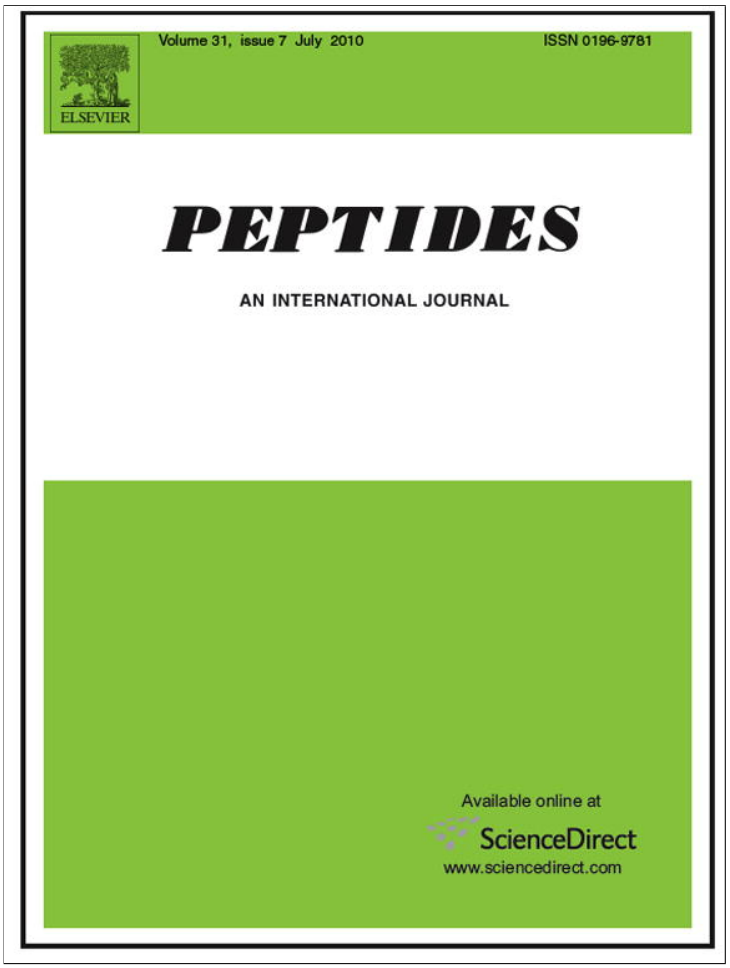

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C-type natriuretic peptide effects on cardiovascular nitric oxide system in spontaneously hypertensive rats

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ABSTRACT

The aim was to study the effects of C-type natriuretic peptide (CNP) on mean arterial pressure (MAP) and the cardiovascular nitric oxide (NO) system in spontaneously hypertensive rats (SHR), and to investigate the signaling pathways involved in this interaction. SHR and WKY rats were infused with saline or CNP. MAP and nitrites and nitrates excretion (NO_x) were determined. Catalytic NO synthase (NOS) activity and endothelial (eNOS), neuronal (nNOS) and inducible NOS (iNOS) were measured in the heart and aorta artery. NOS activity induced by CNP was determined in presence of: iNOS or nNOS inhibitors, NPR-A/B natriuretic peptide receptors blocker and Gi protein and calmodulin inhibitors. CNP diminished MAP and increased NO_x in both groups. Cardiovascular NOS activity was higher in SHR than in WKY. CNP increased NOS activity, but this activation was lower in SHR. CNP had no effect on NOS isoforms expression. iNOS and nNOS inhibitors did not modify CNP-induced NOS activity. NPR-A/B blockade induced no changes in NOS stimulation via CNP in both tissues. Cardiovascular NOS response to CNP was reduced by Gi protein and calmodulin inhibitors in both groups. CNP interacts with NPR-C receptors, activating Ca–calmodulin eNOS via Gi protein. NOS response to CNP is impaired in the heart and aorta of SHR. Alterations in the interaction between CNP and NO would be involved in the maintenance of high blood pressure in this model of hypertension.

1. Introduction

C-type natriuretic peptide (CNP) belongs to a family of natriuretic peptides and plays an important role in cardiovascular homeostasis [29,37,40,41]. CNP is extensively distributed in the cardiovascular system, particularly in endothelial cells and cardiac myocytes [8,14,39,41].

This peptide would act in paracrine and/or autocrine manner causing local vasodilatation and preventing smooth muscle cell and cardiac fibroblast proliferation [2,36].

The cardiovascular actions of CNP are mediated via activation of natriuretic peptide receptors subtype B and subtype C (NPR-B, NPR-C), which are expressed in cardiac atria and ventricle, as well as in the aorta and peripheral vasculature [30,33]. NPR-B is a membrane guanylyl cyclase (GC) and its activation induces cGMP synthesis, which mediates cellular responses [33]. In contrast, NPR-C is coupled to adenylyl cyclase through an inhibitory guanine nucleotide regulatory (Gi) and/or to phospholipase C (PLC) activation [4,27].

On the other hand, the free radical nitric oxide (NO) is synthesized by a family of nitric oxide synthases (NOS) that involves neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) isoforms. These isoforms are expressed in several tissues, such as endothelial cells, cardiomyocytes and smooth muscle cells [15]. nNOS and eNOS are expressed constitutively and influenced by intracellular concentrations of Ca^{2+}. The complex Ca^{2+}–calmodulin is necessary to maintain the enzyme active [32]. In contrast, iNOS is expressed after transcriptional induction and independent of intracellular Ca^{2+} [3]. With regard to eNOS activity, this isoform is regulated by various kinases [26]. Phosphorylation of eNOS at Ser^{1177} close to the carboxy-terminal is a critical requirement for eNOS activation [26].

It seems that both factors, CNP and NO, are closely related in cardiovascular homeostasis regulation. In coronary and mesenteric resistance vessels, CNP acts as an endothelial derived hyperpolarizing factor inducing relaxation mediated by the NPR-C pathway [7,19]. Moreover, it has been demonstrated that CNP induces coronary vasculature relaxation partially mediated via NO-cGMP [6]. In addition, CNP inhibits the proliferation of smooth muscle cells [1,22] as well as cardiomyocytes hypertrophy, an effect associated with the increase in cGMP concentration [36]. On the other hand,

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NO blocks hypertrophic response to growth factor stimulation in cultured cardiac myocytes [21].

In previous studies, we demonstrated that CNP induces a hypotensive effect related to an enhancement of vascular NOS activity as well as cardiac NO system activation in normotensive rats. Moreover, we showed that those actions are mediated by coupled G protein NPR-C receptor and subsequently Ca^{2+}–calmodulin dependent NOS activation in the heart and aorta [10].

In addition, Simon et al. showed that CNP hyperpolarizes pulmonary microvascular endothelial cells by activating large-conductance calcium-activated potassium channels mediated by the activation of NPR-B, PKG, eNOS, and sGC [35].

