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NO donors induce vascular relaxation by different cellular mechanisms in hypertensive and normotensive rats

Alice V. Araújo[a,1], Fernanda A. Andrade[b,1], Michele Paulo[b], Tiago D. de Paula[b], Simone R. Potje[b], Amanda C. Pereira[b], Lusiane M. Bendhack[b,*]

[a] School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil
[b] Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

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ABSTRACT

Purpose: This study investigated the intracellular mechanisms involved in the vasodilatation induced by the classic NO donor SNP and the non-classic NO donor cis-[Ru(bpy)2(py)(NO2)](PF6) (or RuBPY) in mesenteric resistance arteries obtained from renal hypertensive (2K-1C) and normotensive (2K) rats.

Methods: On the basis of fluorimetric assays in cultured vascular smooth muscle cells (VSMCs) isolated from 2K-1C and 2K rats, we measured NO release from SNP and RuBPY, cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$), and reactive oxygen species (ROS) with the selective probes DAF-2DA, Fluo-3AM and the more selective probe for peroxynitrite (7-CBA), respectively. We determined isometric tension in mesenteric arteries to assess SNP- and RuBPY-induced relaxation.

Results: SNP and RuBPY released NO in comparable amounts in cultured aortic VSMCs from hypertensive 2K-1C and normotensive 2K rats. The NO^0 scavenger hydroxocobalamin blunted NO release. Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibition with thapsigargin reduced $[Ca^{2+}]c$ in normotensive 2K rat VSMCs only. ROS amounts were greater in hypertensive 2K-1C than in normotensive 2K rat VSMCs, but neither SNP nor RuBPY altered ROS concentrations in any of the groups. SNP and RuBPY induced similar relaxation in hypertensive 2K-1C and normotensive 2K rat mesenteric resistance arteries. The SNP and RuBPY-induced relaxation involves sGC and PKG activation. On the other hand, SNP but not RuBPY activates K^+ channels. Interestingly, SERCA inhibition reduces SNP induced relaxation only in normotensive 2K rat mesenteric arteries whereas RuBPY-induced relaxation does not involve SERCA activation in both normotensive and hypertensive arteries.

Conclusion: Our results indicate that SNP and RuBPY-induced mesenteric resistance artery relaxation involves NO/sGC/cGMP/PKG pathway activation. K^+ channels and SERCA activation is required to SNP but not for RuBPY-induced relaxation. Moreover, SERCA seems to be impaired in hypertensive 2K-1C rat mesenteric resistance arteries although it does not impact SNP- or RuBPY-induced relaxation.

1. Introduction

Nitric oxide (NO) is a water-soluble gas and free radical that helps to regulate and to mediate numerous processes in the nervous, immune, and cardiovascular systems. NO synthase (NOS) isoforms produce NO, which is released by endothelium into vascular cells, to regulate blood flow and to maintain the vascular tone [1].

Reduced NO generation and bioavailability occurs in several cardiovascular diseases, including hypertension [2]. Therefore, pharmacological compounds that release NO have been useful tools to study and to understand the role NO plays in physiology and cardiovascular disorders and the NO mechanism of action [3].

Sodium nitroprusside (SNP) is a classic and potent organic NO donor and rapid-acting nitrovasodilator that is clinically used in hypertensive emergencies, like angina *pectoris* and heart failure. SNP also provides a controlled hypotensive effect during surgery. In addition, SNP is frequently employed as a prototype nitrovasodilator in pharmacological studies [4]. However, SNP has some undesired effects such as reflex tachycardia [4,5] and may promote severe toxicity [6,7].

The compound *cis*-$[Ru(bpy)_2(py)(NO_2)](PF_6)$ (RuBPY) is a nitrite-

* Corresponding author. Laboratório de Farmacologia, Bloco S, 3° andar, Faculdade de Ciências Farmacêuticas de Ribeirão Preto USP, Av. do Café, s/n°, 14040-903, Ribeirão Preto, SP, Brazil.
E-mail address: bendhack@usp.br (L.M. Bendhack).
[1] These authors contributed equally to this study.

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ruthenium that can act as NO donor and which offers advantages over SNP. First, RuBPY releases NO in a tissue enzyme-dependent and controlled manner [8]. Additionally, RuBPY induces hypotensive effect in normotensive animals, and it promotes higher, slower, and longer lasting hypotensive effect than SNP in hypertensive rats, without reflex tachycardia. Moreover, RuBPY displays a vasodilator effect on different blood vessels (conductance and resistance arteries), induces coronary artery relaxation (which is useful in angina), and exerts no effect on basilar artery (so it does not induce headache) [8].

All NO donors somehow produce NO-related activity when they are applied in biological systems, so they are mainly suited to mimicking an endogenous NO response. Nevertheless, individual classes of NO donors release different NO species through distinct pathways and present different chemical reactivity [9]. Although all NO donors release NO, intracellular cascade activation; i.e., participation of diverse enzymes and/or ionic channels, may vary among the several NO donors, animal species, and vascular beds. For example, blood vessel sensitivity to soluble guanylyl cyclase (sGC) inhibition may depend on the NO donor type or the studied vascular bed [10]. Similarly, K^+ channel blockade may inhibit relaxation, reduce NO donor potency, or have no effect depending on the NO donor or the blood vessel type (conductance *versus* resistance) [11,12].

We hypothesized that SNP and RuBPY induce relaxation through different pathways in mesenteric resistance arteries from renal hypertensive (2K-1C) and normotensive (2K) rats due to sGC/cGMP/PKG pathway activation, which then activates K^+ channels and/or sarco/endoplasmic reticulum Ca^{2+}- ATPase (SERCA). Therefore, this study aims to demonstrate that the classic NO donor SNP and the non-classic NO donor RuBPY may induce relaxation by distinct cellular mechanisms that depend on hypertension. To this end, we investigate the SNP- and RuBPY-induced intracellular mechanisms in 2K and 2K-1C rat resistance mesenteric arteries. The reason for using mesenteric resistance arteries is that they play an important role in vascular resistance control and, consequently, in blood pressure control.

