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Central administration of TRV027 improves baroreflex sensitivity and vascular reactivity in spontaneously hypertensive rats

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Abstract

TRV027 is a biased agonist for the Angiotensin (Ang)-II type 1 receptor (AT₁R), able to recruit β -arrestin 2 independently of G-proteins activation. β -arrestin activation in the central nervous system (CNS) was suggested to oppose the effects of Ang-II. The present study evaluates the effect of central infusion of TRV027 on arterial pressure (AP), autonomic function, baroreflex sensitivity (BRS), and peripheral vascular reactivity. Spontaneously hypertensive (SH) and Wistar Kyoto (WKY) rats were treated with TRV027 for 14 days (20 ng/h) delivered to the lateral ventricle via osmotic minipumps. Mechanistic studies were performed in HEK293T cells co-transfected with AT₁R and Ang converting enzyme type 2 (ACE2) treated with TRV027 (100nM) or Ang-II (100nM). TRV027 infusion in SHRats (SHR) reduced AP (~ 20 mmHg, $P < 0.05$), sympathetic vasomotor activity (MAP = -47.2 ± 2.8 compared with -64 ± 5.1 mmHg, $P < 0.05$) and low-frequency (LF) oscillations of AP (1.7 ± 0.2 compared with 5.8 ± 0.4 mmHg, $P < 0.05$) compared with the SHR control group. TRV027 also increased vagal tone, improved BRS, reduced the reactivity of mesenteric arteries to Ang-II and increased vascular sensitivity to phenylephrine (Phe), acetylcholine, (ACh), and sodium nitroprusside (SNP). *In vitro*, TRV027 prevented the Ang-II-induced up-regulation of ADAM17 and in contrast with Ang-II, had no effects on ACE2 activity and expression levels. Furthermore, TRV027 induced lesser interactions between AT₁R and ACE2 compared with Ang-II. Together, these data suggest that due to its

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Author contribution

A.C.-G., E.L., M.S.F.-S., and C.M.F. designed the experiments. A.C.-G., B.O., J.X., C.R.S.-A., L.G.M.-J., D.D.G., J.C.C., T.M.Q., C.M.B., and V.A.B. were responsible for the experiments and data collection. A.C.-G., B.O., J.X., C.R.A.S.-A., L.G.M.-J., T.M.Q., and C.M.B. performed data analysis. A.C.-G., B.O., J.X., L.G.M.-J., J.C.C., T.M.Q., C.M.B. V.A.B., C.M.F., E.L., and M.S.F.-S. conducted the data interpretation. All the authors contributed to and approved the final manuscript.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

biased activity for the β -arrestin pathway, TRV027 has beneficial effects within the CNS on hypertension, autonomic and vascular function, possibly through preserving ACE2 compensatory activity in neurones

Introduction

Arterial hypertension is an important health problem worldwide of difficult treatment due to the complexity of its underlying mechanisms. Diverse endogenous molecules are involved in the (dys)regulation of blood pressure (BP), including the renin-angiotensin (Ang)-aldosterone system (RAAS), whose imbalance is involved in the pathogenesis of hypertension [1,2]. Besides the peripheral effects induced by Ang-II [3], it was established that the hypertensive effects induced by circulating Ang-II are partly mediated by the central nervous system (CNS) [4].

Since classical studies by Bickerton and Buckley (1961) [5], central Ang-II-induced hypertensive outcomes have been extensively studied [6–8]. It was hypothesized that, under normal conditions, circulating Ang-II acts on circumventricular organs (CVO), such as the subfornical organ (SFO) [9] and organum vasculosum of the lamina terminalis (OVLT) [10], which lack a blood–brain barrier and express a high density of peptide receptors. Thus, activation of the Ang-II type 1 receptor (AT_1R) located in these areas stimulate integrative and effector regions of the brain, resulting in central production of Ang-II, including in cardiovascular regulatory areas such as the paraventricular nucleus of the hypothalamus (PVN) and the rostral ventrolateral medulla (RVLM) [11]. The CVO send projections to PVN, a key forebrain nucleus mediating central Ang-II actions [12]. PVN pre-sympathetic neurones project to the RVLM or to the spinal cord, in order to modulate sympathetic nerve activity (SNA) [12,13]. Accordingly, neurogenic hypertension is mediated in part by Ang-II-induced effects along the SFO-PVN-RVLM network [14].

Although Ang-II triggers multiple intracellular signaling mechanisms after binding to AT_1R , excessive Gq/11 activation is the classical pathway described for peripheral and central Ang-II-induced hypertension [15]. Additional pathways have been established, including AT_1R -mediated β -arrestin recruitment and subsequent AT_1R internalization and desensitization [16,17]. Importantly, hypertensive rats have been reported to have lower β -arrestin 2 expression in the RVLM and overexpression of this protein in this region attenuated BP, heart rate (HR), and SNA [18].

However, recent evidence clearly demonstrated that G-protein coupled receptors, including AT_1R , can exert biological effects through Gq-protein independent pathways, including recruitment of β -arrestin. Ligands able to selectively stimulate specific signaling pathways over the others are named biased agonists and several Ang-II analogs, able of selectively engaging the GRK/ β -arrestin axis have been synthesized [19,20].

