Hemodynamic phenotyping of transgenic rats with ubiquitous expression of an angiotensin-(1-7)-producing fusion protein

Daniele T. Alves1,2,3, Luiz Felipe Mendes1, Walkyria O. Sampaio1, Leda M.C. Coimbra-Campos1, Maria Aparecida R. Vieira1, Anderson J. Ferreira4, Almir S. Martins1, Elena Popova2, Mihail Todiras2, Fatimunnisa Qadri2, Natalia Alenina2,3, Michael Bader2,3,5,6, Robson A.S. Santos1 and Maria Jose Campagnole-Santos1

1Department of Physiology and Biophysics and INCT-Nanobiopharmaceutics, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; 2Max-Delbrück Center for Molecular Medicine-MDC, Berlin, Germany; 3DZHK (German Centre for Cardiovascular Research), Partner Site Berlin, Berlin, Germany; 4Department of Morphology, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; 5Institute for Biology, University of Lübeck, Lübeck, Germany; 6Charité, University Medicine Berlin, Berlin, Germany

Correspondence: Maria Jose Campagnole-Santos (mjcampagnole@ufmg.br)

Activation of the angiotensin (Ang)-converting enzyme (ACE) 2/Ang-(1-7)/MAS receptor pathway of the renin–angiotensin system (RAS) induces protective mechanisms in different diseases. Herein, we describe the cardiovascular phenotype of a new transgenic rat line (TG7371) that expresses an Ang-(1-7)-producing fusion protein. The transgene-specific mRNA and the corresponding protein were shown to be present in all evaluated tissues of TG7371 with the highest expression in aorta and brain. Plasma Ang-(1-7) levels, measured by radioimmunoassay (RIA) were similar to control Sprague–Dawley (SD) rats, however high Ang-(1-7) levels were found in the hypothalamus. TG7371 showed lower baseline mean arterial pressure (MAP), assessed in conscious or anesthetized rats by telemetry or short-term recordings, associated with increased plasma atrial natriuretic peptide (ANP) and higher urinary sodium concentration. Moreover, evaluation of regional blood flow and hemodynamic parameters with fluorescent microspheres showed a significant increase in blood flow in different tissues (kidneys, mesentery, muscle, spleen, brown fat, heart and skin), with a resulting decrease in total peripheral resistance (TPR). TG7371 rats, on the other hand, also presented increased cardiac and global sympathetic tone, increased plasma vasopressin (AVP) levels and decreased free water clearance. Altogether, our data show that expression of an Ang-(1-7)-producing fusion protein induced a hypotensive phenotype due to widespread vasodilation and consequent fall in peripheral resistance. This phenotype was associated with an increase in ANP together with an increase in AVP and sympathetic drive, which did not fully compensate the lower blood pressure (BP). Here we present the hemodynamic impact of long-term increase in tissue expression of an Ang-(1-7)-fusion protein and provide a new tool to investigate this peptide in different pathophysiological conditions.

Introduction

The renin–angiotensin system (RAS) is an important systemic, tissue and cellular modulator of the function of basically all organs. Actions of the RAS depend essentially on the balance of two opposing branches: a pressor and proliferative comprising angiotensin (Ang)-converting enzyme (ACE), Ang II, and the AT1 receptor and a hypotensive and anti-proliferative composed of ACE2, Ang-(1-7), and its receptor MAS [1]. Ang II is a potent vasoconstrictor, increases sympathetic activity, induces thirst and attenuates...
baroreflex function. Long-term increase in Ang II is involved in the pathophysiology of many diseases, mainly because of its inflammatory, proliferative and profibrotic actions [2]. On the other hand, Ang-(1-7), known as the protective peptide of the RAS, induces vasodilation, neuromodulation, improves baroreflex function and presents anti-inflammatory, anti-proliferative, anti-hypertrophic, anti-thrombic and anti-fibrotic actions via MAS [1,3].

Different transgenic animals with genetic modifications of RAS components were described and have been contributing to amplify the knowledge of this system over the last 30 years [4,5]. Regarding the ACE2/Ang-(1-7)/MAS branch, mice deficient for MAS and ACE2 and ACE2 overexpressing animals were generated [6–8]. Moreover, in rats, a transgenic strain that expresses an Ang-(1-7)-producing fusion protein mainly in testis, TGR(A1-7)-L3292 (TG3292), was previously generated [9]. In these animals, testis functions as an infusion pump releasing Ang-(1-7) into the circulation. Indeed, plasma level of Ang-(1-7) are 4.5-fold higher in these animals than in Sprague–Dawley (SD) controls [9]. No mRNA expression or increase in Ang-(1-7) levels was detected in other tissues of TG3292 rats, such as kidney, adrenal gland, lung, atrium, ventricle, liver, brain and aorta. Further, these animals presented a normotensive phenotype [9]. The same fusion protein was employed to generate transgenic rats and mice overexpressing Ang-(1-7) specifically in the heart [10], which contributed to demonstrate the cardioprotective function of the ACE2/Ang-(1-7)/MAS axis.

In the present study, we describe a new transgenic rat strain, TGR(A1-7)-L7371 (TG7371), with ubiquitous expression of an Ang-(1-7)-producing fusion protein in different tissues, however with no alteration in the circulating Ang-(1-7) levels. These rats represent a new tool to investigate long-term impact of increased levels of tissue Ang-(1-7) on the pathophysiology of cardiovascular diseases.

Materials and methods

Generation of the transgenic rats, TG7371

Homozygous transgenic rats, TG7371, were generated at the Max Delbrück Center for Molecular Medicine, Berlin, Germany, based on the methodology of Methot et al. [11] and using a modified construct described by Santos et al. [9] to generate TG3292, an animal with increased circulating levels of Ang-(1-7). The construct human glial fibrillary acidic protein (hGFAP)-Ang-(1-7) used in this study was designed to express a fusion protein, containing: (i) a signal peptide from human renin to ensure the transport of the protein into the endoplasmic reticulum for further secretion of this protein to the extracellular medium; (ii) an immunoglobulin fragment from mouse IgG2b linked to a portion of the human prorenin prosegment which promotes mass and exposes the cleavage site for the endoprotease furin; (iii) a furin cleavage site; (iv) coding sequence for Ang-(1-7) followed by a stop codon inserted by the use of a double-stranded oligonucleotide. Furthermore, a polyadenylation cassette of SV40 virus was inserted downstream of the stop codon and 2.2 kb of the hGFAP promoter was inserted upstream of fusion protein coding sequence, as previously reported [12,13]. The construct was used for pronuclear microinjection into fertilized rat zygotes using established methods [14]. Animals were genotyped using primers IG5 (5′-CATCACCCCATCGAGAGAACC-3′) and hRENEX (5′-GGACCAAGCCTGGCCATGTCC-3′) as described [9].