2. Methods

2.1. Animals

This study employed male Wistar rats (180–200 g) maintained in a 12-h light/dark cycle with access to food (standard rat chow) and water *ad libitum*. All experimental protocols were approved by the Animal Care and Use Committee of the University of São Paulo (License CEUA 044/2008) and were conducted in accordance to the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.2. 2K-1C hypertension induction

Rats were anesthetized with tribromoethanol (2.5 mg kg^{-1}, i. p., Sigma-Aldrich, St. Louis, MO-USA). A midline laparotomy was made, and a silver clip with an internal opening of 0.2 mm was placed on the left renal artery. The sham-operated group, 2K, was submitted to the same procedure, except that the silver clip was not placed in the rat. To prevent infection, the rats received a single oxytetracyclin dose (200 mg kg^{-1}, i. m., Zoetis Manufacturing & Research Spain, Vall de Bianya, Girona-Spain). All the rats were used six weeks after the clip was implanted in the renal artery, and systolic blood pressure (SBP) was evaluated by tail plethysmography (Power Lab, AD Instruments, Bella Vista, New South Wales-Australia). 2K-1C and 2K normotensive rats had SBP $\geq$160 mmHg and $\leq$110 mmHg, respectively.

2.3. cis-[Ru(bpy)$_2$(py)(NO$_2$)](PF$_6$) synthesis

The complex *cis*-[Ru(bpy)$_2$(py)(NO$_2$)](PF$_6$), or RuBPY, was synthesized in the Analytical Chemistry Laboratory of the Department of Physics and Chemistry (Faculty of Pharmaceutical Sciences of Ribeirão Preto– USP) as described previously [13].

2.4. Vascular smooth muscle cell culture

A primary culture of rat aortic vascular smooth muscle cells (VSMCs) was obtained as described by Chi et al. (2017). Normotensive 2K rat and renal hypertensive 2K-1C rat VSMCs were maintained in growth medium (Dulbecco's modified eagle's medium, Vitrocell, 00025) with 10% fetal bovine serum (FBS, Gibco, 12657029) and 1% antibiotics/antimycotics (Sigma, St. Louis - United States) and used in passages 4–5. Before the experiments, the VSMCs were cultured in serum-free medium for 24 h. More than 90% of the cells were positive for smooth muscle-specific α-actin (measured by flow citometry), and confocal microscopy images showed typical hill-and-valley morphology of vascular smooth muscle cells as previously described by Chi and cols. (2017).

2.5. NO, reactive oxygen species (ROS), and intracellular Ca^{2+} measurement in VSMCs by fluorescence analysis

To study how VSMCs incubation with SNP or RuBPY affects NO, ROS, and Ca^{2+} concentrations, fluorescence probes were employed, namely DAF-2DA (a selective dye for NO), FLUO-3AM, (a selective dye for Ca^{2+}), and coumarin-7-boronic acid (7-CBA) (a more selective fluorescent dye for peroxynitrite that helps to detect ROS). VSMCs (10^4 cells/well) were incubated with DAF-2DA (5 μmol/L), 7-CBA (5 μmol/L), or FLUO-3AM (10 μmol/L) for 30 min. To measure NO, the cultured cells were stimulated with SNP (100 μmol/L) or RuBPY (10 μmol/L) for 5 min, in the presence or absence of the NO^0 scavenger hydroxocobalamin (0.1 mmol/L). To determine [Ca^{2+}]c, the cells were stimulated with the selective α_1-adrenergic receptor agonist phenylephrine (10 μmol/L) for 5 min, in the absence of or after incubation with thapsigargin (1 μmol/L), the non-competitive inhibitor of SERCA, for 5 min. Then, the cells were stimulated with SNP (100 μmol/L) or RuBPY (10 μmol/L) for 5 min. To measure ROS, the cells were incubated with SNP (100 μmol/L) or RuBPY (10 μmol/L) for 5 min. Fluorometric analyses were performed on a spectrofluorometer (BioTeK®) equipped with a 150-Watt Xenon lamp (Excitation/Emission: DAF-2DA 488/530 nm, 7-CBA 350/450 nm, and FLUO-3AM 506/526 nm). Slit widths were set at 10 nm for both monochromators. All the measurements were carried out at medium sensitivity. The NO donor concentrations do not promote cytotoxicity [8,14].

2.6. Functional studies

Rats were killed by decapitation and had their mesenteric bed removed and cleaned with Krebs solution in a Petri dish at 4 °C (Krebs solution, in mmol/L: NaCl 118.0, KCl 5.9, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, and $C_6H_{12}O_6$ 11.0; pH 7.4). The mesenteric artery second or third branch (internal diameter of 200–300 μm) was removed, cleaned, cut into rings (length of 2 mm), and mounted in a myograph (DMT, AD Instruments, Bella Vista, New South Wales-Australia) as described by Mulvany and Halpern [15]. To check vessel viability, contraction was induced with high extracellular KCl concentration (120 mmol/L) twice. Endothelium was removed by rubbing the vessel internal surface with human hair. Endothelium removal efficiency was assessed by verifying the absence of acetylcholine (10 μmol/L)-induced relaxation in vessels pre-contracted with phenylephrine (10 μmol/L). Then, cumulative concentration-response curves (1 nmol/L to 100 μmol/L) were constructed for SNP and RuBPY in normotensive 2K and hypertensive 2K-1C rat vessels, in arteries pre-contracted with phenylephrine (10 μmol/L).

To investigate the intracellular pathways involved in SNP and RuBPY response, concentration-effect curves were plotted in the absence or presence of (1) the sGC inhibitor (1*H*)-(1,2,4)oxadiazole(4,3-*a*) quinoxalin-1-one (ODQ, 1 μmol/L), (2) the cGMP-dependent protein

kinase inhibitor Rp-8-Br-PET-cGMPS (30 μmol/L), (3) the non-selective K^+ channel blocker tetraethylammonium (TEA, 1 mmol/L), or (4) the SERCA inhibitor thapsigargin (1 μmol/L).

2.7. Data analysis

To normalize relaxation between groups, data are represented as the percentage of the maximum effect (Emax) and are expressed as the mean ± SEM, with *n* indicating the number of different mesenteric arteries rings used in the experiment or the number of cell culture experiments. For the vascular reactivity studies, the Emax was considered as the maximal amplitude response reached in the concentration-effect curves for the relaxant agents SNP and RuBPY. The agent concentration that produced half-maximal relaxation amplitude (EC_{50}) was determined after logarithmic transformation of the normalized concentration-effect curves, and EC_{50} values are reported as the negative logarithm (pD_2) of the mean of individual values for each tissue stimulated with SNP. The RuBPY concentration-effect curves did not fit a sigmoid curve, so the EC_{50} values may not be accurate. Hence, we used the Emax values to express the RuBPY responses. Comparison of two groups was made by Student's t-test and of three or more groups by two-way ANOVA with Bonferroni post-hoc test; the software Prism Graphpad 5.0 was employed. In all cases, a p value < 0.05 was considered statistically significant.

