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Renal actions of atrial natriuretic peptide in spontaneously hypertensive rats: the role of nitric oxide as a key mediator

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Elesgaray R, Caniffi C, Savignano L, Romero M, Mac Laughlin M, Arranz C, Costa MA. Renal actions of atrial natriuretic peptide in spontaneously hypertensive rats: the role of nitric oxide as a key mediator. *Am J Physiol Renal Physiol* 302: F1385–F1394, 2012. First published February 29, 2012; doi:10.1152/ajprenal.00624.2011.—Atrial natriuretic peptide (ANP) is an important regulator of blood pressure (BP). One of the mechanisms whereby ANP impacts BP is by stimulation of nitric oxide (NO) production in different tissues involved in BP control. We hypothesized that ANP-stimulated NO is impaired in the kidneys of spontaneously hypertensive rats (SHR) and this contributes to the development and/or maintenance of high levels of BP. We investigated the effects of ANP on the NO system in SHR, studying the changes in renal nitric oxide synthase (NOS) activity and expression in response to peptide infusion, the signaling pathways implicated in the signaling cascade that activates NOS, and identifying the natriuretic peptide receptors (NPR), guanylyl cyclase receptors (NPR-A and NPR-B) and/or NPR-C, and NOS isoforms involved. In vivo, SHR and Wistar-Kyoto rats (WKY) were infused with saline (0.05 ml/min) or ANP (0.2 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). NOS activity and endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) NOS expression were measured in the renal cortex and medulla. In vitro, ANP-induced renal NOS activity was determined in the presence of iNOS and nNOS inhibitors, NPR-A/B blockers, guanine nucleotide-regulatory (G_i) protein, and calmodulin inhibitors. Renal NOS activity was higher in SHR than in WKY. ANP increased NOS activity, but activation was lower in SHR than in WKY. ANP had no effect on expression of NOS isoforms. ANP-induced NOS activity was not modified by iNOS and nNOS inhibitors. NPR-A/B blockade blunted NOS stimulation via ANP in kidney. The renal NOS response to ANP was reduced by G_i protein and calmodulin inhibitors. We conclude that ANP interacts with NPR-C, activating Ca-calmodulin eNOS through G_i protein. NOS activation also involves NPR-A/B. The NOS response to ANP was diminished in kidneys of SHR. The impaired NO system response to ANP in SHR participates in the maintenance of high blood pressure.

nitric oxide synthase; natriuretic peptide receptors; hypertension

BLOOD PRESSURE IS INFLUENCED by several vasoactive factors which also regulate renal function. An imbalance in the regulation of salt and water reabsorption contributes to hypertension. Two factors involved, nitric oxide (NO) and atrial natriuretic peptide (ANP), are very important because of their hypotensive, diuretic, and natriuretic effects (15, 24, 25, 28). Consequently, alterations in these two systems could be involved in the development and/or maintenance of hypertension.

ANP exerts its biological actions through interaction with two specific membrane-bound guanylyl cyclase receptors: natriuretic peptide receptor A (NPR-A) and B (NPR-B) (21, 27, 30). A third natriuretic receptor subtype, NPR-C, has been proposed primarily as a clearance receptor removing natriuretic peptides from the circulation, but now other biological functions have also been reported (2, 4, 5). In this regard, NPR-C is coupled to adenylyl cyclase through an inhibitory guanine nucleotide-regulatory protein (G_i) and/or to activation of phospholipase C (PLC) (2, 30).

On the other hand, the free radical nitric oxide (NO) is synthesized by a family of nitric oxide synthases (NOS) that comprises neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) isoforms which are expressed in many tissues, including the endothelium, vascular smooth muscle, and specific segments of the nephron, etc. (1, 13). Both nNOS and eNOS are expressed constitutively. The Ca^{2+} -calmodulin complex is necessary to maintain the enzyme active. In contrast, iNOS is expressed after transcriptional induction and it is independent of intracellular Ca^{2+} (1). With regard to eNOS activity, this isoform is regulated by various kinases and phosphorylation of eNOS at Ser¹¹⁷⁷ close to the carboxy terminal is a critical requirement for eNOS activation (26).

In previous studies, we have demonstrated that ANP exerts its hypotensive, natriuretic, and diuretic effects, at least in part, through activation of cardiovascular and renal NOS (7, 9). Furthermore, we have reported that the NPR-C natriuretic receptor mediates activation of NOS by ANP in the kidney (4, 11). In contrast, McLay et al. (22) have shown that, in cultures of proximal tubular cells, ANP inhibits iNOS activity via NPR-C and cGMP. Additionally, we have also shown that ANP interacts with NPR-A and/or B natriuretic receptors in the kidney, resulting in NOS activation (4, 11). These results are in accordance with studies performed in primary cultures of human proximal tubular cells, which show that NO synthesis can be stimulated by ANP via NPR-A receptors (23).