Spontaneously hypertensive rats (SHR) are genetic models of hypertension that show endothelial dysfunction, augmented oxidative stress, enhanced vasoconstrictor factors and decreased bioavailability of NO [15,18,26].

In view of these previous findings and the fact that a link exists between both NO and CNP and arterial blood pressure regulation, we can speculate that alterations in the interaction between both systems are involved in the maintenance of high levels of blood pressure in this model of hypertension.

Bearing in mind this hypothesis, in the present study we therefore set out to investigate the effects of CNP on the NO system in SHR, studying the changes in cardiovascular NOS activity and expression in response to peptide infusion. In addition, the signaling pathways implicated in the interaction between CNP and NOS were investigated, identifying natriuretic peptide receptors and NOS isoforms involved in this model of hypertension.

2. Materials and methods

2.1. Animals

Sixteen-week-old male SHR and WKY animals were purchased from the Instituto de Investigaciones Médicas A. Lanari (UBA, Argentina). Rats were housed in a humidity and temperature-controlled environment with an automatic 12-h light/dark cycle. They were fed standard rat chow from Nutrimentos Purina (Buenos Aires, Argentina) and tap water ad libitum up to the day of the experiments.

Animals were care according to the Argentina's National Drug, Food and Medical Technology Administration (ANMAT) (Regulation 6344/96) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and experimental procedures were approved by the Ethic Committee of the School of Biochemistry and Pharmacy (CEFFB), Buenos Aires University, Argentina.

2.2. Experimental design

2.2.1. Protocol 1

2.2.1.1. Effects of CNP infusion on mean arterial pressure and the NO system. Rats were anaesthetized with urethane (1 g/kg bodyweight, i.p.; Sigma, St. Louis, MO, USA). The femoral vein and artery and the urinary bladder were cannulated with a polyethylene catheter for drug administration (saline or CNP infusion), mean arterial pressure (MAP; Power Lab and Lab Chart soft from ADInstruments, Bella Vista, NSW, Australia) recording and urine collection, respectively.

After surgery, an isotonic saline infusion (NaCl 0.9% w/v) was started at a rate of 0.05 ml/min and maintained for 40 min to allow stabilization of hemodynamic and renal parameters. The first 30 min following stabilization were considered the control period, during which the rate of saline infusion was maintained at 0.05 ml/min. At the end of this period, one group of SHR and WKY rats received first a bolus of CNP (10 $\mu g\,kg^{-1}$; Sigma, St. Louis, MO, USA) and then an infusion with CNP (1 $\mu g\,kg^{-1}\,min^{-1}$) over 1 h, and the other group received first a bolus of saline and then continued with the isotonic saline infusion.

Experimental groups:

WKY + NaCl ($n = 8$): control period (NaCl infusion) and experimental period (bolus of saline + NaCl infusion over 1 h).

SHR + NaCl ($n = 8$): control period (NaCl infusion) and experimental period (bolus of saline + NaCl infusion over 1 h).

WKY + CNP ($n = 8$): control period (NaCl infusion) and experimental period (bolus CNP + infusion CNP over 1 h).

SHR + CNP ($n = 8$): control period (NaCl infusion) and experimental period (bolus CNP + infusion CNP over 1 h).

Taking into account dose response curve on blood pressure of CNP, we selected the lower dose of CNP (bolus + infusion) that induced the maximum hypotensive effect (data not shown).

MAP was recorded and urine samples were collected at the end of the control and experimental periods in all groups.

The concentration of nitrites and nitrates (NO_x), end products derived from NO metabolism, was determined in urine samples according to the procedure described by Verdon et al. [39].

At the end of the experimental period animals were sacrificed by decapitation and the right atria, left ventricle and aorta artery were removed in order to determine NOS activity and expression, and eNOS phosphorylation.

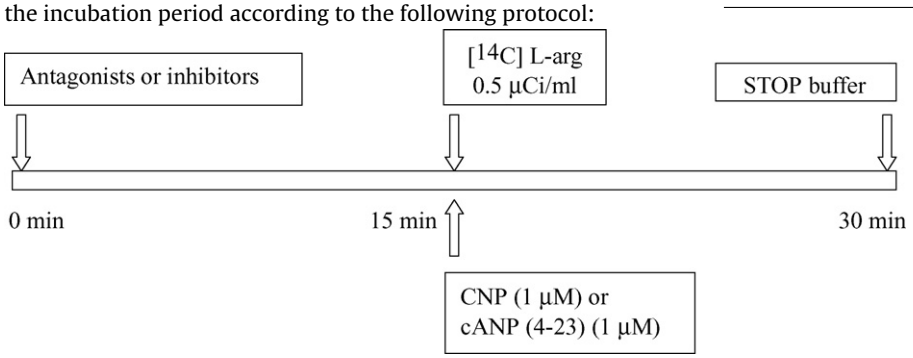
2.2.1.2. Determination of NOS activity. Tissue NOS activity was measured, using [^{14}C] L-arginine (PerkinElmer, Boston, MA, USA) as substrate as described previously [12]. Tissue slices (2–3 mm thick) were incubated 30 min at 37 °C in Krebs solution with 0.5 μCi/mL [^{14}C] L-arginine. The reaction was stopped by adding 500 μL stop buffer (0.5 mmol/L EGTA, 0.5 mmol/L EDTA, 20 mmol/L HEPES, pH 5.5). Tissue samples were homogenized in the stop solution. The homogenates were centrifuged at 12,000 × *g* for 20 min. The supernatants were then applied to a 1 mL Dowex AG 50W-X8 column (Na^+ form, Bio-Rad), hydrated with the stop buffer and eluted with 2 mL distilled water. The amount of [^{14}C] L-citrulline was determined with a liquid scintillation counter (Wallac 1414 WinSpectral). Specific NOS activity was assessed in the presence of 10^{-4} M L-N-nitro-arginine methyl ester (L-NAME; Sigma, MO, USA). Nitric oxide production (measured as pmol of [^{14}C] L-citrulline) in each tube was normalized to the weight of the tissue slices incubated with the substrate during equal periods of time and thus expressed as pmol/g wet weight min. All chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2.1.3. NOS expression and eNOS phosphorylation. NOS expression and eNOS phosphorylation were determined by Western blot. Samples of different tissues containing equal amounts of protein (0.10 mg protein/lane) were separated by electrophoresis in 7.5% SDS–polyacrylamide gels, transferred to a nitrocellulose membrane and then incubated with rabbit polyclonal anti-NOS antibodies (1/500 dilution: anti-iNOS, anti-eNOS, anti-nNOS) or rabbit anti-phospho-eNOS Ser^{1177} (1/500 dilution) and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1/5000 dilution). Samples were revealed by chemiluminescence using the enhanced chemiluminescence reagent for 2–4 min. Quantification of the bands was performed by digital image analysis using a Hewlett-Packard scanner and Totallab analyzer software. All antibodies and Western blot reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2.2. Protocol 2

2.2.2.1. Effects of CNP on NOS activity in isolated heart and aorta artery. Signaling cascade involved in CNP and NOS interaction in SHR. SHR and WKY rats were sacrificed by decapitation and NOS activity

was measured in the aorta, right atria and left ventricle using [^{14}C] L-arginine as substrate. Tissue slices (2–3 mm thick) were incubated 30 min at 37 °C as described in protocol 1. Agonists (15 min after incubation was started) and/or antagonists or inhibitors (at the beginning of the 30 min incubation period) were added during the incubation period according to the following protocol:



cANP(4-23), NPR-C receptor selective agonist (Sigma, St. Louis, MO, USA).