3. Results

3.1. Primary VSMC cultures

The cells culture used in this study was stained with α-smooth muscle actin (α-SMA) antibody to confirm the vascular smooth muscle cells population. As shown in Fig. 1, VSMCs at third passage demonstrated parallel organization of elongated actin filament with a randomized distribution. At least 90% (in these representative results, 97.7%) of the cells culture used in this study was positive for α-smooth muscle cell actin.

3.2. NO released in SNP- or RuBPY-stimulated VSMCs partially depends on NO0

We examined NO release stimulated with 100 μmol/L SNP or 10 μmol/L RuBPY in 2K and 2K-1C rat VSMCs by applying the DAF-2DA probe to VSMCs extracted from 2K and 2K-1C rat aortas (Fig. 2). SNP (Fig. 2A) and RuBPY (Fig. 2B) increased DAF-2DA fluorescence in 2K and 2K-1C rat VSMCs. Fluorescence was decreased in cells pre-treated with the NO^0 scavenger hydroxocobalamin (0.1 mmol/L), indicating that NO released by SNP and RuBPY partially depends on NO^0.

3.3. SNP and RuBPY induce similar relaxation in 2K and 2K-1C rat mesenteric resistance arteries

SNP and RuBPY induced concentration-dependent vasodilator effect in 2K and 2K-1C rat endothelium-denuded mesenteric artery rings. The pre-contraction induced by phenylephrine was not different between 2K (11.67 ± 1.19 mN) and 2K-1C (12.92 ± 0.61 mN). Fig. 3 shows that SNP and RuBPY induced similar relaxation in 2K and 2K-1C rats. Tables 1 and 2 list the Emax and pD_2 values for SNP and RuBPY, respectively.

3.4. sGC activation is involved in SNP and RuBPY-induced relaxation

Fig. 4A and B shows that ODQ (1 μmol/L) dramatically reduced SNP-induced relaxation in 2K and 2K-1C rat mesenteric resistance arteries as compared to the control response. Similarly, sGC inhibition

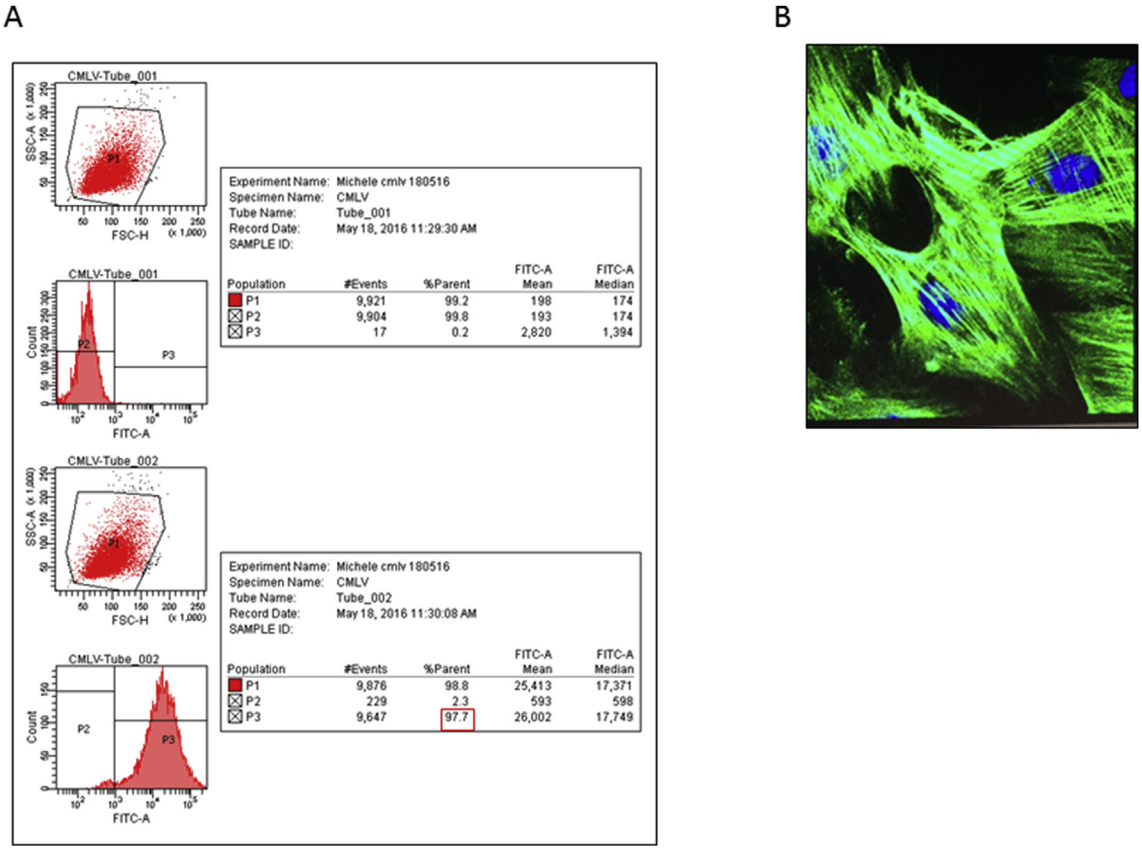

Fig. 1. Vascular smooth muscle cells (VSMC) phenotype. (A) Represents the purity analysis of isolated VSMC by flow cytometry (FACS, DIVA- BD). (B) Represents uniform immunestaining of α-smooth muscle cell actin (green) and cell nuclei with DAPI staining (blue).

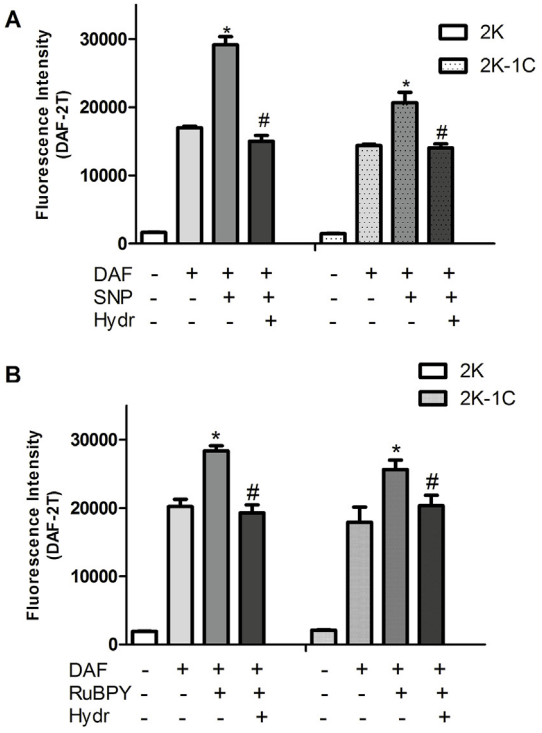

Fig. 2. NO generation by 100 μmol/L SNP (A) or 10 μmol/L RuBPY (B) in 2K or 2K-1C rat vascular smooth muscle cells (VSMCs). Fluorescence intensity emitted by the NO-sensitive probe DAF-2DA in 2K or 2K-1C rat aorta VSMCs in response to SNP or RuBPY stimulus in the presence or absence of hydroxocobalamin (Hydr, 0.1 mmol/L). Data are the mean ± SEM, n = 4. $^{*}p < 0.05$ indicates difference in relation to the control (DAF), $^{\#}p < 0.05$ indicates difference in relation to SNP or RuBPY. Two-way ANOVA with Bonferroni post-hoc analysis.