The spontaneously hypertensive rat (SHR) is a genetic model of hypertension that shows endothelial dysfunction, augmented oxidative stress, enhanced vasoconstrictor factors, and decreased bioavailability of NO (20, 29). Moreover, high plasma levels of ANP and increased NPR-A expression, associated with cGMP overproduction, have been observed in this model of hypertension (19, 21, 24). On the other hand, it has been shown that NO synthesis could be enhanced in SHR, probably as a counterregulatory mechanism activated to compensate the increase in blood pressure (20, 33). Several studies

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have found evidence suggesting that the activity and/or expression of the different NOS isoforms in the kidney are altered in this model and that a differential expression of NOS isoforms is age dependent (12, 17, 18, 20, 31, 33).

We have recently found evidence that demonstrates that the cardiovascular NOS response to ANP is impaired in SHR (6). In view of previous findings and existence of a link between both NO and ANP (3) and the role of the kidney in arterial blood pressure regulation, we hypothesize that the alterations in the interaction between both systems are involved in the development and/or maintenance of high levels of blood pressure in this experimental model of spontaneous hypertension.

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

MATERIALS AND METHODS

Animals

Sixteen-week-old male SHR and Wistar-Kyoto (WKY) animals were purchased from the Instituto de Investigaciones Médicas A. Lanari,

Facultad de Medicina (Universidad de Buenos Aires, Buenos Aires, Argentina). Rats were housed in a humidity- and temperature-controlled environment with an automatic 12:12-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.

Experimental Design

Animals were cared for according to the standards set out by 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 ethics committee of the School of Pharmacy and Biochemistry (CEFFB), Universidad de Buenos Aires, Buenos Aires, Argentina.

Protocol 1: effects of ANP infusion on mean arterial pressure and the NO system. Rats were anesthetized with urethane (1 g/kg body weight ip; Sigma-Aldrich, St. Louis, MO), and the femoral vein and artery and the urinary bladder were cannulated with a polyethylene catheter for drug administration (NaCl or ANP infusion), mean arterial pressure (MAP) recording, and urine collection, respectively.

The protocol is described in Fig. 1A. After surgery, an infusion of isotonic sodium chloride (0.9% NaCl) 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, and the rate of isotonic NaCl infusion was maintained

