A peptide fraction from hardened common beans (*Phaseolus vulgaris*) induces endothelium-dependent antihypertensive and renal effects in rats

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ABSTRACT

Beans reached the research spotlight as a source of bioactive compounds capable of modulating different functions. Recently, we reported antioxidant and oxidonitrergic effect of a low molecular weight peptide fraction (<3 kDa) from hardened bean (*Phaseolus vulgaris*) *in vitro* and *ex vivo*, which necessitate further *in vivo* assessments. This work aimed to evaluate the hypotensive effect and the involved physiological mechanisms of the hardened common bean peptide *(Phaseolus vulgaris)* in normotensive (Wistar) and hypertensive (SHR) animals. Bean flour was combined with a solution containing acetonitrile, water and formic acid (25: 24: 1). Protein extract (PV3) was fractioned (3 kDa membrane). We assessed PV3 effects on renal function and hemodynamics of wistar (WT–normotensive) and spontaneously hypertensive rats (SHR) and measured systemic arterial pressure and flow in aortic and renal beds. The potential endothelial and oxidonitrergic involvements were tested in isolated renal artery rings. As results, we found that PV3: I) decreased food consumption in SHR, increased water intake and urinary volume in WT, increased glomerular filtration rate in WT and SHR, caused natriuresis in SHR; II) caused NO- and endothelium-dependent vasorelaxation in renal artery rings; III) reduced arterial pressure and resistance in aortic and renal vascular beds; IV) caused antihypertensive effects in a dose-dependent manner. Current findings support PV3 as a source of bioactive peptides and raise the potential of composing nutraceutical formulations to treat renal and cardiovascular diseases.

1. Introduction

The common bean (*Phaseolus vulgaris*) is a legume widely consumed and cultivated throughout the world. The largest producers are India, Myanmar and Brazil (Coêlho, 2019; Ribeiro, 2017). The high consumption of beans occurs mainly in underdeveloped countries as it is considered a low-cost protein when compared to animal sources (Batista et al., 2010). The nutritional facts show high contents of proteins,

minerals, vitamins, phenolic compounds, tannins, carbohydrates and fibers (Batista, 2014; Ribeiro, 2017; Ribeiro et al., 2019; Valencia-Mejía et al., 2019). However, beans are susceptible to hardening due to inadequate storage, likely resulting from exposure to high temperature and humidity that change physicochemical features and nutrient availability, thus turning the bean more resistant to cooking: the well-known Hard-to-Cook effect (HTC) (Siqueira et al., 2018). Considering the high protein content of hardened beans, it is feasible proposing this legume as a source of bioactive peptides, since the hardening process may rely on protease activation that hydrolyzes many proteins in smaller fragments capable of displaying biological activities (Costa et al., 2006; Luna-Vital et al., 2015; Rodrigues et al., 2022). Many peptides are found in their encrypted form and need to undergo hydrolysis processes to perform their functions (Lemes et al., 2016; Rodrigues et al., 2022; Valencia-Mejía et al., 2019). The possibility that peptides from HTC beans promote beneficial effects to health is supported by recent evidence obtained in different experimental models (Graziani et al., 2021; Ribeiro et al., 2019; Rodrigues et al., 2022).

Several sources of compounds that may have biological activities are considered health-promoting foods; such effects are likely reached by the bioactions of proteins and peptides on molecular targets expressed in different physiological systems (Durak et al., 2013; Gianfranceschi et al., 2018; Martínez-Sánchez et al., 2020). The biological activities of each peptide are settled by the amino acid composition, sequence, size, structure, type of amino acid found in the C and N terminal region, hydrophobicity and charge (Gianfranceschi et al., 2018; Lemes et al., 2016; Martínez-Sánchez et al., 2020). Due to their rich composition, peptides with anti-hypertensive potential are among the most studied molecules in the functional foods field (Martínez-Sánchez et al., 2020). Recently, we reported that a low molecular weight peptide fraction (<3 kDa) from *Phaseolus Vulgaris* beans (PV3) is antioxidant and is able to induce nitric oxide (NO) release in endothelial cells, thus evoking vasorelaxation in isolated aortic rings (Graziani et al., 2021). This necessitates further assessments using *in vivo* experimental models of hypertension and renal dysfunctions. Therefore, this study was aimed at evaluating the PV3 effects on renal and cardiovascular functions, and the mechanisms potentially involved in the hemodynamic effects of this pool of peptides extracted from hardened common bean. We also tested whether endothelial NO release contributes to the renal and antihypertensive effects of PV3.

2. Material and methods

2.1. Material

The material used in this study was supplied by the Brazilian Agricultural Research Corporation (EMBRAPA) Rice and Beans, Santo Antônio de Goiás, Goiás. The beans supplied belong to the species *Phaseolus vulgaris*, cultivar Pontal, from the carioca commercial group. The methods described by Ribeiro et al. (2019) were of choice to harden the beans. From these HTC beans, the flours were produced with manually dehulled beans, passed in a knife mill and later sieved (500 μm). The flours were stored in sealed plastic bags and placed in a refrigerator at −20 °C.

2.2. Extraction of proteins and peptides

To extract the protein fractions, the methodology described by Ribeiro et al. (2019) was used. The material obtained, called extract 1 (E1), was lyophilized and stored at room temperature. Protein extracts were subjected to ultrafiltration in porous membrane ("cut-off" 3 kDa) (Amico Bioseparations). The ultrafiltration was conducted under nitrogen gas pressure (50 kgf/cm^2). The filtrate with molecular weight below 3 kDa will be identified by the acronym PV3, as refereed to the peptide fraction smaller than 3 kDa obtained from *Phaseolus vulgaris* hardened beans.

2.3. Systematic review

In order to check for a possible homology among the PV3 sequences recently reported by our group (Graziani et al., 2021) with other peptide sequences already reported in the literature, a comparative systematic review was performed. A data search was done using the principal indexing sources in the field: PubMed and ScienceDirect. The retrieving articles containing identifications or purifications of food-borne peptides with antihypertensive activity by using the keywords: "bioactive peptides", "food peptides", "bean peptides", "antihypertensive peptides" and "peptides sequencing" were selected. Following, we performed a comparison among sequences and amino acid fragments that were homologous to those reported by Graziani et al. (2021). As inclusion criteria, the motifs bigger than 5 amino acids were considered for peptide homology comparisons.

2.4. PV3 osmolarity

To perform the assay, 50 μl of PV3 in the concentration range (50–1000 μg mL^1) and 150 μl of deionized water were used, and then the solution was placed in the equipment to take the reading. The osmolarity of the PV3 solution was determined after its freezing, by the micro-osmette equipment (Precision Systems).

2.5. Determination of ions in protein extract

To determine the amount of sodium and potassium ions, enzymatic analysis sets (BioClin) were used, and the reaction was carried out in an automated biochemical analysis equipment (Wiener Lab Group-CM200).

2.6. Experimental design - in vivo and ex vivo assays

2.6.1. Animals

The experimental protocols were performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and with the Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA) Experimental procedures involving animals were approved by the local Ethics Committee on Animal Use (Protocol CEUA/UFG 84/2018). We made all efforts to minimize the number of animals used. All experiments were performed in adult male Wistar (WT) and in spontaneously hypertensive rats (SHR) with 250–350 g and with the age group from 12th to 15th week, bred at the Central Animal Facility of the Federal University of Goiás.

2.6.2. Drugs

The drugs used were Sodium Heparin (Liquemine, 5000U.I mL^{-1} – Roche Laboratories, Brazil); Sodium thiopental (THIOPENTAX®) (2.5%) (Cristália Ltda); Krebs-Henseleit nutrient solution (pH 7.4; composition in mM: NaCl - 130; NaHCO$_3$ - 14.9; KCl - 4.7; KH$_2$PO$_4$ - 1.18; MgSO$_4$ 7H$_2$O - 1.17; CaCl$_2$ 2H$_2$O - 1.6; glucose – 5.5); Sodium nitroprusside (1 μM); Phenylephrine (1 μM); Acetylcholine (10 μM), Nω-nitro-L-arginine methyl ester (100 μM); Halothane (2% in 100% O$_2$; Cristália, Ltda).