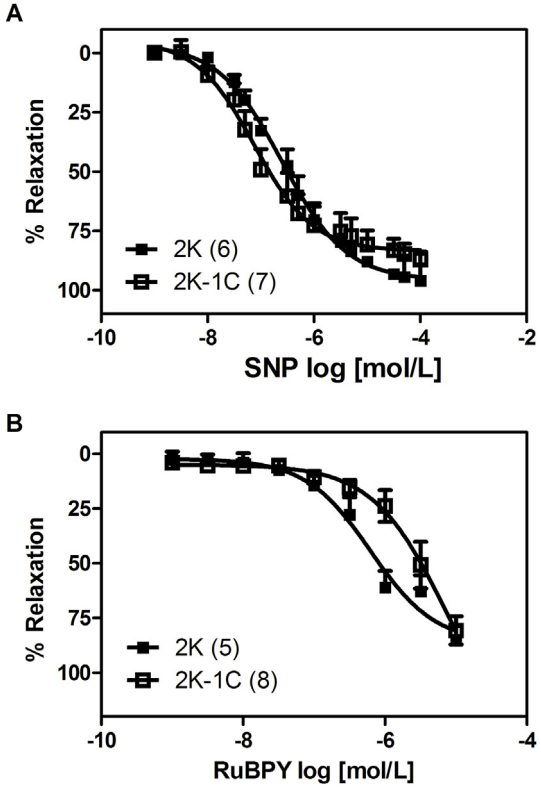

Fig. 3. SNP- (A) or RuBPY- (B) induced relaxation in 2K and 2K-1C rat endothelium-denuded mesenteric resistance arteries. Concentration-effect curves represent relaxation induced by SNP or RuBPY in arteries pre-contracted with phenylephrine (10 μmol/L). Points represent the mean ± SEM of experiments performed on different animals. The number in parenthesis means the number of animals in each group.

with ODQ (1 μmol/L) also diminished RuBPY-induced relaxation (Fig. 4C and D) in 2K and 2K-1C rats. Tables 1 and 2 summarize the Emax and pD_2 values after sGC inhibition with ODQ for SNP and RuBPY, respectively.

3.5. Protein kinase G inhibition reduces SNP- and RuBPY-induced relaxation

Rp-8-Br-PET-cGMPS (30 μmol/L) decreased SNP potency in 2K and 2K-1C rat mesenteric resistance arteries as compared to the control response (Fig. 5A and B, Table 1). Rp-8-Br-PET-cGMPS (30 μmol/L) attenuated the maximum relaxant effect (Emax) induced by RuBPY in 2K and 2K-1C rat mesenteric artery rings (Fig. 5C and D, Table 2).

3.6. K^+ channels blockade impairs SNP-induced relaxation

TEA (1 mmol/L) decreased SNP-induced relaxation response in 2K and 2K-1C rat mesenteric resistance arteries as compared to the control (Fig. 6A and B, Table 1). On the other hand, 1 mmol/L TEA did not change RuBPY-induced relaxation in 2K or 2K-1C rat mesenteric resistance arteries (Fig. 6C and D, Table 2).

3.7. SERCA activation contributes to SNP-induced relaxation in 2K rats only

Thapsigargin inhibited SNP-induced relaxation in 2K, but not in 2K-1C rat mesenteric resistance arteries as compared to the control response (Fig. 7A and B, Table 1). However, thapsigargin did not alter RuBY-induced relaxation in 2K or 2K-1C rat mesenteric artery rings (Fig. 7C and D, Table 2).

3.8. NO donors diminish $[Ca^{2+}]c$ by SERCA activation in normotensive VSMCs

We examined how SNP and RuBPY affect 2K and 2K-1C rat VSMCs pre-loaded with Fluo-3 AM, as well as their effect on Ca^{2+} levels after stimulation with phenylephrine (Fig. 8). Phenylephrine increased Ca^{2+} levels in 2K (Figs. 8A) and 2K-1C (Fig. 8B) rat VSMCs, but Ca^{2+} levels decreased after stimulation with SNP or RuBPY. To investigate whether SERCA is involved in this effect, we studied the impact of the non-competitive inhibitor thapsigargin (1 μmol/L). Thapsigargin (1 μmol/L) reduced SNP- and RuBPY-induced Ca^{2+} levels in 2K rat VSMCs only.

3.9. 2K-1C rat VSMCs have increased ROS production

We examined SNP- and RuBPY-stimulated ROS release in 2K and 2K-1C rat VSMCs with the aid of the probe 7CBA (Fig. 9). Non-stimulated 2K-1C rat VSMCs had higher 7CBA fluorescence as compared to 2K rat VSMCs. After SNP or RuBPY stimulus, there were no differences in 7CBA fluorescence.

4. Discussion

Our results demonstrate that SNP and RuBPY share the relaxing mechanisms of the classic NO/sGC/cGMP/PKG pathway. However, in terms of K^+ channels activation and reduced cytosolic Ca^{2+} levels, SNP and RuBPY follow distinct mechanisms, and 2K-1C and 2K rat vessels differ. Moreover, all the effects seem to derive from released NO given that SNP and RuBPY do not modify ROS levels.

Taking into account that NO donors are mainly used to reduce arterial pressure in the clinical setting, we studied SNP- and RuBPY-

Table 1
Effect of the inhibitors on maximum relaxant effect (Emax) and potency (pD_2) induced by sodium nitroprusside (SNP) in mesenteric arteries rings of normotensive (2K) and renal hypertensive rats (2K-1C).