Animals

Experiments were performed in male, 12–16 weeks of age, SD (n=57; 350–450 g of body weight; control group-SD) and transgenic (n=59; 300–400 g of body weight; TG7371) rats kept at the animal facilities of the Laboratory of Hypertension, ICB, Federal University of Minas Gerais, Brazil and Max Delbrück Center for Molecular Medicine, Berlin, Germany. Rats were maintained in a temperature-controlled room (22–24°C) on 12/12-h light–dark cycle. All experimental protocols were performed in accordance with the NIH guidelines (Guide for the Care and Use of Laboratory Animals) and approved by both institutional animal committees (UFMG-CEUA protocol #303/2012, #400/2017; MDC, Y9001/2016).

Quantitative real-time reverse-transcription polymerase chain reaction

For quantitative real-time reverse-transcription polymerase chain reaction (qPCR), different brain areas [nucleus tractus solitarii (NTS), rostral ventrolateral medulla (RVLM), caudal ventrolateral medulla (CVLM), paraventricular nucleus of the hypothalamus (PVN), supraoptic nucleus (SON), periaqueductal gray area (PAG), cortex, striatum and hippocampus], heart, lung, liver, testis, kidney, aorta and adrenal gland were quickly isolated from TG7371 rats (n=5) killed by anesthesia (2.5% Isofluran CP, CP-Pharma, Germany) followed by decapitation. Tissues were frozen in dry ice and stored at −80°C. Tissue total mRNA was extracted using the TRIzol reagent (Invitrogen Life Technologies), treated with DNase (AMP-D1, Sigma), and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Thermo Fisher). Both endogenous ribosomal protein S26
and the transgenic Ang-(1-7) cDNAs were amplified using specific primers (BioteZ Berlin-Buch, Germany) and SYBR green reagent (QuantiNova Qiagen, Hilden, Germany). Relative gene expression analysis of targets was performed in the Applied Biosystems ViiA 7 qPCR system (Applied Biosystems, Foster City, U.S.A.). Transgene-specific primers were: forward primer IG5 (5'-TTGCTCTTCTAGACGTGA-3') located in IgG fragment and the reverse primer hRENEX (5'-CTTCAAGGCCTTTCCGATG-3') located in the human prorenin fragment of the transgene. S26 was used as a housekeeping gene for the normalization of the transgene expression (forward primer 5'-CGATTCCTGACACCTTGTAG-3' and reverse primer 5'-CGTGGTCCCAAGCTCTATGTG-3').

Analysis was performed using ‘Design and Analysis Application qPCR (DA)’ and Thermo Fisher Connect software. A single peak obtained by the dissociation curve was observed to confirm specificity and identity of each qPCR target. The medulla oblongata of an SD rat was used as a negative control (n=1).

Identification of transgene-specific mRNA by in situ hybridization using RNAscope

In situ hybridization (ISH) was performed using RNAscope technology to locate the RNA of the mouse immunoglobulin fragment (IgG2b) present in TG7371 and to identify the expressing cell type. For ISH, SD and TG7371 rats (n=3 each) were killed with an overdose of inhaled anesthetic (5% Isoflurane CP 1 ml/ml, CP-Pharma, Germany) and 1 min after breathing stopped, brain, aorta, lung, heart and kidney were isolated from animals fixed in 4% formalin in 4% formalin and embedded in paraffin. Briefly, five micrometer tissue sections mounted on super frost glass slides were deparaffinized with xylene and rehydrated with a series of ethanol washes. Endogenous peroxidase was blocked by incubating the sections with H2O2. Sections were then heated in antigen retrieval buffer (RNAscope Target Retrieval, Ref. #322001, ACD) in a steamer and digested by protease solution at 40°C for 30 min (RNAscope H2O2 and Protease Plus Reagents, Ref. #322330, ACD). Sections were incubated with ISH target probe pairs at 40°C in a hybridization oven for 2 h. The eight ZZ-ISH probes targeting mouse IgG2b RNA were designed and synthesized by Advanced Cell Diagnostics (ACD, Newark, U.S.A.; Mm-Igg2b-cust, Ref. #827371, Lot #19330C). Sections were washed with buffer (RNAscope Washing Buffer, Ref. #320058, ACD). The signal was amplified using the pre-amplifier and amplifier conjugated to alkaline phosphatase and incubated with a Fast-Red substrate solution for 10 min at room temperature, according to the instructions of the manufacturer (RNAscope 2.5 HD Detection Reagent-RED, ACD). Sections were then stained with Hematoxylin for 10 s, air-dried, incubated in xylene for 30 min and coverslipped using xylene-based mounting medium (EcoMount, Biocare Medical, Ref. #EM897L). On the next day, images were taken using the inverse Keyence bright light fluorescence microscope (Keyence BZ9000, Germany) and evaluated.

Evaluation of the expression of the fusion protein by Western blotting

For Western blotting, TG7371 rats (n=2) were killed by excess anesthesia (2.5% Isoflurane CP 1 ml/ml, CP-Pharma, Germany) and decapitated. Next, tissues were isolated and homogenized into a lysis buffer (RIPA buffer 1×, Cell Signaling Technology, Germany) containing a mixture of protease inhibitors (Roche, U.S.A.). Protein concentrations in the crude homogenates was determined by the Bradford method [15]. Ang-(1-7) and Ang II were extracted from plasma or supernatant with Strata® C18-E column (Phenomenex, USA). Briefly, columns were preactivated by sequential washes with 10 ml of 99.9% acetonitrile/0.1% heptafluorobutyric acid (HFBA) and 10 ml of 0.1% HFBA. Sequential washes with 10 ml of 99.9% acetonitrile/0.1% HFBA, 10 ml of 0.1% HFBA, 3 ml of 0.1% HFBA containing 0.1%
Blockade. Recording period of at least 1 h was ensured to obtain baseline levels before the beginning of each protocol.