β -arrestin biased-recruitment may require another G-protein, such as G_{α_i} , required for AT_1R biased activation triggered by stretch [21] or biased agonist [Sar1, Ile4, Ile8]-AngII (SII) [22]. Recruitment of this G-protein has not been shown for biased compounds such as TRV023 and TRV026, as described by Wang et al. (2018) [21].

TRV027 ([Sar1, d-Ala8]-Ang-II) is a β -arrestin biased ligand at the AT₁R which antagonizes Ang-II-stimulated Gq/11 protein signaling [21]. *In vitro* studies have shown that TRV027 activates MAP kinase signaling pathways and endothelial nitric oxide synthase (eNOS) phosphorylation. Interestingly, in anesthetized rats, TRV027 reduced BP while stimulating cardiac contractility [23].

In the present study, we used TRV027, to distinguish between the roles of Gq-protein activation compared with β -arrestin recruitment in AT₁R-mediated neurogenic hypertension. We hypothesized that central β -arrestin 2 activation could induce beneficial cardiovascular effects in neurogenic hypertension. To test our hypothesis, we infused TRV027 centrally for 14 days followed by a combination of studies to evaluate arterial pressure (AP), HR, autonomic and baroreflex functions, as well as vascular reactivity in Wistar Kyoto (WKY) and spontaneously hypertensive (SH) rats (SHR), as this animal model is the most similar to human essential hypertension [24]. Additional cell culture studies were designed to clarify the mechanisms of action of TRV027 with regard to the compensatory RAAS.

Methods

Animals

Ten-week-old male SH and WKY rats were obtained from the University of São Paulo and housed in controlled temperature conditions (21 ± 1 °C), 12:12-h light–dark cycle with access to standard chow (Labina®, Purina, Brazil) and water *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Federal University of Paraíba (CEUA – protocol # 090/2016). The study followed the ARRIVE guidelines. Animals were randomly divided into four groups: WKY and SHR treated or not with TRV027 by intracerebroventricular (ICV) infusion.

Drugs and solutions

The following drugs were used: TRV027 (GenScript, Piscataway, NJ); acetylcholine (ACh) chloride; Ang-II; atropine; l-phenylephrine (Phe) chloride; propranolol hydrochloride and sodium nitroprusside (SNP) were obtained from Sigma–Aldrich (São Paulo, SP, Brazil); heparin sodium salt (Roche Brazil, São Paulo, Brazil); ketamine and xylazine (Sespo, Paulinia, SP, Brazil). All other compounds were dissolved in saline solution and stored at 0–4°C. Tyrode's solution contained (in mM): NaCl: 158.3; KCl: 4.0; CaCl₂: 2.0; MgCl₂: 1.05; NaH₂PO₄: 0.42; NaHCO₃: 10.0, and glucose: 5.6; pH 7.4. A depolarizing Tyrode's solution (K⁺: 60 mM) was obtained by adjusting Na⁺ and K⁺ concentrations.

ICV infusion

Rats anesthetized with ketamine and xylazine (80 and 10 mg/kg, i.p., respectively) were instrumented with a rat brain infusion kit (ALZET pump 1002, infusion rate, 0.25 μ l/h) for chronic ICV infusion of TRV027 (20 ng/h) for 14 days. The flow moderator of the minipump was connected with a polyethylene catheter to a metal cannula (30-gauge) to allow for long-term ICV infusion. Implantation of the infusion kit was performed using a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The skull was exposed by a midline sagittal incision through the scalp and a subcutaneous pocket was prepared on the

back of the rat. The mini osmotic pump was placed into the pocket, and the metal cannula was inserted in the left lateral cerebral ventricle, according to the Paxinos and Watson brain atlas [25], with the following co-ordinates relative to bregma: +0.5 mm rostral, -1.4 mm lateral, and 3.9 mm ventral.

Catheters implantation and hemodynamic measurements

After 13 days, rats were anesthetized as described above and polyethylene catheters were inserted into the lower abdominal aorta and inferior vena cava through left femoral artery and vein, respectively. Both catheters were filled with heparinized saline, tunneled subcutaneously, exteriorized, and sutured at the dorsal surface of the neck. Twenty-four hours after surgical procedures, experiments were performed in awake rats. AP and HR were recorded using a pressure transducer (MLT0380/D, ADInstruments, Sydney, Australia) and connected to a computer running the LabChart software (ADInstruments, Bella Vista, Australia).

Physiological recordings

Baroreflex sensitivity (BRS) was evaluated after a 24-h recovery from the catheter implantation surgery. After 40 min of AP and HR baseline recordings, reflex responses were obtained using the vasoactive drugs Phe (8 µg/kg) and SNP (25 µg/kg) according to the modified Oxford method, as previously described [26]. Spontaneous baroreceptor reflex sensitivity (SBRS), which reflects the physiological baroreflex control of BP was calculated using baseline recording and the sequence method [26].

Autonomic function was assessed in conscious freely moving rats after treatment, using a pharmacological method involving iv injections of propranolol (β-blocker, 5 mg/kg), atropine (muscarinic receptor blocker, 2 mg/kg), and hexamethonium (ganglionic blocker, 30 mg/kg) [27]. Each injection was separated by a 3-h recovery period. Changes in HR (HR) or mean AP (MAP) were calculated following administration of these blockers. At the end of the protocol, the rats were killed and superior mesenteric arteries were collected for vascular reactivity experiments.

