinghamshire, UK). The resolved bands were scanned using a ChemiDoc MP System

Software and quantified using Image Lab Software, version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

5.11 STATISTICAL ANALYSIS

All data were expressed as mean ± SEM. and were analyzed using unpaired Student's t-test, one way or two-way ANOVA. When ANOVA test showed a significant treatment effect, Bonferroni's *post hoc* test was used to compare individual means (GraphPad Prism Software, San Diego, CA, USA). Differences were considered statistically significant at $P < 0.05$.

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APPENDICE A: FUNDINGS

This study was financed by the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior”, Brazil (CAPES, Finance code 001, Grant number: 88881.068093/214-01) and the “Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco”. Brazil (FACEPE, grant number: 16/2012 APQ-1133-2.07/12) and by FIRB Grant to Fabio Naro, University of Roma, Italy (grant number – FIRB2010RBAP109BLT_003).

This thesis is a result of an international cooperation between the ‘Laboratório de Fisiologia e Farmacologia Cardiovascular e Laboratório de Reatividade Vascular’ - Universidade Federal de Pernambuco - Brazil and the ‘Laboratorio Meccanismi Regolativi della Morfogenisi Normale e Patologica and Locomotor’ - University of Rome, 'La Sapienza' – Italy.

APPENDICE B: ARTICLE PUBLISHED IN LIFE SCIENCES

Teixeira-da-Silva, J. J., Nunes-Moreira, H. S., Silva, C. O., Lahlou, S., Naro, F., Xavier, F. E., & Duarte, G. P. (2019). Chronic administration of sildenafil improves endothelial function in spontaneously hypertensive rats by decreasing COX-2 expression and oxidative stress. Life Sciences, 225. https://doi.org/10.1016/j.lfs.2019.03.074

Life Sciences 225 (2019) 29–38

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Chronic administration of sildenafil improves endothelial function in spontaneously hypertensive rats by decreasing COX-2 expression and oxidative stress

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ARTICLE INFO

Keywords:
Endothelial dysfunction
Hypertension
Phosphodiesterase
Sildenafil
Vasorelaxation

ABSTRACT

Aims: Spontaneously hypertensive rats (SHR) exhibit impaired endothelial vasodilation and enhanced vasoconstriction. The phosphodiesterase 5 (PDE5) inhibitor sildenafil (Sild) potentiates the nitric oxide (NO)-mediated effects exerting antioxidative and anti-inflammatory actions. In the present study, we hypothesized that Sild could improve endothelial function in SHR.

Materials and methods: Male rats were treated daily for 60 days by oral gavage with Sild (45 mg/kg) before the onset of the hypertensive state (pre-hypertensive protocol). The aortic relaxation to acetylcholine (ACh), sodium nitroprusside (SNP) and the phenylephrine (Phe)-induced contraction was evaluated in SHR. Protein expression of eNOS, p-eNOS, caveolin, COX-1, COX-2, ERK and p-ERK was measured by Western blot.

Key findings: Resting blood pressure was not modified by Sild administration. Treatment with Sild did not alter the relaxation response to SNP but improved the ACh-induced relaxation and reduced Phe-induced contraction in aortic rings from SHR. This protective effect of Sild could be attributed to reduced superoxide anions (O_2^-) generation, cyclooxygenase type 2 (COX-2) protein downregulation and increased NO bioavailability.

Significance: Sild improves endothelial function in SHR aorta without affecting resting blood pressure values. These results indicate that PDE5 inhibition has a potential role in the improvement of vascular function and could be an adjuvant in the treatment of essential hypertension.

1. Introduction

Hypertension is commonly associated with structural and functional vascular abnormalities. These include increase in arterial stiffness, wall to lumen ratio, vasoconstrictor responses and endothelial dysfunction [1], which themselves are associated with highest cardiovascular risk. Endothelial dysfunction results from impairment of nitric oxide (NO) bioavailability. In hypertension, NO deficiency is a multifactorial process involving the decrease of NO production and the increase of NO degradation by reactive oxygen species (ROS) [2]. The intracellular effects of NO are mediated by generation of cyclic 3′5′-guanosine monophosphate (cGMP) and its increased breakdown is associated with the development of endothelial dysfunction [3].

Phosphodiesterase 5 (PDE5) is one of the responsible factors for selective degradation of cGMP levels in various tissues. Its activity modulates the intensity and duration of cGMP mediated intracellular signal [4,5]. Increased activity of cGMP-hydrolyzing PDE5 is associated with arterial hypertension [6,7].

At vascular level, PDE5 inhibitors potentiate the NO-mediated effects by increasing cGMP levels leading to activation of protein kinase G (PKG) [3,4,7]. In addition to its vasodilatory effect related to increased cGMP/PKG signaling, sildenafil (Sild) presents antioxidative and antigenotoxic activity [8], increases the number and function of endothelial progenitor cells [9] and decreases lipid deposition in conductance arteries [8]. In a recent study, Leal et al. [10] demonstrated that chronic Sild administration to atherosclerotic (apoE$^{-/-}$) mice decreases pro-inflammatory cytokines and O_2^- production and antagonizing the vascular dysfunction induced by COX-derived thromboxane A_2 (TxA_2). The

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https://doi.org/10.1016/j.lfs.2019.03.074
Received 5 January 2019; Received in revised form 20 March 2019; Accepted 29 March 2019
Available online 30 March 2019

anti-inflammatory mechanisms proposed for Sild comprise interference with mitogen-activated protein kinase (MAPK) activation and/or with downstream NF-κB transactivation [11].

Oxidative stress and inflammation are a key feature in the initiation, progression and clinical implications of hypertension-associated vascular disorders [2]. Increased pro-inflammatory cytokines levels, vascular COX-2 expression and ROS generation, together with reduced NO bioavailability are well-established characteristics of hypertension [12]. In hypertensive rats, COX-2-dependent prostanoids stimulates NADPH oxidase and ROS generation, which in turn activate COX-2 in a circuitous relationship. These events stimulate the production of vasoconstrictor prostanoids, such as prostaglandin E_2 (PGE_2) and TxA_2, reducing the endothelium-dependent relaxation and increasing vascular smooth muscle contraction [12]. O_2^- upregulates PDE5 protein expression leading to decreased cGMP levels and further impairment in NO-mediated vasodilation [13]. All together, these vascular alterations participate in hypertension development. Therefore, pharmacological strategies are needed to interrupt this vicious cycle cascade by reducing ROS generation, inflammation and/or increasing NO activity, to ameliorate the cardiovascular damage associated to the hypertension.

Among the several experimental models used to investigate the impact of PDE5 inhibition in hypertensive conditions [14,15], the most commonly used animal model of essential or primary hypertension is the spontaneously hypertensive rat (SHR) [16,17]. Functional and structural mechanisms that result from increased blood pressure in SHR begin at 5 weeks of age. Blood pressure steadily increases to reach a systolic arterial pressure (SAP) value of ~180–200 mm Hg [17]. However, the long-term effects of treatment with the PDE5 inhibitor Sild during the advance of hypertension in SHR remain controversial. Moreover, to our knowledge, no study has yet investigated the effects of chronic Sild treatment on endothelial dysfunction which occurs in young SHR. Thus, the present study investigated how chronic PDE5 inhibition affects the resting blood pressure and the functional vascular properties in SHR as well as the role of NO and COX pathways in the vascular effects exerted by Sild treatment.