	SNP					
	2K		n	2K-1C		n
	pD_2	Emax (%)		pD_2	Emax (%)	
Control	6.71 ± 0.12	96.2 ± 0.9	5	6.89 ± 0.30	87.3 ± 3.5	7
ODQ	–	10.6 ± 5.4*	5	–	14.6 ± 2.9*	5
Rp-8-Br	5.02 ± 0.22*	83.31 ± 7.4	5	5.94 ± 0.31*	94.5 ± 2.0	4
TEA	5.70 ± 0.10*	69.2 ± 6.2*	4	6.30 ± 0.12	77.1 ± 6.6*	7
Thapsigargin	5.95 ± 0.30	51.7 ± 12.9*	4	6.36 ± 0.30	74.6 ± 6.7	8

*$p < 0.05$ denotes difference between the values obtained after incubation with the given inhibitor and the control response.

Table 2
Effect of the inhibitors on maximum relaxant effect (Emax) induced by the complex cis-$[Ru(bpy)_2(py)(NO_2)](PF_6)$ (designated RuBPY) in mesenteric arteries rings of normotensive (2K) and renal hypertensive rats (2K-1C).

	RuBPY			
	2K		2K-1C	
	Emax (%)	n	Emax (%)	n
Control	76.7 ± 9.8	5	80.7 ± 6.5	8
ODQ	29.9 ± 6.9*	5	16.4 ± 9.3*	6
Rp-8-Br	43.9 ± 4.2*	5	50.9 ± 8.1*	5
TEA	77.1 ± 6.2	4	61.4 ± 9.5	6
Thapsigargin	70.3 ± 3.4	5	64.0 ± 7.3	5

*$p < 0.05$ denotes difference between the values obtained after incubation with the given inhibitor and the control response.

induced relaxation in 2K and 2K-1C rat resistance arteries.

Endothelial dysfunction is a common feature in hypertension and occurs in different experimental hypertension models [16–18] including the renal hypertension 2K-1C model [19,20]. Endothelial dysfunction is mainly characterized by reduced endothelium-dependent vascular relaxation due to altered production or release of relaxing and/or constricting endothelial factors [21,22]. Endothelial cells can positively [23] or negatively [24] modulate relaxation induced by NO donors. To avoid possible endothelial cell interference and to investigate the molecular mechanisms that SNP and RuBPY induce, we performed all the experiments in endothelium-denuded arteries or cultured VSMCs.

Initially, we investigated whether SNP and RuBPY release NO in 2K and 2K-1C rat VSMCs. Using DAF-2DA as a probe to detect NO, we verified that incubation with SNP or RuBPY for 5 min increases cytosolic NO concentration. NO^0 is the NO species related to sGC activation [25,26]. Furthermore, NO^0 species is the most important NO specie released by SNP and RuBPY because the NO^0 scavenger hydroxycobalamin decreases the NO levels induced by SNP and RuBPy. SNP and RuBPY have similar NO release profile. DAF-2DA only reacts with NO inside the cells [27], so SNP and RuBPY could release NO after chemical reactions take place inside the cells. In fact, we have already demonstrated that RuBPY does not release NO spontaneously: it requires that tissues release NO [28].

Although the SNP [29] and RuBPY [8] hypotensive effects are well described, there are gaps in the knowledge about the intracellular signaling cascade involved in SNP- and RuBPY-induced relaxation. The NO donor mechanism of action involves NO release. Nevertheless, the activated intracellular cascades may vary among NO donors, species, cell type, and disease condition.

To investigate the role that sGC activation plays in SNP- and RuBPY-induced relaxation, we used the selective inhibitor ODQ [30]. ODQ exerts its inhibitory effect through changes in the sGC heme group

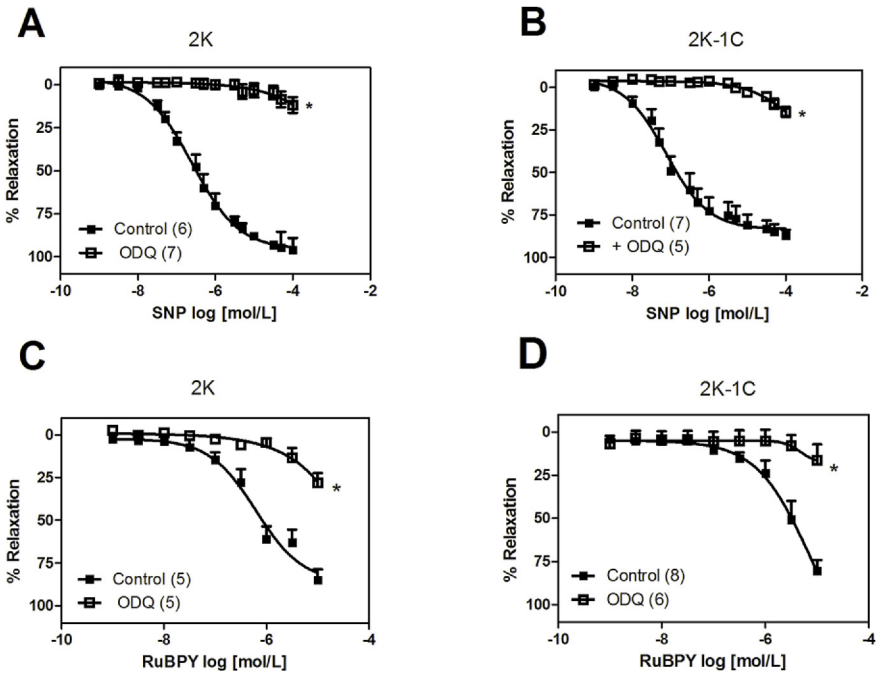

Fig. 4. sGC inhibition effect on SNP- (A,B) or RuBPY- (C,D) induced relaxation in 2K and 2K-1C rat endothelium-denuded mesenteric resistance arteries. Concentration-effect curves represent relaxation induced by SNP or RuBPY in mesenteric rings pre-contracted with phenylephrine (10 μmol/L) in the absence (black squares) or presence (white squares) of the sGC inhibitor ODQ (1 μmol/L). Points represent mean ± SEM of experiments performed on different animals. The number in parenthesis means the number of animals in each group. *$p < 0.05$ statistically different in relation to the maximum effect (Emax) of the control group, Student's *t*-Test.