2.6.3. Non-invasive systolic arterial pressure measurement

Tail plethysmography technique was used to assess the systolic arterial pressure (SBP) daily, during 3 days before starting treatments, in order check for the hypertensive and normotensive states of SHR and WT, respectively. Samplings were as follows. Animals were housed in a warming box at a temperature of approximately 36 °C for 15 min for caudal artery dilatation. The animal was placed in an individual box and a rubber cuff was placed at the base of the tail along with a pulse receiver, both coupled to a transducer and connected to the data acquisition system (Powerlab 4/25, ML0380/AD Instruments, Bella

Vista, Australia). From the inflation and deflation of the cuff at regular intervals, the SBP was determined by the arithmetic mean of triplicate values from the same record. An average of these triplicates was considered the daily SBP value. Mean SBP resulted from the 3 days outcomes.

2.6.4. Experiment 1: PV3 effects on renal function, hydroelectrolytic balance and ingestive behaviors

WT and SHR (250g–350g) were distributed into 4 groups (n = 8 animals per group, according to their treatments) and housed in individual metabolic cages, with water and food *ad libitum,* similar to the used in our previous studies (Rodrigues et al., 2022; Sales da Silva et al., 2020). After 24 h of adaptation, water and food were replaced for experimental analysis. Following, animals received intraperitoneal injection with vehicle (NaCl 0.9%) or PV3 at doses of 50 µg kg^{-1}, 2.5 and 5 mg kg^{-1} that were an extrapolation of those from Valencia-Mejía et al. (2019). In the next day after treatments, urine volumes were measured at the end of the stage and stored for biochemical tests, feces and food were weighed, followed by water volume measurement. Animals were then euthanized through an overdose of anesthesia (40 mg kg^{-1}, i.p) with sodium thiopental (2.5%), followed by blood sampling (5 mL) with heparinized syringes and transferred to heparinized microtubes (0.1 mL heparin - 5000 I.U. mL^{-1}). The collected blood was centrifuged at 5000 rpm and the plasma was taken for biochemical analysis.

The uric acid, creatinine, urea, sodium, and potassium were analyzed in plasma and urine samples using a commercial kit (Bioclin) and chemical reactions were performed in an automated clinical analysis equipment (Wiener Lab - CM 200). The following calculations were performed: Creatinine Clearance (mL min^{-1}) = [Urinary Creatinine (mg dL^{-1}) x *DF x Urinary Volume/min/Plasma Creatinine (mg dL^{-1})]; *DF = Dilution factor. Glomerular filtration rate (mL min kg^{-1}) = [Creatinine clearance (mL/min)/Weight (Kg)].

2.6.5. Experiment 2: PV3 effects on vascular reactivity

This experimental series aimed to assess the PV3 effects on vasomotion of renal arteries. Renovascular reactivity was determined in an organ bath setting, as previously described by Machado et al. (2021). Animals were euthanized by decapitation using a guillotine. Then the renal arteries were carefully dissected, quickly removed, and placed in ice-cold modified Krebs-Henseleit solution (4 °C) [composition (in mM): 130 NaCl, 14.9 NaHCO$_3$, 4.7 KCl, 1, 18 KH$_2$PO$_4$, 1.17 MgSO$_4$ · 7H$_2$O, 5.5 glucose, 1.56 CaCl$_2$ · 2H$_2$O and 0.026 EDTA], gassed with 5% CO$_2$/95% O$_2$ to maintain a pH of 7.4. Arteries were cleared of adherent fat and connective tissue and were mounted on a force transducer placed in an organ bath with modified Krebs-Henseleit solution at temperature adjusted to 37 °C. Rings were initially loaded with 1.5 g of tension (basal tension) by incremental application over 30 min and then equilibrated for a further 30–40 min before the start of the studies. The Krebs-Henseleit solution was changed every 15 min. Changes in basal tension were recorded by isometric transducers connected to an AQCAD data acquisition system (AVS Projetos, São Carlos, SP, Brazil).

To verify the contractile status of the renal artery, endothelial function was evaluated by testing the relaxing effect of acetylcholine (ACh, 10 µM) in vessels contracted with phenylephrine (Phe, 1 µM). Renal arteries with a vasodilator response to ACh greater than 90% were considered vessels with intact endothelium. In another set of experiments, renal arteries were subjected to intimal surface friction and a maximum of 10% relaxation to ACh in the vessel rings were considered endothelium denuded.

After 30 min of equilibration, cumulative concentration-response curves for PV3 were made (0.0001–10 mg mL^{-1}) and preparation was performed with intact endothelium (E +) and endothelium - denuded (E −). To assess the endothelial pathway of nitric oxide synthases (eNOS), renal arteries were pretreated with the NOS inhibitor nitroarginine methyl ester (L-NAME, 100 µM) for 30 min. After incubation, concentration-response curves were tested for PV3 (0.0001–10 mg

mL^{-1}), when the contractile response reached a plateau, the CCE curve for PV3 was constructed.

2.6.6. Experiment 3: PV3 effects on hemodynamics

WT and SHR were anesthetized initially with halothane (2% in O$_2$ 100%; Tanohalo; Cristalia, Itapira, SP, Brazil). The right femoral vein was cannulated for infusion of urethane (1.2 g kg^{-1}, iv., Sigma-Aldrich, St. Louis, MO, USA). The right femoral artery was catheterized to record pulsatile arterial pressure, from which mean arterial pressure (MAP) and heart rate (HR) were calculated. After trichotomy of the left lateral flank, the retroperitoneal region was surgically accessed to expose the abdominal aorta, left kidney and renal artery. Miniature probes (T206 Transonic Systems, Inc., Ithaca, NY, USA) were set around aorta (2.0 R B) and left renal artery (1.5 R B) to record aortic (ABF) and renal blood flow (RBF), respectively. Throughout the experiment, the body temperature was maintained between 36 and 37 °C with the aid of a heating pad.

The RBF and ABF were recorded by transit time flowmetry as described by Pedrino et al. (2006). Miniature probes were connected to a T206 flowmeter (Transonic Systems, Inc., Ithaca, NY, USA), which allows determining the flow in absolute values (mL.min^{-1}). The signals obtained were sent to the MP150 data acquisition and analysis system (Biopac Systems, Inc., Goleta, CA, USA). To calculate vascular resistance (RV), mean arterial pressure (MAP) and blood flow (BF) values were considered for the aorta and renal arteries. The RV calculation equation is shown below:

$$VR = MAP/BF \qquad (Eq.1)$$

Initially doses of 50 µg kg^{-1}, 2.5 mg kg^{-1} and 5 mg kg^{-1} of PV3 were injected i.v, and the parameters MAP, HR, renal vascular resistance (RVR) and aortic vascular resistance (AVR) were sampled for 5 min. After PV3 i.v injection, these hemodynamic parameters were recorded for the subsequent 30 min. Points for analyses were from segments collected every 10 s.

2.7. Statistical analyses

Results were expressed as mean ± error of the mean. We used two-way analysis of variance (Two-Way ANOVA) as appropriate (see legend of each figure), using the GraphPad Prism 8 software. Statistical significance was considered when *P < 0.05.*

3. Results

3.1. Comparison with database (Pre-molecular modeling)

Homologous sequences were considered for peptides with antihypertensive activities and composed by residues of up to 5 amino acids, with coincident motifs placed at either the end or within the chain. Descriptions on polarity were not a criterion. Literature search, as of 2021/December, brought up peptides that are considerably homologous to the PV3-containing amino acid primary sequences, as reported in our previous study (Graziani et al., 2021). These homologies were found in peptides from other food sources (Table 1).

3.2. PV3 osmolarity

Hyperosmotic fluids are known for changing tonicity and cell responses (Usach et al., 2019; Wang, 2015; Wermeling et al., 1985). Therefore, we assessed the osmolarity of crescent PV3 concentrations, ranging from that of choice for treatments, to 1000 µg mL^{-1}. The results showed that the PV3 extract has low osmolarity, with values lower than that of body fluids even at the highest concentration tested (Fig. 1).

Table 1
Comparison of amino acid sequences found by our study group with amino acid sequences found by other authors.