Spectral analysis of systolic BP variability

An indirect evaluation of the autonomic modulation of vascular resistance was performed through an analysis of AP variability in the frequency domain, as previously described [28]. Briefly, pulsatile AP (PAP) recordings (10 min) obtained at baseline were processed with a computer software (LabChart, ADInstruments) that employs an algorithm to detect beat-to-beat inflection points in the pulse pressure signal. Beat-by-beat series with systolic BP (SBP) values were generated and loaded into a customized software (CardioSeries V2.4, <http://www.danielpenteado.com>).

The power of the oscillatory components obtained from the rats belonging to the WKY and SH groups was integrated into low-frequency (LF; 0.2–0.75 Hz) and high-frequency (HF; 0.75–3 Hz) bands, and results were expressed in absolute values (mmHg²). AP oscillations at the LF range are representative of the modulatory effects of sympathetic activity

controlling vascular tone, whereas oscillations at the HF range are associated with a respiratory modulation [28].

Vascular reactivity studies

The superior mesenteric artery was collected, cleaned from connective and adipose tissues, and rings (1–2 mm) were obtained. The rings were placed in physiological Tyrode's solution, maintained to 37°C, gassed with a carbogenic mixture (95% O₂ and 5% CO₂) at pH 7.4. All preparations were stabilized under a resting tension of 0.75 g for 1 h. The solution was replaced every 15 min to prevent the accumulation of metabolites. The tension was recorded by a force transducer (PowerLabTM, ADInstruments, MA, U.S.A.).

In order to confirm the presence of functional endothelium, the rings were pre-contracted with Phe (10 µM) and exposed to ACh (10 µM). Relaxations exceeding 80% indicated a functional endothelium and were included in the study.

A maximum contraction was induced using a high potassium concentration (K⁺: 60 mM). After wash out, Ang-II (10 µM) was added to the bath to evaluate the contractile response. In addition, we performed concentration–response curves to Phe (10^{−10} to 10^{−4} M), ACh (10^{−10} to 10^{−4} M), or SNP (10^{−12} to 10^{−5} M), the last two in rings pre-contracted with Phe (10 µM). Contractile responses to Phe and Ang-II were compared with the maximum contraction obtained following tissue stimulation with high [K⁺]. Relaxation responses to cumulative concentrations of ACh and SNP were calculated as percentage of inhibition of Phe-induced maximal contraction. Results were expressed as maximum effect (ME) and pD₂ (negative logarithm to base 10 of the EC₅₀).

Cell culture and activity assays

HEK293T and Neuro2A cells were obtained from the American Type Culture Collection (Manassas, VA). Experiments were performed in passages 16–24. The cells were cultured in Dulbecco's modified Eagle's medium and supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin, as described [29].

Ang converting enzyme type 2 (ACE2) enzymatic activity was determined in HEK293T cells in six wells at a density of 2×10^6 cells/well transfected with ACE2 and AT₁R plasmids (0.5 µg/well) for 2 days. One day before the experiment, cells were starved in DMEM without FBS and incubated with Ang-II or TRV027 (100 nM each) for 4 h. Subsequently, the cells were lysed, suspended in ACE2 reaction buffer, sonicated, and used for activity measurements, as described [30]. The assay consisted of 0.5 µg protein extract and 50 µM Mca-YVADAPK(Dnp) substrate. Fluorescence emission was measured at 405 nm with excitation at 320 nm in a Biotek3 spectrophotometer. Blank values were obtained from wells containing only the extraction buffer. The determinations were repeated from at least three independent transfections and the results are presented as % of control values in untreated cells.

Neuro2A cells treated with TRV027 (100 nM) or vehicle groups were processed for ADAM17 activity, as reported previously [31]. Data were collected as fluorescence units

(amount of substrate converted into fluorescent product) per minute, normalized for protein content.

Bioluminescence resonance energy transfer

To determine AT₁R/ACE2 interactions, we used a NanoBret assay (Promega). Human ACE2 and AT₁R were cloned in NLuc and HaloTag vectors, respectively. HEK293T cells, grown in six-well plates at a density of 2×10^6 cells/well, were transfected using Lipofectamine 2000 with 1 µg of each construct. After 6 h, the cells were trypsinized and plated on black 96-well plates at a density of 25×10^4 cells/well for 24 h. One day before measurement, the cells were incubated in DMEM without Phenol Red or FBS. HaloTag NanoBRET 618 Ligand (100 nmol/l final concentration) or DMSO (negative control) were added 16 h before final determinations. The cells were treated with 100 nM Ang-II or TRV027 for the indicated periods. Nano-Glo Substrate was added for 30 min and donor emission (460 nm) and acceptor emission (618 nm) were measured in a Biotek3 spectrophotometer. The final values were calculated as ratio of 618 nm/460 nm after subtraction of the negative control values (DMSO). Each experiment was repeated in four different transfections.

Western blot

The experiments were carried out as described previously [29]. In brief, the cell lysates were separated by SDS/PAGE and transferred on to PVDF membranes. The signal was detected using ECL Plus (PerkinElmer) and a luminescent image analyzer (LI-COR) and quantitated using the Image Studio software (LI-COR). Anti-ACE2 antibody (sc-73668) and anti-β-actin antibody (sc-8432) were from Santa Cruz Biotechnology, Inc. (Dallas, TX).