2. Materials and methods

2.1. Animals

Animals were obtained from the animal facility of the Federal University of Pernambuco (UFPE). Rats were housed in individually ventilated cages (425 mm × 266 mm × 185 mm; Tecniplast, Buguggiate, Italy), three animals were hosted in each cage. The room had a 12 h–12 h light-dark cycle, 60% of humidity and the temperature was regulated within the range of 22–24 °C. All experimental protocols were performed in accordance with the Ethical Principles in Animal Research set forth by US National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications, 2011), and all the experimental procedure was approved by the Institutional Animal Care and Use Committee (approval reference number: 0046/2016).

2.2. Experimental design

Male SHR (n = 8 per group) were treated orally by gavage with Sild (45 mg/kg/day) [18]. Chronic PDE5 inhibition was started in the pre-hypertensive period (4 weeks-old) until 12 weeks of age when the establishment of hypertension occurs in these animals. According to the treatment, rats were divided into two experimental groups: SHR, which were treated with vehicle (saline, 0.9%) and SHR-Sild, which were treated with the PDE5 inhibitor Sild. Body weight as well as water and food intake were weekly evaluated. After 8 weeks of treatment, animals were anesthetized with sodium pentobarbital (50 mg/kg) and euthanized.

2.3. Arterial blood pressure measurement

Twelve-week-old SHRs were anesthetized with intraperitoneally (i.p.) injected sodium pentobarbital (50 mg/kg) and submitted to catheterization procedure [19]. A polyethylene (PE) catheter [PE10 coupled to PE50, filled with heparin (125 UI·mL^{-1})] was inserted into the abdominal aorta via the left femoral artery. Postoperatively, animals were kept in individual cages and treated with Penicillin (1.200.000 UI). Twenty-hours later, SAP, diastolic arterial pressure (DAP), mean arterial pressure (MAP) and heart rate (HR) were measured for 60 min in conscious, freely moving rats. For this purpose, the arterial catheter was connected to a blood pressure transducer (MLT0380; ADInstruments Pty Ltd., Castle Hill, New South Wales, Australia) connected to an amplifier (Bridge Amp, ML224; ADInstruments, Australia) and an acquisition system (PowerLab 4/30, ML866; ADInstruments, Australia).

2.4. Vascular reactivity study

Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and euthanized by exsanguination. The descending thoracic aorta was carefully excised and cleaned of fat and connective tissues. Segments of aorta (~3 mm length each one) were placed in cold (4 °C) Krebs-Henseleit Solution (KHS) of the following composition (in mmol/L): 115 NaCl, 2.5 $CaCl_2$, 4.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4{\cdot}7H_2O$, 25 $NaHCO_3$, 11 glucose and 0.03 EDTA (Sigma, St. Louis, MO, USA). The aortic rings were mounted on stainless steel triangles, placed and suspended in vertical organ baths containing warmed (37 °C) KHS (5 mL), continuously bubbled with a mixture of O_2 (95%) and CO_2 (5%), under a resting tension of 1 g for 60 min. Isometric tension was recorded using an isometric force transducer (Letica TRI 201, Panlab, S.L., Barcelona, Spain) connected to an acquisition system (PowerLab 8/35, ML870/P ADInstruments, Australia).

2.4.1. Experimental protocols

After 60-min of equilibration period, aortic rings were challenged twice with 75 mmol/L KCl to check their functional integrity and to assess the maximum contractility. After a washout period of 30 min, cumulative concentration-response curves to ACh (0.1 nmol/L to 100 μmol/L; Sigma, St. Louis, MO, USA) were performed in vessels pre-contracted with Phe, (1 μmol/L; Sigma, St. Louis, MO, USA). After a washout period of 60 min, the contractile responses to Phe (0.1 nmol/L to 100 μmol/L) were evaluated. Furthermore, aortic responsivity to Phe was assessed in the presence of a nonselective NO synthase inhibitor N-nitro-L-arginine methyl ester, (L-NAME, 100 μmol/L, Sigma, St. Louis, MO, USA), a specific inhibitor of soluble guanylate cyclase (sGC), [1H-[1,2,4] oxadiazolo- [4,3-a]quinoxalin-1-one] (ODQ; 0.1 μmol/L, Tocris®, USA), or a non-selective COXs inhibitor, indomethacin (10 μmol/L; Sigma, St. Louis, MO, USA). All drugs were added 30 min before generating the concentration-response curve. Endothelium-independent relaxation was evaluated by adding cumulative concentrations of the NO donor, sodium nitroprusside (0.1 nmol/L to 100 μmol/L; Sigma, St. Louis, MO, USA) in aortic rings precontracted with Phe (1 μmol/L). In some experiments, vascular endothelial was removed by gently rubbing the intimal surface with a stainless-steel rod. The effectiveness of removal of the endothelium was confirmed by the absence of relaxation in response to ACh (1 μmol/L) in pre-contracted rings.

2.5. Western blot analysis

Aorta and heart were homogenized using an extraction buffer (pH = 7.4) composed by KCl (3 mmol/L), HEPES (1 mmol/L; Sigma, St. Louis, MO, USA), $MgCl_2$ (1 mmol/L; Sigma, St. Louis, MO, USA), EDTA (0.5 mmol/L; Sigma, St. Louis, MO, USA), 1,4-dithiothreitol (DTT; 1 mmol/L, Bio-Rad, Hercules, CA, USA), glycerol 10% (Sigma, St. Louis,

MO, USA) and sodium dodecyl sulfate (SDS; 10%, Sigma, St. Louis, MO, USA), in the presence of protease inhibitors such as: sodium orthovanadate (OTV), phenylmethylsulfonyl fluoride (PMSF), pepstatin, aprotinin and leupeptin, all at 1 mg/mL (Sigma, St. Louis, MO, USA). Samples were centrifuged for 10 min at 10,000*g*, (0 °C). Protein amount was measured according to the methodology described by Bradford [20].