Peptide sequence present in PV3	Peptides found in the literature		
	Sequence	Food	Reference
DYGAELPPR	DY	Bamboo shoot	Liu et al. (2013)
DPLPDKNR	PLP	Milk	Sánchez-Rivera et al. (2016)
DFSETSGPPGSDK DPGPPPGETPR	GPP	Soybean	Ma et al. (2006b)
NKFYGWR	FY	wakame	Sato et al. (2002)
QHAEGLPPDQQR	GLP	Salmon skin	Lee et al. (2014)
TSLVGEEEESQDR	EEEES	Common Bean	Tagliazucchi et al. (2015)
TVGEVSEEGQQRK EQEVSEEGKQRDK	VSE	Common Bean	Tagliazucchi et al. (2015)
TVGEVSEEGQQRK	EV	Common Bean	Tagliazucchi et al. (2015)
QPVKVSTPTLVEVSR	EV	Common Bean	Tagliazucchi et al. (2015)
EQEVSEEGKQRDK	EV	Common Bean	Tagliazucchi et al. (2015)
QDEVKEVQR	EV	Common Bean	Tagliazucchi et al. (2015)
QLAVTQVPTLVEVSR	EV	Common Bean	Tagliazucchi et al. (2015)
EQEVSEEGKQRDK	EV	Common Bean	Tagliazucchi et al. (2015)
EMEVR	EV	Common Bean	Tagliazucchi et al. (2015)
KEVESEETDPR	EV	Common Bean	Tagliazucchi et al. (2015)
QDEVKQVER	EV	Common Bean	Tagliazucchi et al. (2015)
TAFNAAEVNSK	EV	Common Bean	Tagliazucchi et al. (2015)
EYLTPDNK	EY	Common Bean	Tagliazucchi et al. (2015)
EYNKATGR	EY	Common Bean	Tagliazucchi et al. (2015)
QLAVTQVPTLVEVSR	AVT	Common Bean	Tagliazucchi et al. (2015)
DFDTEEEPVDK	DF	Common Bean	Tagliazucchi et al. (2015)
SSSTEDFFSK	DF	Common Bean	Tagliazucchi et al. (2015)
DFSETSGPPGSDK	DF	Common Bean	Tagliazucchi et al. (2015)
QKTALVELLK	ELL	Common Bean	Tagliazucchi et al. (2015)
ELLER	ELL	Common Bean	Tagliazucchi et al. (2015)
LHVFSFDHEQRR	SF	Common Bean	Tagliazucchi et al. (2015)
VAAFALVPVKGAADR	AF	Common Bean	Tagliazucchi et al. (2015)
TAFNAAVNSK	AF	Common Bean	Tagliazucchi et al. (2015)
WLGYYAVSVLLLR	VLL	Common Bean	Tagliazucchi et al. (2015)
MNGPLLR	PLL	Common Bean	Tagliazucchi et al. (2015)
SYLQGFSK	GF	Common Bean	Tagliazucchi et al. (2015)
GFVLELR	GF	Common Bean	Tagliazucchi et al. (2015)
NKYFGWR	KY	Wakame seaweed	Suetsuna et al. (2004)
NKFYGWR	FY	Wakame	Suetsuna et al. (2004)
VLSPNRGDGLK	DG	Soybean	Wu and Ding (2002)
SSSEEPNVDGSK	DG	Soybean	Wu and Ding (2002)
NKFYGWR	KFYG	Wakame	Suetsuna and Nakano (2000)
QDSSLSPEPTPAGK	LSP	Corn	Puchalska et al. (2012)
VLSPNRGDGLK	LSP	Corn	Puchalska et al. (2012)
QAAYFGWR	AY	Corn flour	Yang et al. (2007)

Table 1 (*continued*)

| Peptide sequence present in PV3	Peptides found in the literature		
	Sequence	Food	Reference
NKFYGWR	GW	Soy sauce	Nakahara et al. (2010)
QAAYFGWR	AY/GW	Soy sauce	Nakahara et al. (2010)
SYLQGFSK	SY	Soy sauce	Nakahara et al. (2010)
WLGYYAVSVLLR	GY	Soy sauce	Nakahara et al. (2010)
RPMTVGYK	GY	Soy sauce	Nakahara et al. (2010)
VAAFALVPVKGAADR	AF	Soy sauce	(Nakahara et al., 2010; Zhu et al., 2008)
TAFNAAEVNSK	AF	Soy sauce	(Nakahara et al., 2010; Zhu et al., 2008)
TSLVGEEEESQDR	VG	Soy sauce	Nakahara et al. (2010)
RPMTVGYK	VG	Soy sauce	Nakahara et al. (2010)
LVGGELHDVPK	VG	Soy sauce	Nakahara et al. (2010)
NTFDEEEHSSR	TF	Wheat bran	Nogata et al. (2009)
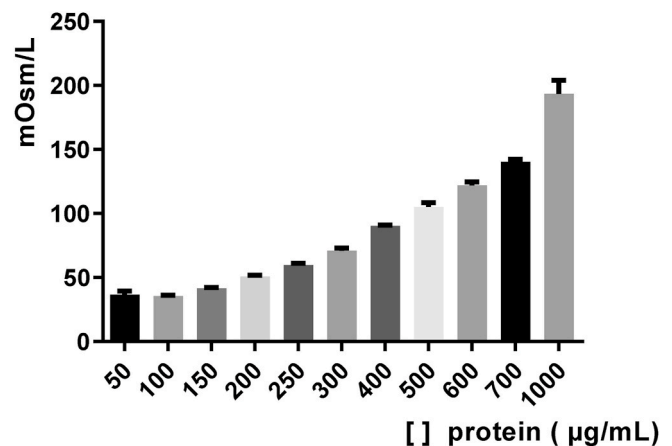
Fig. 1. Evaluation of PV3 osmolarity in the concentrations ranging from 50 to 1000 µg mL^{-1} following dilution in deionized water. Values are expressed as mean $\pm$ standard error.

3.3. Quantification of sodium and potassium ions in PV3

PV3 at concentration of 313 µg mL^{-1} was chosen to assess the amount of sodium and potassium in the solution. We found 251.9 mmol L^{-1} of sodium ions and 69.6 mmol L^{-1} of potassium ions within PV3 extract.

3.4. PV3 effects on kidney function

Tail plethysmography was attempted before starting experiments to confirm the hypertensive state of SHR, as an inclusion criterion. As expected, all SHR were indeed hypertensive, as shown by the mean values of their systolic pressure (192.5 $\pm$ 7.9 mmHg). These values significantly differ ($P < 0.05$) from those sampled in WT (117 $\pm$ 8 mmHg).

Fig. 2 shows the results obtained in WT and SHR treated with PV3 at different doses. PV3 (50 mg kg^{-1}) reduced food intake in SHR (Fig. 2A), which reflected in reduction in feces excretion in this same group (Fig. 2B). Water intake was increased in WT treated with PV3 (Fig. 2C) and this resulted in augmentations of urinary excretion (Fig. 2D). As expected, body weight was not changed by a single acute PV3 injection (Fig. 2E).