Animals were separated in different groups to evaluate baroreflex sensitivity or cardiac and ganglionic blockade. Twenty to twenty-four hours later, pulsatile arterial pressure, MAP and HR were continuously recorded with suture thread. After telemetry probe implantation in the abdominal aorta under xylazine (7.5 mg/kg) and ketamine (80 mg/kg) anesthesia, rats were housed in individual cages and allowed 8–10 days for recovery before transmitters were turned on. After telemetry recordings indicate re-establishment of a regular 24-h oscillation of MAP and HR, data were sampled (500 Hz) for 10 s, every 10 min, 24 h for 3 consecutive days. In additional experiments, short-term monitoring of MAP and HR were performed in conscious freely moving rats with a data acquisition system (MP100/150—Acqknowledge IV; Biopac System; Santa Barbara, CA). Anesthetized (xylazine 7.5 mg/kg and ketamine 80 mg/kg i.p.) rats were subjected to catheterization of the femoral artery and vein with polyethylene tubing (PE10/PE50) for arterial pressure recordings and drug injections, respectively. Catheters were tunneled through the subcutaneous to the interscapular region and fixed with suture thread. Twenty to twenty-four hours later, pulsatile arterial pressure, MAP and HR were continuously recorded. Animals were separated in different groups to evaluate baroreflex sensitivity or cardiac and ganglionic blockade. Recording period of at least 1 h was ensured to obtain baseline levels before the beginning of each protocol.

Evaluation of the baroreflex control of HR and cardiac autonomic control
Baroreflex bradycardia was measured by the reflex HR responses that were triggered by changes in MAP (in mmHg) induced by increasing doses of the α1-adrenergic agonist, phenylephrine (0.25–5 μg per rat, i.v.), as in previous studies [17–20]. HR responses were converted into pulse interval (PI, ms) and the sensitivity index was calculated in each rat by the average of the DPI/DMAP obtained with each dose of phenylephrine. For illustrative purposes the best fit line that correlates mean changes in PI and mean changes in MAP for the entire group was plotted, as well.

Evaluation of the cardiac autonomic control
Thirty minutes after the baroreflex test, cardiac autonomic tonus was determined in the same animals as described in previous studies [20]. First, rats were subjected to muscarinic blockade with methyl-atropine (3 mg/kg, i.v.) and the maximum HR change in a period of 15–20 min was collected. Next, rats were subjected to β-adrenergic blockade with propranolol (4 mg/kg, i.v.) and the HR value after 15–20 min was collected, which corresponded to the intrinsic HR. The difference of the maximum HR and the intrinsic HR was the sympathetic tonus. On the next day, autonomic blockers were injected in the reverse order, i.e., first propranolol to obtain the minimal HR value and next methyl-atropine. The difference between intrinsic HR and the minimum HR was the parasympathetic tonus.

Evaluation of peripheral sympathetic activity by ganglionic blockade
After recording the baseline values for MAP and HR for at least 30 min, conscious SD control (n=5) and TG7371 (n=4) rats received a dose of hexamethonium (50 mg/kg, i.v.), a ganglionic blocker, in order to evaluate the contribution of the sympathetic activity. The effect of hexamethonium on MAP were determined by averaging 1 min of the maximal response.

Evaluation of renal parameters
To measure urine volume and water intake, animals were individually housed in metabolic cages (Nalgene, U.S.A.) for 72 h with free access to standard chow and tap water. The first and second days were used for adaptation to the cages. Samples (24-h urine and tail blood) were collected during the following 24 h (third day). Water balance was calculated by difference between water intake and urinary volume (UV). Serum sodium (Na+) and potassium (K+), urine sodium and potassium were measured by chemistry analyzer (ROCHE Cobas Mira Plus CC, U.S.A.) and plasma and urinary osmolality was determined by freezing point in an Osmometer (5004 MICRO- OSMETTE™, U.S.A.). Renal parameters were calculated as follows: Urinary excretion = [Na+] or [K+] × UV (mEq/day/100 g); Osmolar clearance = urine osmolality × UV ÷ plasma osmolality (ml/min/100 g); and Free water clearance = UV − osmolar clearance (μl/min/100 g). Glomerular filtration rate (GFR) was estimated through creatinine clearance.
Commercially available Jaffe colorimetric method was used to measure plasma and urinary creatinine concentration (Creatinine - K016-1, Bioclin-Brazil). Creatinine clearance was calculated by the formula: \[\text{Creatinine}_{\text{urine}} \times \frac{\text{UV}}{\text{Creatinine}_{\text{plasma}}} \text{ (ml/min/100 g of body weight)}.\]

**Measurement of plasma levels of vasopressin and atrial natriuretic peptides**

Commercially available enzyme immune or enzyme-linked immunosorbent assays were used to measure plasma levels of vasopressin (Arg8-Vasopressin; ELISA kit, Abcam®, U.S.A.) and atrial natriuretic peptide (ANP; EIA Kit, Phoenix Pharmaceuticals, U.S.A.) according to the manufacturers’ protocols.

**Hemodynamic measurements using fluorescent microspheres**

For surgical preparation, the rats were anesthetized with urethane (1.2 g/kg i.p.; Sigma–Aldrich, U.S.A.) and the trachea was cannulated to keep spontaneous breathing. The left brachial artery was cannulated with PE-10/PE-50 tubing for blood pressure (BP) and HR recordings (MP100/150—Acqknowledge IV; Biopac System; CA, U.S.A.). For administration of fluorescent microspheres, the right carotid artery was exposed, cannulated with PE-50 tubing and connected to a pressure transducer. The cannula was guided through the common carotid artery into the left ventricle and its placement was confirmed by recording the typical ventricular pressure. The right femoral artery was cannulated and connected to a pump (Minipuls 3, Gilson; Villiers le Bel, France) for blood withdrawal. Hemodynamics parameters and regional blood flow were determined using 15-μm fluorescent polystyrene microspheres (FluoSpheres Blood Flow Determination, Molecular Probes; Eugene, OR) as previously reported by Glenny et al. [21], Gervais et al. [22], and in our laboratory [23–25]. Briefly, a color of the microspheres was selected, sonicated and vigorously vortexed for 2 min to avoid sedimentation. After the mixing was completed, 300000 (0.3 ml) fluorescent microspheres were infused into the left ventricle over a 10-s period and flushed out with 0.3 ml of saline over an additional 10-s period. To calculate the blood flow, arterial blood was withdrawn at a rate of 0.85 ml/min through the right femoral artery. Blood sample for reference was withdrawn for 90 s starting 10 s before the microsphere injection. At the end of the experiment, animals were killed with an overdose of anesthetic (three-times the anesthetic dose, urethane 1.2 g/kg i.p.; Sigma–Aldrich, U.S.A.) and organs (kidneys, brain, mesentery, adrenals, spleen, abdominal skin, gastrocnemius muscle, lungs, testis, left ventricle, brown and white fat) were dissected 1 min after breathing stopped, weighed and stored in individual vials. Using a solution of 4 M ethanolic potassium hydroxide (KOH) containing 2% Tween-80, tissues and reference blood samples were digested in a hot bath (50°C) overnight. Thereafter, microspheres were recovered through sedimentation methodology described by Van Oosterhout et al. [26] and the dye was extracted in 4 ml of organic solvent, ethyl acetate. Fluorescence intensity of the dye was measured using a spectrophotometer (Cary Eclipse Fluorescence Spectrophotometer, Varian; U.S.A.). The following parameters were calculated: cardiac output (CO), stroke volume, total peripheral resistance (TPR), cardiac index and regional vascular resistance, as previously described [21–26].