Statistical analysis

Data were expressed as mean ± S.E.M. Data were analyzed by one-way or two-way ANOVA, followed by Tukey for multiple comparisons between means when appropriate. Statistical comparisons were performed using Prism 6 (GraphPad Software, San Diego, CA). Difference was considered significant when $P < 0.05$.

Results

TRV027 reduces AP, improves autonomic function and BRS in SHR

As expected, the SH group exhibited MAP levels higher than the WKY rats (Figure 1A,B). ICV administration of TRV027 for 14 days reduced MAP in SH (160.0 ± 5.5 compared with 181.3 ± 3.5 mmHg, $P < 0.05$, Figure 1B) but not in WKY (108.0 ± 5.0 compared with 119.3 ± 6.0 mmHg, $P < 0.05$, Figure 1B) rats. The same effect was observed when we evaluated SBP and diastolic BP (DBP) separately between the SHR and WKY control groups (SBP: 219.3 ± 7 compared with 140.2 ± 8 mmHg; DAP: 143.5 ± 5 compared with 104.0 ± 4 mmHg, respectively, $P < 0.05$). TRV027 administration reduced only SBP in SHRs (194.0 ± 8 mmHg, $P < 0.05$) (Figure 1C,D). HR was not significantly different amongst the groups (WKY = 319 ± 11 bpm; WKY + TRV = 327 ± 18 bpm; SHR = 345 ± 12 bpm and SHR + TRV = 350 ± 5 bpm; Figure 1E).

Vagal tone was blunted in SH compared with WKY rats ($P<0.05$, Figure 2A) while both vascular and cardiac sympathetic drive were increased ($P<0.05$, Figure 2B,C). TRV027 treatment reduced dysautonomia in SHR when compared with untreated SHR, as evidenced by a higher vagal tone (tachycardia to atropine: $+83 \pm 1$ compared with $+54 \pm 4$ bpm, $P<0.05$, Figure 2A), a reduced vasomotor sympathetic drive (hypotension to hexamethonium: -47 ± 3 compared with -64 ± 5 mmHg, $P<0.05$, Figure 2B) and a blunted cardiac sympathetic drive (bradycardia to propranolol: -249 ± 24 compared with -282 ± 15 bpm, $P<0.05$, Figure 2C). To confirm the changes in vasomotor sympathetic modulation within the groups, we assessed SBP variability. Representative spectra (Figure 2D) highlight the expected increase in oscillation in the LF range in SH compared with WKY rats (5.8 ± 0.4 compared with 1.7 ± 0.2 mmHg², $P<0.05$; respectively, Figure 2E). Treatment with TRV027 decreased the magnitude of oscillation in the LF range in SH (3.8 ± 0.5 compared with 5.8 ± 0.4 mmHg², $P<0.05$, Figure 2E) and did not produce significant changes in WKY rats. There were no changes at the HF range between groups ($P=0.59$; Figure 2F).

To determine the role of TRV027 on BRS, baroreceptor reflex responses were initially evaluated after injections of vasoactive drugs Phe and SNP. Rats from the SH group presented a reduction in BRS when compared with WKY rats (-1.4 ± 0.2 compared with -3.3 ± 0.3 bpm.mmHg⁻¹, $P<0.05$). Of note, after TRV027 ICV infusion, the baroreflex gain was restored in SHR (-2.4 ± 0.3 compared with -1.4 ± 0.2 bpm.mmHg⁻¹, $P<0.05$, Figure 3A). Unsurprisingly, SBRS was significantly impaired in SH compared with the WKY group (0.5 ± 0.1 compared with 1.6 ± 0.1 ms.mmHg⁻¹, $P<0.05$) and treatment with TRV027 prevented the reduction in SBRS in SHR (1.8 ± 0.2 compared with 0.5 ± 0.1 ms.mmHg⁻¹, $P<0.05$, Figure 3B). Altogether, these data suggest that treatment with this biased AT₁R agonist reduces hypertension by preventing autonomic dysfunction and improving BRS.

TRV027 decreases vascular reactivity to Ang-II and increases sensitivity to other vasoactive agents in SHR mesenteric arteries

To determine whether activation of β -arrestin in the CNS by the AT₁R-biased agonist would affect peripheral vasoconstriction, superior mesenteric arteries were contracted with Ang-II and Phe. Vascular reactivity to Ang-II was greater in SHR compared with WKY animals, as observed by the of contraction (0.4 ± 0.03 compared with 0.09 ± 0.02 g, respectively; $P<0.05$, Figure 4A) or percentage of contraction to Ang-II in relation to contraction induced by high [K⁺] (61.3 ± 6.2 compared with $14.6 \pm 2.8\%$, respectively, $P<0.05$, Figure 4B). ICV infusion of TRV027 reduced vascular reactivity to Ang-II in SHR (0.17 ± 0.03 compared with 0.4 ± 0.03 g; $P<0.05$, Figure 4A) and (38.2 ± 8.5 compared with $61.3 \pm 6.2\%$, respectively; $P<0.05$, Figure 4B). In addition, the time course for Ang-II contraction was altered in SHR compared with WKY (227.5 ± 22.7 compared with 150.0 ± 14.6 s; $P<0.05$, Figure 4C) and treatment with TRV027 tended to reduce the contraction time to Ang-II in SHR (178.8 ± 19.4 compared with 227.5 ± 22.7 s; $P=0.139$, Figure 4C). The response to Ang-II was also plotted as the product between change of contraction by time divided by 2 (g.ms/2). As expected, this relation was enhanced in SHR and treatment with TRV027 reduced this value as observed in Figure 4D. Together, these parameters indicate that the central activation of β -arrestin induced by TRV027 was able to reduce peripheral vascular reactivity to Ang-II, which in turn was found to be elevated in SHR.