Supernatant samples (25 μg of protein) were run on 10% SDS-PAGE (SDS- Polyacrylamide Gel Electrophoresis), transferred to polyvinylidene fluoride (PVDF) membranes (GE HealthCare, Little Chalfont-Buckinghamshire, UK) using the Mini Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). Membranes were previously blocked with of nonfat dry milk (5%, Sigma, St. Louis, MO, USA) dissolved in Tris-buffered saline Tween 20 (TBST, 0.1%) (0.05 M Tris, 0.15 M NaCl, pH 7.5 and 1% Tween-20). Subsequently the membranes were incubated overnight in 4 °C on a shaker, with anti-PKG-1 (1:1000, Cell Signaling Technology, Danvers, MA), anti-phospho-eNOS Ser1177 (1:1000, Cell Signaling Technology, Danvers, MA), anti-eNOS (1:1000, Cell Signaling), anti-phospho-Akt 1/2/3 Ser 473-R (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Akt 1/2/3 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-ERK p44/42 (1:1000, Cell Signaling Technology, Danvers, MA), anti-ERK2 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), anti-COX1 (1:500, Cayman Chemical; Ann Arbor, MI), anti-COX2 (1:500, Cell Signaling Technology, Danvers, MA), anti-caveolin (1:1000, Abcam) and anti-alpha actin (1:3000, Sigma, St. Louis, MO, USA). Membranes were washed three times with TBST 0.1% and incubated with specifics peroxidase-conjugated secondary antibodies (GE HealthCare, Little Chalfont-Buckinghamshire, UK). The resolved bands were scanned using a ChemiDoc MP System Software and quantified using Image Lab Software, version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Reactive oxygen species quantification

Aortas were homogenized using a Tris-base (50 mmol/L) containing EDTA (1 mmol/L) buffer, with protease inhibitors: PMSF and OTV (1 mmol/L). Samples were centrifuged at 1000*g*, 10 min at 4 °C. The relative levels of ROS were measured through a fluorometric reaction between the aortic supernatant samples (50 μL) and the 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) reagent (50 μL) in black polystyrene 96-well plate incubated for 45 min at 37 °C. The final product was converted into highly fluorescent compost DCF in presence of ROS [21]. Blank readings were subtracted from loaded sample readings and fluorescence was measured at λ-excitation = 504 nm and λ-emission = 522 nm. Values were reported as fluorescence units per milligram of tissue (FU/mg).

2.7. Determination of cardiac hypertrophy

Hearts were removed from animals of both groups and extra-cardiac and atrial tissue was trimmed. Cardiac ventricular wet weight (mg) was measured and normalized to the body weight in order to quantify cardiac ventricular hypertrophy.

2.8. Statistical analysis

The relaxation responses were expressed as a percentage of the contraction induced by Phe. Contractile responses were expressed as a percentage of the response to KCl. To compare the magnitude of effect of endothelium-removal, L-NAME, ODQ or indomethacin on the vascular responses to ACh and Phe, some results were expressed as "differences" of the area under the concentration-response curves (dAUC) in control (in absence of L-NAME, ODQ or indomethacin) and stimulated conditions (in presence of L-NAME, ODQ or indomethacin), as was performed in our previous investigations [22–24]. AUC was calculated from the individual concentration-response curve plot by using GraphPad Prism software (Software, San Diego, CA, USA) and the differences were expressed as a percentage of AUC of the corresponding control situation. All data were expressed as mean ± S.E.M. and were analyzed using Student's *t*-test and one- or two-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test as appropriate (GraphPad Prism Software, San Diego, CA, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of sild treatment on biometric parameters

At the end of the treatment period with Sild, body weight did not differ among the two groups (SHR-Sild: 260 ± 7.90 g vs. SHR: 275 ± 5.70 g, $P > 0.05$). There are no differences in water and food intake between the two groups (data not shown). Chronic PDE5 inhibition by Sild prevented cardiac hypertrophy expressed as the wet heart weight/body weight ratio (mg/g) (SHR-Sild: 3.60 ± 0.05 vs. SHR: 4.20 ± 0.09; $P < 0.05$).

3.2. Effects of sild treatment on baseline blood pressure and heart rate

With respect to untreated rats, chronic treatment with Sild for 60 days did not alter significantly baseline SAP, DAP, MAP and HR values (Table 1, $P > 0.05$).

3.3. Vascular effects of sildenafil treatment

In endothelium-intact aortic rings from SHR, Sild treatment promoted a significant increase in ACh-induced relaxation (Fig. 1A), without altering the relaxation evoked by SNP (Fig. 1B). In these arteries, chronic Sild administration did not affect the contraction induced by KCl (SHR: 1.90 ± 0.14 g vs. SHR-Sild: 2.00 ± 0.16 g, $P > 0.05$), but reduced the contractile response induced by the α_1-adrenoceptor agonist Phe (Fig. 1C). In isolated aorta rings from both groups, the endothelium removal significantly increased Phe-induced contraction (Fig. 2A). However, this effect was greater in arteries from Sild-treated SHR as evidenced in Fig. 2D.

To investigate the involvement of NO/sGC/PKG pathway on vascular effects of Sild in SHR aorta, experiments were performed in endothelium-intact aortic rings in the presence of L-NAME or ODQ. Pretreatment with theses inhibitors abolished the vasorelaxation induced by ACh in both groups (data not shown). In the presence of L-NAME or ODQ, Phe-induced contraction was increased in arteries from treated and untreated animals (Fig. 2B and C). In SHR-Sild aorta, the effect of L-NAME and ODQ was higher compared to the effect recorded in the aorta of SHR animals (Fig. 2E and F). Besides that, protein expression of eNOS, phosphorylated eNOS at Ser^{1177}, AKT 1/2/3, phospo-AKT 1/2/3, PKG, and caveolin were unchanged by Sild treatment (Fig. 3).

To assess the possible contribution of COX-derived prostaglandins to the ACh and Phe responses, arteries were preincubated with the COX inhibitor indomethacin. COX inhibition increased relaxation response

Table 1
Baseline blood pressure and heart rate values in sildenafil-treated (SHR-Sild) and untreated SHR.

	SHR	SHR-Sild	P value
SAP (mm Hg)	180 ± 10	190 ± 14	0.84
DAP (mm Hg)	129 ± 6	134 ± 6	0.60
MAP (mm Hg)	150 ± 8	153 ± 11	0.81
HR (bpm)	327 ± 6	325 ± 13	0.90

Values are means ± SEM (n = 5 rats per group). SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MAP, mean arterial pressure; HR, heart rate. Data were analyzed by Student's *t*-test.