**Hypertension model**

Six male heterozygous hypertensive rats were obtained by cross-breeding homozygous female TG7371 with male homozygous transgenic (mREN2)27 rats carrying the mouse renin gene, Ren-2 [27]. The presence of both transgenes was verified by genotyping PCRs. These male double heterozygous TG7371/(mREN2)27 rats and heterozygous (mREN2)27 controls were subjected to catheterization of the femoral artery for short-term BP and HR monitoring, under xylazine/ketamine anesthesia (7.5 mg/kg and 80 mg/kg i.p., respectively). The recording lasted at least 30 min after parameter stabilization and were performed in a data acquisition system (MP100/150—Acqknowledge IV; Biopac System; Santa Barbara, CA), in conscious rats 20–24 h after catheterization.

**Statistical analysis**

Data were expressed as mean ± SEM. Statistical differences were assessed by non-paired Student’s t test or one or two-way analysis of variance (ANOVA) followed by Bonferroni’s or Sidak’s multiple post-hoc tests, where appropriated and indicated in each figure legend. The criterion for statistical significance was set at \(P < 0.05\). Statistical analysis and graphics were performed using GraphPad Prism software (version 9.1.2).
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Figure 1. Transgene expression by qPCR and Western Blotting and Ang-(1-7) levels
(A) Expression of the mRNA encoding Ang-(1-7)-producing fusion protein (arbitrary units) measured by qPCR in tissues from TG7371 rats (n=2 for NTS; n=3 for CVLM; n=5 for all the rest). (B) Expression of the Ang-(1-7)-producing fusion protein by Western blotting in different tissues of TG7371 rats (n=2). Two immunoreactive bands were detected, one with the expected size of ∼32 kDa (band #1) and a shorter one, probably lacking the Ang-(1-7) after furin-mediated processing (band #2). SD rat tissues (medulla oblongata and ventricles) were used as a negative control (n=1). (C) Ang II-Ir and Ang-(1-7)-Ir determined by RIA in the plasma and hypothalamus of SD (n=4–7) and TG7371 (n=5–7) rats. Values are means ± SEM.

Results
Characterization of Ang-(1-7) expression in TG7371 rats
We first evaluated expression of the transgene in newly generated TG7371 rats at the mRNA level. Transgene expression was detectable in all tissues of the transgenic rats analyzed and quantified by qPCR. Highest mRNA expression for the transgene was found in the aorta, followed by brain areas, such as RVLM, CVLM, NTS in the medulla, as well as hippocampus, PAG, PVN and cortex (Figure 1A). Low amounts of transgene mRNA were found in other organs, e.g., lung, heart, kidney, liver, testis and adrenal gland. This result shows a ubiquitous mRNA expression of the transgene with the highest expression in aorta and brain. In order to identify in which cell type the transgene was expressed, RNAscope technique was performed. Based on the expression profile determined by qPCR, we chose to examine aorta, heart, lung, kidney and brain. Transgene expression was detectable preferentially in endothelial cells in aorta, heart, lung and kidney (Figure 2A). Low expression was also observed in aortic smooth muscle cells and Bowman capsule (Figure 2A). In the brain, the transgenic mRNA was present in glial cells (Figure 2B). In addition, to verify that mRNA was effectively translated into the fusion protein, we performed Western blotting analysis using an anti-mouse-IgG2b antibody in different tissues of TG7371. The fusion protein migrates with a molecular mass of ∼32 kDa [9]. Western blotting images showed the appearance of two immunoreactive bands in all evaluated tissues:
Figure 2. RNAscope detection of the mRNA encoding the Ang-(1-7)-producing fusion protein in sections (5 μm) from formalin-fixed, paraffin-embedded tissues

Detection of transgene expression in the aorta, heart, lung, kidney (A) and hypothalamus, cerebral cortex and medulla oblongata (B) in TG7371 rats is shown by the arrowheads. Tissues of SD rats were used as negative control. n=3 each group. Nuclei were counterstained with Hematoxylin. Abbreviations: A, alveolus; B, bronchiole; BC, Bowman capsule; C, capillary; G, glomerulus; L, vascular lumen. Images were taken using the inverse Keyence bright light/Fluorescence microscope (Keyence BZ9000).
one band with the expected size of ∼32 kDa and another slightly smaller, which likely represents the residual protein after the release of the Ang-(1-7) peptide (Figure 1B).

Next, hypothalamus and plasma Ang-(1-7) and Ang II levels were measured by RIA. As shown in Figure 1C, hypothalamus levels of Ang-(1-7) were significantly higher in TG7371 (29.7 ± 3.8 pg/mg protein, n=7) in comparison to SD rats (19.5 ± 2.7 pg/mg protein, n=5). Ang II levels were not significantly different in the hypothalamus of TG7371 (45.1 ± 4.8 pg/mg protein; n=7) in comparison to SD rats (55.9 ± 10.6 pg/mg protein; n=7; Figure 1C). These changes result in a 1.8-times higher Ang-(1-7)/Ang II ratio in TG7371 (ratio = 0.66) in comparison to that in SD rats (ratio = 0.35). However, plasma Ang-(1-7) and Ang II levels were similar in TG7371 and SD rats (Figure 1C). Thus, although there is a significant expression of the fusion protein in tissues, especially in brain and aorta, little or no spillover of Ang-(1-7) appears into the circulation, arguing for the local action of the released peptide.

Baseline arterial pressure
We compared arterial pressure of TG7371 (n=6) with SD control (n=7) rats with a long-term method to monitor BP, 24 h per day, using a radiotelemetry system. TG7371 rats showed a significant lower MAP both during the day (97 ± 2.3 vs 106.5 ± 1.7 mmHg in SD; Figure 3) and during the night (104 ± 2.4 vs 113 ± 1.5 mmHg in SD; Figure 3). No significant difference in baseline HR was observed between TG7371 and SD rats. Further, circadian variation of MAP and HR was not altered in TG7371 in comparison to SD rats (Figure 3). Although the arterial pressure alteration was
small in TG7371, this phenotype indicates an important cardiovascular effect resulting from increased Ang-(1-7) in tissues. Considering the normotensive background of these animals, the hypotensive phenotype of TG7371 is notable.