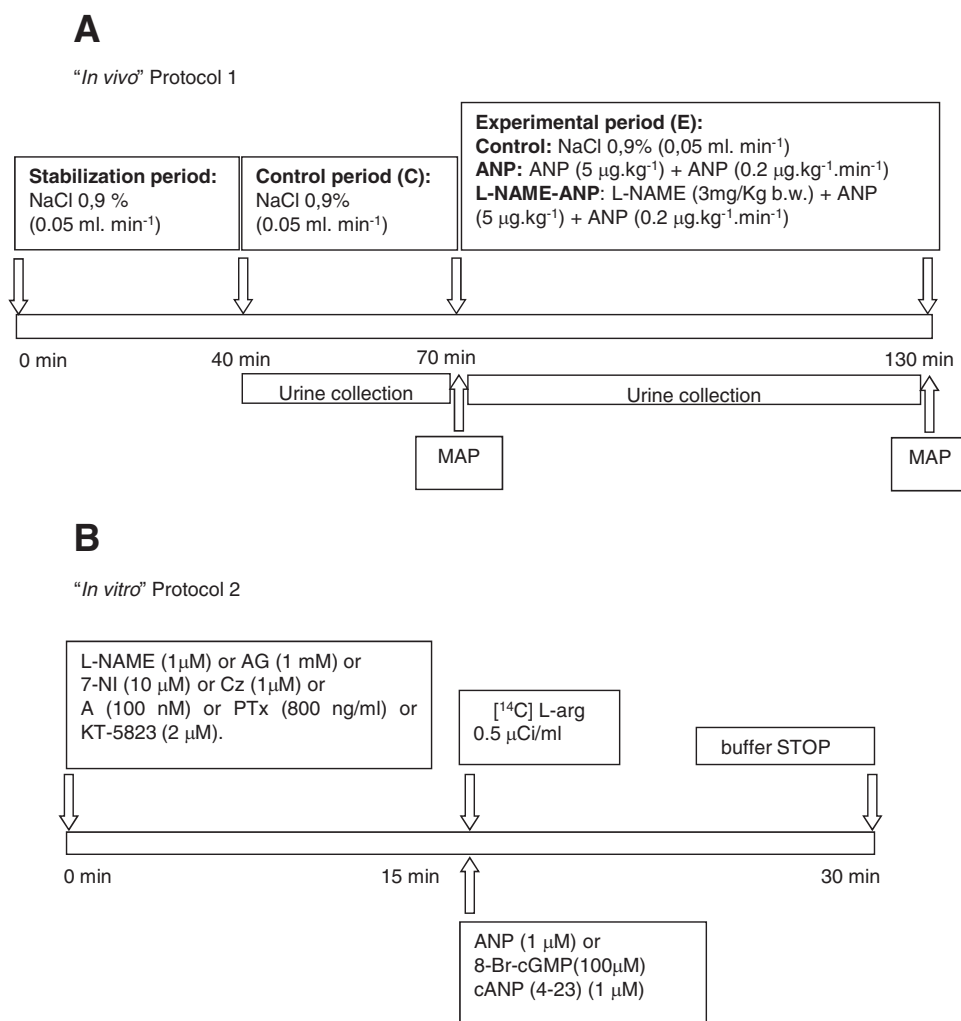


Fig. 1. A: *in vivo* protocol 1. MAP, mean arterial pressure; ANP, atrial natriuretic peptide; L-NAME, L-nitro arginine methyl ester; nitric oxide synthase (NOS) inhibitor. B: *in vitro* protocol 2. L-Nitro arginine methyl ester (L-NAME); NOS inhibitor; AG, aminoguanidine; inducible NOS (iNOS) inhibitor; 7-NI, 7-nitro-indazole; neuronal NOS (nNOS)-specific inhibitor; Cz, calmidazolium; calmodulin antagonist; cANP(4-23); natriuretic peptide receptor (NPR)-C-selective agonist; anantin (A); NPR-A/B receptor antagonist; 8-bromo (Br)-cGMP; stable analog of cGMP; KT-5823, cGMP-dependent protein kinase G (PKG) inhibitor; PTx, pertussis toxin; Gi₁₋₂ protein inhibitor.

at 0.05 ml/min. At the end of this period, one group of SHR and WKY rats received first a bolus of ANP (5 µg/kg, Sigma; vehicle, 0.9% NaCl, stored at -20°C) and then an infusion with ANP (0.2 µg·kg⁻¹·min⁻¹) over 1 h, and another group continued with the isotonic NaCl infusion. L-Nitro arginine methyl ester (L-NAME; a NOS inhibitor) was injected subcutaneously (3 mg/kg body wt) into another group of SHR and WKY animals 1 h before bolus administration and ANP infusion (5 µg/kg and 0.2 µg·kg⁻¹·min⁻¹, respectively). The L-NAME dose used in this protocol induced no changes in MAP (36).

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

Arterial blood pressure was measured with a Statham P23 ID pressure transducer (Gould Instruments, Cleveland, OH) and recorded with a polygraph (Physiograph E & M, Houston, TX). Data were obtained using data acquisition software (Labtech Notebook, Laboratory Technologies, Wilmington, MA).

Urine volume was determined gravimetrically, and urinary sodium concentration was evaluated with an ion analyzer (Tecnolab, model T-412).

The concentration of nitrites and nitrates (NOx), end products derived from NO metabolism, in urine samples was determined according to the procedure described by Verdon et al. (34).

At the end of the experimental period, animals were euthanized by decapitation, and kidneys were removed to determine NOS activity and expression.

DETERMINATION OF NOS ACTIVITY. Tissue NOS activity was measured using L-[¹⁴C]arginine as a substrate, as described previously (8, 10). Tissue slices (2–3 mm thick) were incubated 30 min at 37°C in Krebs solution with 0.5 µCi/ml L-[¹⁴C]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, and 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 L-[¹⁴C]citrulline was determined with a liquid scintillation counter (Wallac 1414 WinSpectral). Specific NOS activity was assessed in the presence of 10⁻⁴ M L-NAME (Sigma). NO production (measured as pmol of L-[¹⁴C]citrulline) in each tube was normalized to the weight of the tissue slices incubated with the substrate during equal periods of time and expressed as picomoles per gram wet weight per minute.

NOS EXPRESSION AND ENOS PHOSPHORYLATION. NOS expression and eNOS phosphorylation were determined by Western blotting.

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 Ser¹¹⁷⁷ (1:500 dilution) and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000 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).