It was observed that PV3 reduced plasma creatinine at the doses tested in WT and SHR (Fig. 3A). Regarding urinary creatinine levels, it was observed that PV3 increased creatinine values at a dose of 5 mg kg^{-1} in SHR. (Fig. 3B). Results from creatinine clearance evidenced that PV3 increased clearance at doses of 50 µg kg^{-1}, 2.5 and 5 mg kg^{-1} in the normotensive condition. PV3 increased creatinine clearance at a dose of 50 µg kg^{-1} in the hypertensive condition (Fig. 3C).
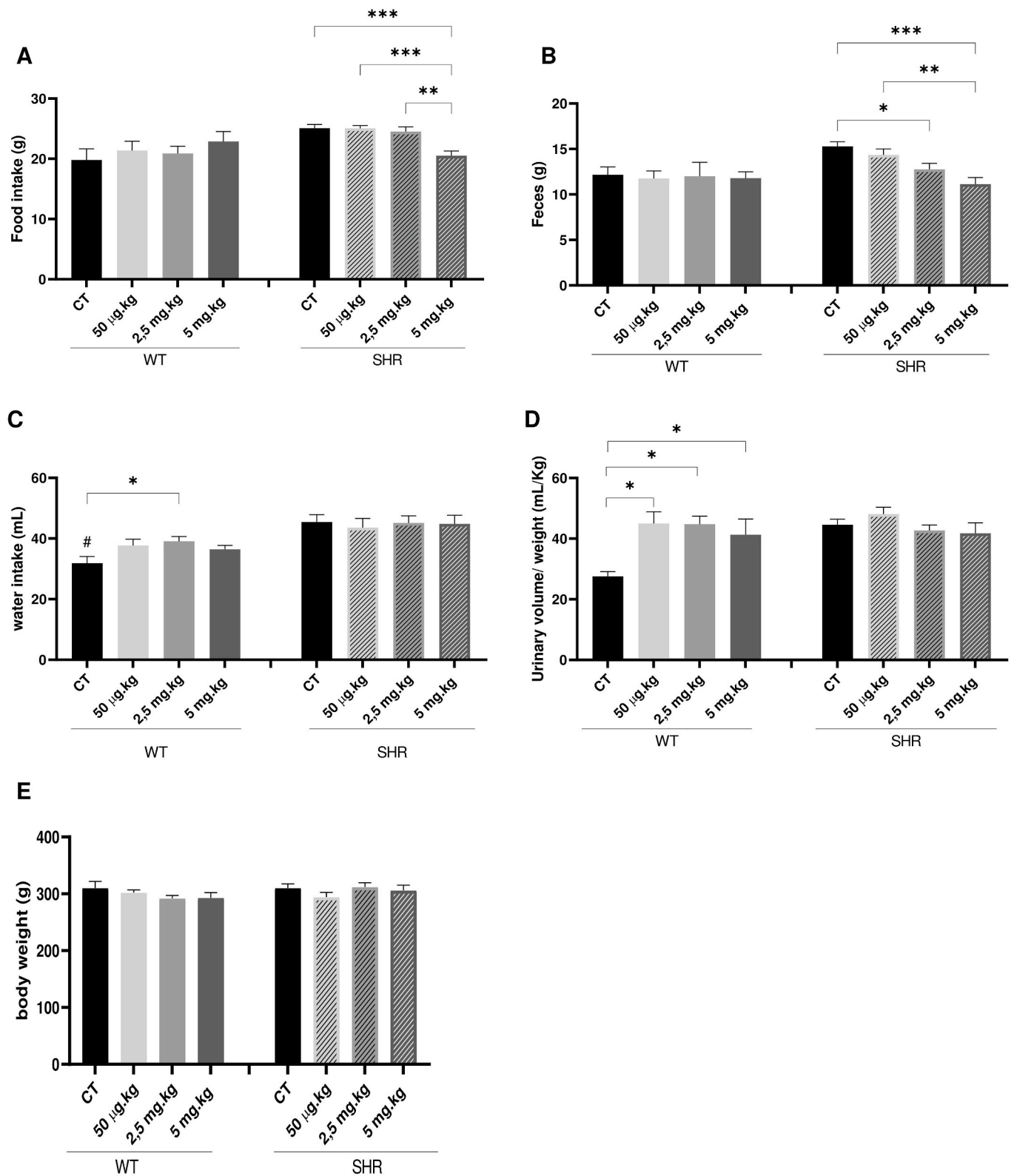
Fig. 2. Metabolic parameters measured 24 h after injections of vehicle (CT), PV3 at doses of 50 μg kg^{-1}, 2,5 mg kg^{-1}, 5 mg kg^{-1} in WT and SHR. **A** – Food intake (g); **B** – Feces (g); **C** – Water intake (mL); **D** – Urinary volume (mL) **E** – Body weight (g). *$P < 0.05$ indicates statistical difference between the bars indicated by the bracket extremities. *$P < 0.05$ indicates statistical difference between the bars indicated by the bracket extremities. #$P < 0.05$ indicates that the bar of SHR with # symbol differs statistically from WT that underwent the same experimental condition. Two-way ANOVA followed by Tukey's post hoc test.
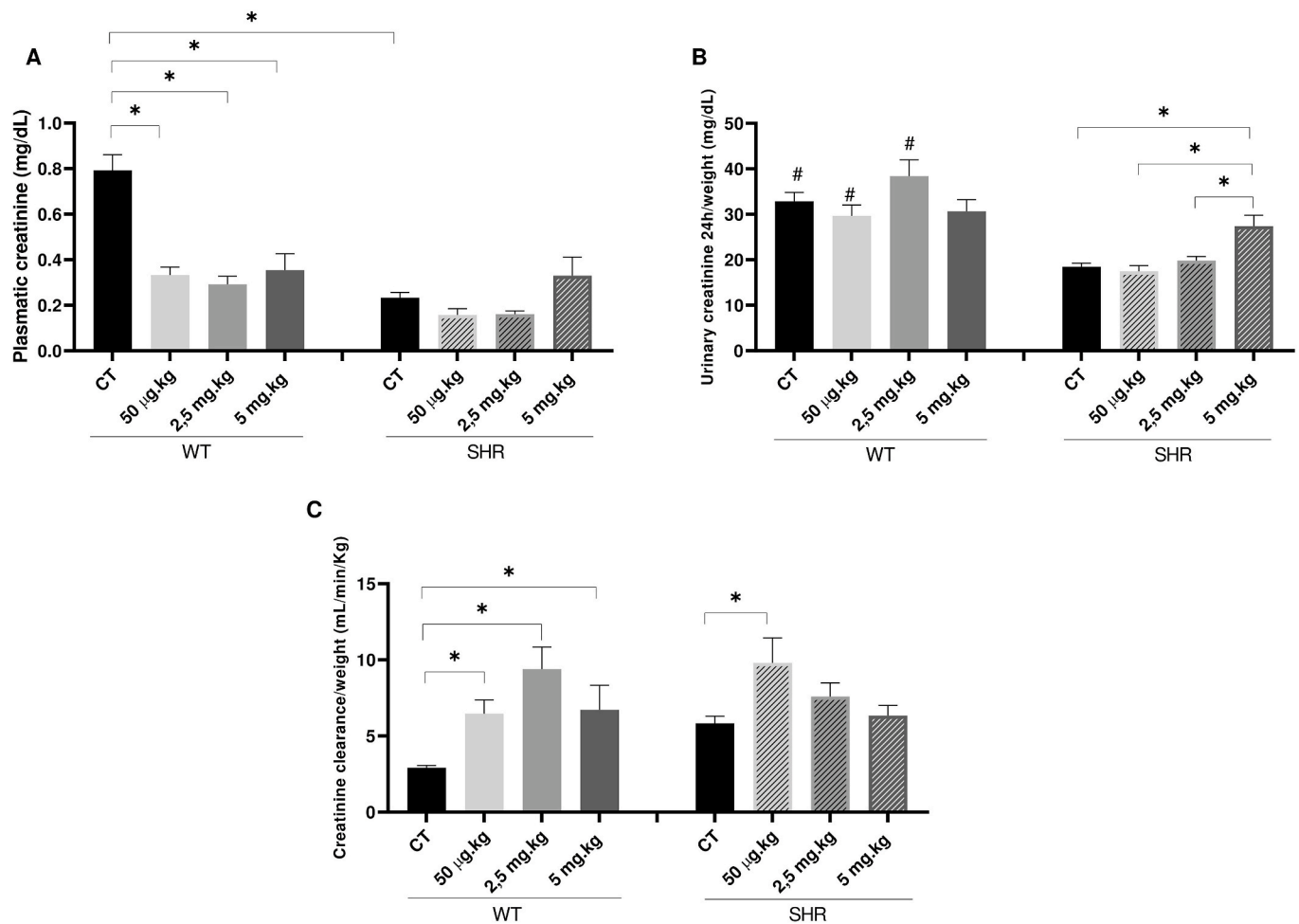
Fig. 3. Plasma and urinary creatinine levels measured 24 h after injections of control (CT), PV3 at doses of 50 µg kg^{-1}, 2,5 mg kg^{-1}, 5 mg kg^{-1} in WT and SHR. **A** – Plasma creatinine (mg dL^{-1}); **B** – Urinary creatinine 24 h/weight (mg dL^{-1}); **C** – Creatinine clearance weight 24 h*$P < 0.05$ indicates statistical difference between the bars indicated by the bracket extremities. #$P < 0.05$ indicates that the bar of SHR with # symbol differs statistically from WT that underwent the same experimental condition. Two-way ANOVA followed by Tukey's post hoc test.

Interestingly, PV3 reduced plasma sodium levels in SHR at doses of 2.5 and 5 mg kg^{-1} (Fig. 4A), which was concomitant to an augmented sodium excretion in SHR (Fig. 4B). Plasma and urinary potassium levels were reduced by PV3 at doses of 2.5 and 5 mg kg^{-1} in the hypertensive condition. PV3 increased SHR potassium excretion when compared to the effects in WT at a dose of 2.5 mg kg^{-1} (Fig. 4D), indicating a natriuretic effect of PV3.