Baroreflex control of HR
One of the most well-reproduced effects induced by Ang-(1-7) is the improvement in baroreflex function, especially the baroreflex control of HR [1,17,20]. Since the transgene was highly expressed in the brain, we next evaluated the sensitivity of the baroreflex bradycardia in TG7371. For this protocol, we used a short-term method to record BP by an intraarterial catheter. Corroborating data from telemetry measurements, TG7371 showed lower MAP (105 ± 1.5 mmHg; n=5) than SD rats (113 ± 2.9 mmHg, n=6; Supplementary Figure S1), however, accompanied with an increased baseline HR (400 ± 11 vs 351 ± 14 beats/min in SD; Supplementary Figure S1). Further, TG7371 showed similar baroreflex bradycardia as SD rats (Figure 4A,B), indicating that baroreflex function was normal.

Cardiac autonomic activity
Due to the alteration in baseline HR observed with short-term BP recordings, we next assessed the cardiac autonomic tone using pharmacological blockade of post-ganglionic receptors of the autonomic nervous system in the heart. To our surprise, sympathetic cardiac tone was significantly increased in TG7371 (145 ± 12 beats/min, n=5; Figure 4C) in comparison to SD rats (100 ± 11 beats/min, n=5; Figure 4C). Vagal tone in TG7371 (47 ± 7 beats/min; Figure 4D) was not significantly different from that in SD (62 ± 9 beats/min in SD; P=0.23; Figure 4D). When we compared the ratio between sympathetic and parasympathetic tone of TG7371 and SD rats (Figure 4F), the predominance of cardiac sympathetic tone in TG7371 is even stronger. There was no significant difference between TG7371 and SD rats in relation to maximum HR (507 ± 4 vs 454 ± 23 beats/min in SD; data not shown) and minimum HR (315 ± 10 vs 307 ± 6 beats/min in SD; data not shown). Intrinsic HR (Figure 4E) was also not different, suggesting that there is no alteration in the pacemaker cell firing.

Overall sympathetic activity
Considering the increased level of sympathetic tone to the heart, we next attempted to evaluate the global net sympathetic tone through the BP response induced by the ganglionic blocker, hexamethonium. A greater fall in BP in TG7371 (−45.5 ± 1.3 mmHg, n=4; Figure 4G,H) was observed as compared with SD rats (−36.9 ± 2.6 mmHg, n=5; Figure 4G,H). Considering that TG7371 had lower baseline MAP, this effect is very impressive. Taken together, our data show that TG7371 present an increase in cardiac and global net sympathetic activity, however, with lower baseline BP.

Renal parameters
Results described above prompted us to evaluate other parameters that could affect regulation of BP, such as the ability of TG7371 for volume handling. Figure 5 shows that TG7371 presented increase in water intake (9.2 ± 0.5 vs 7.2 ± 0.3 ml/day/100 g in SD; Figure 5A), without significant difference in UV, resulting in a more positive water balance (6.8 ± 0.5 vs 4.5 ± 0.4 ml/day/100 g in SD; Figure 5C). Moreover, TG7371 showed increased urinary sodium excretion (0.63 ± 0.12 vs 0.34 ± 0.04 mEq/l in SD; Figure 5D) accompanied by an increase in urinary osmolality (2093 ± 137 vs 1641 ± 116 mOsm/kg H2O in SD; Figure 5F) and a reduction in free water clearance (−10.5 ± 0.5 vs −8.2 ± 0.8 μl/min/100 g in SD; Figure 5K). In addition, TG7371 presented increased plasma potassium concentration (5.30 ± 0.17 vs 4.15 ± 0.23 mEq/l in SD; Figure 5H). TG7371 showed similar plasma osmolality, potassium excretion in the urine and sodium concentration in the plasma as SD rats (Figure 5G–I). GFR, measured through creatinine clearance, showed a downward tendency in TG7371 (0.38 ± 0.05 vs 0.53 ± 0.12 ml/min/100 g in SD; Supplementary Figure S2), that was not statistically significant. These data prompted us to evaluate plasmatic levels of ANP and vasopressin (AVP).

Plasma levels of vasopressin and ANP
TG7371 exhibited, indeed, a significant increase in plasma levels of ANP (253 ± 44 vs 135 ± 20 pg/ml in SD; Figure 5M). In addition, we also observed a significant increase in plasma AVP (4.6 ± 0.9 vs 2.2 ± 0.3 pg/ml in SD; Figure 5L). These data showed that mechanisms activated directly (direct action on tissues, mainly in the central nervous system (CNS)) or indirectly (triggered by lower BP) by Ang-(1-7) are contributing to the urinary parameters reported above.
Figure 4. Baroreflex control of HR and peripheral autonomic control in TG7371 rats

(A) Index of the sensitivity of baroreflex bradycardia (ΔPI/ΔMAP, ms/mmHg) and (B) the best-fit regression line between the changes in HR (ΔPI, ms) and changes in MAP (ΔMAP, mmHg) induced by different doses of phenylephrine in TG7371 (n=5) and SD (n=6) rats. (C) Cardiac sympathetic tone (in beats/min), (D) vagal tonus (in beats/min), (E) intrinsic HR (in beats/min) and (F) the ratio between cardiac sympathetic and vagal tone in TG7371 (n=5) and SD (n=5) rats. (G) Baseline and (H) changes in MAP (mmHg) in response to hexamethonium (50 mg/kg; i.v.) in TG7371 (n=5) and SD (n=4) rats. Values are mean ± SEM. *P<0.05 vs SD (Student’s t test); #P<0.05 vs baseline (two-way ANOVA followed by Bonferroni’s test).
Figure 5. Urinary parameters and hormone levels in TG7371 rats

(A–K) Bar graphs showing water intake, urine volume (ml/day/100 g) and water balance in 24 h (%); sodium and potassium concentration/excretion in plasma (mEq/day/100 g of body weight) and urine (mEq/l); plasma and urine osmolarity (mOsm/kg H₂O); osmolar clearance (ml/min/100 g) and free water clearance (μl/min/100 g) in TG7371 (n=5) and SD (n=6) rats. Plasma levels of Vasopressin (pg/ml; n=7–9; (L)); and ANP (pg/ml; n=6–7; (M)). Values are mean ± SEM. *P<0.05 vs SD (Student’s t test).
Hemodynamic measurements