Protocol 2: ANP effects on NOS activity in isolated renal medulla and cortex. SIGNALING CASCADE INVOLVED IN THE INTERACTION BETWEEN ANP AND NOS IN SHR. SHR and WKY rats were euthanized by decapitation, and NOS activity was measured in the renal cortex and medulla (inner and outer medulla without papilla) using L-[¹⁴C]arginine as substrate. Tissue slices (2–3 mm thick) were incubated for 30 min at 37°C as described in *protocol 1*, but agonists (15 min after incubation was started) and/or antagonists (at the beginning of the 30-min incubation period) were added during the incubation period according to the protocol described in Fig. 1B.

Concentration-dependent stimulation of NOS activity by ANP in all tissues studied had been performed in previous studies (Costa MA, unpublished observations). The lowest concentration of ANP that induced the maximum effect on NOS was used in the experimental protocol.

Statistical Analysis

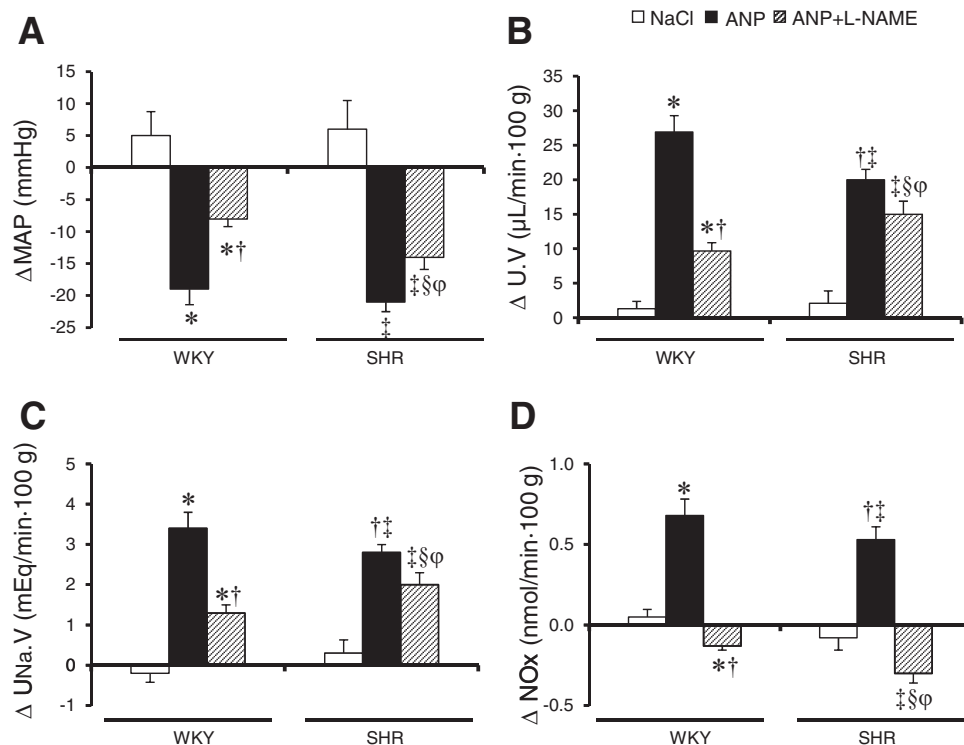
All values are expressed as means ± SE. The Prism program (GraphPad Software, San Diego, CA) was used for statistical analysis. The mean and standard deviation or median values of each variable were calculated for each group. The results of each variable for each experimental group were analyzed with a two-way ANOVA, where one factor was the different treatments and the other was the genotype (WKY or SHR). The effects of one factor were tested independently of the effects of the other, as well as the interaction between both factors. We found no interaction between treatments and genotype. Multiple comparisons were performed using a Bonferroni post hoc test. A *P* value <0.01 was considered a significant difference.

Table 1. Changes in mean arterial pressure, diuresis, natriuresis, and nitrite and nitrate excretion induced by ANP infusion with or without L-NAME pretreatment in SHR and WKY