Antagonists or inhibitors: L-nitro arginine methyl ester (L-NAME, 1 µM), NOS inhibitor; aminoguanidine (AG, 1 mM), iNOS inhibitor; 7-nitroindazole (7-NI, 10 µM), nNOS specific inhibitor; calmidazolium (Cz, 1 µM), calmodulin antagonist; anantin (AN, 100 nM), NPR-A/B receptor antagonist; pertussis toxin (PTx, 800 ng/ml), Gi_{1-2} protein inhibitor; U-73122 (10 µM), phospholipase C inhibitor; GF-109203 (100 nM), protein kinase C inhibitor; wortmannin (10 µM) PI3-K/Akt pathway inhibitor; LY-294002 (10 µM), PI_3K inhibitor; LY-294002 (10 µM), PI_3K inhibitor; U-0126 (10 µM), p42/p44 mitogen-activated protein kinase (MAPK) inhibitor; SB-203580 (10 µM), p38 MAPK inhibitor; SP-600125 (10 µM), JNK 1/2 inhibitor; PD-98059 (10 µM), MEK1/ERK 1 2 inhibitor. All antagonists above described were purchased from Sigma (St. Louis, MO, USA). Each in vitro experiment was performed with a slice of tissue (atria, ventricle or artery) from both groups, SHR and WKY ($n = 8$).

Concentration-dependent stimulation of NOS activity by CNP in all studied tissues in SHR and WKY had been performed in previous studies (data not shown). The lowest concentration of CNP that induced the maximum effect on NOS was used in this experimental protocol.

2.3. Statistical analysis

All values are expressed as means ± SEM. The program Prism (Graph Pad Software, Inc., San Diego, CA, USA) was used for statistical analysis. The mean and standard deviation or median values of each variable for each group were calculated. The results of each variable for each experimental group were analyzed with a two-way analysis of variance (ANOVA), where one factor was the different treatments and the other genotypes (WKY or SHR). The effects of each factor were tested independently of the effect of the other, as well as the interaction between both factors. We did not found interaction between treatments and genotype. Multiple comparisons were performed using Bonferroni post hoc test. p value <0.01 was considered a significant difference.

3. Results

3.1. Protocol 1: effects of CNP infusion on MAP and the NO system

In this model of hypertension, we found that MAP and NO_x excretion were significantly higher in SHR than in WKY (Table 1). CNP infusion reduced MAP in both groups of animals and this reduction was higher in normotensive than in hypertensive animals. The reduction in MAP was accompanied by a rise in NO_x excretion in both groups, but the increase in NO_x was more marked in WKY than in SHR, indicating a lower response to the peptide in hypertensive animals.

Then we verified whether the increase in NO_x excretion was associated with increased NOS activity in the heart and aorta. NOS activity in response to saline or CNP infusion is shown in Fig. 1. In all studied tissues, NOS activity was higher in SHR than in WKY. CNP infusion enhanced this activity in both groups of animals. NOS stimulation induced by CNP was lower in SHR than in WKY, suggesting an impaired response of the enzyme to CNP in this hypertensive model.

eNOS and iNOS isoforms were expressed in the right atria, left ventricle and aorta (Fig. 2). SHR showed greater protein levels of both isoforms in all tissues compared with WKY. Reactivity against the antibody for the neuronal isoform was observed in the heart but not in the aorta in both groups of animals. Heart nNOS expression was more marked in SHR than in WKY. CNP induced no changes in protein level of eNOS, nNOS and iNOS isoforms, neither in the heart nor artery in both, normotensive and hypertensive, animals (Fig. 2).

Given that phosphorylation of eNOS at Ser^{1177} close to the carboxy-terminal is a critical requirement for eNOS activation, the role of CNP in eNOS activation was evaluated by measuring eNOS phosphorylation at Ser^{1177}. As shown in Fig. 3, CNP infusion significantly increased Ser^{1177} phosphorylation in ventricle.

3.2. Protocol 2: effects of CNP on NOS activity in isolated heart and aorta artery. Signaling cascade involved in CNP and NOS interaction.

In vitro experiments showed that CNP increases cardiac and vascular NOS activity in both groups of animals. These effects of CNP on NOS activity were abolished when L-NAME was previ-

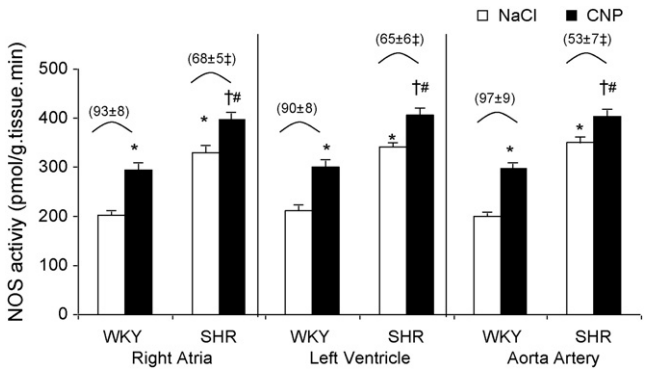


Fig. 1. Effect of NaCl and CNP infusion on cardiac and vascular NOS activity in SHR and WKY. Data corresponding to the difference (Δ) between saline and CNP treatment in each group are shown between brackets. Data are mean ± SEM, $n = 8$ rats/group. *$p < 0.01$ vs. WKY + NaCl; †$p < 0.01$ vs. SHR + NaCl; #$p < 0.01$ vs. WKY + CNP; ‡$p < 0.01$ vs. ΔWKY.

Table 1
Effects of CNP infusion on mean arterial pressure and nitrites and nitrates excretion in SHR and WKY.