3.5. PV3 effects on arterial renovascular reactivity

The results show that PV3 dilated the isolated renal artery rings with preserved endothelium from WT (47.8 ± 2.5%) and SHR (41 ± 8.7%) (Fig. 5A and B). The results also show a significant difference when comparing the two animal models used, where renal artery rings from WT rats were more prone to vasorelaxation than those from SHR (Fig. 5A).

Following the assessment of PV3 vasorelaxant effects on renal artery rings, we investigated whether such effects would depend on constitutive pathways of the endothelium. Endothelium denuded rings from WT and SHR revealed a loss of PV3 effect (E.max: 3 ± 1.8% in WT and 6.6 ± 3.3% in SHR) (Fig. 6A and B), thus demonstrating that the PV3 actions depend on the endothelium. The next step was to test for the oxidonitrergic pathways involvement. The incubation of renal artery rings with the NOS enzyme inhibitor (L-NAME, 100 µM) abolished the PV3-induced vasorelaxation (E max: 2.8 ± 1.2% in WT and 8.9 ± 4.66% in

SHR) (Fig. 6C and D).

3.6. PV3 effects on hemodynamics

After a 10-min period of stabilization of cardiovascular parameters, MAP levels sampled in SHR were higher than WT, confirming the hypertensive state of this strain. Just after PV3 injection, a transient MAP reduction was observed in WT and SHR (Fig. 7A). In WT, the mean maximal reduction was 36.8 ± 9% for the dose 2.5 mg kg^{-1} and 43.3 ± 4.5% for dose the 5 mg kg^{-1}, while for SHR the reduction was 14.5 ± 7.1% for the 2.5 mg kg^{-1} dose and 22 ± 8.4% for the 5 mg kg^{-1} dose (Fig. 7B). Converse, PV3 was unable to cause a significant difference in cardiac chronotropy of WT and SHR strains (Fig. 7C and D).

PV3 reduced vascular resistance in both renal artery and aorta of WT and SHR throughout the recording period (Fig. 8A and C). When evaluating the renal vascular resistance, a reduction of 18.6 ± 4.6% and 29.1 ± 2.5% was observed for the doses of 2.5 and 5 mg kg^{-1} in WT, respectively. SHR, in turn, displayed reductions of 23.88 ± 2.9% and 36.8 ± 6.2% for the same aforesaid doses, respectively (Fig. 8B). In WT, the magnitude of aortic resistance reductions was 32.4 ± 4.7% and 40.5 ± 2.5% for the doses of 2.5 and 5 mg kg^{-1}, while in SHR the two doses caused reductions of 23.5 ± 3.6% and 30.3 ± 6.8% respectively (Fig. 8D).
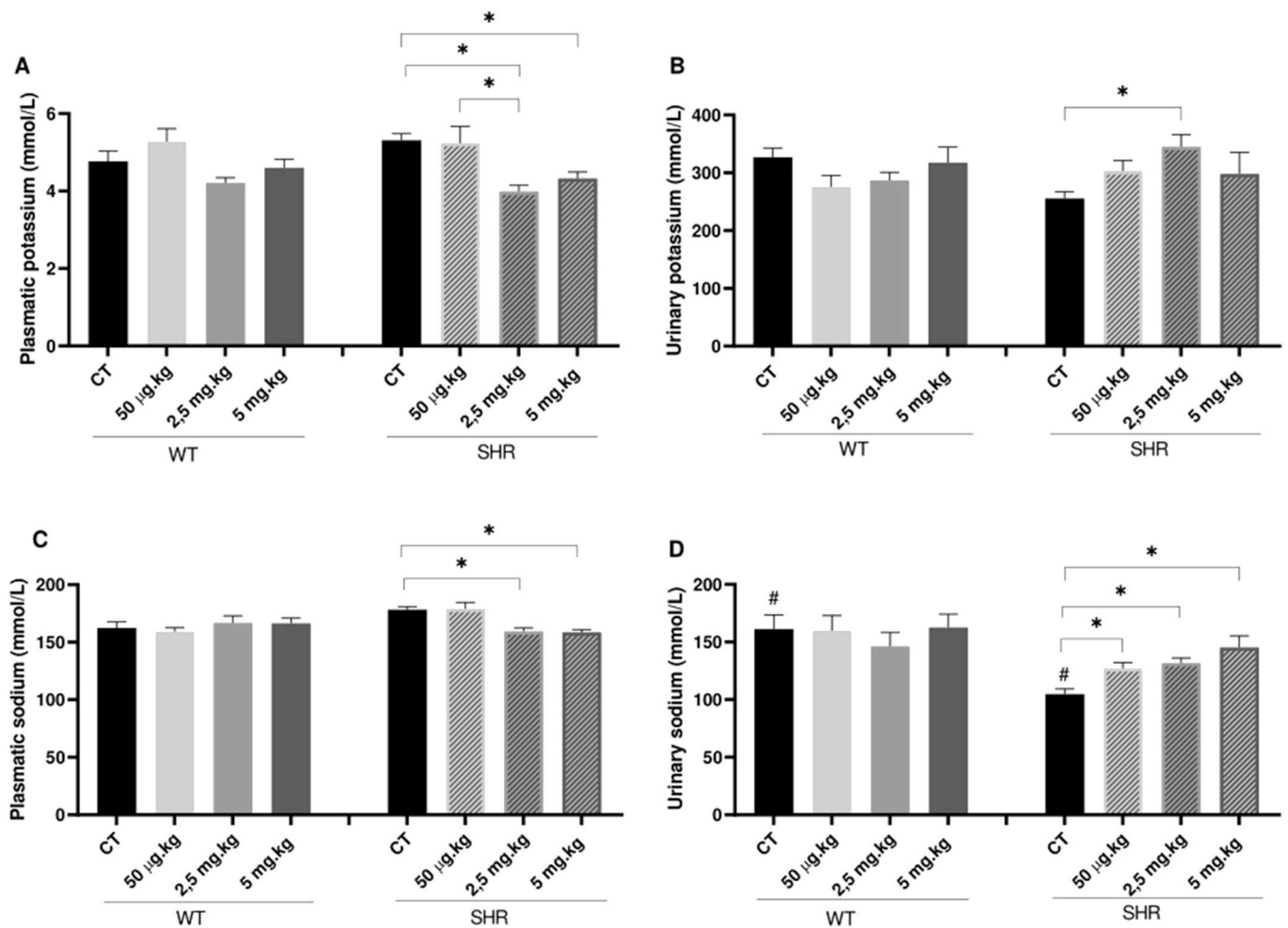
Fig. 4. Plasma and urinary levels of Na$^+$ and K$^+$ measured 24 h after injections of control (CT), PV3 at doses of 50 µg kg^{-1}, 2,5 mg kg^{-1}, 5 mg kg^{-1} in WT and SHR. **A** – Plasma sodium (Na$^+$) (mmol L^{-1}); **B** – Plasma potassium (K$^+$) (mmol L^{-1}); **C** – Urinary sodium (Na$^+$) (mmol L^{-1}); **D** – Urinary potassium (K$^+$) (mmol L^{-1}). *$P <$ 0.05* indicates statistical difference between the bars indicated by the bracket extremities. #$P <$ 0.05 indicates that the bar of SHR with # symbol differs statistically from WT that underwent the same experimental condition. Two-way ANOVA followed by Tukey's post hoc test.
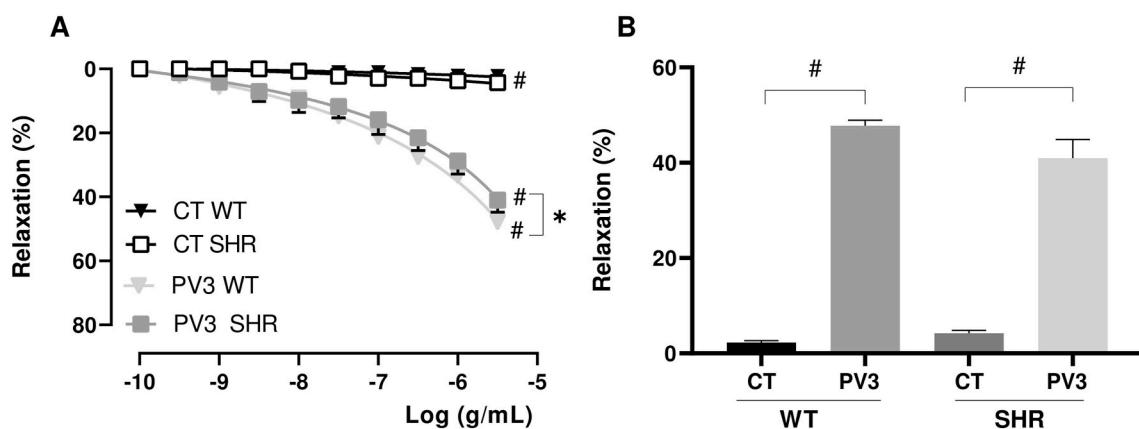

