Hypotensive and vasorelaxing effects of the new NO-donor [Ru(terpy)(bdq)NO$^+$]$^{3+}$ in spontaneously hypertensive rats

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ABSTRACT

Drugs that release nitric oxide (NO) usually have limitations due to their harmful effects. Sodium nitroprusside (SNP) induces a rapid hypotension that leads to reflex tachycardia, which could be an undesirable effect in patients with heart disease, a common feature of hypertension. The nitrosyl ruthenium complex [Ru(terpy)(bdq)NO$^+$]$^{3+}$ (TERPY) is a NO donor that is less potent than SNP in denuded aortic rings. This study evaluated the hypotension and vasorelaxation induced by this NO donor in Wistar (W) and spontaneously hypertensive rats (SHR) and compared to the results obtained with SNP. Differently from the hypotension induced by SNP, the action of TERPY was slow, long lasting and it did not lead to reflex tachycardia in both groups. The hypotension induced by the NO-donors was more potent in SHR than in W. TERPY induced relaxation with similar efficacy to SNP, although its potency is lower in both strains. The relaxation induced by TERPY is similar in W and SHR, but SNP is more potent and efficient in SHR. The relaxation induced by TERPY is partially dependent on guanylate cyclase in SHR aorta. The NO released from the NO donors measured with DAF-2 DA by confocal microscopy shows that TERPY releases similar amounts of NO in W and SHR, while SNP releases more NO in SHR aortic rings.

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Introduction

The exogenous administration of nitric oxide (NO) is an attractive pharmacological alternative in the study and treatment of hypertension. The chemical versatility of this molecule allows the synthesis of a wide variety of NO-donors, each one with different rates of NO release [1–3]. The vasodilatation induced by NO donors occurs in different ways, depending on the localization where the NO is released and the mechanism involved in this process [4,5]. The amount of NO released by a donor is a very important factor, since its cardiovascular actions only occur in very low concentrations, being very toxic in higher concentrations [6,7].

Sodium nitroprusside (SNP) is a classic NO-donor, often used to verify the relaxation of vascular smooth muscle (VSM) via NO-cGMP. The molecule of NO is coordinated to an iron metal, forming an octahedral complex with five cytotoxic cyanide anions [8–12]. The rapid hypotension evoked by SNP makes it useful in hypertensive emergencies, but the reflex tachycardia could be undesirable for patients with heart disease, a common feature in hypertension.

In this case, NO donors with slower release of NO would minimize these effects [3,5,13].

Trying to overcome these limitations, new NO-donors have been developed. Metal complexes of ruthenium have the advantage of releasing NO in a specific biological target and the time to reach maximum relaxation can be modulated according to the structure and the particular characteristic of each compound [14]. The nitrosyl ruthenium complex [Ru(terpy)(bdq)NO$^+$]$^{3+}$ (TERPY, Fig. 1) is a NO donor that is not toxic in the concentrations used to cause maximum relaxation in isolated aortic rings [15]. In denuded aorta of normotensive or hypertensive rats, TERPY is less potent than SNP and the vasodilatation induced by both donors is more pronounced in the aortas of 2K-1C rats [16]. It could be caused by a decrease in the bioavailability of the NO released by TERPY in 2K-1C rat aorta, compared to the normotensive control [17,18].

In the few studies conducted to evaluate the hypotensive response of ruthenium compounds, the blood pressure decrease was higher in hypertensive (SHR, 2K-1C) in relation to normotensive rats [19,20]. There is no published data regarding the hypotensive effects of TERPY. We hypothesized that in spontaneously hypertensive rats, the hypotensive and vasorelaxing effects of TERPY were altered. Therefore, the aim of the present study was to evaluate the hypotensive and vasodilatory effects of TERPY in SHR and to assess the NO released by TERPY in the isolated aorta of these animals.

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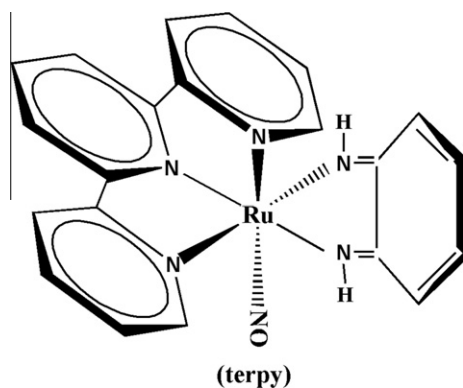E-mail address: crisant@foa.unesp.br (C. Antoniali).

Fig. 1. Molecular structure of $[Ru(terpy)(bdq)NO^+]^{3+}$.

Methods

All experiments were approved by the Animal Research Ethics Committee (CEEA) at the Faculty of Dentistry of Araçatuba, UNESP (process number 001776-2010).