Given the results described above, we went further in order to explain the lower BP of TG7371 measuring hemodynamic parameters. Regional blood flow was measured by fluorescent microspheres method and stroke volume, CO, TPR, cardiac index and regional vascular resistance were calculated in urethane-anesthetized rats. As shown in Figure 6, TG7371 showed a significantly lower TPR (2.28 ± 0.18 mmHg.ml⁻¹.min.100 g) than SD rats (3.71 ± 0.56 mmHg.ml⁻¹.min.100 g). Lower TPR was accompanied by a significantly higher cardiac index in TG7371 (42.0 ± 4.6 ml.min⁻¹.100 g⁻¹) in comparison to SD rats (29.0 ± 3.1 ml.min⁻¹.100 g⁻¹). There was no significant difference in the CO (ml.min⁻¹) and stroke volume (ml) in TG7371 compared with SD rats (Figure 6). MAP in anesthetized TG7371 was also lower (89 ± 4 mmHg; Figure 6) in comparison to SD rats (102 ± 2 mmHg; Figure 6). However, HR was not significantly different between the two groups of rats (TG7371 = 281 ± 8 beats/min and SD = 264 ± 9 beats/min; Figure 6). Figure 6 and Supplementary Table S1 present values of blood flow and regional resistance in different organs evaluated in SD and TG7371 rats. Blood flow in kidney, muscle, heart, mesentery, brown fat and skin were substantially higher in TG7371 rats as compared with their age-matched controls. The increases in blood flow in testis, brain, lung, white fat, spleen and adrenal were not significantly altered. Thus, these data indicate that overexpression of Ang-(1-7) decreases TPR due to important reduction in blood flow in several organs/tissues, which results in the lower baseline BP of TG7371 rats. The increased in cardiac index did not compensate the lower TPR.

Hypertension model

Based on these findings, we evaluated whether overexpression of Ang-(1-7) could rescue the hypertensive phenotype of hypertensive rats overexpressing renin. Transgenic rats expressing both, mouse renin-2 and the Ang-(1-7)-producing fusion protein, TG7371/(mREN2)27, presented a significant lower MAP (150 ± 1 mmHg; n=6; Supplementary Figure S3) in comparison with heterozygous hypertensive transgenic (mREN2)27 rats (175 ± 2 mmHg; n=4; Supplementary Figure S3). No significant difference in baseline HR was observed in these animals (Supplementary Figure S3).

Discussion

In the present study, we described the cardiovascular and hemodynamic phenotype of a transgenic rat strain overexpressing of an Ang-(1-7)-fusion protein in tissues. The main peculiarity of this new transgenic line, TG7371, is the increased tissue expression of Ang-(1-7) fusion protein, associated with absence of changes in the circulating levels of the peptide or Ang II. Our previously described transgenic rat, TG3292, presents increased levels of plasma Ang-(1-7), however, associated with normal tissue levels [9]. While TG3292 are normotensive, TG7371 are hypertensive. Of note to highlight, this is an important phenotype difference, considering the normotensive genetic background of these animals. More importantly, the lower BP of TG7371 shows the strength of long-term overexpression of Ang-(1-7) in tissues versus in the circulation in modulating arterial pressure.

The lower MAP of TG7371 was consistently observed with different methods of BP measurements, radiotelemetry or short-term recordings in conscious or anesthetized animals. Moreover, TG7371 presented increased plasma ANP, increased blood flow in different organs/tissues, which resulted in a significant decrease in TPR, accounting for the hypertensive phenotype of these rats. However, TG7371 also showed alterations in parameters or hormones that in general increase BP, such as, increase in cardiac index, increase in cardiac and global net sympathetic tone and increase in plasma levels of AVP. These alterations suggest that multiple partially compensating mechanisms are triggered by Ang-(1-7)-fusion protein tissue overexpression, with a net result of a reduced baseline BP. The BP reducing effect of this tissue Ang-(1-7)-fusion protein was even more pronounced in rats with a hypertensive background since offspring of TG7371 and the RAS-dependent hypertensive rats, (mREN2)27, presented markedly attenuated hypertension.

Considering the protein and mRNA expression of the Ang-(1-7)-producing fusion protein in different tissues, it is most likely that changes observed in TG7371 are mainly related to alterations in Ang-(1-7) levels in the brain and vascular endothelial cells. Actually, Ang-(1-7) was found to be increased in the hypothalamus of TG7371. Future studies will have to show the level of Ang-(1-7) in different tissues of TG7371, as well as, the activity of the RAS enzymes (ACE, ACE2, renin, pro-renin) and the expression of angiotensin receptors (MAS, AT1 and AT2). Previous studies showed that short-term [1,28] or long-term [1,20,28,29] ICV infusion of Ang-(1-7) attenuated the elevated level of BP in different models of hypertension or in animals subjected to a model of metabolic syndrome [30]. Cerrato et al. [31] also showed that injection of Ang-(1-7) in the anterior hypothalamus promotes reduction in MAP in spontaneously hypertensive rats (SHRs). Furthermore, Yamazato et al. [32] showed that overexpression of ACE2 in the RVLM lowers BP of SHR. ACE2 overexpression in the brain also attenuated the development of neurogenic hypertension, partially
Figure 6. Hemodynamic parameters in TG7371 rats

Mean Arterial Pressure

Total Peripheral Resistance

Cardiac Index

Heart Rate

Stroke Volume

Cardiac Output

Regional Blood Flow

MAP (mmHg), TPR (mmHg.ml⁻¹.min⁻¹.100 g of body weight), Cardiac Index (ml. min⁻¹.100 g⁻¹ of body weight), HR (beats/min), Stroke Volume (ml), CO (ml.min⁻¹) and Regional Blood Flow (ml.min⁻¹.g⁻¹ of tissue) in urethane-anesthetized SD (n=7) and TG7371 (n=7) rats. Blood flow was measured in different organs with fluorescent polystyrene microspheres. Values are mean ± SEM. *P<0.05 vs SD (Student’s t test).
by preventing the decrease in both spontaneous baroreflex sensitivity and parasympathetic tone [8]. In addition, targeted overexpression of ACE2 in vascular smooth muscle cells also reduced the BP in SHR [33]. It is important to point out, however, that the effects observed with overexpression of ACE2 can also be ascribed to a decrease in Ang II and not only to an increase in Ang-(1-7). On the other hand, peripheral short-term infusion of Ang-(1-7) in Wistar rats induced increase in vascular conductance in several tissues resulting in a decreased TPR [23]. The animals in this study also presented increased CO, which seems to have compensated for the fall in TPR resulting in no substantial changes in BP [23]. Similar results were observed in the transgenic rats with lifetime overproduction of circulating Ang-(1-7), TG3292 [24]. These animals showed pronounced increase in regional blood flow with increased vascular conductance, with no changes in BP. TG3292 also presented an attenuated hypertension after been subjected to a DOCA-salt model [34]. In keeping, chronic treatment with an oral formulation of Ang-(1-7) induced attenuation in high BP in SHR [35]. In addition, a direct endothelial-dependent vasorelaxation effect of Ang-(1-7) has been reported by several authors [1,36–38].