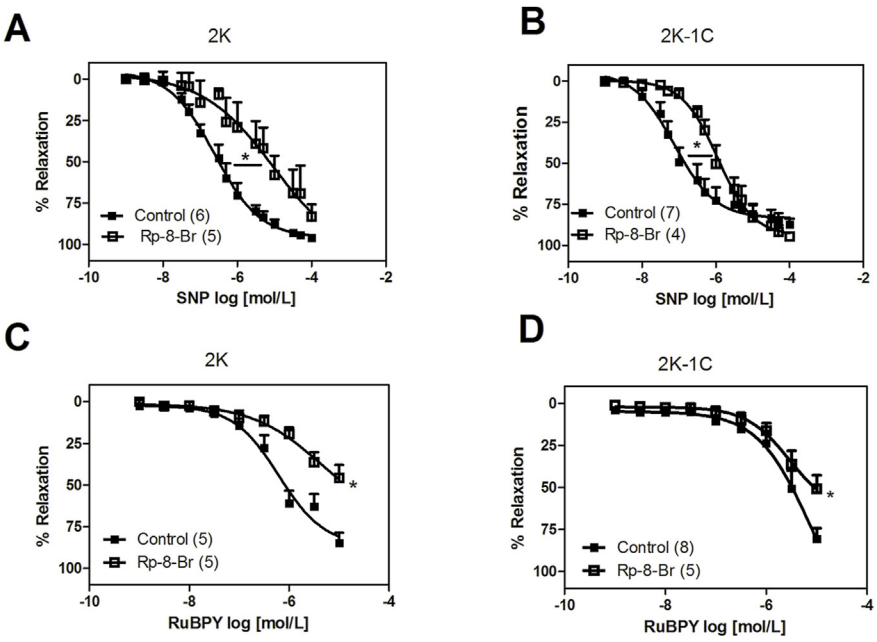

Fig. 5. Protein kinase G inhibition effect on SNP- (A,B) or RuBPY- (C,D) induced relaxation in 2K and 2K-1C rat endothelium-denuded mesenteric resistance arteries. Concentration-effect curves represent relaxation induced by SNP or RuBPY in mesenteric rings pre-contracted with phenylephrine (10 μmol/L) in the absence (black squares) or presence (white squares) of the PKG inhibitor Rp-8-Br-PET-cGMPs (30 μmol/L). Points represent the mean ± SEM of experiments performed on different animals. The number in parenthesis means the number of animals in each group. *$p < 0.05$ statistically different in relation to the maximum effect (ME) or pD_2 values of the control group, Student's *t*-Test.

oxidation state [31]. sGC is the major NO target and participates in smooth muscle relaxation induced by exogenous NO and NO provided by endothelial cells [32,33]. Here, we demonstrate an important dependence of SNP- and RuBPY-induced relaxation on sGC. According to Homer and cols. [10], sGC activation has a pivotal part in SNP-induced relaxation in rat pulmonary artery. Moreover, these authors observed the same situation for other NO donors that also require tissue activation to generate NO (i.e., nitroglycerin and isosorbide dinitrate). In contrast, NO donors that do not require tissue activation (FK409, SIN-1, MAHMANONOate, and spermine NONOate) only have their potency diminished. Other studies have also shown that ODQ inhibits relaxation induced by SNP [34,35] and other NO donors [26,36]. ODQ attenuates RuPBY-induced relaxation in almost the same way that it affects relaxation induced by SNP and another non-classic NO donor (TERPY) in rats with renovascular hypertension [37]. Therefore, our data indicate that NO species released by SNP and RuBPY operate similarly and share relaxing mechanisms at the level of sGC activation.

sGC activation by NO generates cGMP, which in turn activates the PKG enzyme [32,33]. PKG is a serine/threonine kinase that is present in various cell types, including VSMCs. PKG activation induces vasorelaxation through phosphorylation of proteins that control intracellular Ca^{2+} levels [38]. Here, we verified that PKG inhibition abates the potency of SNP-induced relaxation and the maximum effect induced by RuBPY. Using the same PKG inhibitor, Homer and Wanstall [39] showed minimal inhibition of spermine NONOate-induced relaxation but considerable impairment of glyceryl trinitrate- or isosorbide dinitrate-induced rat pulmonary artery relaxation. This PKG inhibitor prevents NO- and cGMP-stimulated increase in PKG activity in some ovine and porcine blood vessels [40–42]. On the other hand, Gao et al. [43] reported that this PKG inhibitor does not change DETA NONOate-

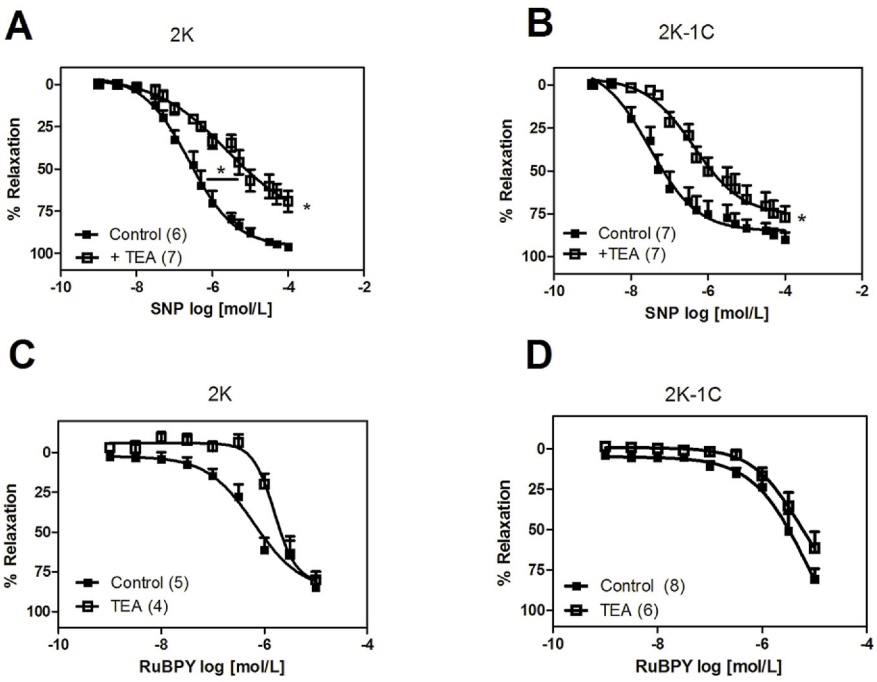

Fig. 6. Non-selective K^+ channel blockade effect on SNP- (A,B) or RuBPY- (C,D) induced relaxation in 2K and 2K-1C rat endothelium-denuded mesenteric resistance arteries. Concentration-effect curves represent relaxation induced by SNP or RuBPY in rings pre-contracted with phenylephrine (10 μmol/L) in the absence (black squares) or presence of the non-selective potassium channel blocker tetraethylammonium (TEA 1 mmol/L, white squares). Points represent the mean ± SEM of experiments performed on different animals. The number in parenthesis means the number of animals in each group. *$p < 0.05$ statistically different in relation to the maximum effect (Emax) or pD_2 values of the control group, Student's *t*-Test.