Animals

Spontaneously hypertensive rats (SHR) and normotensive Wistar rats (W) with 120 days were housed under standard laboratory conditions (12 h light/dark cycle at 21 °C with free access to food and water). The systolic blood pressure (SBP) was evaluated by tail pletismograph (Narco Biosystems–Houston, Texas, USA). Only SHR with SBP $\geqslant$ 150mm Hg were used in this study.

Cannulae implantation surgery

Animals anesthetized with Ketamin (45 mg/kg) and Xilasin (5 mg/kg) had a polyethylene cannula (PE10 connected to PE50) filled with heparinized saline implanted into the abdominal aorta, throughout the femoral artery, for blood pressure recording and into the femoral vein for administration of the NO-donors. The cannulae were exteriorized in the back of the neck and the animals were kept in standard laboratory conditions for 24 h before the experiments.

Mean arterial pressure measurement

The blood pressure was continuously recorded in conscious rats using a pressure transducer and an amplifier (ADInstruments) attached to the intra-arterial cannula. The mean arterial pressure (MAP) in basal conditions and during NO donor administration was calculated using the software 4 Chart (ADInstruments).

Evaluation of the hypotensive effects of the NO-donors

TERPY was dissolved in sodium chloride 0.15 M, protected from light and stored at -20 °C. Before the drug administration, basal arterial pressure and heart rate were determined by recording the MAP during 30 min until pressure stabilization. In the same animal, 5 mg/kg (T_5) and 7 mg/kg (T_7) of TERPY and 35 µg/kg of SNP were injected in a random order. These doses were used based on previous observations from our research group. The ΔMAP was calculated using Chart 4 software, based on the difference on the MAP values at basal condition and until the maximum hypotensive effect after the drugs administration. The elapsed time from the initial of the BP decrease up to the final hypotensive effect achieved (T) was also calculated. The heart rate variation (ΔHR) was calculated through the difference of the basal heart rate values and the mean of the heart rate after the infusion of the drugs.

Vascular reactivity studies

Animals were decapitated and the thoracic aorta was quickly removed, dissected and cut in 4–5 mm rings. The endothelium was mechanically removed. The aortic rings were placed between two stainless-steel stirrups and connected to an isometric force transducer (Letica Scientific Instruments, Barcelona, Spain) for tension measurement. They were kept in a chamber containing Krebs solution with the following composition (mmol/L): NaCl 130.0; KCl 4.7; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 14.9; glucose 5.5 and $CaCl_2$ 1.6; pH 7.4 gassed with 95% O_2 and 5% CO_2 at 37 °C. Each ring was stretched to a 1.5 g resting tension during 60 min for stabilization. During this period, tissues were washed every 15 min. The absence of the endothelium was qualitatively assessed by the lack of acetylcholine (1 µM) induced relaxation in the presence of contractile tone induced by phenylephrine (Phe, 0.1 µM). After washing and stabilizing periods, the rings were stimulated with Phe and cumulative concentrations of TERPY (1 nM–100 µM) and SNP (0.1 nM–10 µM) were added.

The participation of the enzyme soluble guanylate cyclase (sGC) on TERPY and SNP effects was evaluated by incubation of the aortic rings with the selective sGC inhibitor 1H-(1,2,4)oxadizolo(4,3-a)-quinoxalin-1-one (ODQ, 1 µM) for 30 min, before Phe contraction. Then, cumulative concentration–response curves to TERPY (1 nM–100 µM) or SNP (0.1 nM–10 µM) were obtained in the presence of ODQ.