Treatment		NaCl			CNP		
Period		C	E	Δ	C	E	Δ
MAP	WKY	103 ± 6	102 ± 5	4 ± 2	104 ± 4	86 ± 5*	18 ± 3
	SHR	180 ± 8*	185 ± 6	5 ± 4	179 ± 4*	168 ± 5#,†	11 ± 3‡
NO_x	WKY	0.93 ± 0.11	1.01 ± 0.09	0.08 ± 0.06	0.99 ± 0.10	1.38 ± 0.08*	0.40 ± 0.05
	SHR	1.54 ± 0.10*	1.48 ± 0.10	0.06 ± 0.05	1.52 ± 0.07*	1.80 ± 0.11#,†	0.28 ± 0.04‡

C: control period (NaCl infusion at 0.05 ml/min for 30 min); E: experimental period (saline bolus + NaCl infusion or bolus CNP + infusion CNP over 1 h).
MAP, mean arterial pressure (mm Hg); NO_x, nitrites and nitrates excretion (nmol/min 100 g body weight); Δ, change induced by each treatment on MAP and NO_x.
Data are mean ± SEM, $n = 8$ rats/group.
* $p < 0.01$ vs. WKY control period.
# $p < 0.01$ vs. NaCl infused SHR.
† $p < 0.01$ vs. CNP infused WKY.
‡ $p < 0.01$ vs. ΔCNP infused WKY.

Table 2
Effect of NOS blockade with L-NAME on basal or CNP-induced NOS activity in isolated heart and aorta artery in SHR and WKY.

		Basal	L-NAME (1 μM)	CNP (1 μM)	CNP + L-NAME
Right atria	WKY	224.9 ± 9.2	115.2 ± 6.9*	322.1 ± 14.3*	122.3 ± 8.7*†
	SHR	345.1 ± 15.4*	206.5 ± 8.3#	424.7 ± 19.6#	212.9 ± 9.1#‡
Left ventricle	WKY	228.5 ± 7.2	110.1 ± 5.8*	304.4 ± 16.1*	115.1 ± 7.5*,†
	SHR	351.9 ± 13.8*	212.7 ± 9.6#	417.1 ± 20.4#	202.6 ± 10.1#,‡
Aorta artery	WKY	208.8 ± 7.1	104.9 ± 7.1*	311.5 ± 15.4*	110.2 ± 5.9*,†
	SHR	365.7 ± 14.9*	128.4 ± 10.5#	429.4 ± 18.7#	122.5 ± 4.2#,‡

L-NAME, L-nitro arginine methyl ester (L-NAME); NOS activity, pmol/g tissue min.
Data are mean ± SEM, $n = 8$ rats/group.
* $p < 0.01$ vs. basal activity WKY.
# $p < 0.01$ vs. basal activity SHR.
† $p < 0.01$ vs. CNP-induced activity WKY.
‡ $p < 0.01$ vs. CNP-induced activity SHR.

ously added, verifying that measured activity was NOS specific (Table 2).

Similar to in vivo experiments, NOS activation induced by CNP in the heart and aorta was lower in SHR than in WKY.

In order to analyze the isoform involved in NOS activation via CNP in this model, the experiments were performed in the presence of an inhibitor of iNOS (AG) or an inhibitor of nNOS (7-NI). In all tissues in both, WKY and SHR, nNOS inhibition did not modify either basal or CNP induced NOS activity (Fig. 4). Meanwhile, the blockade of iNOS provoked a decrease in basal NOS activity in both groups. This reduction was higher in SHR than in WKY in all tissues (Table 3).

In addition, the decrease in basal NOS activity induced by Ca–calmodulin inhibition was more marked in WKY than in SHR, which confirms that iNOS is the main isoform involved in basal NOS activity observed in SHR (Fig. 4). Then the effect of iNOS blockade on NOS activity was similar in basal and in CNP-stimulated conditions in all studied tissues, indicating that the inducible isoform was not stimulated by CNP.

Table 3
Effect of iNOS blockade on basal or CNP-induced NOS activity in isolated heart and aorta artery in SHR and WKY, with aminoguanidine.

		Δ[Basal-AG]	Δ[CNP − (CNP + AG)]
Right atria	WKY	−21.8 ± 3.9	−16.4 ± 6.5
	SHR	−35.7 ± 3.1*	−39.8 ± 6.9#
Left ventricle	WKY	−30.0 ± 4.8	−38.1 ± 5.7
	SHR	−62.6 ± 7.7*	−57.9 ± 11.8#
Aorta artery	WKY	−26.3 ± 3.4	−22.2 ± 3.7
	SHR	−60.2 ± 10.7*	−58.7 ± 10.1#

AG, aminoguanidine; Δ NOS activity, pmol/g tissue min.
Data are mean ± SEM, $n = 8$ rats/group.
* $p < 0.01$ vs. Δ[Basal-AG] WKY.
# $p < 0.01$ vs. Δ[CNP − (CNP + AG)] WKY.

The increase in NOS activity induced by CNP was abolished by calmidazolium, a calmodulin inhibitor, in the heart and aorta artery of both SHR and WKY, implying a Ca^{2+}–calmodulin-dependent NOS pathway (Fig. 4).

On the other hand, the NPR-A/B receptor antagonist, anantin, did not modify NOS basal activity in the studied tissues in both groups. NPR-A/B receptor blockade induced no changes in CNP-stimulated NOS activity in cardiac atria and ventricle and in aorta in both groups. This latter finding suggests that the NPR-A/B receptors are not involved in NOS activation via CNP in these tissues in WKY and SHR (Fig. 5). In addition, the selective agonist of the NPR-C receptor, cANP(4-23), increased NOS activity in all studied tissues, provoking similar stimulation as CNP (Fig. 5).

As NPR-C receptors are Gi-protein coupled receptors, the effect of CNP on NOS activity in the presence of pertussis toxin, a Gi_{1-2} protein inhibitor, was investigated. The toxin did not modify basal NOS activity in the heart and aorta in neither group (data not shown). Uncouple NPR-C receptor/Gi protein complexes abolished CNP induced NOS activity in atria, ventricle and aorta artery (Fig. 5).

In view of these observations, we sought to establish the participation of the PLC/PKC pathway by using selective inhibitors of PLC (U-73122) and PKC (GF-109203). In all tissues of WKY and SHR, the inhibitors did not modify the effects of CNP on NOS activity (Fig. 5).

Recent studies have revealed that the PI3 kinase/Akt pathway may participate in the signaling cascade that mediates eNOS activation in vascular endothelial cells [31]. In order to evaluate the participation of this pathway in NOS activation via CNP, experiments were carried out in the presence of wortmannin, an inhibitor of the PI3-kinase/Akt pathway, and LY-294002, PI_3K inhibitor. Results showed that these inhibitors do not affect NOS activity induced by CNP in all tissues of WKY and SHR (Fig. 6).

