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

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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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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.

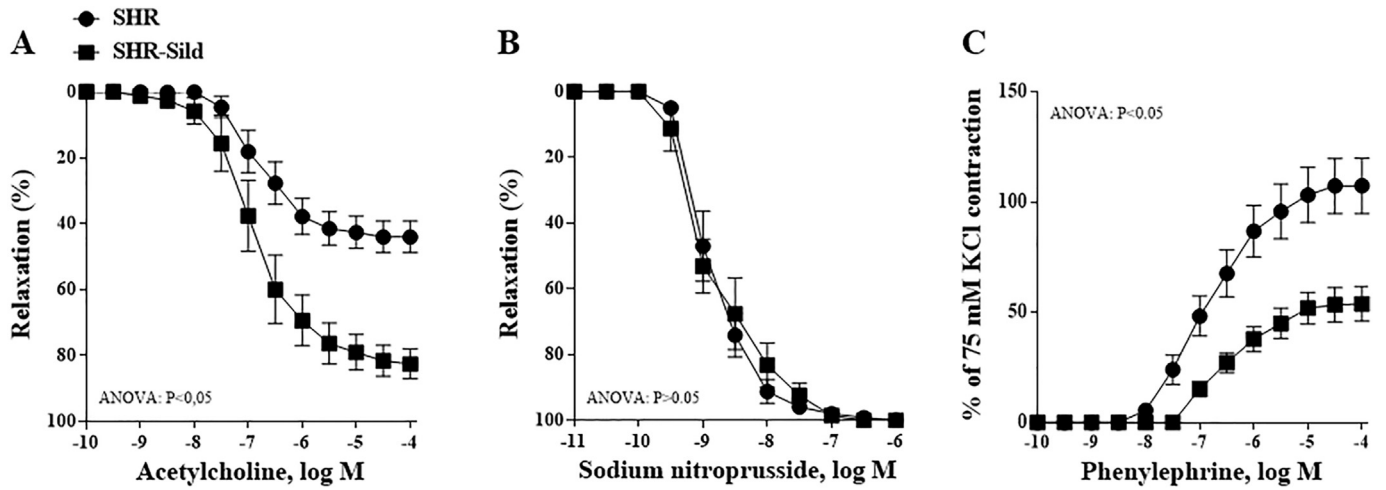

Fig. 1. Effects of chronic PDE5 inhibition on endothelium-dependent and endothelium-independent relaxation response to acetylcholine (A) and sodium nitroprusside (B), respectively and on the vasoconstriction response to phenylephrine (C) in aortic rings from sildenafil-treated (SHR-Sild) and untreated 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