TRV027 had no impact on vascular reactivity to Phe in WKY rats. Although the percentage of contraction to Phe was higher in SHR compared with WKY group at the highest concentration, a decrease in potency was observed in vessels isolated from SHR, as can be seen by pD₂ values. TRV027 infusion enhanced the contraction induced by Phe in SHR + TRV027 group when compared with SHR group (Table 1 and Figure 5A).

Relaxation to ACh was attenuated in SHR when compared with WKY group, suggesting impaired endothelial function. In SHR + TRV027 group there was a shift of the curve to the left, suggesting an increased sensitivity to ACh. There were no changes in WKY + TRV group compared with the WKY (Table 1 and Figure 5B).

The response to SNP, a NO donor, was also attenuated in SHR when compared with WKY, suggesting impairment also in the vascular smooth muscle response. TRV027 prevented this deficit in SHR + TRV027 group. Similar to ACh, there were no changes in WKY + TRV group compared with the normotensive control group (Table 1 and Figure 5C).

TRV027 preserves ACE2 compensatory activity in SHR

To clarify the mechanisms involved in the beneficial effects of TRV027 in hypertensive rats, we treated both HEK293T and Neuro2A cells with the biased agonist and assessed its effects on ACE2 compensatory activity. As reported previously [29], incubation with Ang-II reduced ACE2 activity in HEK293T cells co-transfected with ACE2 and AT₁R. In contrast, we found that treatment with TRV027 did not have similar effects, suggesting that TRV027, by binding to AT₁R, is able to prevent or antagonize the effects of Ang-II on ACE2 activity in HEK293T (Figure 6A). Similar results were observed for ACE2 expression (Figure 6B). Since Ang-II-mediated AT₁R signaling leads to ADAM17 activation and ACE2 shedding, ADAM17 activity was assessed in Neuro2A cells. Our data (Figure 6C) clearly indicate that the biased agonist is preserving ACE2 compensatory activity by failing to engage the sheddase. Previously, we demonstrated that AT₁R activation reduced ACE2 activity by a mechanism dependent on Ang-II-induced AT₁R/ACE2 interactions. To demonstrate that preserved ACE2 enzymatic activity is due to a lack of ACE2/AT₁R interactions, we performed NanoBret experiments. We observed that TRV027 induced significantly less interactions between the two proteins compared with Ang-II (Figure 6D). Together, our data suggest that these different ligands modulate ACE2 protein activity in a specific manner and TRV027 appears to prevent AT₁R-mediated shedding and internalization of ACE2.

Discussion

In the present study, we found that ICV administration of TRV027 in SHR decreases baseline MAP, reduces vasomotor sympathetic drive, increases cardiac vagal tone, improves BRS, reduces the vascular reactivity to Ang-II and improves vascular sensitivity to other vasoactive agents. In addition, we observed that at the cell level, TRV027 prevents the interaction between AT₁R and ACE2 and preclude the activation of ADAM17, thus preserving the compensatory activity of ACE2.

Violin et al. [23] introduced TRV027 as a biased agonist of the AT₁R capable of promoting β -arrestin 2 recruitment through a G-protein-independent mechanism, leading to receptor

internalization and activation of specific signaling pathways. Although Rockman et al. (2018) suggested that biased agonism of AT₁R due to membrane stretch depends on G α i, it was not observed with TRV compounds [21]. Violin et al. [23] described an attenuation in the AP rise induced by Ang-II infusion and a dose-dependent decrease in MAP after peripheral administration of TRV027.

Our results show for the first time the effects of TRV027 treatment in rats with spontaneous hypertension. Interestingly, the hypotensive effect of TRV027 was restricted to these animals, as central administration of TRV027 for 14 days did not change BP in WKY. As shown by Violin et al. [23] and confirmed here, treatment with TRV027 decreased SBP and MAP, but did not alter the HR.

Similar effects on BP and HR are also well characterized for unbiased antagonists of AT₁R such as losartan and telmisartan. However, unlike these antagonists, TRV027 has been shown to improve cardiac contractility by mechanisms involving β -arrestin 2, pointing to a potential advantage of this biased agonist in heart failure patients.

Boerrigter et al. [32] showed that, after intravenous infusion, TRV027 decreased MAP in healthy and heart failure dogs with a higher impact in the disease group, likely due to sympathetic activation and hyperactivity of the RAAS. In addition, Soergel et al. demonstrated that intravenous administration of TRV027 in humans is safe and tolerable and resulted in reductions on MAP in healthy humans with elevated levels of plasma renin activity and in patients with stable systolic heart failure [33,34]. Our model of hypertension also presents sympathetic hyperactivity [35] as well as RAAS hyperactivation [36], justifying at least in part the pronounced effect by TRV027 in SHR when compared with WKY.