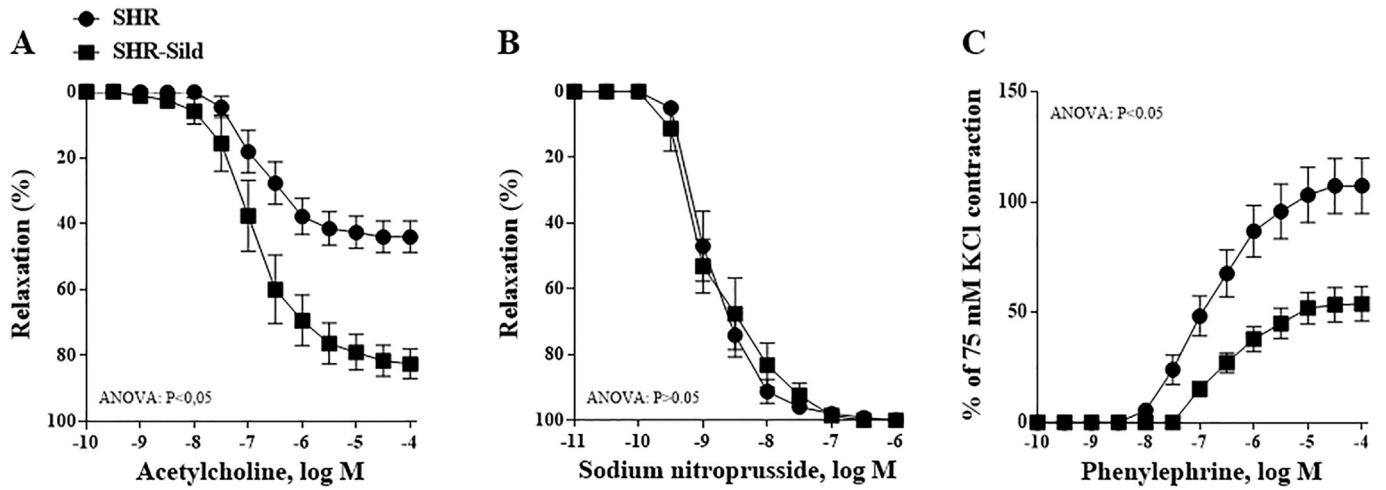

Fig. 1. Effects of chronic PDE5 inhibition on endothelium-dependent and endothelium-independent relaxation response to acetylcholine (A) and sodium nitroprusside (B), respectively and on the vasoconstriction response to phenylephrine (C) in aortic rings from sildenafil-treated (SHR-Sild) and untreated SHR. Results are expressed as mean ± SEM (n = 8 rats per group).

to ACh (Fig. 4A) and decreased contraction response to Phe in segments from both groups (Fig. 4B). However, these effects were lower in arteries isolated from Sild-treated SHR (Fig. 4C and D). As shown in the Fig. 5, treatment with Sild downregulates the content of COX-2 in the aorta of SHR, without affect COX-1 protein expression. Furthermore, SHR-Sild group showed a significant decrease in DCF fluorescence intensity in aortic tissues when compared with fluorescence intensity of aorta in untreated SHR group, suggesting a significant reduction of ROS levels in animals chronically treated with Sild (Fig. 5C).

Several studies suggest that ERK protein expression is upregulated

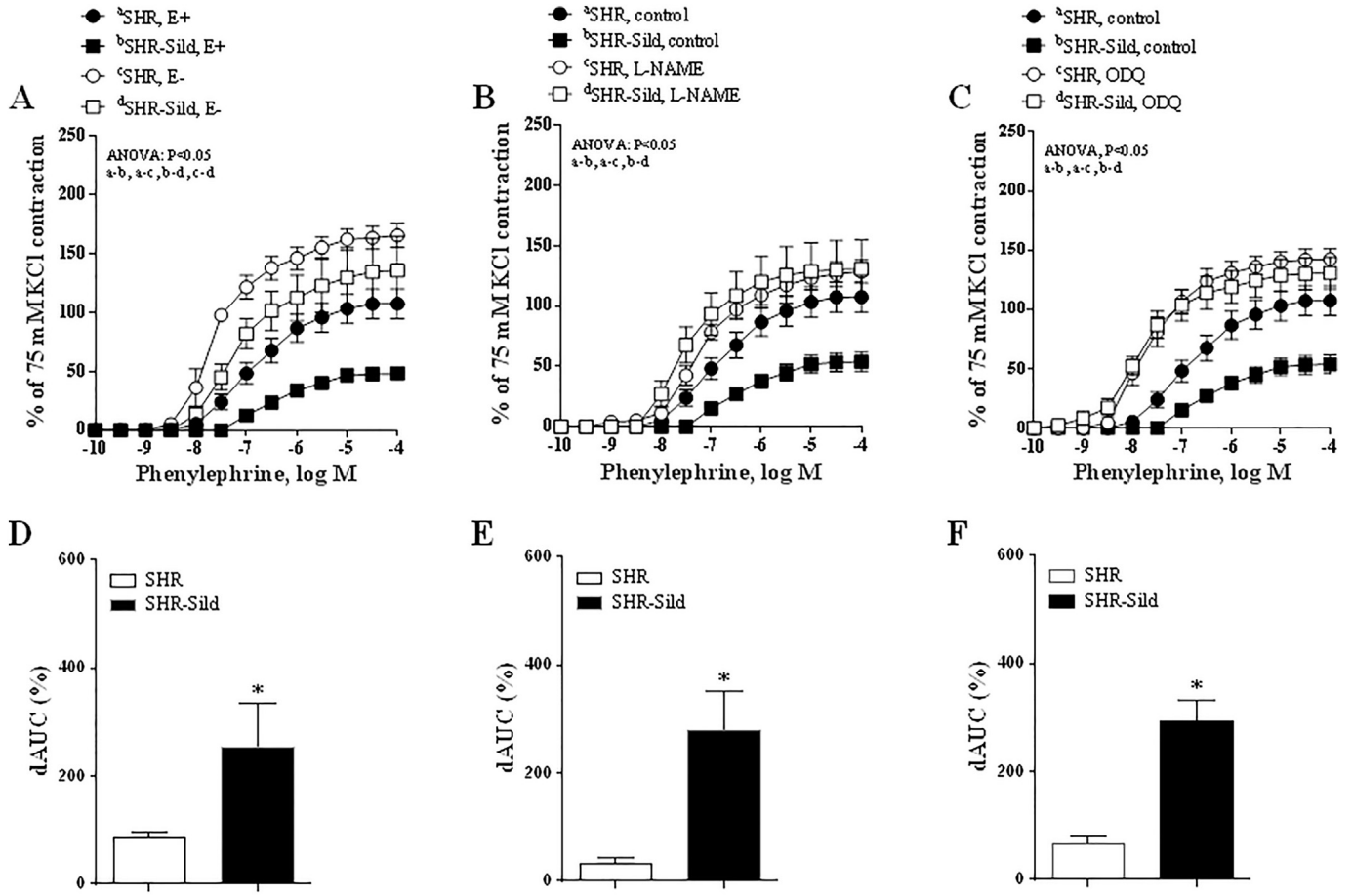

Fig. 2. Effects of endothelium removal (A), pretreatment with L-NAME (B) or ODQ (C) preincubation on contractile response induced by phenylephrine in aortic rings from sildenafil-treated (SHR-Sild) and untreated SHR. Differences in area under the concentration-response curve (dAUC) to phenylephrine in segments with (E +) and without (E−) endothelium, in the absence and presence of L-NAME or ODQ are shown in figures D, E and F, respectively. Results are expressed as mean ± SEM (n = 5–8 rats per group). *P < 0.05 vs. SHR by Student's *t*-test.