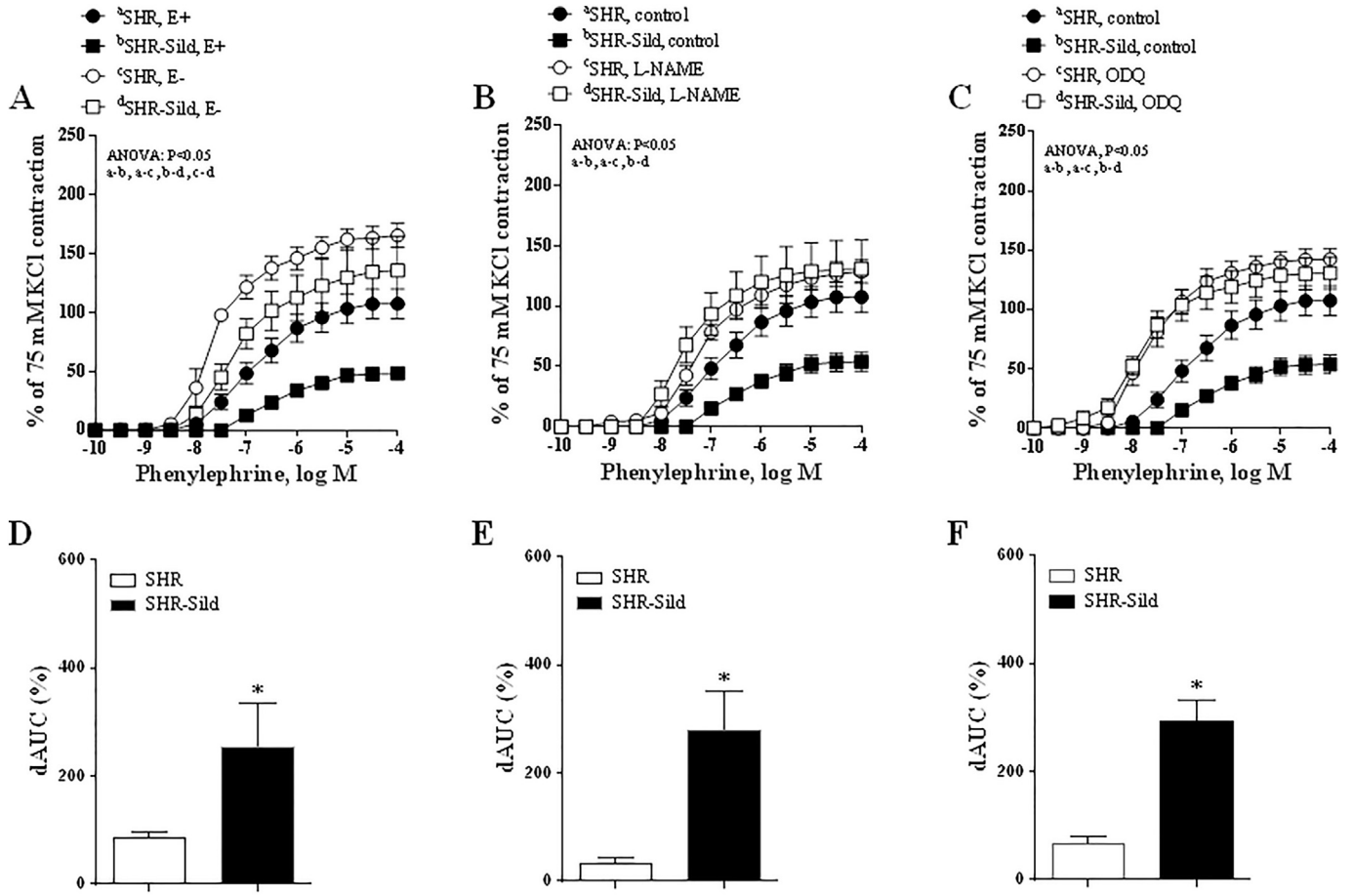

Fig. 2. Effects of endothelium removal (A), pretreatment with L-NAME (B) or ODQ (C) preincubation on contractile response induced by phenylephrine in aortic rings from sildenafil-treated (SHR-Sild) and untreated SHR. Differences in area under the concentration-response curve (dAUC) to phenylephrine in 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.