In the brain, previous studies have shown expression of MAS [1,39] or MAS mRNA [1,40,41] in different areas related to the control of baroreflex or sympathetic tone, such as in the medulla (NTS, RVLM and CVLM) and in the hypothalamus (PVN and SON) and in areas apparently not directly related to cardiovascular function, such as amygdala, hippocampus, and different areas of the cortex. Furthermore, Block et al. [42] showed the neuroanatomical distribution of Ang-(1-7) in the PVN and SON, with the presence of Ang-(1-7) and AVP in magnocellular neurosecretory cells of the PVN suggesting that Ang-(1-7) is part of the secretory hypothalamus pituitary pathway. In addition, previous studies have shown that incubation of the hypothalamic-neurohypophyseal system with Ang-(1-7) stimulates the release of AVP [43]. Thus, the increase in sympathetic drive to periphery and AVP release observed in the present study can be related to a preferential action of Ang-(1-7) at these sites of the brain. Otherwise, increase in peripheral sympathetic tonus and AVP plasma levels might be compensatory mechanisms triggered by the lower BP.

The reduction in free water clearance and a more positive water balance in the transgenic rats are in agreement with the increased AVP, which contributes to the formation of a more concentrated urine. In addition, alterations in water excretion in feces or respiration in TG7371 should be evaluated in the future. The increase in urinary sodium may be related to the renal action of ANP in the distal nephron. ANP leads to inhibition of apical Na\(^+\) channel function and excretion in feces or respiration in TG7371 should be evaluated in the future. The increase in urinary sodium may be the increased AVP, which contributes to the formation of a more concentrated urine. In addition, alterations in water reabsorption from the inner medullary collecting duct and increased urinary excretion of Na\(^+\), natriuresis. In the TG7371, the combined opposite actions of AVP and ANP may have kept urinary volume, i.e., the outcome of a water-absorbing (AVP) and natriuretic/diuretic hormone (ANP) [44,45]. Additionally, we cannot rule out a direct kidney effect of Ang-(1-7) on water handling.

Ang-(1-7) actions in the kidney are controversial and appear to depend on the animal species evaluated, local and systemic concentrations of Ang-(1-7), nephron segment studied and sodium and water balance [46,47]. In vitro and in vivo studies have reported a diuretic and natriuretic action of Ang-(1-7) [48,49], as well as an antidiuretic effect [47,49,50]. Moreover, studies have shown that Ang-(1-7) produced an NO-dependent vasodilation in the kidney using microperfused rabbit afferent arterioles [51] or in the isolated renal artery [52]. These data are in accordance with a previous study showing an increase in renal blood flow after Ang-(1-7) infusion in rats [23]. However, a yet unexplored differential effect of Ang-(1-7) in afferent and efferent renal arterioles and the influence of renal sympathetic activation in TG7371, may contribute to the regulation of the GFR. Thus, a more detailed and systematic study of the renal function of TG7371 should be performed in the future, including the use of a more appropriate method to assess GFR. Additionally, although we have no evidence that plasma renin activity was altered in TG7371, based on normal Ang II plasma levels, the overall increased sympathetic tonus instigates future evaluations on renal sympathetic nerve activity and whether it will have any long term adverse impact in kidney function. Furthermore, while not definitively established yet, there is evidence that chronic elevation of vasopressin can lead to renal failure [53,54]. Thus, future studies will have to monitor TG7371 rats also for kidney damage at older ages.

Lower BP of TG7371 can be attributed to lower TPR, since cardiac index was even higher. TG7371 presented changes in regional blood flow resulting in an increase in the conductance to the kidney, muscle, mesentery, brown fat, skin and left ventricle, which contributed to the reduction in TPR and to the lower baseline BP. The increase in cardiac index can be attributed to both an increase in cardiac sympathetic tone and a possible increase in venous return, which in turn is due to a fall in TPR combined with a possible sympathetic driven reduction in venous capacitance associated to an increase in blood volume. This latter effect is indicated by the increase in water intake and positive water balance. In addition to the direct vasodilator effect of Ang-(1-7), another mechanism that can also contribute to the fall in TPR is the increased level of ANP observed in TG7371. ANP may be contributing to decreased TPR through its well-known vasodilator action [55]. Different studies have shown that individuals with a genetic variant of ANP, which is associated with higher circulating levels of ANP, have lower BP values and lower risk of developing hypertension [56–58]. Thus, the lower BP of TG7371 may also be due to the hypotensive effect of ANP. The increase
in ANP also determined an increase in urinary sodium concentration by its inhibitory effect on the Na⁺/K⁺ ATPase pump and epithelial sodium channels [59], which in turn explains the increase in plasma concentrations of potassium.

The increased ANP in TG7371 rats can be attributed to an increase in venous return due to the hyperdynamic state of the circulation with increased cardiac index, probably associated to an increased blood volume as point out above [60,61]. On the other hand, a direct effect of Ang-(1-7) on cells in the atrium cannot be ruled out. Previous studies have shown that Ang-(1-7) can increase ANP secretion through an MAS/PI3K/Akt/NO signaling pathway [62,63]. Alternatively, and although rather speculative, another possibility is that Ang-(1-7) acting on SON and PVN neurons can indirectly, through the release of oxytocin, induce the release of ANP. This hypothesis is based on studies that showed (i) that oxytocin can act on cardiac receptors promoting secretion of ANP [64], (ii) that in rats with body volume expansion, neuronal excitation in the neurohypophysis elicits oxytocin secretion into the circulation, activating atrial oxytocin receptors and promoting the release of ANP [65], and (iii) that microinjection of Ang-(1-7) into the PVN promotes neuronal excitation [1,43,66], which may induce the release of AVP [43,67] and, probably oxytocin. Future studies will have to be conducted to confirm our hypothesis.