Confocal microscopy

Arterial cross-sections of 100 µm thick for each aortic ring were placed vertically in a glass slide covered with poly-L-lysine. The tissue was loaded with the fluorescent NO dye, diaminofluorescein-2 diacetate (10 µM DAF-2 DA), for 40 min at room temperature in normal Hanks buffer with the following composition in mM: 1.0 $MgCl_2$, 145.0 NaCl, 5.0 KCl, 0.5 NaH_2PO_4, 10.0 dextrose and 10.0 HEPES, pH 7.4. After washing, the segment was placed on the confocal scanning laser microscope (Leica TCS SP2) and viewed from the bottom of the chamber through a water-immersion objective ($63\times$). Images of the aortic ring segment were sequentially taken every 2 s in Hanks buffer (pH 7.4) with 1.6 mM of $CaCl_2$. Nitric oxide was quantified by measuring the fluorescence intensity (FI) by using fluorescent dye DAF-2 DA that intracellularly is converted to DAF-2T. The cytosolic NO concentration ($[NO]_c$) was assessed by exciting the DAF-2 DA fluorophore with the 488 nm line of an argon ion laser and measuring the fluorescence at 515 nm. A time-course software was used to capture images of the cells at intervals of 2 s (xyt) in the Live Data Mode acquisition at 512×512 pixel resolution, at 400 Hz. The protocol was designed to measure $[NO]_c$ in denuded slices of rat aorta. $[NO]_c$ was measured in the slices of aorta before and after addition of the NO donors in the concentration used to cause maximum relaxation in the vascular reactivity studies (TERPY – 1 µM, SNP – 10 µM). By applying the computer software of the LSCM, the intensities of intracellular maximum or minimum fluorescence were measured. In this way, the difference in fluorescence intensity (DFI) between the basal spectrum (F_0) and the spectrum recorded after addition of the NO donor (F) reflects the increase in $[NO]_c$, which was calculated by the formula: $DFI = (F - F_0)$. The fluorescence signal used to calculate $[NO]_c$ was measured in an area of aortic smooth muscle tissue and averaged. The increase in $[NO]_c$ stimulated by each NO-donor was compared between aortic rings of normotensive and hypertensive rats.
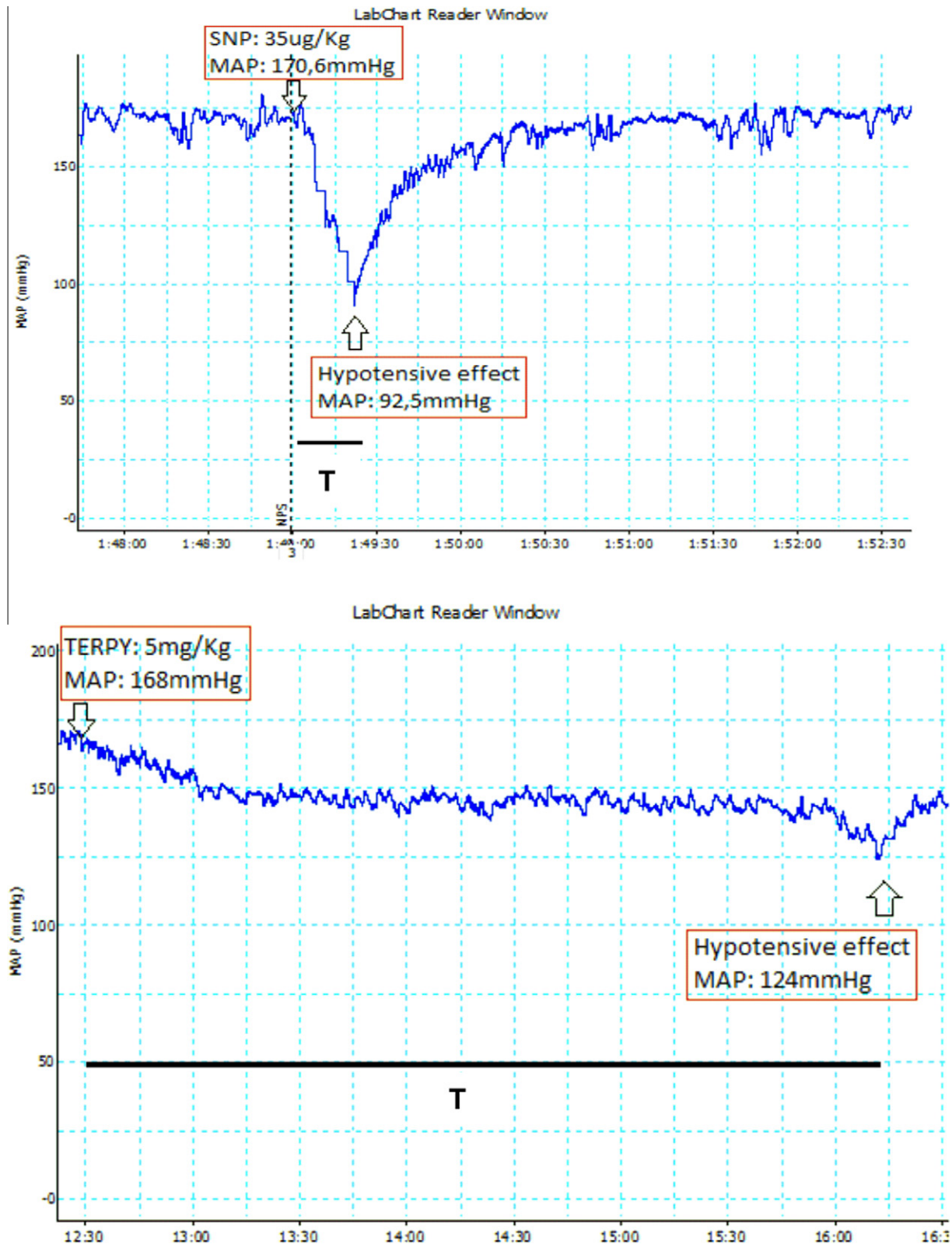
Fig. 2. Representative recording of the mean arterial pressure (MAP) showing the hypotensive effect of SNP (upper) (in seconds) and TERPY (lower) (in minutes) in SHR. *T* indicates the maximum hypotensive effect latency for each NO-donor (Chart 7 – ADInstruments).