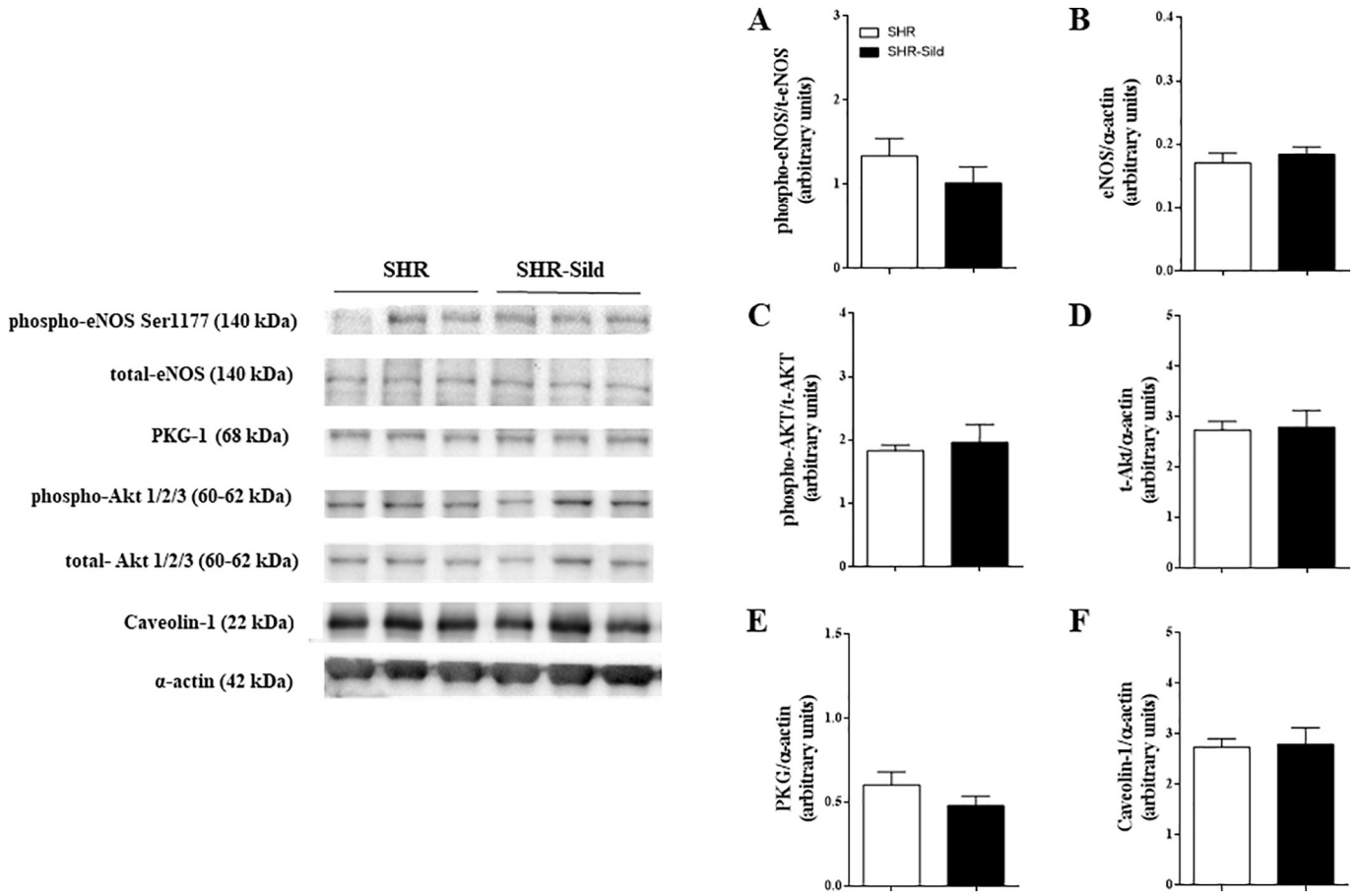

Fig. 3. Left panel: Representative blots of phospho-eNOS Ser^{1177}, total-eNOS, phospho-Akt 1/2/3, total-Akt 1/2/3, PKG and caveolin and α-actin in aorta from sildenafil-treated (SHR-Sild) and untreated SHR. Right panel (A–F): Averaged densitometric data for phospho-eNOS Ser^{1177} (A), total-eNOS (B), 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