Treatment	NaCl		ANP		L-NAME + ANP	
	C	E	C	E	C	E
MAP, mmHg						
WKY	100 ± 6	105 ± 5	104 ± 4	85 ± 3 ^{bd}	105 ± 4	97 ± 3 ^{cde}
SHR	167 ± 8 ^a	173 ± 6 ^d	163 ± 4 ^b	142 ± 5 ^{efg}	165 ± 5 ^c	151 ± 3 ^{ghi}
UV, nl·min ⁻¹ ·100 g ⁻¹						
WKY	16.2 ± 1.1	17.5 ± 1.4	17.6 ± 1.2	44.5 ± 1.4 ^{bd}	18.3 ± 1.4	28.0 ± 1.1 ^{cde}
SHR	61.3 ± 2.9 ^a	63.3 ± 3.5 ^d	62.1 ± 2.1 ^b	82.2 ± 3.7 ^{efg}	60.2 ± 2.6 ^c	75.1 ± 3.1 ^{ghi}
U _{Na} V, µeq·min ⁻¹ ·100 g ⁻¹						
WKY	2.0 ± 0.2	1.80 ± 0.2	1.7 ± 0.1	5.1 ± 0.4 ^{bd}	1.8 ± 0.2	3.1 ± 0.3 ^{cde}
SHR	6.2 ± 0.7 ^a	6.5 ± 0.8 ^d	6.4 ± 0.9 ^b	9.2 ± 1.4 ^{efg}	6.0 ± 0.7 ^c	8.0 ± 0.8 ^{ghi}
NOx, nmol·min ⁻¹ ·100 g ⁻¹						
WKY	0.97 ± 0.10	1.02 ± 0.12	0.92 ± 0.12	1.60 ± 0.09 ^{bd}	0.92 ± 0.07	0.79 ± 0.05 ^{cde}
SHR	1.50 ± 0.10 ^a	1.42 ± 0.10 ^d	1.48 ± 0.05 ^b	2.01 ± 0.11 ^{efg}	1.48 ± 0.10 ^c	1.18 ± 0.08 ^{ghi}

Values are means ± SE; *n* = 8 rats/group. ANP, atrial natriuretic peptide; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; L-NAME, L-nitro arginine methyl ester; MAP, mean arterial pressure; UV, diuresis; U_{Na}V, natriuresis; NOx, nitrite and nitrate excretion; C, control period, NaCl infusion at 0.05 ml/min for 30 min; E, experimental period, NaCl infusion or bolus ANP (5 µg/kg)+infusion of ANP (0.2 µg·kg⁻¹·min⁻¹) or L-NAME, 3 mg/kg body wt) 30 min before ANP treatment, over 1 h. ^a*P* < 0.01 vs. C NaCl WKY. ^b*P* < 0.01 vs. C ANP WKY. ^c*P* < 0.01 vs. C (ANP+L-NAME) WKY. ^d*P* < 0.01 vs. E NaCl WKY. ^e*P* < 0.01 vs. E ANP WKY. ^f*P* < 0.01 vs. C ANP SHR. ^g*P* < 0.01 vs. E NaCl SHR. ^h*P* < 0.01 vs. E ANP SHR. ⁱ*P* < 0.01 vs. C (ANP+L-NAME) SHR.

Fig. 2. Changes induced by atrial natriuretic peptide (ANP) infusion on MAP, diuresis, natriuresis, and nitrites and nitrates (NOx) excretion in spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats. Data are expressed as delta (Δ) between control and experimental period (E-C). C, control period: NaCl infusion at 0.05 ml/min for 30 min. E, experimental period: NaCl infusion or bolus ANP ($5 \mu\text{g/kg}$)+infusion of ANP ($0.2 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) or L-NAME (3 mg/kg body wt) 30 min before ANP treatment, over 1 h. A: MAP. B: diuresis (UV). C: natriuresis (U_{NaV}). D: NOx. Values are means $\pm$ SE; $n = 8$ rats/group. $*P < 0.01$ vs. NaCl WKY. $\dagger P < 0.01$ vs. ANP WKY. $\ddagger P < 0.01$ vs. NaCl SHR. $\S P < 0.01$ vs. ANP SHR. $\phi P < 0.01$ vs. (ANP+L-NAME) WKY.



RESULTS

Protocol 1: Effects of ANP Infusion on MAP, Renal Function, and the NO System

When MAP, urine flow, natriuresis, and systemic production of NO were determined in this model of hypertension, all parameters were found to be significantly higher in SHR than in WKY (Table 1).

NOS blockade with L-NAME did not modify MAP in either SHR or WKY animals that received NaCl infusion (Costa MA, unpublished observations).

ANP infusion reduced MAP in both groups of animals. When rats were pretreated with L-NAME before ANP infusion, the effect of the peptide on MAP diminished in both the SHR and WKY groups (Table 1, Fig. 2). The drop in blood pressure induced by ANP was similar in both SHR and WKY, but L-NAME pretreatment had a more marked impact in WKY than in SHR. With respect to the magnitude of L-NAME inhibition on ANP-induced BP decrease, Fig. 2 shows that L-NAME treatment reduced the hypotensive effect of ANP in WKY by 58% (from 19 to 11 mmHg) vs. 33% (from 21 to 7 mmHg) in SHR. Notwithstanding, after L-NAME treatment, 67% of the ANP hypotensive effect was still present in SHR vs. 42% in WKY.