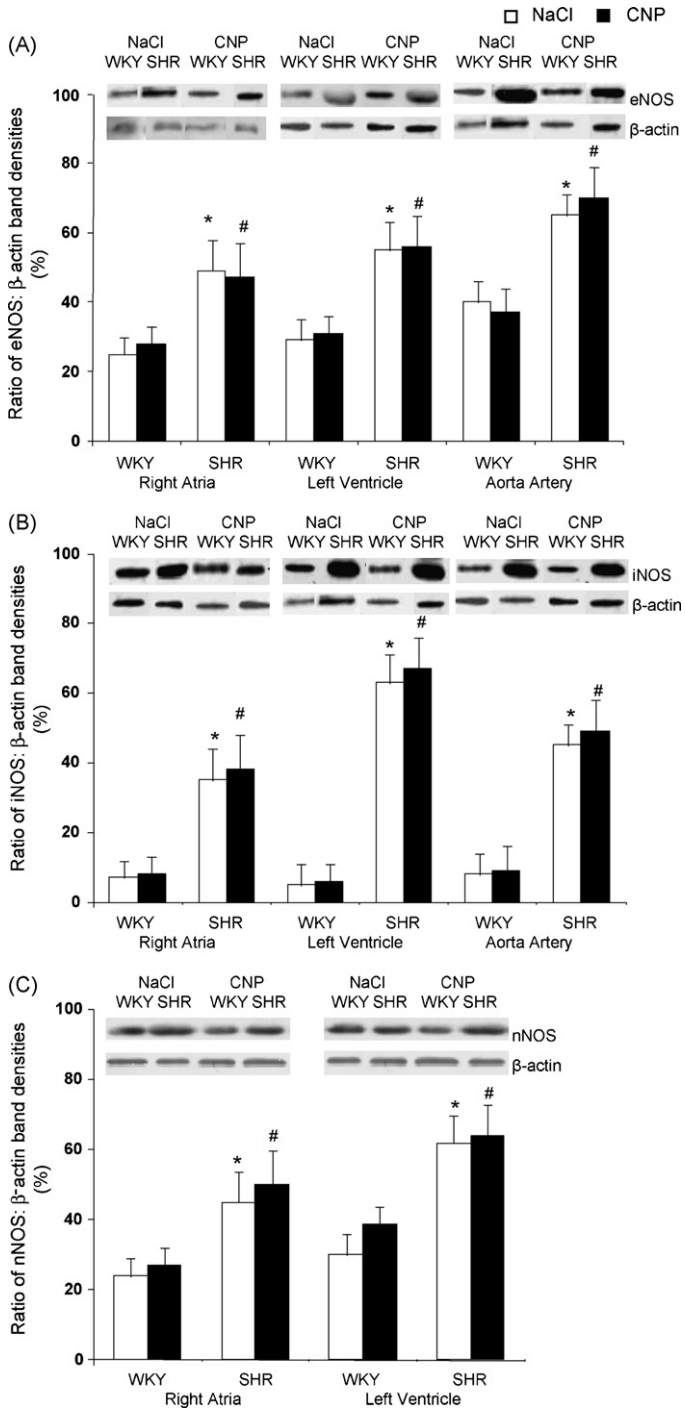


Fig. 2. Representative Western blot analysis of right atria, left ventricle and aorta artery from WKY and SHR, for NaCl and CNP infusion groups to anti-eNOS (A), anti-iNOS (B) and anti-nNOS (C) antibodies. Data are mean ± SEM, $n = 8$ rats/group. All experiments were performed in triplicate. Each blot was normalized with the expression of marker β-actin from the same gels. $^{*}p < 0.001$ vs. WKY + NaCl; $^{\#}p < 0.001$ vs. WKY + CNP.

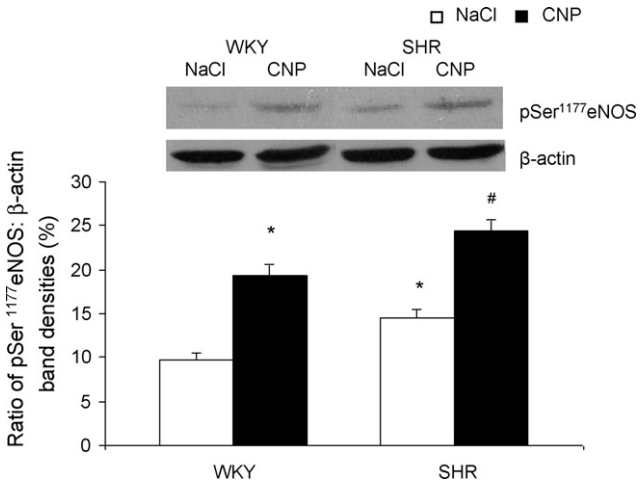


Fig. 3. eNOS phosphorylation at Ser^{1177} in ventricles from WKY and SHR infused with saline or CNP. eNOS phosphorylation at Ser^{1177} was measured by Western blot analysis. Data are mean ± SEM, $n = 8$ rats/group. $^{*}p < 0.01$ vs. WKY + NaCl; $^{\#}p < 0.01$ vs. SHR + NaCl.

4. Discussion

The elevated arterial blood pressure in this model of hypertension is associated with significant increase in urinary excretion of NO metabolites, indicating that the NO system is upregulated in adult SHR in accordance with previous reports [23].

Furthermore, our results showed that basal NOS activity in the heart and aorta artery was higher in hypertensive animals than in normotensive ones. We also showed that the blockade of iNOS diminished basal activity of the enzyme. The fact that the decrease in basal NOS activity induced by Ca–calmodulin inhibition was more marked in WKY than in SHR, confirm that iNOS, a Ca–calmodulin independent enzyme, is the main isoform involved in basal NOS activity in SHR.

This model of hypertension shows controversial results concerning the expression of the different NOS isoforms. Studies performed in different vascular beds showed an increase in the expression of iNOS and eNOS in 8- and 12-week-old SHR [37,38]. Conversely, other authors found a decrease in eNOS and iNOS expression in cardiac myocyte and vascular smooth muscle of hypertensive animals [13,16]. With regards to these findings, our results show that the three isoforms of the enzyme are expressed in the right atria and left ventricle, and that eNOS and iNOS were expressed in the aorta in both groups of animals. Accordingly, tissues from hypertensive rats showed greater protein levels of the three isoforms than normotensive ones. These results suggest that the up-regulation of NOS isoforms in vascular and cardiac tissues may play an important role in the compensatory mechanism in response to elevation of systolic blood pressure during development of hypertension in SHR.

Our in vivo studies showed that CNP treatment reduced MAP in both, normotensive and hypertensive animals. In addition, the hypotensive effect of CNP was lower in hypertensive animals than normotensive ones, indicating an impaired response to the peptide in this model.

Moreover, we also showed that acute infusion with CNP increases NOS activity in the heart and aorta artery in both groups. However, in agreement with our previous results in normotensive rats, the increase in NOS activity is not associated with an increase in NOS protein expression in this model of hypertension. This fact indicates that CNP would exert a positive effect on NOS activity, without modifying expression of the enzyme.

There are many reports that suggest the involving of MAPK pathway in eNOS upregulation [5,20,28]. In this regard, NOS activation induced by CNP was not modified by MAPKs inhibitors, indicating that, in heart and aorta of SHR and WKY, the activation of NOS via CNP does not involve MAPK pathway (Fig. 6).