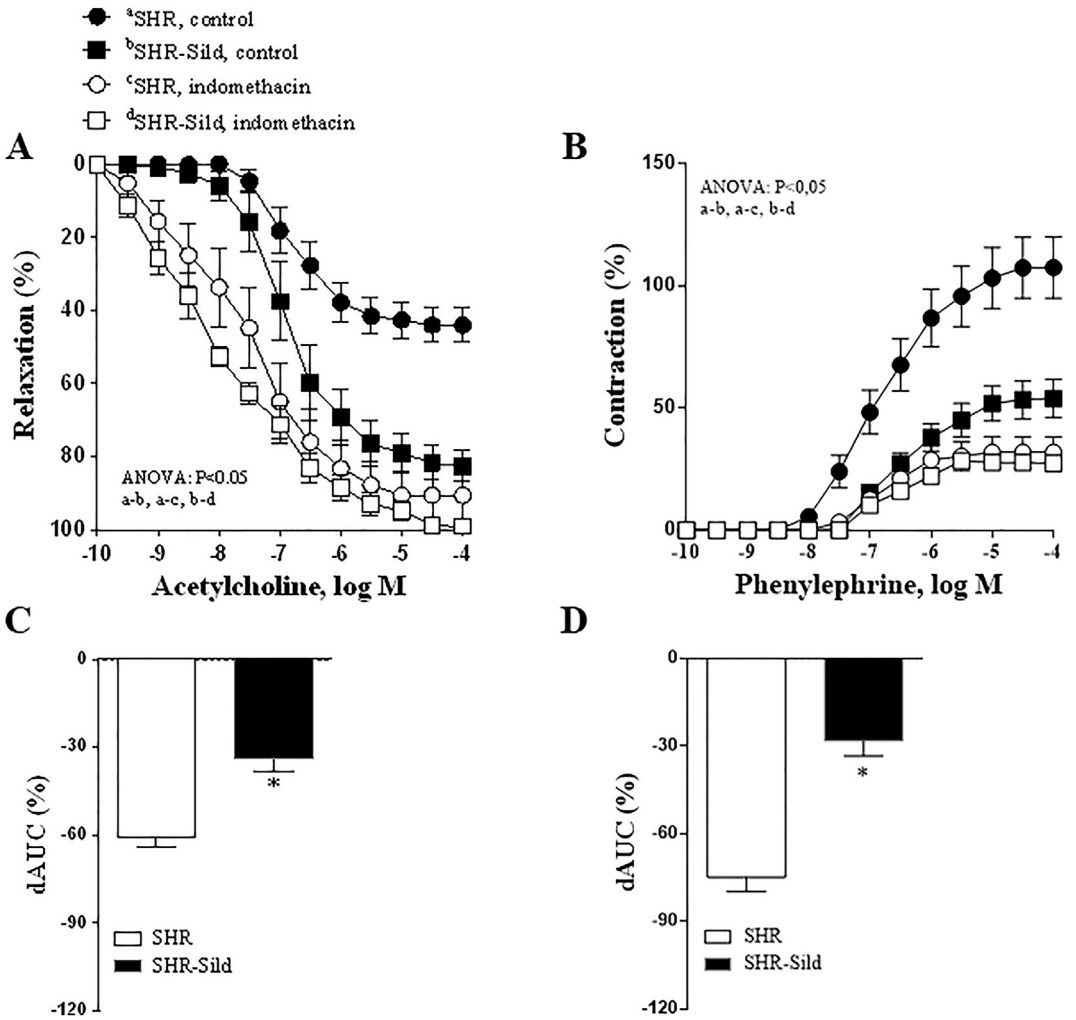

Fig. 4. Effect of COX inhibition by indomethacin on relaxation response to acetylcholine (A) and contractile response to phenylephrine (B) in aortic rings from sildenafil-treated (SHR-Sild) and untreated SHR. Differences in area under the concentration-response curve (dAUC) to acetylcholine (C) and phenylephrine (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