Sympathetic hyperactivity in SHR can be reduced by administration of hexamethonium, a ganglionic blocker. Li et al. [37] have shown that intravenous administration of hexamethonium resulted in a greater reduction in MAP and renal SNA (RSNA) in SH compared with Wistar rats, drawing a positive correlation between the alterations in RSNA and MAP in both groups. In our study, hexamethonium promoted a reduction in MAP in normotensive and hypertensive rats, but to a higher degree in SHR, as reported by Li et al. [37]. Treatment with TRV027 significantly reduced the increase in sympathetic drive in SHR. An increase in cardiac sympathetic tone was observed in SH when compared with WKY rats after propranolol, however, TRV027 did not significantly reduce sympathetic cardiac tone. Supporting the pharmacological tests, the SH group presented increased LF bands for SBP compared with the WKY rats, indicating a sympathetic predominance on the vascular bed of these animals, as suggested previously [28,38]. ICV infusion of TRV027 was able to reduce the oscillations in the LF range in hypertensive animals. These data support the report by Wang et al. [18], in which they attributed to central β -arrestin 2 the ability to reduce BP and SNA in SHR.

When assessing vagal tone with atropine, we observed that in adult SHR the parasympathetic component was impaired when compared with WKY, as previously shown [39], and animals treated with TRV027 demonstrated an increased cardiac vagal tone when

compared with SH controls. Together, these data suggest that the antihypertensive effect of TRV027 is associated, at least in part, with the improvement in autonomic cardiovascular modulation.

Besides autonomic dysfunction, several studies have demonstrated the decrease in BRS in spontaneous hypertension [40,41]. In this study, we observed that there was a reduction in BRS in SHR and the ICV infusion of TRV027 was able to restore it. Similar effects were found in other studies after ICV injection of AT₁R antagonists [42,43].

Several studies have demonstrated that Ang-II-induced actions in brain areas involved with cardiovascular control, mainly in the SFO-PVN-RVLM axis, are responsible for sympathetic hyperactivity and decreased BRS in SHR, and this effect is due to oxidative stress dependent on the activation of Gq/11 protein coupled to AT₁R [8,9,40]. Although we have not evaluated the central oxidative stress, we speculate that the biased ligand TRV027, by antagonizing the G-protein-dependent pathway coupled to AT₁R [23] might reduce Ang-II-induced central oxidative stress in SFO-PVN-RVLM axis, consequently decreasing sympathetic activity and restoring the BRS in these animals [8,9].

Sympathetic activity is closely related to changes in vascular reactivity, which in turn contributes to the progression of hypertension. It is well known that the increase in BP induced by central Ang-II is due to the facilitation of sympathetic nervous activity, causing tachycardia, catecholamine release from the adrenal medulla, and increase in vascular resistance, amongst other effects [44–46]. Furthermore, sympathetic activity stimulates renin production by the juxtaglomerular apparatus, which raises circulating Ang-II levels, increasing the signaling mediated by this peptide in the periphery, such as vascular contraction induced by AT₁R [47]. Having observed that the central AT₁R biased activation promoted by TRV027 was associated with the reduction in sympathetic activity in the SH animals, we aimed to investigate the impact of the central infusion of TRV027 on vascular response mediated by peripheral Ang-II.

In accordance with the literature, our SHR presented hyper-responsiveness to Ang-II when compared with WKY rats. ICV infusion of TRV027 was able to reverse this effect, suggesting that the reduction in Ang-II-mediated effects in the brain contributes to a reduction in the peripheral effects mediated by this peptide. Because of the low rate of infusion (500 nl/h), it is unlikely that TRV027 could have diffused to the periphery to promote the effects observed and it seems more probable that central effects are driving the BP reduction. This is also supported by the structural similarity between TRV027 and Ang-II, which would result in a short half-life (less than 2 min) [23,32].

Plasmatic Ang-II levels in normotensive and SHRs are in the order of 500–1250 picomoles [48,49]. These concentrations to which vessels are exposed *in vivo* are much lower than that used in our study, 10 μ M. While this concentration would seem excessive, it was the lowest concentration able to induce a significant contraction in our vascular preparations from all the groups evaluated. In addition, one cannot directly compare this experimental condition to the physiological environment, as 5 min or less exposure *in vitro* is not quantitatively

comparable with an elevation of plasma levels over a period of hours or several days as it occurs *in vivo*.

Despite sympathetic vasomotor hyperactivity in hypertension in SH control rats, the Phe-induced contraction was attenuated when compared with WKY, as illustrated by pD_2 values and TRV027 infusion, shifting the curve to the left, suggesting an increased sensitivity to this contracting agent. Impaired vascular reactivity to Phe in SHR was also observed in other studies [50,51]. Kojima et al. (1989) [52] reported a decrease in α_1 -adrenoceptor reserve in SHR mesenteric artery compared with WKY, which may be responsible for the reduction in Phe-induced contractions. This effect may be due to a compensatory response to sympathetic hyperactivity directed to the vessels in hypertension, thus ICV infusion of the biased AT_1R agonist, by promoting a down-regulation of central AT_1R , reduces sympathetic activity and downstream vascular compensatory responses. In WKY animals, pD_2 values for Phe were not different in the absence or presence of TRV027.