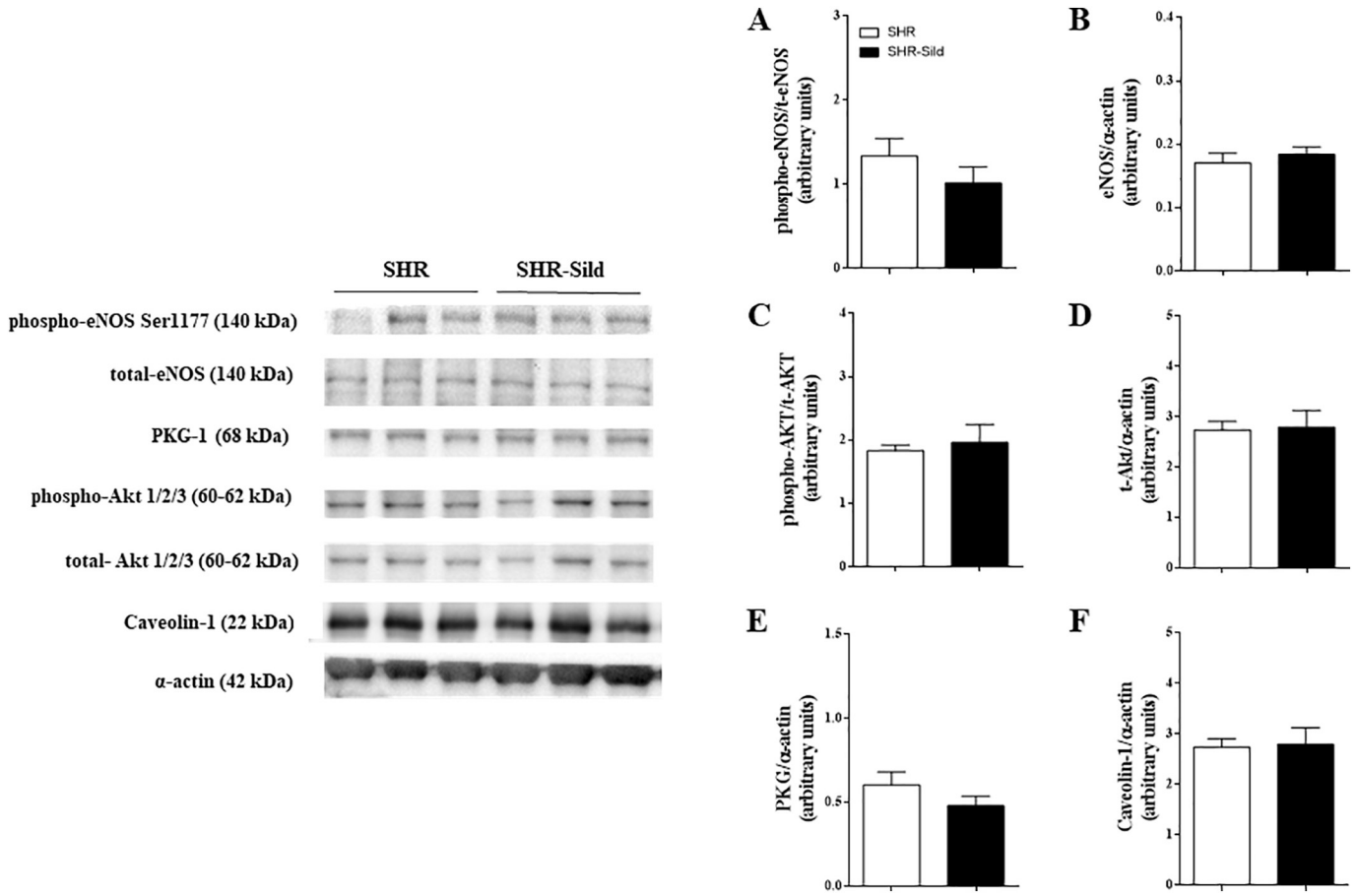

Fig. 3. Left panel: Representative blots of phospho-eNOS Ser^{1177}, total-eNOS, phospho-Akt 1/2/3, total-Akt 1/2/3, PKG and caveolin and α-actin in aorta from sildenafil-treated (SHR-Sild) and untreated SHR. Right panel (A–F): Averaged densitometric data for phospho-eNOS Ser^{1177} (A), total-eNOS (B), phospho-Akt 1/2/3 (C), total-Akt 1/2/3 (D), PKG (E) and caveolin (F) expression in aortic tissues from SHR and SHR-Sild groups. Densitometry of proteins were normalized by α-actin levels. Results were expressed as mean ± SEM (n = 6 rats per group).

in hypertension [25,26] and activation of ERK 1/2 is associated with increased COX-2 expression [27–29]. As shown in Fig. 6A, total ERK protein expression did not change in the aorta of SHR after Sild treatment. A decrease of ERK1/2 phosphorylation was observed in the aorta from animals treated with Sild (Fig. 6B). Furthermore, although Sild chronic treatment was unable to modify the increased resting blood pressure in conscious SHR, it significantly reduced cardiac hypertrophy (Fig. 7A) and ERK1/2 phosphorylation levels (Fig. 7C) in heart tissues of treated animals compared those obtained in the hearts of untreated SHR.

4. Discussion

Hypertension is one of the most important risk factors for cardiovascular morbidity and mortality [1]. It is well known that increased activity of cGMP-hydrolyzing PDE5 contributed to the development of hypertension [6,7] and vascular endothelial dysfunction [3]. The present study investigated the effect of chronic treatment with PDE5 inhibitor Sild on vascular dysfunction which is present in SHR. Our results reveal that treatment with Sild improved the endothelium-dependent vasodilation and decreased the vasoconstriction to Phe in aorta from SHR by increasing endothelial NO and inhibiting COX-2 expression.

SHR is a genetic hypertensive model with a progressive elevation of blood pressure starting from the fifth week of age [17]. In the current study, chronic Sild treatment was started in a pre-hypertensive period (4-week-old) until 12-week-old. At this age, baseline SAP, DAP and MAP values were not different between Sild-treated and untreated SHR suggesting that PDE5 inhibition was not able to prevent the development of hypertension.

Similarly, Kristek et al. [16] in a preventive protocol (4–9 weeks) also showed that PDE5 inhibition by Sild (10 mg/kg/day) did not produce any significant changes on SAP in SHR. Furthermore, clinical [30] and experimental [31] studies suggest that baseline blood pressure and HR remained unaltered or suffered little changes after chronic PDE5 inhibition. Likewise, the absence of significant effects of PDE5 inhibition by Sild on baseline blood pressure was previously described together with the improvement of diabetic cardiomyopathy in humans [32]. On the contrary, chronic antihypertensive effect of Sild administration was observed in SHR (different doses and period of treatment) [14,33], and in clinical trials involving human patients [7,34]. The divergence of data regarding the effects of PDE5 inhibition in essential hypertension led us to investigate its potential role in young SHR. Our initial hypothesis was that the PDE5 inhibition in SHR would be directly related with the enhancement of the cGMP/sGC/PKG signaling cascade, as well NO bioavailability. It is important to highlight that these mechanisms are dependent of components of blood vessels wall, specially the vascular endothelium and vascular smooth muscle cells (VSMCs) [35].