The ANP-induced reduction in MAP was accompanied by a rise in diuresis, natriuresis, and NOx excretion; the increase in these parameters was also more marked in WKY than in SHR, indicating a lower renal response to ANP in hypertensive than in normotensive animals (Fig. 2 and Table 1). When rats were treated with L-NAME before ANP infusion, the effect of the peptide on urine flow and sodium excretion diminished in both the SHR and WKY groups. However, L-NAME had a greater effect on the renal response to ANP in WKY than in SHR (Fig. 2).

As expected, L-NAME treatment reduced NOx excretion in all experimental conditions.

We then verified whether the increase in NOx excretion was associated with the stimulation of renal NOS. NOS activity in response to saline or ANP infusion is shown in Fig. 3. In the renal cortex and medulla, NOS activity was higher in SHR than in WKY. ANP infusion enhanced this activity in both groups of animals. ANP-induced NOS stimulation was lower in SHR than in WKY, suggesting an impaired response to ANP in this model of hypertension.

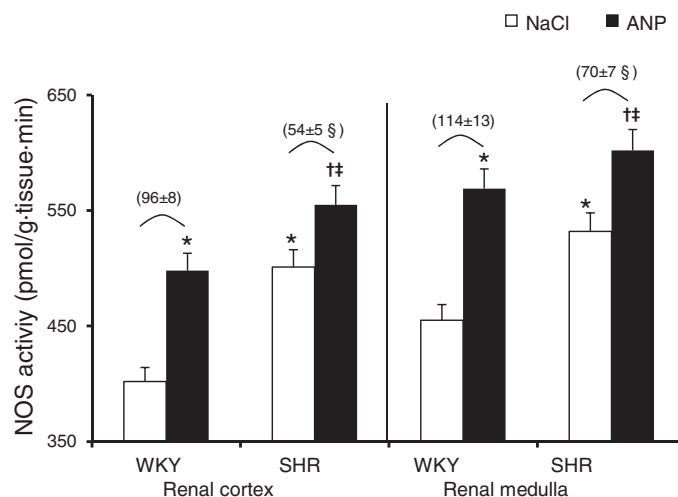


Fig. 3. Effect of NaCl and ANP infusion on renal NOS activity in SHR and WKY. Data corresponding to the difference (Δ) between NaCl (0.9% NaCl, 0.05 ml/min) and ANP treatment [bolus ANP ($5 \mu\text{g/kg}$)+infusion ANP ($0.2 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)] in each group are shown between brackets. Values are means $\pm$ SE; $n = 8$ rats/group. $*P < 0.001$ vs. WKY+NaCl. $\dagger P < 0.01$ vs. SHR+NaCl. $\ddagger P < 0.01$ vs. WKY+ANP. $\S P < 0.01$ vs. Δ WKY.

The three isoforms of NOS, i.e., eNOS, iNOS, and nNOS, were expressed in the renal cortex and medulla (Fig. 4). Greater protein levels of all isoforms were present in the kidney of SHR compared with WKY. ANP induced no changes in protein level of eNOS, nNOS, and iNOS isoforms in the kidneys in both groups (Fig. 4).

Given that phosphorylation of eNOS at Ser¹¹⁷⁷ close to the carboxy terminal is a critical requirement for eNOS activation, the role of ANP in eNOS activation was evaluated by measuring eNOS phosphorylation at Ser¹¹⁷⁷. As shown in Fig. 5, ANP infusion significantly increased Ser¹¹⁷⁷ phosphorylation in the renal cortex and medulla.

Protocol 2: Effects of ANP on NOS Activity in the Isolated Kidney

Signaling cascade involved in the interaction between ANP and NOS. In vitro experiments showed that ANP increased renal NOS activity in both groups (Table 2, Fig. 6). ANP-induced NOS activity was blunted when L-NAME was added previously, verifying that activity measured was NOS specific (Costa MA, unpublished observations).

Similar to in vivo experiments, ANP-induced renal NOS activation was lower in SHR than in WKY (Table 2, Fig. 6).

To analyze the isoform involved in NOS activation via ANP in this model, the experiments were performed in the presence