Data analysis

Data are expressed as the mean ± SEM. The *in vivo* experiments analyzed the ΔMAP and the time to reach the maximum effect (*T*) and compared between groups. In the vascular reactivity studies

the maximum effect (Emax) and the potency (pD_2) were evaluated. Emax is defined as the maximal amplitude response reached in the concentration–effect curves for each relaxant agent. The concentration of the agent producing the half-maximal relaxation amplitude (EC_{50}) was determined after logit transformation of the normalized
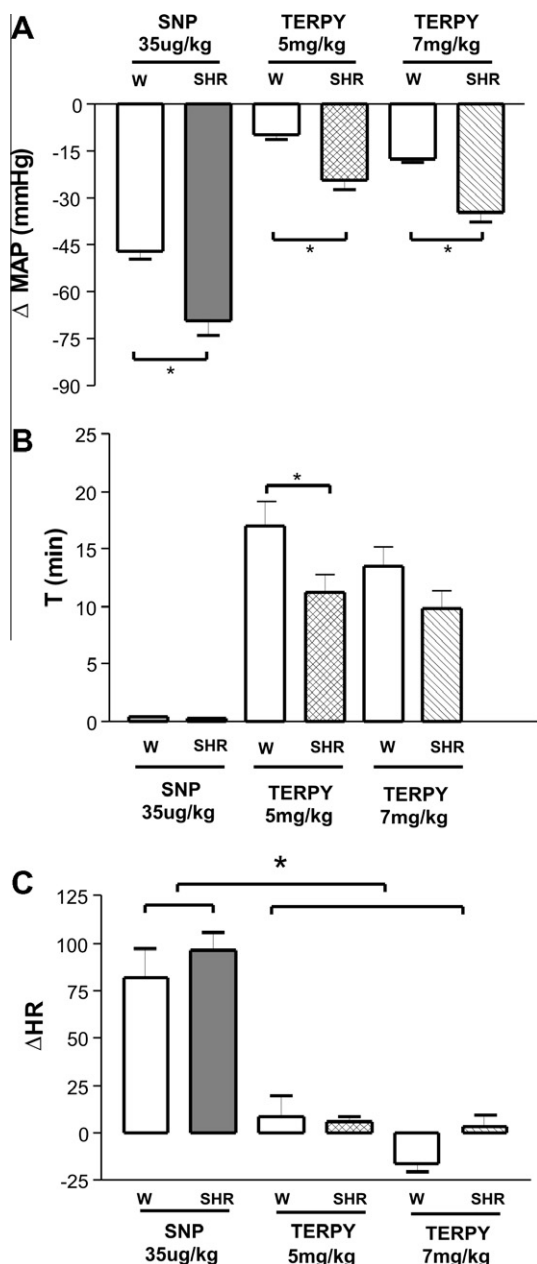
Fig. 3. (A) Magnitude of the hypotensive effect of intravenous *in bolus* injections of SNP (35 μg/kg) and TERPY (5, 7 mg/kg) on the variation of mean arterial pressure (ΔMAP) of conscious Wistar (*W*) and SHR. (B) The maximum effect latency (*T*) refers to the elapsed time from the initial of the MAP decrease up to the maximum hypotensive effect achieved. (C) Heart rate variation (ΔHR) during the infusion of the drugs until maximum effect. Data reported as mean ± SEM. *$p < 0.05$.

concentration–response curves and the EC_{50} values are reported as the negative logarithm (pD_2) of the mean of individual values for each tissue. For the confocal studies, the DFI of SNP and TERPY was evaluated. All the results obtained were compared between normotensive (W) and hypertensive animals (SHR). Statistical analysis was performed by Student's *t* test using the Graph Pad Prism 5.0 program. Differences were considered statistically significant when $p < 0.05$.

Drugs

ACh, Phe, SNP, ODQ and DAF-2DA were obtained from Sigma Chemical (St Louis, MO, USA). TERPY was synthesized in the laboratory of Dr. Roberto Santana da Silva (Faculty of Pharmaceutical Science of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil).