Fig. 5. Vasorelaxant effect of PV3 on renal artery rings of WT and SHR. **A**-vasorelaxant effect of PV3 on endothelium-preserved renal artery rings of WT and SHR (E+). **B** - maximum effect values. Values are expressed as mean ± SEM of 4–5 animals per group. Results were expressed as mean and standard error for 4–5 animals per group. Comparison of models used by Two-Way ANOVA analysis followed by Sidak post-test. *$P <$ 0.*05 WT vs. SHR. #$P <$ 0.05 Control vs PV3.

4. Discussion

The World Health Organization (WHO) emphasizes the importance of consuming foods that are rich in bioactive molecules for the control of

cardiovascular diseases and comorbidities (WHO, 2020). These desired health promoting effects may be reached by consuming beans, which upkeeps the interest in the field (Hartmann and Meisel, 2007; Los et al., 2018). The main results of this study are: I) PV3 sequences are
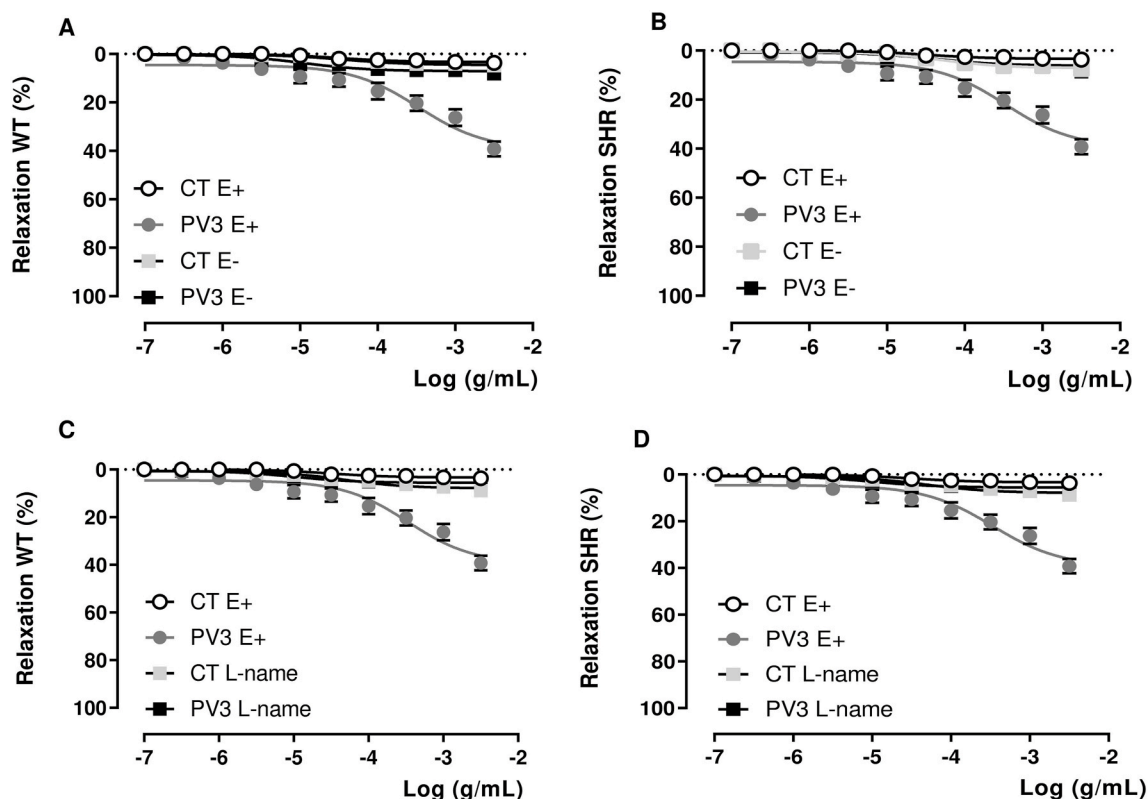
Fig. 6. Evaluation of endothelial involvement in the PV3 vasorelaxant effect in renal artery rings. **A** and **B-** evaluation of endothelial involvement in the PV3 vasorelaxant effect in renal artery rings extracted from WT and SHR. The abbreviations E+ represents the preservation of the endothelium and E−represents the preservation of the endothelium. **C** and **D**-assessment of NOS pathway involvement in endothelium-dependent vasorelaxant effects evoked by PV3 in renal artery rings extracted from WT and SHR. Values are expressed as mean ± SEM of 4–5 animals per group. Results were expressed as mean and standard error for 4–5 animals per group. Comparison of models used by Two-Way ANOVA analysis followed by Sidak post-test. *$P < 0.05$ WT vs. SHR.*

homologous to several amino acid sequences from food sources already known for their antihypertensive activities; II) PV3 is not hyperosmolar; III) PV3 caused a relaxant effect on renal artery that is dependent on the endothelium and relies on oxidonitrergic pathways; IV) PV3 caused a natriuretic effect in the hypertensive strain; V) PV3 increased creatinine clearance; VI) PV3 reduced the arterial pressure and this was concomitant to a reduction in vascular resistance in the aortic and renal vascular beds.

Proteins and peptides have a unique amino acid sequence that confers a three-dimensional structure that favors a binding for molecular targets, consequently exerting their biological functions (Rutherfurd-Markwick, 2012). According to Nelson and Cox (2014), the comparison of functionally similar proteins and peptides from different species may indicate the presence of motifs likely conserved throughout evolution. Thus, there is an intimate link between the protein's primary structure and its function, thus supporting the search for homologies in peptide from different sources. As reported by Nakahara et al. (2010), the dipeptides GW, AY, SY, GY and VG showed a hypotensive effect via ACE inhibition, with IC50 values ranging between 30 μg ml^{-1} (for GW peptide) and 1100 μg ml^{-1} for VG. This varying effectiveness of ACE inhibition was related to the amino acid primary structure and is closely associated with the presence of amino acids with hydrophobic groups in their side chains (Aluko, 2015; Tagliazucchi et al., 2015; Yokoyama et al., 1992). Since the aforesaid dipeptide are within some of the bean-derived previously sequenced (NKFYGWR, QAAYFGWR, SYLQGFSK, WLGYYAVSVLLR, RPMTVGYK, TSLVGEEEESQDR, RPMTVGYK and LVGGELHDVPK) it is worth proposing an ACE inhibitory effect resulting in the depressor effect herein reported. From the PV3 sequencing made by Graziani et al. (2021), tripeptides and peptides homologous to other reports in the literature were obtained, such as the

scrambled sequences GPP, LSP, KFYG, GLP and PLL present in the peptides DFSETSGPPGSDK, DPGPPPGETPR, QDSSLSPEPTPAGK, VLSPNRGDGLK, NKFYNGPLPDWRQ, MNGPLLR. These are also correlated to different mechanisms resulting in antihypertensive activity. Ma et al. (2006a) studied peptides from Buckwheat (*Fagopyrum esculentum*) and observed that the tripeptide GPP has an ACE inhibitory activity. Puchalska et al. (2012) reported that the LSP sequence reduced blood pressure. However, KFYG seem to be unrelated to cardiovascular effects (Suetsuna et al., 2004). The essential characteristics for a peptide to present antihypertensive activity support the pool of peptides present in PV3 as good candidates for the control of this cardiovascular disease.