Endothelial cells synthetize and release several vasodilator factors, such as NO, EDHF and prostacyclin (PGI_2), as well as vasoconstrictor factors, such as TxA_2, endothelin-1 (ET-1), prostaglandin H_2 (PGH_2) and O_2^-. The balance between vasodilator and vasoconstrictor mediators is essential in maintenance of vascular tone and regulation of blood pressure [1,35]. In humans and animals, the increment on blood pressure causes vascular alterations, characterized by impaired endothelial function, increased vasoconstriction and vascular remodeling

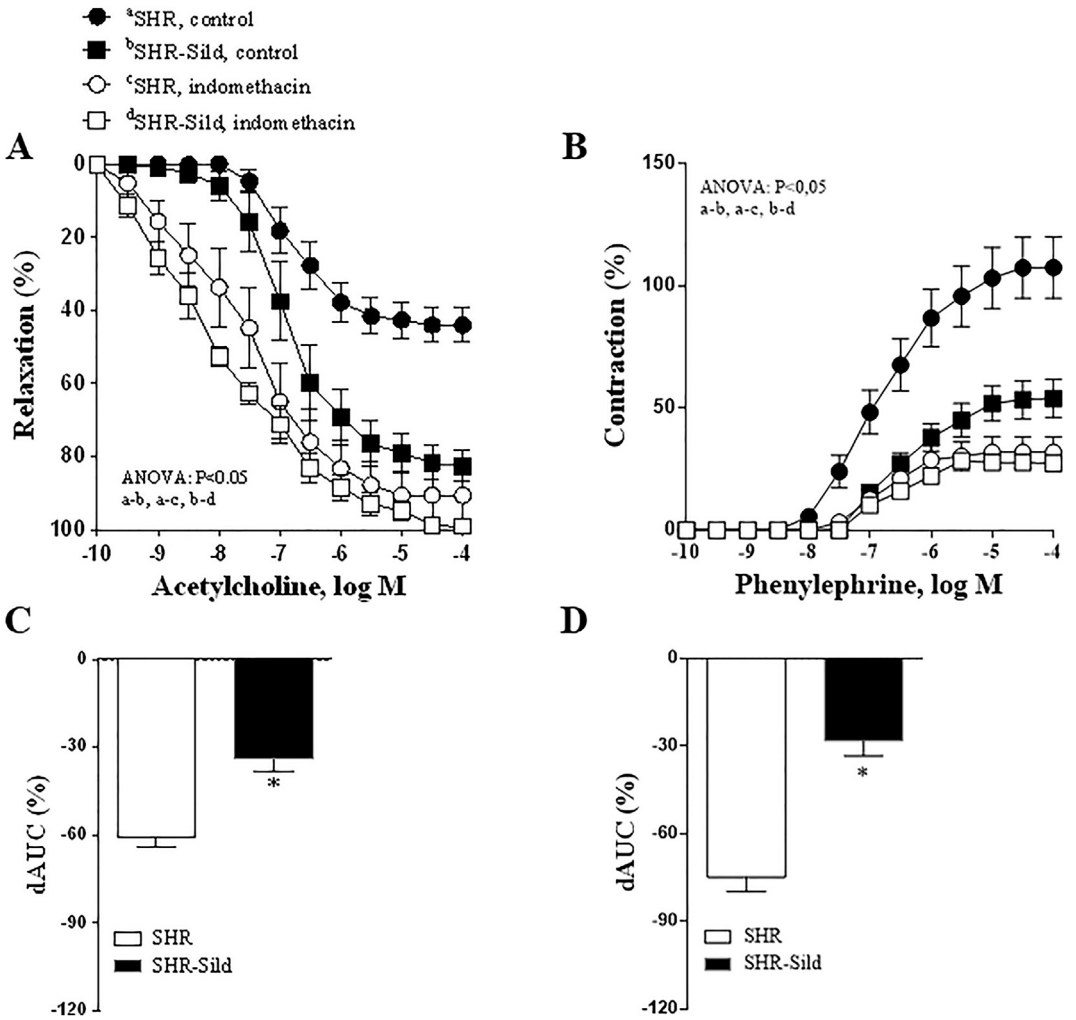

Fig. 4. Effect of COX inhibition by indomethacin on relaxation response to acetylcholine (A) and contractile response to phenylephrine (B) in aortic rings from sildenafil-treated (SHR-Sild) and untreated SHR. Differences in area under the concentration-response curve (dAUC) to acetylcholine (C) and phenylephrine (D) in segments in the absence and in the presence of indomethacin. Results are expressed as mean ± SEM (n = 5–8 rats per group). *P < 0.05 vs. SHR by Student's *t*-test.

[1,36]. The present study confirms the presence of endothelial dysfunction in hypertension, as evidenced by the substantial decrease in endothelium-dependent relaxation to ACh (maximum relaxation response was reduced to ~40% in comparison with normotensive rats, where this response is commonly between 80 and 100%) [37]. The endothelial dysfunction was prevented by PDE5 inhibition in SHR, as demonstrated by the increased vasodilation to ACh following Sild treatment. This effect was limited to the endothelium-mediated relaxation since the response to SNP remained unaltered in aortic rings from Sild-treated rats. These results confirm previous results obtained by Yaguas et al. [14] showing that 24-weeks Sild administration reversed endothelial dysfunction and ameliorated the severity of hypertension in SHR.

Hypertension is commonly associated with increased VSMCs contractility in response to several vasoactive agents [1]. In the current study, chronic Sild administration reduced aortic contractility to Phe, without affecting K^+-induced vasoconstriction. This effect was observed in both endothelium-intact and denuded arteries indicating that chronic Sild affects mechanisms associated to α_1-adrenoceptor-mediated vascular contraction.

To date, it is quite debatable whether endothelial dysfunction during hypertension is cause or consequence of increased blood pressure [38]. Moreover, there are some contradictory results about the NO production in cardiovascular system of hypertensive animals [16,39]. It is well established that NO plays a crucial role for the cardiovascular function and its impaired bioavailability is involved on genesis and/or maintenance of hypertensive state [39]. In rat aorta, NO is the main endothelial relaxant factor [40]. Therefore, the involvement of NO was investigated in the vascular effects of chronic Sild administration in the aorta of SHR. After eNOS or sGC blockade by L-NAME or ODQ, respectively, ACh-induced vasodilation was abolished in aortic vessels from both Sild-treated and untreated SHR. L-NAME or ODQ similarly increased Phe-induced vasoconstriction in arteries from both groups, but this effect was greater in aortic rings from Sild-treated SHR suggesting an increased NO bioavailability and cGMP/sGC pathway in these preparations. Despite this, aortic protein expression of components directly involved with NO production (eNOS, phosphorylated eNOS at Ser^{1177}, AKT 1/2/3, phospo-AKT 1/2/3, PKG, and caveolin) were not changed by chronic Sild treatment. Our results showed that Sild treatment did not affect the relaxation induced by SNP in SHR aorta. Therefore, if Sild affects cGMP levels in SHR aorta, this may not reflected in changes in cGMP/PKG-dependent relaxation at VSMCs. Thus, given these functional results we postulate that Sild could improve vascular dysfunction in SHR aorta by increasing NO bioavailability. As was observed in the present study, chronically administered Sild reduces vascular ROS generation [41], which in turn may increase NO bioavailability and relaxation [2].