In the present study we showed no alteration in the sensitivity of baroreflex control of HR in TG7371 rats. This result is opposite to those observed in several studies in which an increase in Ang-(1-7) improved baroreflex control. This beneficial effect of Ang-(1-7) was shown after microinjection of the peptide into the NTS of normotensive rats or after short- or long-term ICV infusions [1,17,20,28–31]. However, TG7371 also presented an increase in AVP. Michelini and Bonagamba [68] showed that microinjection of AVP into the NTS attenuated baroreflex bradycardia suggesting that a central vasopressinergic system is a negative modulator of the baroreflex. Thus, it is possible that the alterations in AVP triggered by Ang-(1-7) or other mechanisms triggered by the lower BP are counteracting the direct effect of Ang-(1-7) in areas involved in baroreflex control. Future studies will have to evaluate these possibilities. It was also unexpected that TG7371 presented an increase in water intake. Previous studies were unable to show changes in drinking behavior after central administration of Ang-(1-7) [1,17,67]. More recently, however, studies have shown that under certain conditions Ang-(1-7) can increase water intake. Joyner et al. [69] using intravenous infusion of the MAS antagonist, A779, in pregnant rats suggested that Ang-(1-7) contributes to enhance water intake during pregnancy. In addition, dos Santos et al. [70] showed that after water deprivation or salt loading, ICV Ang-(1-7) increased water intake. The expression of MAS in the parvocellular area of PVN and in other nuclei that control water intake [1,39] form the basis to support a dipsogenic effect of Ang-(1-7), at least in particular conditions.

Finally, we would like to point out that the TG7371 expresses an Ang-(1-7)-producing fusion protein under the control of the human GFAP promoter. This promoter was expected to be astrocyte-specific. Transgene mRNA was however abundant also in endothelial cells, which explains the expression observed in most analyzed organs. Indeed, in a previous study we have already observed some expression of a GFAP-driven transgene in endothelial cells in rats [13]. Further, a recent study may have shed some light on this unexpected expression pattern. Osman et al. [71] showed that GFAP-positive progenitors act as stem cells for the development of both vascular smooth muscle and endothelial cells in mice.

**Study limitations**

A limitation of our study is that it did not include phenotyping of female TG7371 rats. There are important gender differences related to the RAS components in response to different stimuli. Thus, it is crucial and perhaps even mandatory, that in future studies gender comparisons are performed in order to improve the treatment and control of diseases for women, in particular cardiovascular diseases. Another limitation is that in the present study we have not evaluated a potential variability of transgene expression among animals, which may account for the variability observed in several parameters. Finally, we have not assessed the effect of the overexpression of the construct without the Ang-(1-7) sequence. However, it is very unlikely that the phenotype observed in TG7371 is due to the sequence of the construct without the peptide. Different models of transgenic mice and rats expressing a similar fusion protein containing angiotensin peptide or other peptide sequence, developed by us and other groups [4,9,72–75], have shown distinct phenotypes and the ones expected for the peptide added. Furthermore, Methot et al. [72] showed that the expression of the same protein without the fusion-peptide in cardiomyocytes, in vitro and in the heart, in vivo, of SD rats had no significant effect.

**Conclusion**

In summary, we showed that transgenic rats presenting tissue overexpression of an Ang-(1-7)-producing fusion protein have lower BP due to a decrease in TPR associated with an increase in ANP. Ang-(1-7)-induced or BP-compensatory mechanisms, such as, increase in peripheral sympathetic drive and increase in plasma AVP, were
also observed and are probably counteracting mechanisms to maintain hemodynamic homeostasis. TG7371 is a good animal model to further explore the contribution of tissue Ang-(1-7) to the pathophysiology of different diseases. It is the first and single transgenic rat model to evaluate the effects induced by tissue Ang-(1-7) without significant alterations in circulating RAS peptide levels. It also represents an important tool to investigate the long-term effect of increased levels of Ang-(1-7) in different systems and under different pathophysiological conditions.

Clinical perspectives

- The RAS is present in several tissues, where angiotensins modulate different functions. However, the relative contribution of local vs blood-borne angiotensins is not completely understood. Here we took advantage of a transgenic model to explore the effect of increasing tissue Ang-(1-7).

- Transgenic rats overexpressing an Ang-(1-7)-producing fusion protein in neurons and endothelial cells (TG7371) presented lower BP due to a decrease in TPR associated with an increase in ANP with normal circulating levels of Ang-(1-7). They also presented an increase in peripheral sympathetic drive and plasma AVP, probably related to a direct effect of Ang-(1-7) or to the lower BP. Moreover, the BP reducing effect of tissue Ang-(1-7) was even more pronounced in hypertensive rats since offspring of TG7371 and the RAS-dependent hypertensive rats, (mREN2)27, presented markedly attenuated hypertension.

- This transgenic rat represents a new tool to investigate the long-term impact of increased levels of tissue Ang-(1-7) on the pathophysiology of cardiovascular and other diseases. Moreover, these data give support for the development of pharmacological therapies targeted to induce tissue-specific increase in Ang-(1-7) for the treatment of cardiovascular and inflammatory diseases.

Data Availability

All key supporting data are included in the main article and its supplementary files. The data underlying this article will be available upon reasonable request to the corresponding author.

Competing Interests

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

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CRediT Author Contribution

Daniele T. Alves: Conceptualization, Formal analysis, Investigation, Methodology, Writing—original draft, Project administration, Writing—review and editing. Luiz Felipe Mendes: Investigation. Walkyria O. Sampaio: Investigation, Methodology. Leda M.C. Coimbra: Investigation, Methodology. Almir S. Martins: Visualization, Methodology. Elena Popova: Methodology. Mihail Todiras: Investigation, Methodology. Fatimunnisa Qadri: Investigation, Methodology. Natalia Alenina: Resources, Supervision, Funding acquisition, Writing—review and editing. Michael Bader: Conceptualization, Resources, Supervision, Funding acquisition, Visualization, Writing—review and editing. Robson A.S. Santos: Conceptualization, Resources, Supervision, Funding acquisition, Writing—review and editing. Maria Jose Campagnole-Santos: Conceptualization, Resources, Supervision, Funding acquisition, Project administration, Writing—review and editing.
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Abbreviations

ACE, angiotensin-converting enzyme; Ang, angiotensin; ANP, atrial natriuretic peptide; AVP, vasopressin; BP, blood pressure; CO, cardiac output; CVLM, caudal ventrolateral medulla; GFR, glomerular filtration rate; HFEBA, heptafluorobutyric acid; hGFAP, human glial fibrillary acidic protein; HR, heart rate; ISH, in situ hybridization; MAP, mean arterial pressure; NTS, nucleus tractus solitarii; PAG, periaqueductal gray area; PI, pulse interval; PVN, paraventricular nucleus; qPCR, quantitative real-time reverse-transcription polymerase chain reaction; RAS, renin-angiotensin system; RIA, radioimmunoassay; RVLM, rostral ventrolateral medulla; SD, Sprague–Dawley; SHR, spontaneously hypertensive rat; SON, supraoptic nucleus; TPR, total peripheral resistance; UV, urinary volume.

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