Osmolarity was measured in order to exclude the possibility that hypertonicity would aid PV3 effects. We found that PV3 is hypoosmotic, thus knocking down the chances that a possible hyperosmotic fluid would underly the effects that indeed rely only on PV3 peptidergic content. Wang stated that the solutions to be injected in the intramuscular or subcutaneous routes must present an osmolarity below 600 mOsm kg^{-1} and for intravenous injections, it must be below 1000 mOsm kg^{-1} (Wang, 2015). In this regard, the PV3 doses and concentrations are within the recommended range, since 1 mg mL^{-1} of PV3 contains 180 mOsm L^{-1}. Another factor regarding the PV3 tonicity was the sodium and potassium contents: the values of sodium and potassium in PV3 (1 mg mL^{-1}) were 251.9 and 69.6 mmol L^{-1}, respectively, which are still considered within the isotonic range (Wermeling et al., 1985).

PV3 at dose of 5 mg kg^{-1} affected the food intake of SHR. This finding is similar to that found by Marczak et al. (2006), which reported that orally given hydrolyzed canola peptides reduced food intake in mice through cholecystokinin (CCK) system mechanisms. Another report shows that soybean peptides display similar effects. These peptides are called soy morphine that display anxiolytic and anorexigenic
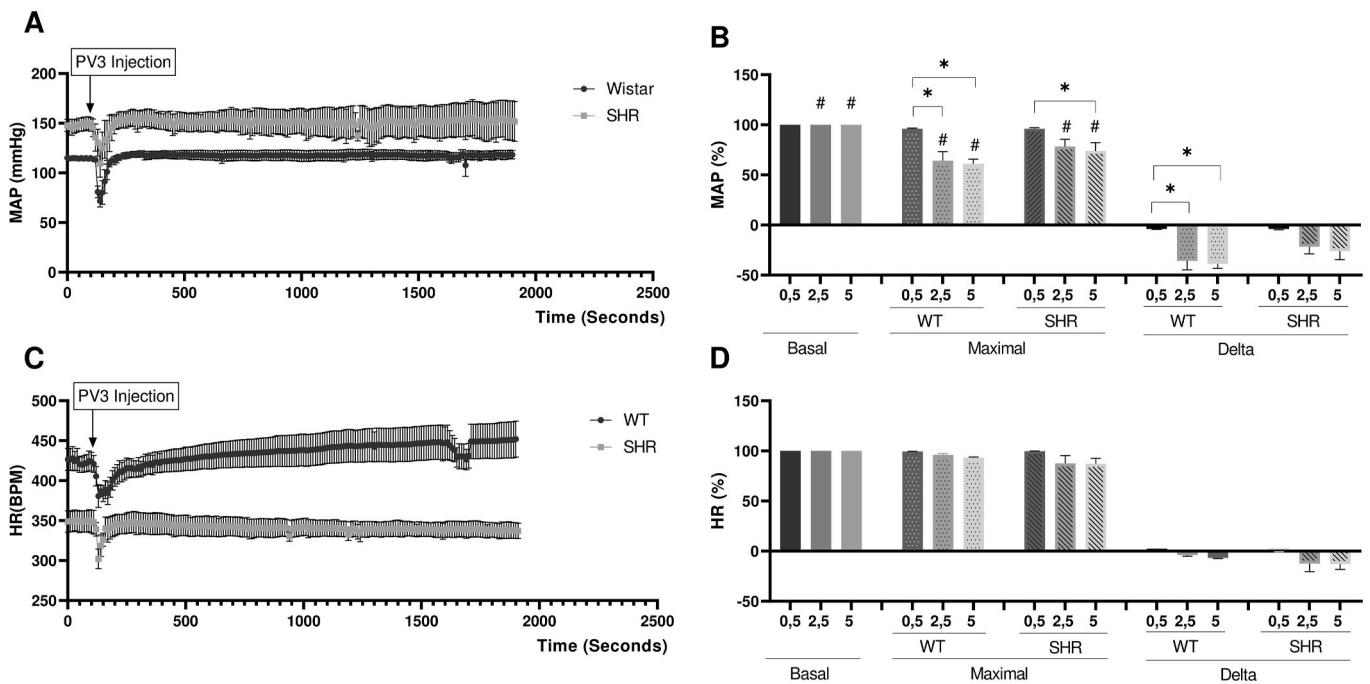
Fig. 7. Changes in mean arterial pressure (MAP) and heart rate (HR) changes caused by PV3 injection in WT and SHR. **A** - PV3 effects on the mean arterial pressure over the time of recording at a dose of 5 mg kg^{-1} in WT and SHR, and in **B**, the basal values, maximum responses, and deltas of the doses of 0.5; 2.5 and 5 mg kg^{-1} on blood pressure (%) of WT and SHR. In **C**, the effect of PV3 on heart rate over time of recording at a dose of 5 mg kg^{-1} in WT and SHR, and in **D**, the basal values, maximum responses, and deltas of PV3 0.5; 2.5 and 5 mg kg^{-1} on heart rate (%) of WT and SHR. Values are expressed as mean ± standard error (n = 5). *$P < 0.05$* indicates statistical difference between the bars indicated by the bracket extremities. #$P < 0.05$ indicates that the bar of SHR with # symbol differs statistically from WT that underwent the same experimental condition. Two-way ANOVA followed by Tukey's post hoc test.
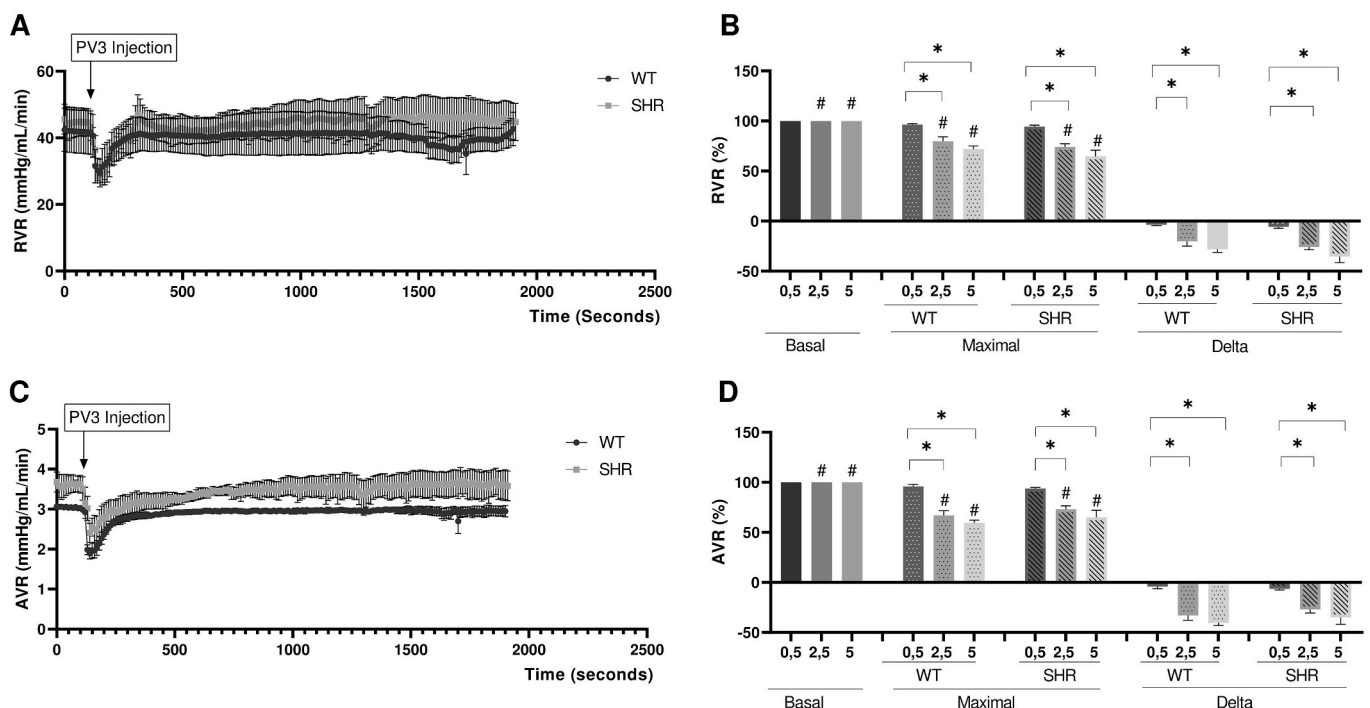