COX overexpression exerts several effects in the vasculature, such as highest synthesis of arachidonic acid-derived metabolites and enhanced vascular inflammation [12]. Both endothelial cells and, to a lesser

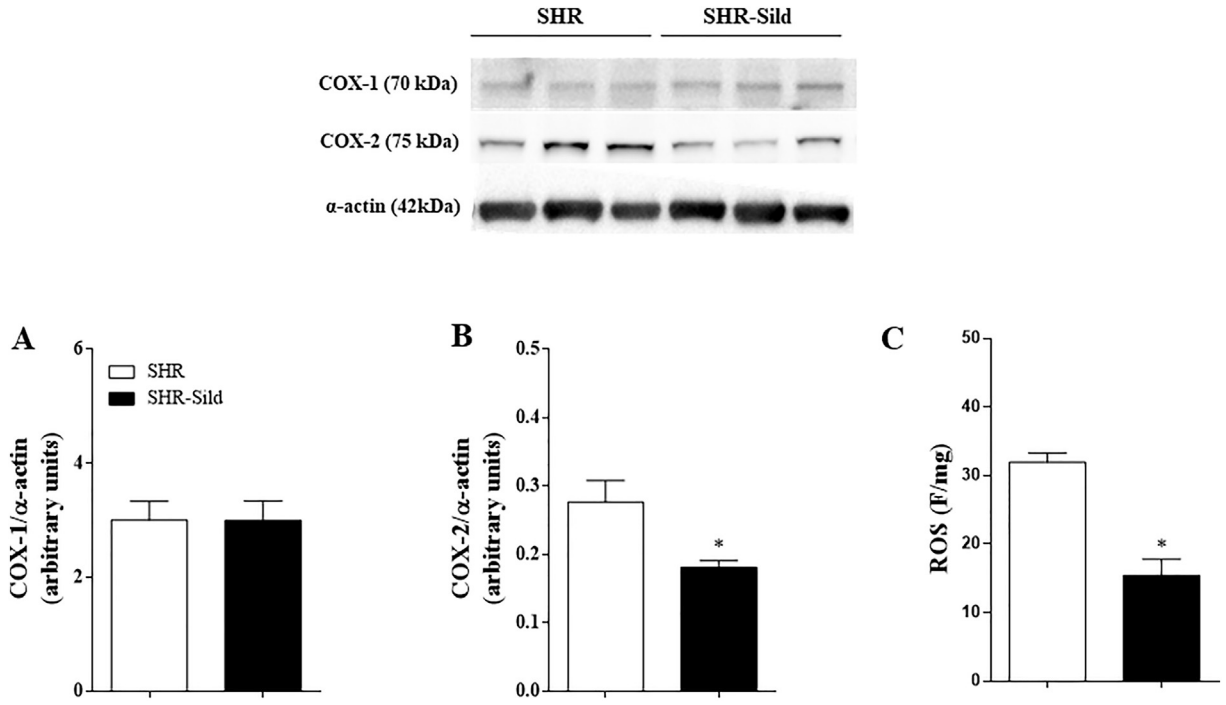

Fig. 5. Left top panel: Representative blots of COX-1, COX-2 and α-actin in aorta from sildenafil-treated (SHR-Sild) and untreated SHR. Averaged densitometric data for COX-1 (A) and COX-2 (B) expression in aortic tissues from SHR and SHR-Sild groups. Densitometry of proteins were normalized by α-actin levels. Aortic relative reactive oxygen species levels (C) in SHR and SHR-Sild groups. Results are expressed as mean ± SEM (n = 6–8 rats per group). *P < 0.05 vs. SHR by Student's *t*-test.

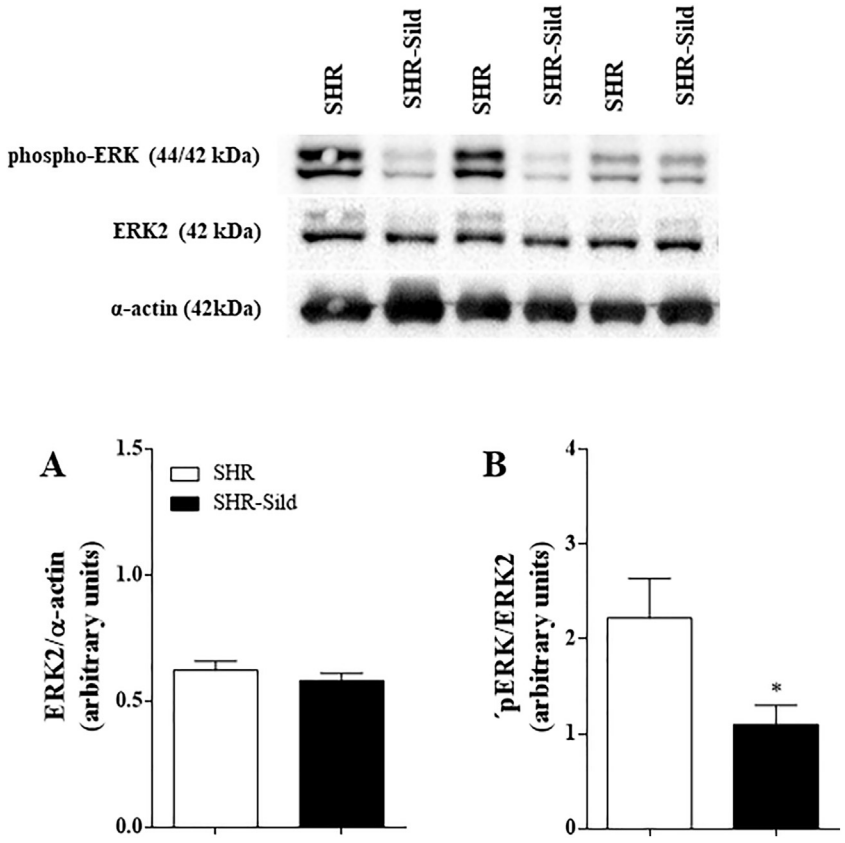

Fig. 6. Western blot analysis of protein expression of total-ERK (A) and phospho-ERK (B) in aorta from sildenafil-treated (SHR-Sild) and untreated SHR. Densitometry of proteins was normalized by α-actin levels. Results are expressed as mean ± SEM (n = 6 rats per group). *P < 0.05 vs. SHR by Student's *t*-test.