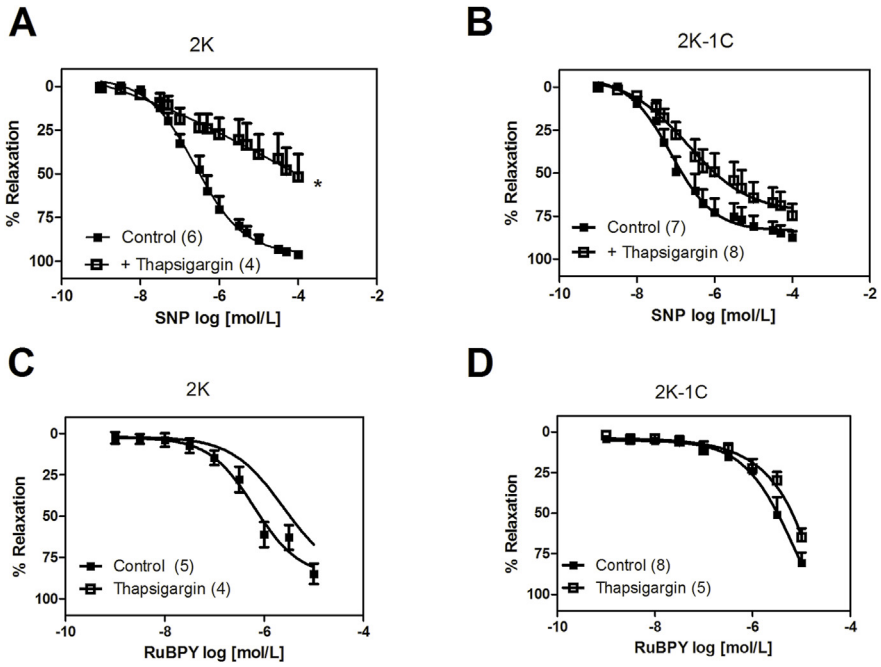

Fig. 7. SERCA inhibition effect on SNP- (A,B) or RuBPY- (C,D) induced relaxation in 2K and 2K-1C rat endothelium-denuded mesenteric resistance arteries. Concentration-effect curves represent relaxation induced by SNP or RuBPY in mesenteric rings pre-contracted with phenylephrine (PE 10 μmol/L) in the absence (black squares) or presence (white squares) of the SERCA inhibitor Thapsigargin (Thaps 1 μmol/L). Points represent the mean ± SEM of experiments performed on different animals. The number in parenthesis means the number of animals in each group. $^{\#}p < 0.05$ statistically different in relation to the maximum effect (Emax) of the control group, Student's *t*-Test.

induced relaxation in fetal lamb pulmonary arteries and veins. In summary, PKG participation varies depending on the NO donor and the vascular bed, but it underlies relaxation induced by most NO donors, including SNP and RuBPY.

Potassium channels may also participate in NO donor-induced relaxation. The PKG catalytic subunit can activate K^+ channels [43]. Here, we found that SNP- and RuBPY-induced relaxation is similar in 2K and 2K-1C rat denuded mesenteric arteries. In aortic rings, relaxation induced by SNP and another NO donor (15-ane) is less intense in 2K-1C than in 2K rats due to impaired K^+ channel activation [44]. However, SNP-induced relaxation is not impaired in mesenteric resistance arteries from Angiotensin II-infused or genetically hypertensive rats [45,46]. In contrast, Zhou et al. [47] proposed that despite channel phosphorylation, PKG activates a protein phosphatase that activates K^+ channels by dephosphorylation. PKG can directly or indirectly activate K^+ channels. In addition, NO can directly activate K^+ channels [48]. The non-selective K^+ channel blocker TEA reduces the potency of SNP and another NO donor, $[Ru(terpy)(bdq)NO]^{3+}$, in rat aortic rings [36]. On the basis of our data, the non-selective K^+ channel blocker TEA reduces SNP-induced relaxation. Thus, the alteration mediated by hypertension in K^+ channel activity could not be present in 2K-1C rat mesenteric arteries. In fact, we verified that SNP-induced relaxation depends on K^+ channel activation. On the other hand, RuBPY seems to overwhelm K^+ channel dependence to induce its effects.

SERCA is another enzyme that may participate in NO-induced relaxation. SERCA phosphorylation together with ATP hydrolysis results in Ca^{2+} translocation to the sarcoplasmic reticulum lumen [49]. Consequently, cytosolic Ca^{2+} concentration diminishes, to culminate in vascular relaxation. NO can activate this mechanism [50], which may occur via PKG activation [51]. The SERCA inhibitor thapsigargin inhibits SNP-induced relaxation in piglet pulmonary and mesenteric arteries and mouse aorta [50,52], suggesting that this relaxation involves SERCA activation. In our study, we observed that thapsigargin inhibits SNP-induced relaxation in 2K mesenteric arteries only. Moreover, thapsigargin does not affect RuBPY-induced relaxation in 2K or 2K-1C mesenteric arteries.

Fluorometric assays conducted on 2K rat VSMCs revealed that reduction in cytosolic Ca^{2+} levels induced by SNP and RuBPY depends on SERCA. However, in 2K-1C rat vessels, reduction in cytosolic Ca^{2+} levels induced by RuBPY and dependent on SERCA is not mediated by the same mechanism as in 2K rat vessels.

SNP and RuBPY lower Ca^{2+} levels in a SERCA-independent way in 2K-1C rat VSMCs. The fact that SNP acts to diminish Ca^{2+} levels irrespective of SERCA activity could indicate altered SERCA activity. Some studies have demonstrated that SERCA function can be either normal or increased in hypertension [53–56]. On the other hand, it was reported that SERCA activity or expression can be reduced in hypertension. According to Webb and Bhalla [57], the microsomal fraction of aorta obtained from spontaneously hypertensive rats (SHRs) has smaller calcium-sequestering ability as compared to normotensive rat aortas. Similarly, Ceron and Bendhack [58] verified diminished SERCA activity in aortas from renal hypertensive rats (1K-1C), whereas Li et al. [59] saw reduced $SERCA_{2a}$ protein expression in SHR cardiomyocytes.