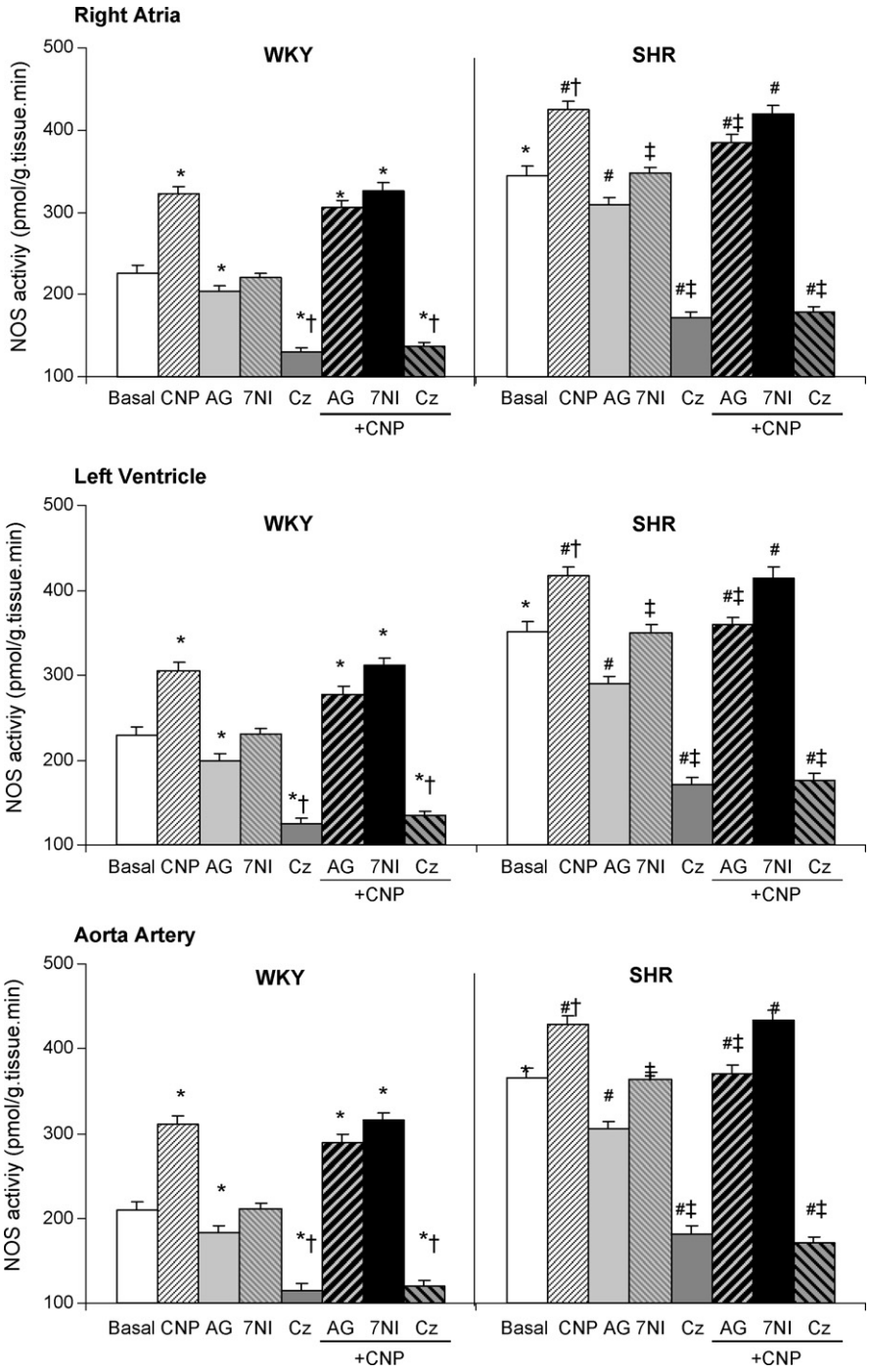


Fig. 4. Changes in CNP-induced cardiovascular NOS activity provoked by aminoguanidine (AG, iNOS inhibitor), 7-nitroindazole (7NI, nNOS specific inhibitor) or calmidazolium (Cz, calmodulin antagonist) in SHR and WKY. Data are mean ± SEM, $n = 8$ rats/group. $^{*}p < 0.01$ vs. basal activity WKY; $^{\dagger}p < 0.01$ vs. CNP-induced activity WKY; $^{\#}p < 0.01$ vs. basal activity SHR; $^{\ddagger}p < 0.01$ vs. CNP-induced activity SHR.

Additionally, we have also shown that CNP increased NOS activity in cardiac atria and ventricle and in aorta in vivo as well as in vitro in both groups, suggesting that NOS stimulation induced by CNP infusion is independent of the hemodynamic changes induced by the peptide in this model of hypertension.

With regard to the NOS isoform involved in CNP effects, nNOS did not participate since its inhibition did not modify activation of the enzyme induced by CNP. Moreover, the blockade of iNOS induced a similar drop in basal NOS activity than in CNP-stimulated one. These findings suggest that neither nNOS nor iNOS participate in the interaction between CNP and the NO system in this model of hypertension. Moreover, increased NOS activity induced by CNP was abolished by the antagonist of calmodulin, suggesting that the signaling cascade is mediated by Ca^{2+}/calmodulin-dependent NOS. The present findings indicate that eNOS is the isoform involved in the effects of CNP on NO-system in hypertensive animals.

With respect to the natriuretic peptide receptor involved in this mechanism, our results showed that the specific NPR-C receptor agonist, cANP(4-23), in the same way as CNP, induced an increase in NOS activity, indicating that this receptor is involved in NOS activation by CNP in the heart and aorta in both groups of animals. Although, Simon et al. showed in pulmonary endothelial cells