Results

Hypotensive effects of NO-donors

The basal MAP was increased in SHR (W: 112.7 ± 2.6 mm Hg, $n = 5$; SHR: 160.3 ± 3.1 mm Hg, $n = 7$). SNP and TERPY evoked hypotensive effects on conscious hypertensive and normotensive rats. TERPY evoked a slow and long lasting hypotensive effect, in contrast to the fast and potent effect of SNP (Fig. 2). The effect of SNP was also higher in SHR (−69.3 ± 4.5 mm Hg, $n = 7$) than in Wistar (−46.7 ± 2.8 mm Hg, $n = 5$). The magnitude of the hypotensive responses of TERPY was more accentuated in SHR: (T_5: −24.5 ± 2.9 mm Hg; T_7: −34.8 ± 2.7 mm Hg, $n = 7$) than in Wistar rats (T_5: −9.7 ± 1.6 mm Hg, $n = 5$; T_7: −17.3 ± 1.3 mm Hg, $n = 4$) (Fig. 3A). There were no differences in the time needed to reach the maximum hypotensive effect of SNP between the groups (0.5 min in both groups). The time to reach the maximum effect (T) of TERPY 5 mg/kg was reduced in SHR, however there were no differences in the T of TERPY 7 mg/kg between the groups (in min, T_5: W: 17.0 ± 2.1; SHR: 11.3 ± 1.5; T_7: W: 13.5 ± 1.7; SHR: 9.8 ± 1.5) (Fig. 3B). SNP hypotensive effect was associated with an increase on heart rate of SHR and Wistar rats (ΔHR W: 81.8 ± 15.3 bpm, SHR: 96.3 ± 9.1 bpm). However, no alteration of heart rate was observed during the hypotensive effect of TERPY (ΔHR T_5: W: 8.9 ± 10.9 bpm, SHR: 6.0 ± 2.8 bpm, T_7: W: −16.20 ± 3.88 bpm, SHR: 3.18 ± 6.07 bpm) (Fig. 3C).

Relaxing effect of TERPY in denuded aortic rings

SNP and TERPY evoked vasorelaxation on aortic rings of hypertensive and normotensive rats. TERPY was less potent than SNP in aortic rings of both groups (Fig. 4A). The Emax induced by SNP is increased in SHR aortic rings (W: 105.9 ± 3.3%; $n = 5$; SHR: 118.8 ± 7.3%; $n = 5$, $p < 0.05$). The potency of SNP was higher in the aorta of SHR (pD_2: 8.74 ± 0.15; $n = 5$, $p < 0.05$) than in Wistar (pD_2: 7.97 ± 0.07; $n = 5$). However, the vasodilator effect of TERPY was not different between Wistar (Emax: 103.5 ± 1.1%, pD_2: 6.60 ± 0.08, $n = 5$) and SHR (Emax: 106.3 ± 4.8%, pD_2: 6.25 ± 0.07, $n = 5$; Fig. 4B).

Effect of ODQ on the relaxation induced by TERPY in SHR denuded aortas

In the presence of ODQ (1 μM), the relaxation–response curves to TERPY were shifted to the right in aortic rings of Wistar (pD_2: 4.8 ± 0.08, $n = 5$; Fig. 5A) and SHR (pD_2: 4.9 ± 0.4, $n = 5$; Fig. 5B). ODQ reduced the efficacy of TERPY in aortas from Wistar (Emax: 90.2 ± 3.01%, $n = 5$), but not from SHR (Emax: 97.7 ± 2.7%, $n = 5$). ODQ incubation shifted to the right the curves of SNP in Wistar (pD_2: 6.22 ± 0.2, $n = 5$; Fig. 5C) and SHR (pD_2: 7.18 ± 0.13, $n = 5$; Fig. 5D). There was no difference in the Emax of SNP in Wistar aortic rings (Emax: 96.57 ± 1.90%, $n = 5$). In SHR aorta, it reached more than 100% (Emax: 104.64 ± 7.3%, $n = 5$) of relaxation, but it was still not as effective as in the control group (Emax: 142.85 ± 10,7%, $n = 5$).