It has been described by Salomone et al. [60] that NO and cGMP pathway facilitates the inhibitory effects of Ca^{2+} channel blockers on KCl-induced contraction of rat aortas. The authors suggested that it is due to the decreased sensitivity of the contractile machinery to Ca^{2+} by cGMP. Our results are suggestive that even with no reduction in cytosolic Ca^{2+} levels, RuBPY induced relaxation due to increased cGMP levels that could decrease the contractile machinery sensitivity to Ca^{2+}. In contrast, SNP relies on diminished Ca^{2+} levels as an important relaxation mechanism, which changes in hypertension. This information indicates that NO released from different NO donors can induce differential PKG activity. Moreover, PKG could lower Ca^{2+} levels in a SERCA-independent way as a compensatory mechanism to overwhelm a possible alteration or requirement in SERCA activity to promote smooth muscle relaxation in hypertension.

ROS have an essential role in normal [61] and in pathological situations. Higher ROS load has been investigated and correlated with cardiovascular diseases, especially hypertension [22,62]. The superoxide anion could react with NO, raising the peroxynitrite levels, and consequently decreasing NO bioavailability. In renovascular hypertension, oxidative stress is high and its reduction reverts some hypertension features like endothelial dysfunction [63–65]. Our data demonstrate that neither SNP nor RuBPY modifies the high peroxynitrite levels in 2K-1C rat VSMCs. These effects could be related to NO derived from SNP or RuBPY rather than to indirect effects of peroxynitrite levels.

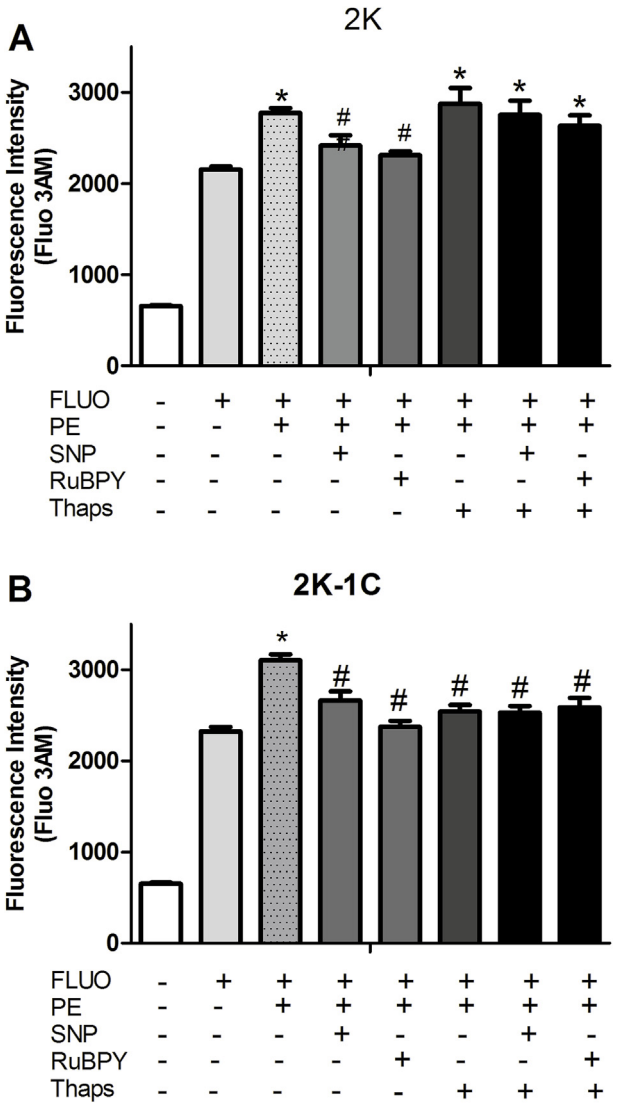

Fig. 8. Effect of SNP or RUBPY on cytosolic Ca^{2+} concentration [(Ca^{2+})] in 2K (A) and 2K-1C (B) rat aorta VSMCs. Fluorescence intensity emitted by the [(Ca^{2+})]-sensitive probe FLUO3-AM in 2K or 2K-1C rat aorta VSMCs in response to 100 μmol/L SNP or 10 μmol/L RuBPY after pre-incubation with phenylephrine (PE 10 μmol/L) in the presence or absence of Thapsigargin (Thaps 1 μmol/L). Data mean ± SEM, n = 4. $^{*}p < 0.05$ indicates difference in relation to the control (FLUO-3AM). $^{\#}p < 0.05$ indicates difference in relation to FLUO + PE, Two-way ANOVA with Bonferroni post-hoc analysis.

5. Conclusion

Our results demonstrate that the classic NO donor SNP and the non-classic NO donor RuBPY can induce different molecular mechanisms even though they have similar relaxation actions. Moreover, the normal pattern of molecular mechanism activation to elicit NO release from NO donors and induce relaxation can be altered during hypertension.

Author contributions

Alice V. Araújo, Fernanda A. Andrade and Lusiane M. Bendhack conceived and designed the experiments. Alice V. Araújo, Amanda C. Pereira and Fernanda A. Andrade conducted the functional experiments and analyzed the data. Michele Paulo, Tiago Dal-Cin de Paula and Simone R. Potje conducted the fluorimetric assays and analyzed the data. All the authors wrote and revised the manuscript.

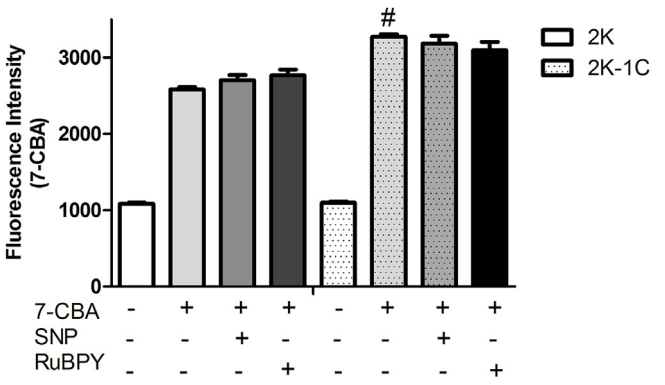

Fig. 9. ROS generation by 100 μmol/L SNP (A) or 10 μmol/L RuBPY (B) in 2K or 2K-1C rat aorta VSMCs. Fluorescence intensity emitted by the ROS-sensitive probe 7-CBA in 2K or 2K-1C VSMCs in response to SNP or RuBPY. Data mean ± SEM, n = 4. $^{\#}p < 0.05$ indicates difference between 2K + 7-CBA, Two-way ANOVA with Bonferroni post-hoc analysis.

Conflicts of interest

The authors declare no conflicts of interest.

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