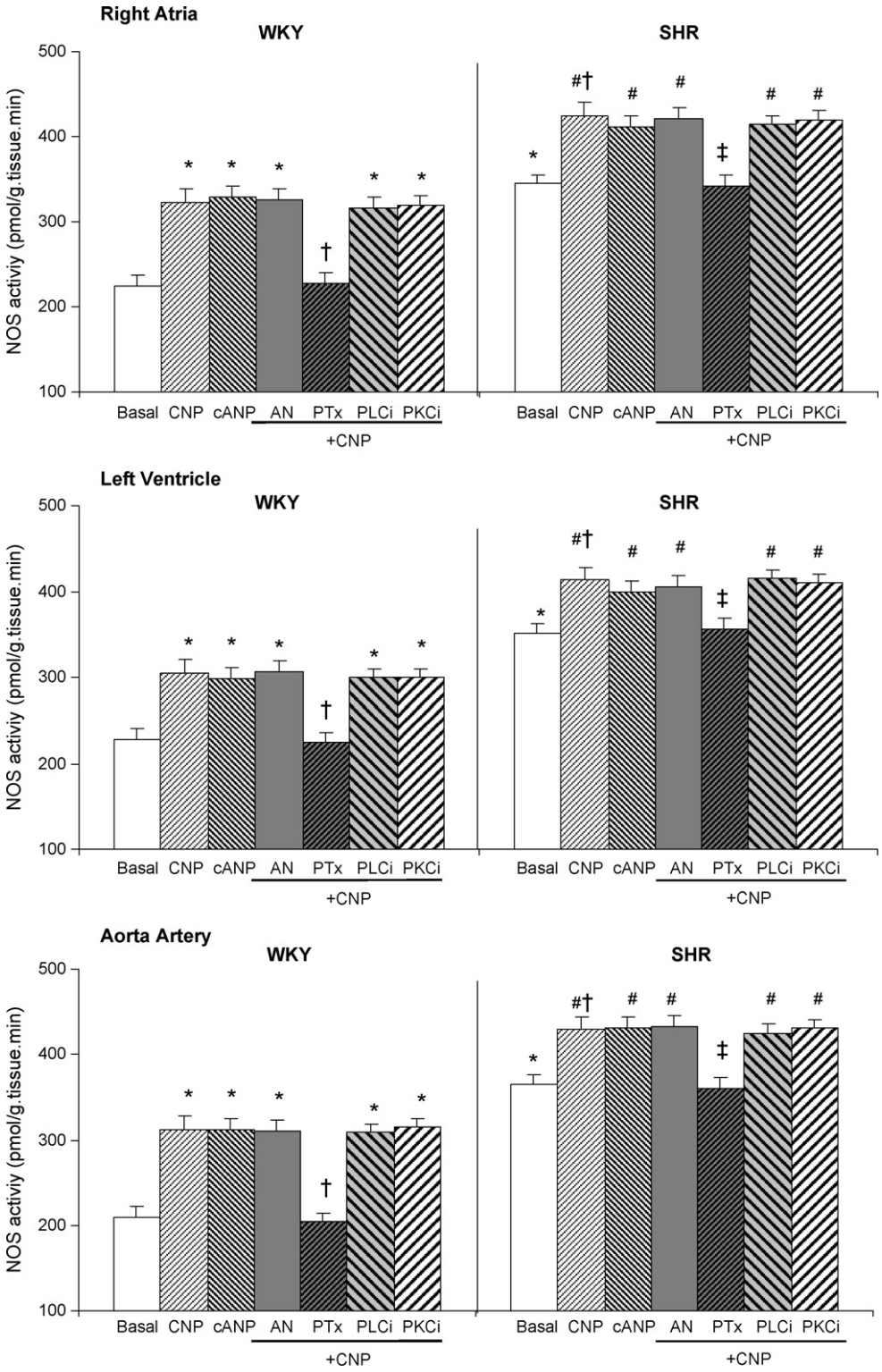


Fig. 5. Changes in CNP-induced cardiovascular NOS activity provoked by cANP(4-23), anantin (AN, NPR-C receptor selective agonist), pertussis toxin (PTx, Gi_{1-2} protein inhibitor), U-73122 (PLCi, phospholipase C inhibitor) or GF-109203 (PKCi, protein kinase C inhibitor) in SHR and WKY. Data are mean ± SEM, $n = 8$ rats/group. $^{*}p < 0.01$ vs. basal activity WKY; $^{\dagger}p < 0.01$ vs. CNP-induced activity WKY; $^{\#}p < 0.01$ vs. basal activity SHR; $^{\ddagger}p < 0.01$ vs. CNP-induced activity SHR.

the hyperpolarization induced by CNP involved NPR-B receptors to activate eNOS, we demonstrated that the blockade of NPR-A/NPR-B did not modify the effect of CNP on cardiovascular NOS [35]. This fact confirms that NPR-C is the unique natriuretic peptide receptor involved in the interaction between CNP and NOS in heart and aorta of normotensive and hypertensive animals.

With regard to NPR-C signaling pathway and NOS stimulation, previous results showed that CNP interacts with Gi coupled NPR-C receptor, but another G protein could also participate in this pathway, and we also reported that renal NOS stimulation via atrial natriuretic peptide is blocked when G protein is inhibited in normotensive rats [10,17]. Accordingly, our present results show that