Confocal microscopy

NO measurement in the cell milieu was accomplished by confocal microscopy. The cytosolic NO concentration ([NO]$_c$) in the vascular smooth muscle of aortic rings was obtained in the absence and after the addition of SNP (1 μM) or TERPY (10 μM). The DFI after
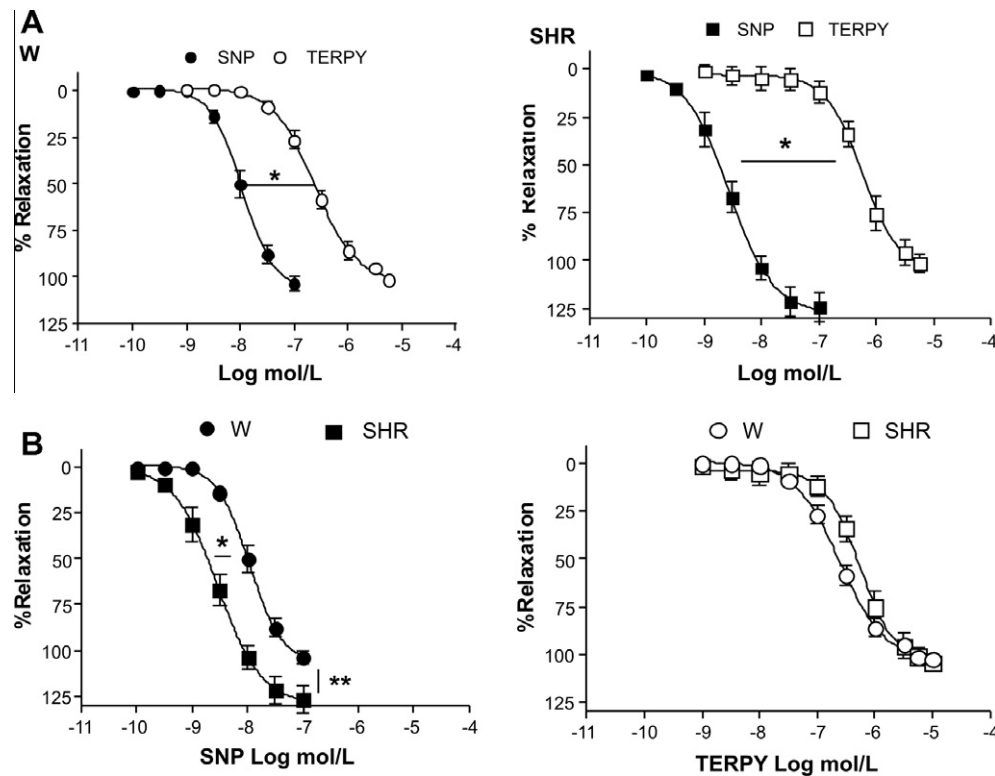
Fig. 4. The NO donors were cumulatively added in isolated rings previously contracted with phenylephrine (100 nM). (A) Vasodilatation induced by SNP (0.01 nm–1 μM) and TERPY (1 nm–100 μM) in denuded aortic rings of Wistar (W) and SHR. (B) Comparison of the effect of the drugs between Wistar (W) and SHR denuded aortic rings. The data are presented as mean ± SEM and presents the percentage of relaxation in relation to the value of the initial contraction. Differences in the pD_2 and the Emax were considered significant when $p < 0.05$.
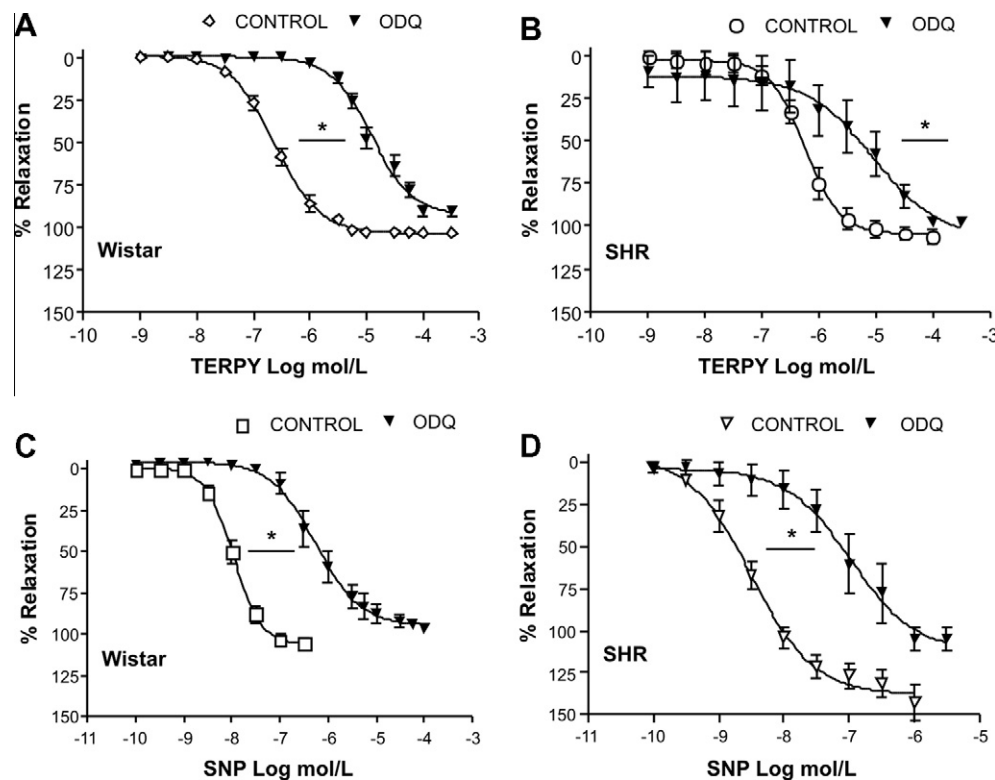