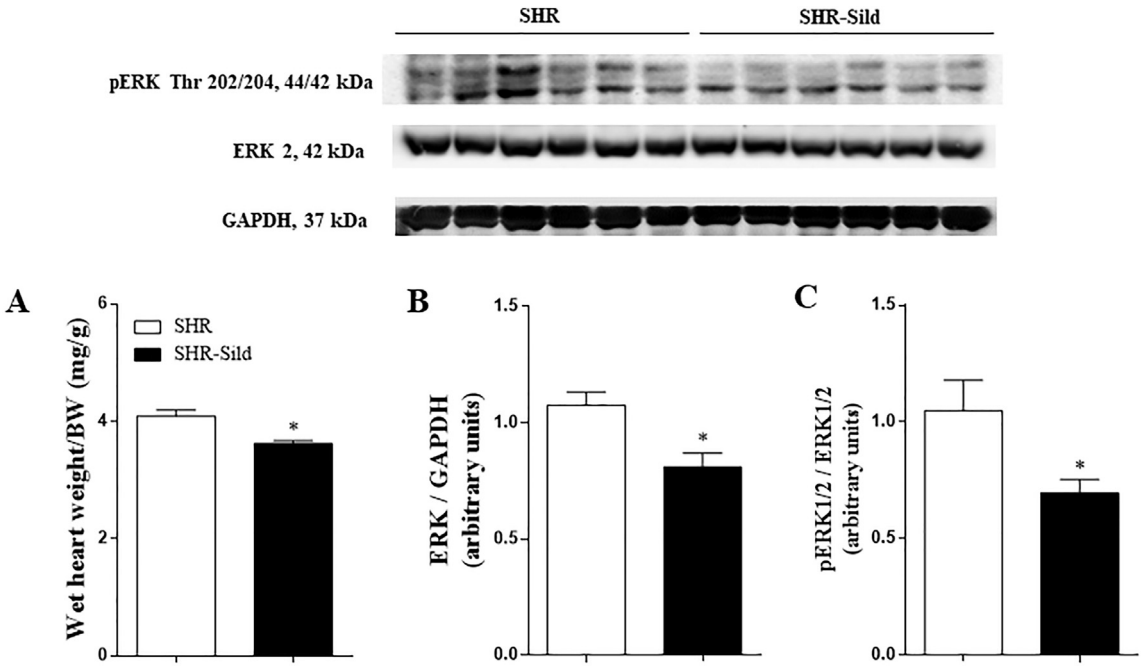

Fig. 7. Top panel: Representative blots of cardiac phospho-ERK, total-ERK and GAPDH expression in sildenafil-treated (SHR-Sild) and untreated SHR groups. Wet heart weight/body weight (BW) ratio (A) and western blot analysis of left ventricular cardiac protein expression of phospho-ERK (B) and total-ERK (C) from sildenafil-treated (SHR-Sild) and untreated SHR. Densitometry of proteins was normalized by GAPDH levels. Results are expressed as mean ± SEM (n = 6 rats per group). *P < 0.05 vs. SHR by Student's *t*-test.

extent, VSMCs express the two isoforms of COX. COX-1 is expressed constitutively but it is modulated in some situations, COX-2 is usually expressed at sites of inflammation [42]. In several cardiovascular diseases, the widespread vascular and tissue inflammation and the associated oxidative stress induce COX-2 expression and shift their metabolism from vasodilatation and anti-thrombosis to vasoconstriction, pro-thrombosis and increased inflammation and oxidative stress [12,42]. It was widely demonstrated that COX-2-derived contractile prostanoids production contributes to endothelial dysfunction and vascular remodeling observed in SHR [43].

To investigate the participation of COX-derived products in vascular effects of Sild in SHR aorta, arteries were preincubated with the COX inhibitor indomethacin. In agreement with a previous study [44], our results show that inhibition of COX restores the impaired endothelium-dependent relaxation, as well as inhibits the endothelium-dependent contractions in SHR. Alvarez et al. [43] demonstrated that the selective COX-2 inhibitor NS398 reduced the concentration-response curves to Phe more efficiently in segments of aorta obtained from SHR than in those isolated from Wistar-Kyoto rats. These findings are in line with our present results showing that contraction to Phe was markedly diminished and ACh relaxation was increased in indomethacin-preincubated arteries. However, these responses were lower in aorta from Sild-treated SHR suggesting a decreased participation of COX-derived products in SHR aorta after Sild administration. This hypothesis is corroborated by the findings that although treatment with Sild did not alter the COX-1 expression, it significantly reduced the expression of COX-2 in aortic tissues obtained from SHR. A previous study performed in diabetic mice also showed that PDE5 inhibition by Sild downregulated COX-2 expression in the heart tissues [45].

COX-2 activity is regulated at post-transcriptional levels by MAPK-ERK signaling [27–29]. ERK1/ERK2 are activated through the Ras/Raf/MEK/ERK cascade. ERK protein overexpression is involved in both vasoconstriction and VSMCs proliferation in hypertension [46]. Basal levels of p44/42 ERK are augmented in hypertension [25,47]. Moreover, Touyz et al. [25] showed that MEK inhibition restores endothelial function in mesenteric arteries from SHR without influencing blood pressure. The present finding of decreased ERK1/2 phosphorylation in aortic tissues from SHR-Sild group suggests that inhibition of MAPK-ERK signaling is involved in the downregulation of COX-2 observed in this group.

Experiments in cultured VSMCs from SHR aorta demonstrated that MAPK-ERK signaling activity is increased in SHR in association to a higher VSMC reactivity to angiotensin II and endothelin-1. Moreover, inhibition of MAPK/ERK abolished sustained contraction and normalized angiotensin II effects in VSMC from SHR [48]. In the present study, chronic Sild administration decreased ERK1/2 phosphorylation in SHR aorta suggesting that ERK-dependent signaling pathways may decrease aortic contractility.

Even in the absence of significant effects on blood pressure, PDE5 inhibition by Sild was able to modulate p44/42 MAPK phosphorylation not only in aorta, but also in the left ventricular cardiac mass. Furthermore, animals treated with Sild showed reduction of wet heart weight/body weight ratio suggesting a possible anti-hypertrophic effect of Sild. Reduced cardiac mass was observed in both PDE5 inhibition [16] and ERK1/2 pathway inhibition [49].

Our study has some limitations. The first one was the non-quantification of vascular tissue cGMP levels after Sild treatment. Second, signaling and ROS assays were performed using whole aorta which make their correlation with endothelial dysfunction of some weakness. Further experiments are needed to strengthen this correlation by performing signaling and ROS assays in the endothelial cells or VSMCs. Finally, we showed that Sild treatment reduced vascular ROS generation which was measured through a fluorometric reaction. To provide more insight into the mechanisms by which Sild induces this effect, measurements of expression and/or activity of ROS-generating enzymes would be necessary.

5. Conclusions

In summary, this study showed that PDE5 inhibition by Sild not only attenuates the development of endothelial dysfunction, but also modulate vascular inflammatory activation pathways through COX-2 and

ERK1/2 in aorta from SHR during the development of hypertension. These findings, which are novel, may be of a putative clinical relevance in the use of Sild as an adjunct therapy in essential hypertension. Further experiments are needed to give more insight in this issue such as investigating the reactivity of resistance vessels and cardiac performance in this strain.

Acknowledgements

We are grateful to José Antonio de Albuquerque for his technical assistance. Expert reviewing of the manuscript by Professor Mohamed Latif is acknowledged.

Funding

This study was financed partly by the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior”, Brazil (CAPES, Finance code 001, Grant number: 88881.068093/214-01) and the “Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco”. Brazil (FACEPE, grant number: 16/2012 APQ-1133-2.07/12) and partly by the University of Roma, Italy (grant number: FIRB2010RBAP109BLT_003). Gloria P. Duarte, Fabiano E. Xavier and Saad Lahlou are recipients of research fellowship from the “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPq/Brazil).

Conflicts of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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