In parallel, ACh-mediated relaxation was attenuated in the SHR group, where TRV027 seems to shift the curve to the left, suggesting an increased sensitivity to this vasodilator. The reduced reactivity to ACh in SHR is classically accepted as consequence of the endothelial dysfunction present in this model, characterized by increased oxidative stress, reduction in NO bioavailability, eNOS uncoupling and hyper-responsiveness to contracting agents such as Ang-II and endothelin [53]. These effects were most likely prevented by treatment with TRV027 by reducing the hypertensive process.

Endothelium-independent relaxation, examined with SNP was impaired in arteries isolated from SHR. Similar results were found in obese SHR by Mendizábal et al. (2011) [51]. The effects related to endothelial dysfunction which counterbalanced NO-induced relaxation, such as excess of reactive oxygen species and increased response to some vasoconstrictors agents, probably attenuates the SNP-induced relaxation, TRV027 infusion reverted this effect.

Our data suggest that TRV027-mediated biased activation of β -arrestin coupled to AT_1R within the CNS extended its beneficial effects to the periphery with an overall improvement of arterial hypertension, by reducing the vascular hyperactivity to Ang-II and impaired endothelial and vascular smooth muscle function. These peripheral effects were accompanied by an improvement in autonomic function with restoration of BRS. The molecular mechanisms mediating these effects appear to involve reduced interactions between AT_1R and ACE2 by TRV027 compared with Ang-II, leading to preservation of ACE2 expression levels and enzymatic activity. In addition, by failing to engage the traditional G-protein pathway downstream of AT_1R , TRV027 resulted in a reduction in AT_1R -mediated ADAM17 activity.

A limitation of the present study is our inability to assess the effects of TRV027 on ACE2 and ADAM17 activities in the brain of SHR and WKY. These translational experiments are warranted in our follow-up studies.

This is the first study to assess the effect of TRV027 in hypertension and the effects of central administration of this biased agonist on autonomic modulation and its implications in

periphery. It is a mechanistic study that broadens our knowledge of the effects induced by biased activation of central β -arrestin on cardiovascular parameters. Although some studies have shown that peripheral administration of TRV027 was able to reduce BP and exert several beneficial effects on the cardiovascular system of normotensive rats, healthy and heart failure canines and humans [23,32–34], in our study, peripheral administration would not allow us to evaluate the link between central and peripheral effects after treatment with TRV027. We do not have data related to the effect of peripheral administration on the parameters evaluated, especially on vascular reactivity, so we cannot say if the peripheral treatment would be more effective or not. These data encourage us to evaluate these parameters after peripheral administration in future studies and also to deepen the mechanisms by which TRV027 exerts its effects in hypertension.

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Abbreviations

ACE2	angiotensin converting enzyme type 2
Ach	acetylcholine
Ang	angiotensin
AP	arterial pressure
AT₁R	Ang-II type 1 receptor
BP	blood pressure
BRS	baroreflex sensitivity
CNS	central nervous system
CVO	circumventricular organ
DBP	diastolic BP
eNOS	endothelial nitric oxide synthase
HF	high frequency
HR	heart rate
ICV	intracerebroventricular

LF	low frequency
MAP	mean AP
pD₂	negative logarithm to base 10 of the EC ₅₀
Phe	phenylephrine
PVN	paraventricular nucleus of the hypothalamus
RAAS	renin-angiotensin-aldosterone system
RSNA	renal sympathetic nerve activity
RVLM	rostral ventrolateral medulla
SBP	systolic BP
SBRS	spontaneous baroreceptor reflex sensitivity
SFO	subfornical organ
SH	spontaneously hypertensive
SHR	SH rat
SNA	sympathetic nerve activity
SNP	sodium nitroprusside
WKY	Wistar Kyoto

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Clinical perspectives

- The administration of biased agonists of AT₁R, such as TRV027, have shown beneficial effects over unbiased antagonists in heart failure. However, the potential of these drugs in hypertension, a multifactorial cardiovascular disorder, has not yet been addressed.
- In the present study, we found that ICV infusion of TRV027 induced several beneficial effects that resulted in BP reduction. These included decreases in sympathetic vasomotor drive, increase in vagal cardiac tone, restoration of BRS, and decrease in vascular response to Ang-II. At the cellular level, these effects may result not only from the decreased interaction of central Ang-II to AT₁R but also from the preservation of ACE2 compensatory activity.
- Our data highlight the beneficial role of biased activation of central β -arrestin in hypertension. Considering that hypertension is a health problem worldwide, presenting a predicted prevalence of 1/3 of global population by 2025 and that its multifactorial origin makes it difficult to treat, the present study broadened our knowledge about the effects of TRV027 on the cardiovascular system during hypertension, pointing to β -arrestin as a possible target in the search for therapeutic alternatives to this disorder.