Fig. 5. The rings were incubated with 1H-(1,2,4)oxadizolo(4,3-a)-quinoxalin-1-one (ODQ) (1 μmol) for 30 min prior to the application of phenylephrine (0.1 μmol). The effect of (ODQ) on the relaxation induced by the TERPY in denuded rat aortic rings pre-contracted with phenylephrine was studied in Wistar (A) and SHR (B). The effects of ODQ in the relaxation induced by SNP were also studied in Wistar (C) and SHR (D). Data are means ± SEM of experiments performed on preparations obtained from different animals. *$p < 0.05$ between pD_2 values.
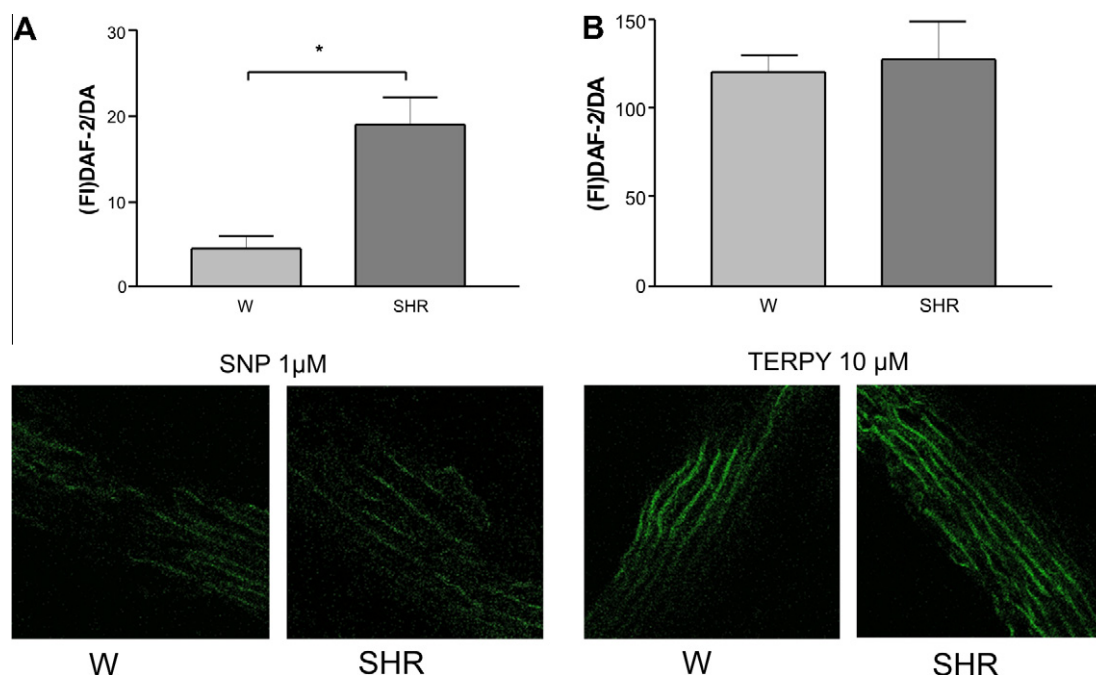
Fig. 6. Cytosolic concentration of NO in denuded aortic rings of Wistar (W) and SHR. Fluorescence of DAF-2 DA (10 μM) was recorded in slices of aortas before and after the addition of the NO donors: (A) SNP (1 μM); (B) TERPY (10 μM). The difference in fluorescence intensity (DFI) between the F_0 (basal spectrum) and F (spectrum recorded after addition of the NO-donors) reflects the increase in $[NO]_c$, which was calculated by the formula: DFI = $(F−F_0)$. Fluorescent images of cytosolic concentration of nitric oxide ($[NO]_c$) in a representative slice of rats denuded aorta preloaded with DAF-2 DA. Data are mean ± SEM of experiments performed. Difference between the values of DFI obtained in aortic rings of SHR and Wistar was considered significant when $*p < 0.05$.

stimulation with SNP was increased in SHR aorta (18.94 ± 3.19, $n = 4$) when compared to Wistar (4.48 ± 1.56, $n = 6$, Fig. 6A). There were no differences in DFI of TERPY between Wistar (120.34 ± 9.44, $n = 5$) and SHR (127.01 ± 21.85, $n = 4$, Fig. 6B).

Discussion

The present data bring the first evidence of the hypotensive effect of TERPY in rats. Opposite to the observed SNP effect, the hypotension induced by this new NO-donor is slow and long lasting and does not evoke changes in the heart rate (Fig. 2). TERPY is less potent than SNP to induce hypotensive effect. The magnitude of the hypotensive response to the NO-donors used was more accentuated in SHR than in Wistar rats. There were no significant differences in the duration of the hypotension of the NO-donors in Wistar or SHR. The only difference found was in the T of TERPY 5 mg/kg, that was slower in SHR (Fig. 3). Few studies evaluated the hypotensive effect of ruthenium complexes in conscious animals. de Gaitani et al. [20] have observed that the hypotensive effect of *trans*-[RuCl([15]aneN4)NO]$^{2+}$ (15-ANE) was significantly increased in hypertensive rats (2K-1C) and that was higher in severely hypertensive than in moderately hypertensive 2K-1C rats. In a study with SHR, de Barros et al. [19] verified that the hypotensive response of the NO-donor (*trans*-[Ru(NO)(NH$_3$)$_4$(POEt)$_3$](PF$_6$)$_3$) was also increased in SHR when compared to Wistar rats. However, in SHR, the hypotensive effect of Ru(NO)(NH$_3$)$_4$(POEt)$_3$(PF$_6$)$_3$ was higher than the effect of SNP, which differs from the results obtained with TERPY.