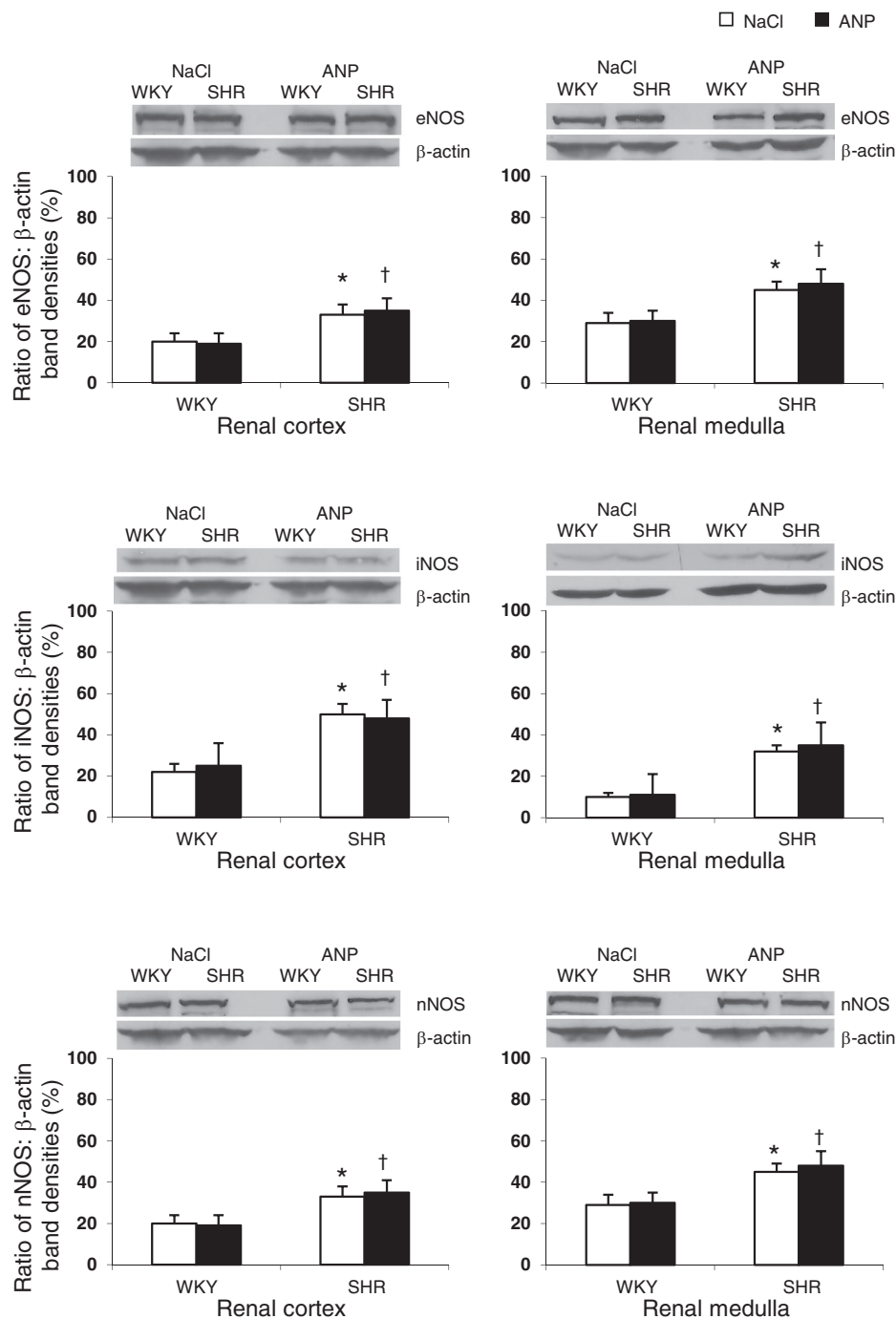
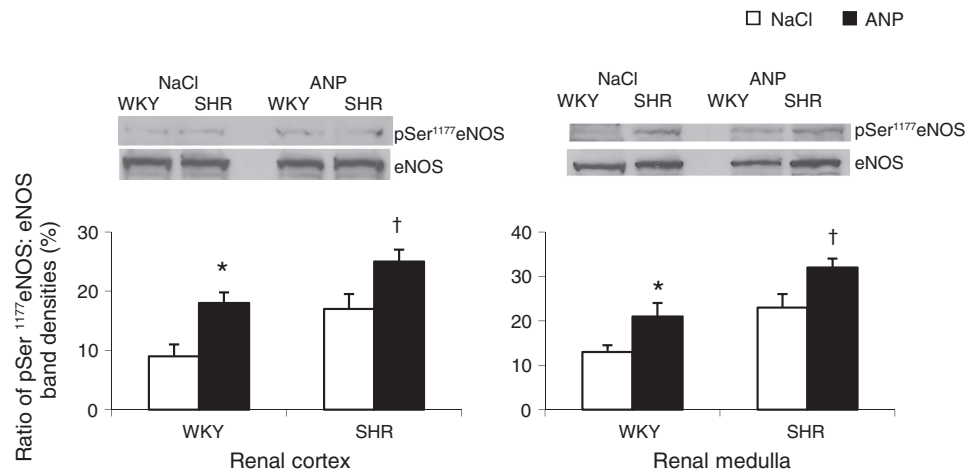


Fig. 4. Representative Western blot analysis of renal cortex and medulla from WKY and SHR for NaCl (0.9% NaCl, 0.05 ml/min) and ANP infusion [bolus ANP (5 μ g/kg)+infusion ANP (0.2 μ g·kg⁻¹·min⁻¹)] groups to anti-eNOS, anti-iNOS, and anti-nNOS antibodies. Values are means $\pm$ SE; n = 8 rats/group. All experiments were performed in triplicate. β -Actin was used as a loading control, and data are normalized to actin expression. White spaces demarcate noncontiguous gel lanes. * P < 0.001 vs. WKY+NaCl. † P < 0.001 vs. WKY+ANP.