Figure 1. Effect of ICV administration of TRV027 in rats

(A) Typical PAP, MAP, and HR traces from conscious and freely moving rats treated or not with TRV027. TRV027 administration induces a reduction in MAP (B) and SBP (C) in SHR without affecting DBP (D) and HR (E). * $P < 0.05$ compared with WKY control, and # $P < 0.05$ compared with SHR. All data are mean $\pm$ S.E.M. WKY: $n=9$; WKY + TRV: $n=7$; SHR and SHR + TRV: $n=6$ / group.

Figure 2. Evaluation of autonomic function after treatment with TRV027

SHR treated with TRV027 had improved cardiac vagal tone (A) and reduced vascular sympathetic tone (B) although cardiac sympathetic drive was not altered (C). Representative spectra of SBP (D), average magnitudes of LF (E) and HF (F) components of SBP from WKY and SHR treated or not with TRV027. * $P < 0.05$ compared with WKY control, and # $P < 0.05$ compared with SHR. All data are mean $\pm$ S.E.M. $n=5$ in all groups.

Figure 3. Evaluation of BRS after treatment with TRV027

Both baroreflex gain using a pharmacological method (A) and the spontaneous baroreflex (B) were improved after 14 days of treatment with TRV027 in hypertensive rats. * $P < 0.05$ compared with WKY control, and # $P < 0.05$ compared with SHR. All data are mean $\pm$ S.E.M. SHR and WKY: $n=6$; SHR + TRV and WKY + TRV: $n=7$; spontaneous baroreflex: $n=5$ in all groups.

Figure 4. TRV027 decreases vascular reactivity to Ang-II in mesenteric arteries from SHR
Mean of group data showing the increase in tension values (g) after Ang-II in SHR and a reduction following TRV027 treatment (A). Percentage of contraction for Ang-II (B); time course response to Ang-II (C), and relative contraction/time to Ang-II (D). * $P < 0.05$ compared with WKY control, and # $P < 0.05$ compared with SHR. All data are mean $\pm$ S.E.M.; $n=7$ in all groups.

Figure 5. Impact of TRV027 treatment on vascular reactivity in SHR
 TRV027 treatment increased vascular sensitivity to Phe (A), ACh (B), and SNP (C) in mesenteric arteries from SHRs. All data are mean $\pm$ S.E.M.; $n=8$ in all groups.

Figure 6. Impact of TRV027 treatment on ACE2 activity and expression, ADAM17 activity and ACE2/AT1R interactions

ACE2 enzymatic activity in ACE2 and AT₁R co-transfected HEK293T cells (**A**). The activity was tested 72 h after transfection, the cells being serum starved 24 h before experiments. Treatments with Ang-II and TRV027 (100 nM each) lasted for 4 h. * $P < 0.05$ compared with control. $n = 9$ from three independent transfections. (**B**) Top: ACE2 total cellular levels in HEK293T cells co-transfected with AT₁R and ACE2 for 72 h determined by Western blot. After serum starvation for 24 h, the cells were treated with Ang-II or TRV027 (100 nM each) for 18 h. Bottom: statistics of two independent experiments in two separate transfections. (**C**) Neuro2A cells were treated with Ang-II (300 nM) and TRV027 (500 nM) for 18 h, then processed for ADAM17 enzymatic assay. In the co-treatment group, Ang-II and TRV027 were added simultaneously to the serum-free medium. (**D**) ACE2/AT₁R interactions determined by nanoBRET. HEK293T cells were co-transfected with ACE2-NLuc and AT₁R-HaloTag and plated in black 96-well plates at a density of 25×10^4 cells/

well for 48 h. One day before measurement, the cells were incubated in DMEM without Phenol Red or FBS. HaloTag NanoBRET 618 Ligand (100 nM final concentration) or DMSO (negative control) were added 16 h before final determination. The cells were treated with 100 nM Ang-II or TRV027 for 4 h. Nano-Glo Substrate was added for 30 min and donor emission (460 nm) and acceptor emission (618 nm) were measured in a Biotek3 spectrophotometer. The final values were calculated as ratio of 618 nm/460 nm after subtraction of the negative control values (DMSO). $n=12$ from three independent transfections.

Table 1

Impact of TRV027 treatment on vascular reactivity to Phe, ACh, and SNP

Experimental groups	Contraction to Phe		Relaxation to ACh		Relaxation to SNP	
	ME (%)	pD ₂	ME (%)	pD ₂	ME (%)	pD ₂
WKY	114.3 ± 24.4	5.9 ± 0.09	94.0 ± 3.7	7.8 ± 0.10	103.3 ± 2.5	9.2 ± 0.14
WKY + TRV027	129.1 ± 11.8	5.7 ± 0.12	99.3 ± 3.3	7.7 ± 0.17	109.7 ± 5.1	9.4 ± 0.32
SHR	185.2 ± 15.1 [*]	4.9 ± 0.08 [*]	92.8 ± 11.4	6.4 ± 0.25 [*]	106.1 ± 8.0	7.9 ± 0.18 [*]
SHR + TRV027	116.3 ± 14.8 [†]	5.5 ± 0.12 [†]	102 ± 4.2	7.3 ± 0.20 [†]	97.7 ± 4.9	9.3 ± 0.31 [†]

TRV027 treatment enhanced vascular reactivity to Phe, ACh, and SNP in superior mesenteric arteries from SHR. All data are mean ± S.E.M.

^{*} $P < 0.05$ compared with WKY control.

[†] $P < 0.05$ compared with SHR.