In order to understand if the hypotensive effect of TERPY could be modulated by the vasodilator effect of this drug, the effect of TERPY was studied in isolated denuded aortic rings. As expected, TERPY induces relaxation in aortic rings pre-contracted with phenylephrine in a concentration-dependent manner, but it is less potent than SNP as demonstrated by previous studies of our

research group [17,18]. These results are also in accordance to Bonaventura et al. [21] and Pereira et al. [22] that have reported a higher potency of SNP compared to other nitrosyl complexes (15-ANE and cis-[Ru(bpy)$_2$(py)NO$_2$](PF$_6$) (RuBPY), respectively). In the present study, the relaxation induced by SNP is more potent in SHR aorta than in the aorta of normotensive rats, however the concentration–effect curves of TERPY in Wistar and SHR are similar (Fig. 4). These results differ from results obtained in other hypertensive model. Bonaventura et al. [21] observed that the effect of the NO-donors SNP and 15-ANE were impaired in 2K-1C rat aorta, and Rodrigues et al. [17,18] also observed an impaired relaxation induced by SNP and TERPY in 2K-1C aorta.

To study the mechanism of action of TERPY, we evaluated its vasorelaxation after incubation with ODQ, a selective sGC inhibitor [23]. The inhibitory effect of ODQ is due to changes in the oxidation state of the heme moiety of the enzyme, without adverse effects on its catalytic activity. Although ODQ has been reported to interfere with heme-protein-dependent processes [24], it remains the most selective and potent inhibitor available of sGC. Vascular relaxation sensitivity to ODQ inhibition is an indicative of a predominant mechanism of heme site mediated activation of sGC, whereas resistance toward ODQ suggests the possible presence of alternative mechanisms of vasorelaxation. In the concentration of 1 μM, ODQ abolished the relaxation induced by TERPY [25] and 15-ANE [21] in 2K-1C aortas. These results differ from what is observed in SHR aortas, where the concentration–response curves to TERPY and SNP, are shifted to the right in the presence of ODQ (1 μM), but were not abolished (Fig. 5). These results suggest that while the sGC seems to be the most important pathway involved in relaxation induced by TERPY in 2K-1C aorta, this NO donor can elicit vascular relaxation in SHR aortas, at least in part, through a pathway that is independent of the activation of sGC.

To investigate if the amount of NO released by TERPY would account for the similar results of vascular reactivity between Wistar and SHR, the NO concentration in the vascular smooth muscle of

aortic rings of both groups after the stimulation with the drugs was determined by using the NO sensitive dye, DAF-2 DA (Fig. 6). It was observed that the NO concentration is enhanced after the NO donors addition. Considering that NO was measured in denuded aortic rings, the enhanced fluorescence from the bound DAF can be attributed to NO release, as demonstrated by previous observations [17,18,22]. Through this technique, it was possible to verify that when SNP was added, the amount of NO sensitive dye was significantly increased in SHR aortic rings, which did not occur when the NO was released from TERPY. SNP is thought to exert its vasodilator action, at least in part, by NO release inside the vascular smooth muscle cell. Thus, a greater amount of NO released by SNP in SHR aortic rings could be associated to the higher potency of this NO-donor in the aortic relaxation of SHR. So, our results suggest that NO concentration released by each NO-donor could be directly related to the vasodilator response of SHR aorta.

The cellular alterations that lead to the differences observed in 2K-1C aorta may not be the same observed in the aorta of SHR. These differences could be related the way that the different NO-donors are reduced, before NO release [1,4] and/or to the mechanism of the relaxing response of the vascular smooth muscle, as activation of potassium channels and cGMP accumulation [18,21]. These possible differences are currently being explored in our laboratory.

The present results still cannot point the reason for the differences observed in the hypotensive effect of the drugs, but these data brings more evidence that drugs that slowly release NO could be used as a target in the comprehension of the mechanisms involved in NO-induced hypotension in SHR, as well as the mechanism of NO release and smooth muscle relaxation evoked by NO-donors, as pharmacological tools in the study of the vasodilatory and hypotensive processes. Besides, NO-donors that do not induce reflex tachycardia could be attractive when alterations in the heart rhythm are undesirable.

Acknowledgments

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