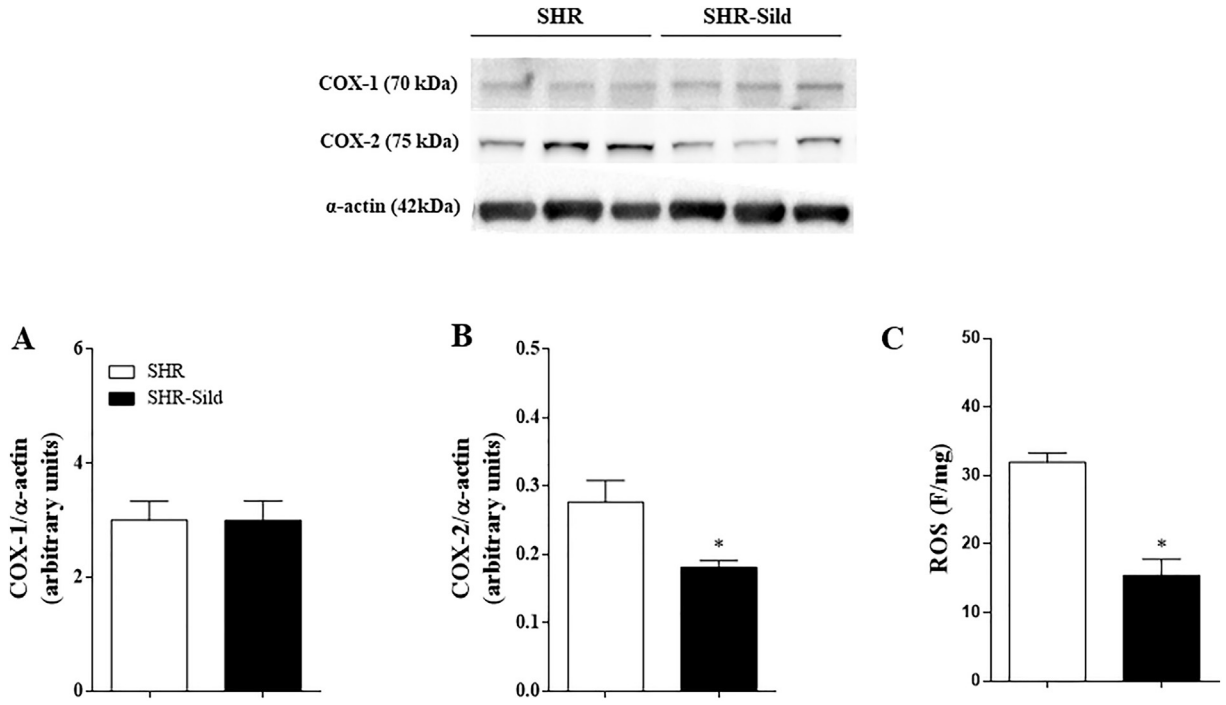

Fig. 5. Left top panel: Representative blots of COX-1, COX-2 and α-actin in aorta from sildenafil-treated (SHR-Sild) and untreated SHR. Averaged densitometric data for COX-1 (A) and COX-2 (B) expression in aortic tissues from SHR and SHR-Sild groups. Densitometry of proteins were normalized by α-actin 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.