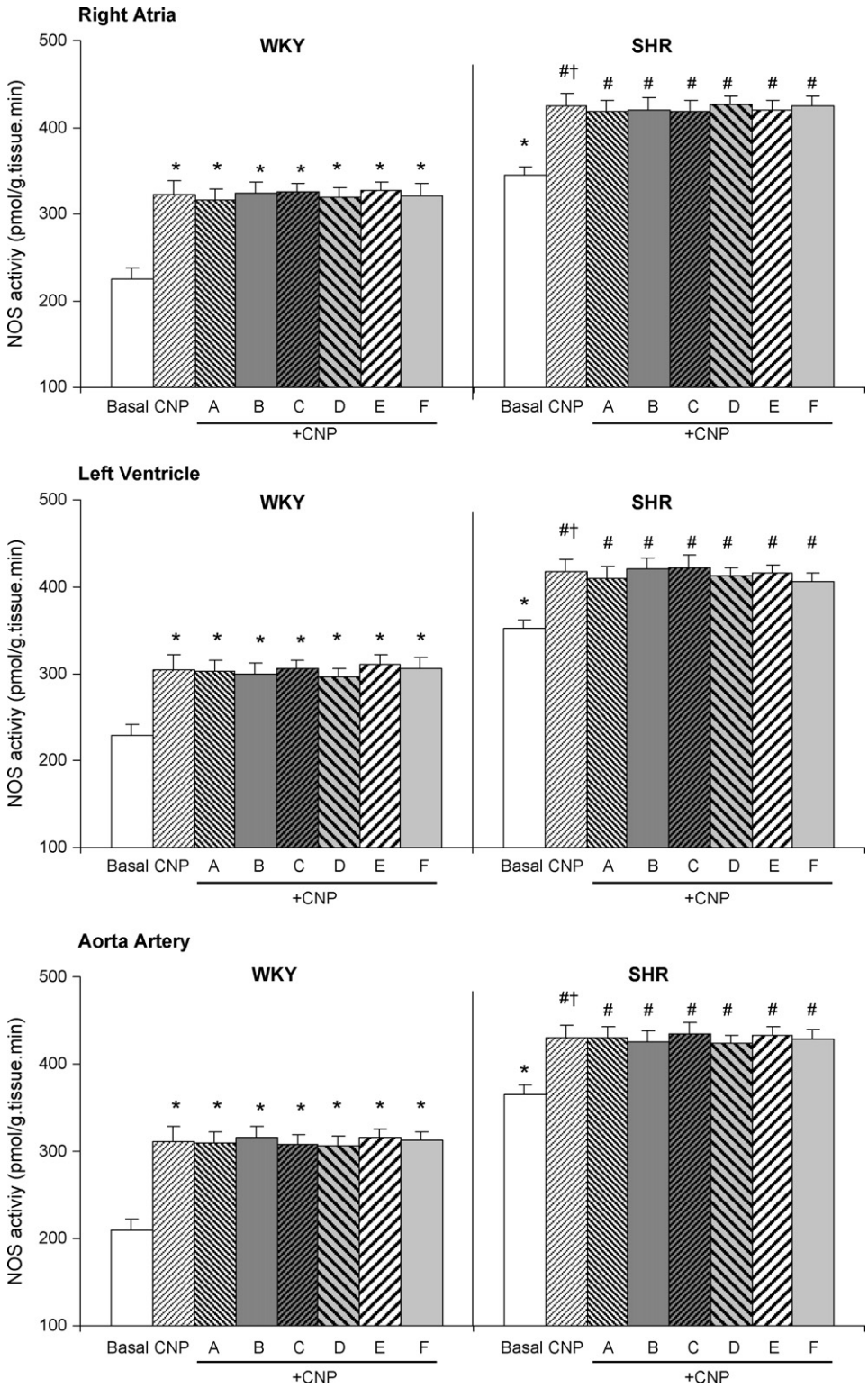


Fig. 6. Changes in CNP-induced cardiovascular NOS activity provoked by MAPKs and PI3K/Akt pathway inhibitors in SHR and WKY. MAPKs: mitogen-activated protein kinases; A: wortmannin (PI3-K/Akt pathway inhibitor); B: LY-294002 (PI_3K inhibitor); C: U-0126 (p42/p44 MAPK inhibitor); D: SB-203580 (p38 MAPK inhibitor); E: SP-600125 (JNK 1/2 inhibitor); F: PD-98059 (MEK1/ERK 1 2 inhibitor). Data are mean ± SEM, $n = 8$ rats/group. $^{*}p < 0.01$ vs. basal activity WKY; $^{\dagger}p < 0.01$ vs. CNP-induced activity WKY; $^{\#}p < 0.01$ vs. basal activity SHR.

cardiovascular NOS activation induced by CNP is inhibited by pertussis toxin, indicating that a Gi is involved in this mechanism in hypertensive animals. Meanwhile, Marcil et al. have demonstrated that there are no differences in Gi protein abundance and mRNA expression in the heart between SHR and WKY [24]. Our studies show that NOS response to CNP in hypertensive animals is impaired in all studied tissues when the interaction involves the NPR-C receptor and Gi protein pathway. In view of this fact we could speculate that this receptor or any of its pathway steps are altered in this model of hypertension.

Recent findings suggest that, in the exocrine pancreas, by activating NPR-C, atrial natriuretic peptide induces the release of Gβγ subunits that lead to PLC stimulation and subsequent PKC activation [25]. However, in the present work the selective inhibition of PLC or PKC failed to alter cardiovascular NOS activation induced by CNP in SHR and WKY.

On the other hand, our results failed to demonstrate participation of the PI3 kinase/Akt pathway in the signaling cascade mediating eNOS activation in the studied tissues while other authors have shown that this pathway is involved in eNOS activation in different experimental conditions [34].

Recent findings of Aregian et al. have shown that NO induces the downregulation of NPR-C [5]. Besides taking into account these findings and our results, we can speculate that the activation of NO production via CNP contributes to modulate CNP actions, through NPR-C receptor. Moreover, Aregian et al. also postulate that NO-induced decreased expression of NPR-C may upregulate the expression of guanylyl cyclase receptor NPR-A which binds with high affinity. In this regard, a cross-talk between NPR-C and NPR-A has been shown [5]. Futhermore, we showed that ANP stimulates cardiovascular eNOS via NPR-A and NPR-C, and this activation is impaired in normotensive and hypertensive animals [9,11]. Then impaired ANP actions in this model of hypertension may be related, almost in part, to a minor NOS activation via CNP.

Taken together, it may be suggested that the NOS impaired response to the natriuretic peptides, CNP and ANP, through linked pathways, would be involved in the maintaining of the high arterial blood pressure levels, in this model of hypertension.

Moreover, we cannot dismiss the possibility that NOS response to CNP in SHR, a model in which basal activity is enhanced may be the upper limit of the enzyme response. This fact could also explain the impaired response to CNP observed in this model of hypertension.

5. Conclusions

It is well known that CNP inhibits hypertrophy of cardiomyocytes and has hyperpolarizing actions on different vascular beds [7,21]. We previously demonstrated that these physiological effects of CNP involve the activation of NOS via NPR-C [10]. The present results show that the cardiovascular effects of CNP would be mediated, at least in part, by the interaction with the NO system in this model of genetic hypertension. According to our results neither nNOS nor iNOS, which activity is increased in this model of hypertension, would participate in NO system stimulation via CNP. In this regard we postulate that CNP induces eNOS stimulation interacting with the NPR-C receptor in heart and aorta artery, activating a pathway that involves Gi_{1-2} protein and Ca^{2+}/calmodulin. Taking these above mentioned findings into account, the impaired NO-system response to CNP in hypertensive animals, could participate in the development and/or maintenance of arterial hypertension in this model of genetic hypertension.

In patients with heart failure, levels of endogenous natriuretic peptides are increased and the body becomes resistant to their vasodilatory, diuretic and natriuretic effects. Nevertheless, exogenous natriuretic peptides infusion appears to overcome this apparent resistance, at least temporarily.

CNP has not yet found a role in the pharmacological treatments of arterial hypertension and associated pathologies. The study of the molecular mechanisms in models of arterial hypertension and their relation with other regulating systems, like the NO system, can contribute to the development of new therapeutic strategies.

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Glossary

7-NI: 7-nitroindazole, nNOS specific inhibitor
AN: anantin, NPR-A/B receptor antagonist
AG: aminoguanidine, iNOS inhibitor
cANP(4-23): NPR-C receptor selective agonist
cGMP: cyclic guanosine monophosphate
CNP: C-type natriuretic peptide
Cz: calmidazolium, calmodulin antagonist
eNOS: endothelial nitric oxide synthase
iNOS: inducible nitric oxide synthase
L-*NAME:* L-nitro arginine methyl ester, NOS inhibitor
MAP: mean arterial pressure
MAPK: mitogen-activated protein kinase
nNOS: neuronal nitric oxide synthase
NO: nitric oxide
NOS: nitric oxide synthase
NO_x*:* nitrites and nitrates excretion
NPR-A: A-type natriuretic peptide receptor
NPR-B: B-type natriuretic peptide receptor
NPR-C: C-type natriuretic peptide receptor
PTx: pertussis toxin, Gi_{1-2} protein inhibitor
SHR: spontaneously hypertensive rats
WKY: Wistar Kyoto rats