Fig. 8. Changes in vascular resistance in renal and aorta arteries following PV3 injection in WT and SHR. **A**- PV3 effects on the renal vascular resistance over the time of recording at a dose of 5 mg kg^{-1} in WT and SHR, and in **B**, the basal values, maximum responses, and deltas of the doses of 0.5; 2.5 and 5 mg kg^{-1} on renal vascular resistance (%) of WT and SHR. In **C**, the effect of PV3 on aorta vascular resistance over time of recording at a dose of 5 mg kg^{-1} in WT and SHR, and in **D**, the basal values, maximum responses, and deltas of PV3 0.5; 2.5 and 5 mg kg^{-1} on vascular resistance aorta (%) of WT and SHR. Values are expressed as mean ± standard error (n = 5). Two-way ANOVA followed by Tukey's post hoc test. *$P < 0.05$* baseline vs. maximum responses.

activities probably by acting on μ-opioid receptors (Kaneko et al., 2010). The reduction in the ingestive behavior caused by PV3 can be also derived from the presence of peptides acting as enzyme inhibitors. Protease inhibitors (i.e., trypsin/chymotrypsin inhibitors) are likely to retard the digestion process and increase the satietogenic hormones (i.e., CCK), which reduces food intake (Samtiya et al., 2020). When analyzing the amount of feces of the animals, the same pattern was noticed: there was a decrease in feces excretion in SHR treated with PV3 at the doses of 2.5 and 5 mg kg^{-1} and this coincided with a reduced ingestive behavior.

Interestingly, water intake was increased in PV3-treated WT and as expected, rats intaking more water had increases in urinary volume. This suggests that PV3 was unable to affect drinking behavior in the hypertensive condition, already known for being altered in the SHR (Sales da Silva et al., 2020) strain that is well reported as an experimental model with modifications in health status (Dickhout and Lee, 1998). On the other hand, it is worth suggesting that PV3 preserves the drinking capacity of normotensive animals, since the changes in arterial pressure linked to diminishments in blood volume and to augmentations in urinary excretion caused by PV3 are expected to produce the dipsogenic responses we found ().

Creatinine clearance virtually reflects the glomerular filtration rate (GFR) (). We observed that GFR was increased in WT and SHR treated with PV3. Our results also show that PV3 increased the vascular conductance in the renal artery. Altogether, these findings are consistent evidence that PV3 is able to increase filtration through renovascular dilatory mechanisms. This combination allows inferring that PV3 effects upon pressure results from reductions in intravascular volume and from direct effects on vasomotion. Again, these positive effects raise PV3 as a potential nutraceutical capable of treating cardiovascular diseases in which nephropathies are present.

SHR strain develops hypertension and the consequent damages in target organs, mainly in kidneys (Hultström, 2012). They also present exaggerated retention of salt and water due to reductions in both GFR and urinary sodium excretion that can be caused by abnormalities in several transport mechanisms, especially those existing in the proximal tubule (Ortiz and Garvin, 2001). In this study, while the hypertensive strain injected with all PV3 doses exhibited increases in Na$^+$ excretion, the potassium excretion was changed only in animals injected with PV3 at the dose of 2.5 mg kg^{-1}. This finding is positive for the treatment of hypertension since ion handling may contribute to fluid tonicity and intravascular volume; the latter is variable directly determining pressure. A possible mechanism determining PV3 effect is through an allosteric modulation of Na$^+$/K$^+$-ATPase pump. Besides, it is worth considering the inhibition of sodium channels and the resulting decreases in sodium reabsorption. Whether PV3 may interfere with the tubular mechanisms such as the activity of Na$^+$/K$^+$-ATPase pump remains to be confirmed in further studies.

The PV3 ability to promote a relaxing effect on the renal artery extends the knowledge reported by Graziani et al. (2021), which investigated similar features in aorta. It is known that increases in pressure and shear stress in the microvasculature lead to premature vascular aging and decreases the potential of relaxant molecules released from endothelium (Anishchenko et al., 2015; Konukoglu; Uzun., 2017). This same study of Graziani et al. (2021) showed PV3 oxidonitrergic effects in endothelial cells, which extends our current data showing that PV3 dilates renal beds. Such effect relies on endothelium-dependent NO pathways, thus revealing the vascular mechanism through which PV3 is reaching cardiovascular and renal effects. This oxidonitrergic recruitment may be related to ACE inhibition (Tagliazucchi et al., 2015) that, by implication, may reduce bradykinin degradation, thus boosting endothelial kininergic NO-related paths. Furthermore, it cannot be neglected the possibility that PV3 antioxidant effects may display a protection against the hypertension-related vascular aging (Graziani et al., 2021; Lee et al., 2021; Los et al., 2018). Nevertheless, additional studies attempting chronic treatments are necessary to unravel whether PV3 is angioprotective.

Following on from the premise that vasorelaxation may be an endothelium-dependent measure of vascular function, the vasoactive effect of PV3 on the renal artery with preserved endothelium was evaluated. The results showed that PV3 induced the dilation of renal artery in WT and SHR; however, the magnitude of this effect was significantly attenuated in SHR. It is necessary to keep in mind that SHR is an experimental model of endothelial dysfunction-related hypertension (Konukoglu and Uzun, 2017). The PV3-evoked vasorelaxation was confined to endothelial preserved arterial rings and this excludes the involvement of pathways other than those dependent on the endothelium and on primary targets expressed in subendothelial vascular smooth muscle layers. Therefore, these effects support the proposition that PV3 can be an alternative therapy to improve vascular function, with the positive circulatory effects that go far beyond those involving the dynamics of fluid handling and pressure.

In this work, the mechanisms involved in vascular tone (vasomotion) modulation and in medium and long-term circulatory regulation through renal function were evaluated. After observing vasorelaxant effects in renal artery (*ex vivo*), natriuretic effect and the GFR increases (*in vivo*), we conducted complementary experiments in WT and SHR to evaluate the PV3 effect on vascular conductance/resistance. The data show that PV3 reduced the MAP of WT and SHR at the doses tested (2.5 and 5 mg kg^{-1}) and did not change the HR at all doses tested. Literature have reported that other positively charged food-borne peptides that have hydrophobic amino acids display hypotensive effect (Martínez-Sánchez et al., 2020). These are some of the chemical features of PV3 peptides (Graziani et al., 2021). Although the PV3 antihypertensive effects coincide with those reported for homologous peptides extracted from other food sources, this study is the first to unravel the effects and the mechanisms underlying hemodynamic, renovascular and endothelial actions of peptides derived from hardened beans.

5. Conclusions

In conclusion, PV3 exerts an antihypertensive activity that is related to the vasorelaxation of renal arteries through endothelial and oxidonitrergic pathways. Furthermore, PV3 provokes natriuresis and increases glomerular filtration rate, which jointly culminate in pressure decreases. Such findings are relevant for the molecular and physiological study of bioactive food-borne peptides, since the literature is scarce about the mechanisms underlying the aforesaid effects. Current results further strength the use of hardened beans considered agro-industrial residues in nutraceutical formulations targeted to the control and management of diseases such as hypertension and associated nephropathies.

Submission declaration

The work has not been published previously, except in the form of an abstract, a published lecture or academic thesis. The submitted version of this manuscript is approved by all authors, is not under consideration for publication elsewhere and, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

CRediT authorship contribution statement

Juliana Vila Verde Ribeiro: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, preparation, Visualization. **Daniel Graziani:** Methodology, Formal analysis, Software, Writing – review & editing. **Jhulle Horrane Moreira Carvalho:** Methodology, Formal analysis, Investigation. **Michelle Mendanha Mendonça:** Methodology, Formal analysis. **Lara Marques Naves:** Methodology, Formal analysis. **Helton Freires Oliveira:** Methodology, Formal analysis. **Hericles Mesquita Campos:** Methodology, Formal analysis. **Maria Clorinda Soares Fioravanti:** Methodology, Formal analysis. **Lilian Fernanda Pacheco:** Resources. **Patricia Maria Ferreira:** Conceptualization, Formal analysis. **Gustavo Rodrigues Pedrino:** Resources, Methodology, Formal analysis. **Paulo César Ghedini:** Resources, Methodology, Formal analysis. **Kátia Flávia Fernandes:** Resources, Conceptualization, Writing – review & editing. **Karla de Aleluia Batista:** Conceptualization, Resources, Project administration, Writing – review & editing. **Carlos Henrique Xavier:** Conceptualization, Resources, Supervision, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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