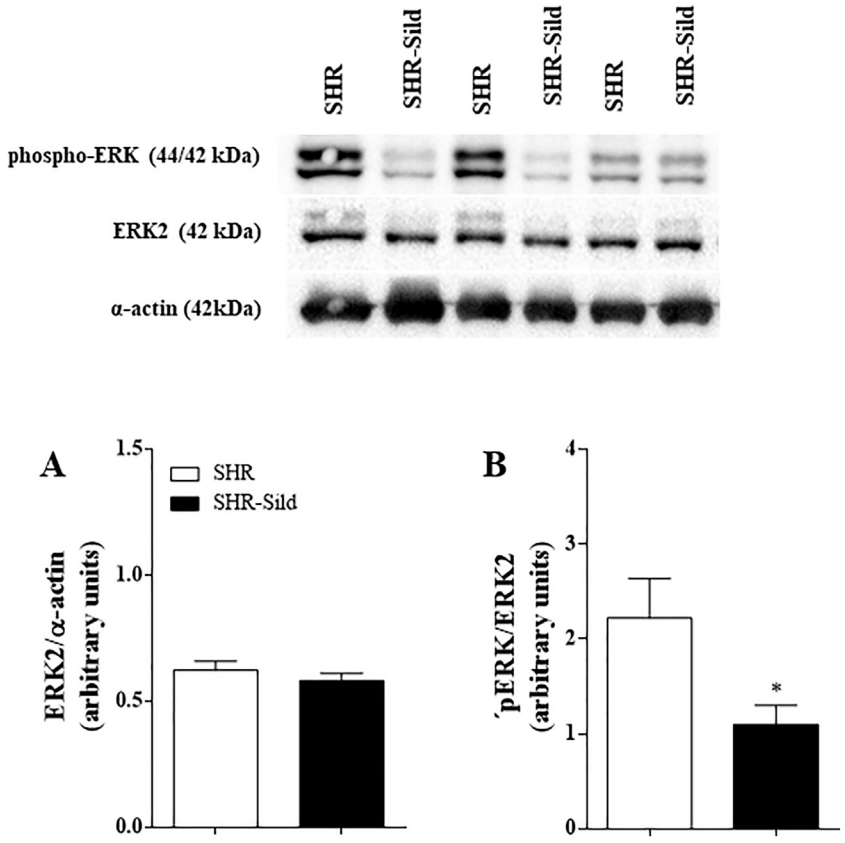

Fig. 6. Western blot analysis of protein expression of total-ERK (A) and phospho-ERK (B) in aorta from sildenafil-treated (SHR-Sild) and untreated SHR. Densitometry of proteins was normalized by α-actin levels. Results are expressed as mean ± SEM (n = 6 rats per group). *P < 0.05 vs. SHR by Student's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

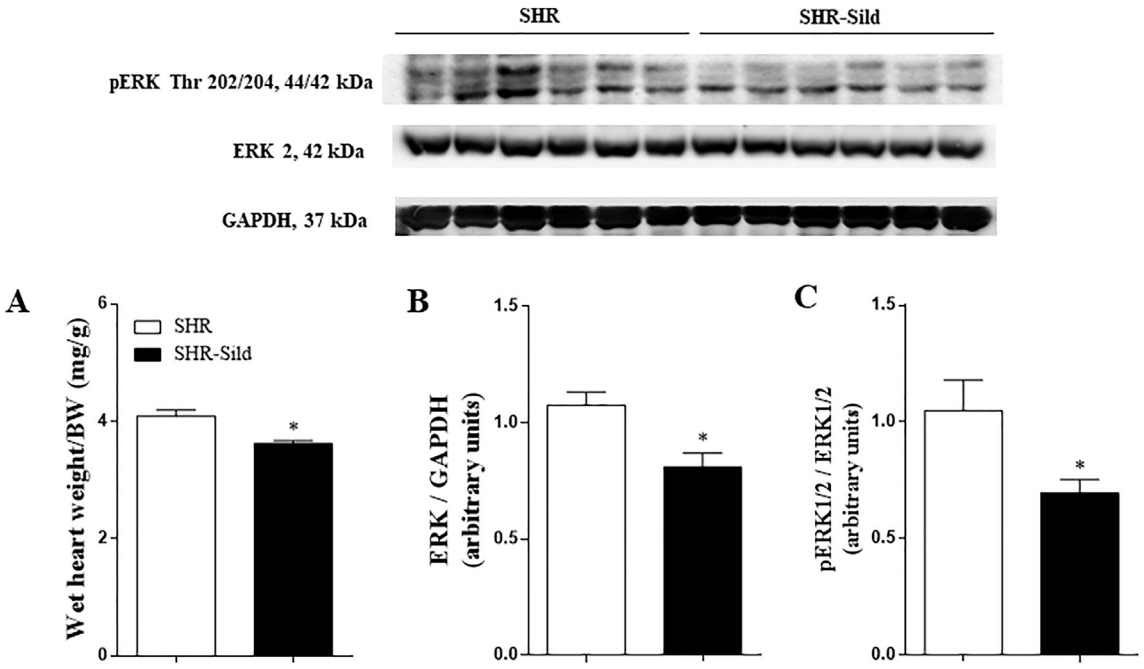

Fig. 7. Top panel: Representative blots of cardiac phospho-ERK, total-ERK and GAPDH expression in sildenafil-treated (SHR-Sild) and untreated SHR groups. Wet heart weight/body weight (BW) ratio (A) and western blot analysis of left ventricular cardiac protein expression of phospho-ERK (B) and total-ERK (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.

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Conflicts of interest

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

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