Fig. 5. eNOS phosphorylation at Ser¹¹⁷⁷ in WKY and SHR renal medulla and cortex infused with isotonic NaCl or ANP [bolus ANP (5 µg/kg)+infusion ANP (0.2 µg·kg⁻¹·min⁻¹)]. eNOS phosphorylation at Ser¹¹⁷⁷ was measured by Western blot analysis. Values are means ± SE; *n* = 8 rats/group. White spaces demarcate noncontiguous gel lanes. **P* < 0.01 vs. WKY+NaCl. †*P* < 0.01 vs. SHR+NaCl.



of an inhibitor of iNOS aminoguanidine (AG) or an inhibitor of nNOS (7-nitroindazole; 7-NI). In all renal tissues, neither basal nor ANP-induced NOS activity was modified by nNOS inhibition (Table 2, Fig. 6) in either group, while iNOS blockade provoked a decrease in basal NOS activity in both groups. This reduction was higher in both the renal cortex and medulla in SHR relative to WKY (Table 3). It is known that Ca-calmodulin is required for eNOS activity (1). Thus our results showed that the inhibition of this complex decreased basal NOS activity in both groups. Combined inhibition of Ca-calmodulin and iNOS blockade abolished NOS activity in the tissues studied, indicating that both isoforms are involved in basal NOS activity observed in the kidney (Table 2, Fig. 6). The effect of iNOS blockade on NOS activity was similar in basal and in ANP-stimulated conditions in both tissues, indicating that the inducible isoform is not stimulated by ANP (Table 3).

The ANP-induced increase in NOS activity was abolished by calmidazolium in both SHR and WKY renal cortex and medulla, implying a Ca-calmodulin-dependent NOS pathway (Table 2, Fig. 6).

Although the selective agonist of the NPR-C receptor, cANP (4–23), increased NOS activity in the renal medulla and cortex, NOS stimulation was lower than the ANP-induced increase (Table 4, Fig. 7).

On the other hand, the NPR-A/B receptor antagonist anantin did not modify NOS basal activity in the tissues studied in either group (Costa MA, unpublished observations). Renal ANP-induced NOS activity was partially abolished by this

natriuretic receptor antagonist in both groups. This latter finding suggests that the NPR-A/B receptor participates in NOS activation via ANP in the renal cortex and medulla in both WKY and SHR (Table 4, Fig. 7).

The action of 8-bromo (Br)-cGMP on NOS activity was determined to verify involvement of guanylyl cyclase-coupled natriuretic receptors in the effect of ANP on NOS activity in the kidney. In all tissues, addition of 8-Br-cGMP partially mimicked the effect of ANP on NOS activity (Table 4, Fig. 7).

The experiments were performed in the presence of KT-5823 to evaluate whether cGMP signaling effects occur through PKG. Basal NOS activity in the kidney was not affected by the presence of the PKG inhibitor in either group (Costa MA, unpublished observations). Inhibition of PKG partially reduced the effect of the peptide on renal NOS activity in both groups (Table 4, Fig. 7).

As NPR-C receptors are G_i-protein coupled receptors, the effect of ANP on NOS activity in the presence of pertussis toxin, a G_{i1-2} protein inhibitor, was investigated. The toxin did not modify basal NOS activity in the kidney in either group (Costa MA, unpublished observations). Results showed that the uncoupled NPR-C receptor/G protein complexes blunted NOS activity elicited by ANP in the renal cortex and medulla in both WKY and SHR (Table 4, Fig. 7). Pertussis toxin completely blocked the effect of the NPR-C agonist cANP on NOS activity. NPR-A/B blockade plus G_{i1-2} protein inhibition abolished NOS activity induced by ANP.

Table 2. Changes in ANP-induced renal NOS activity provoked by aminoguanidine, 7-nitroindazole or calmidazolium in SHR and WKY

	Basal	ANP	ANP				
			AG	7-NI	AG+7-NI	Cz	AG+Cz
Renal cortex							
WKY	118.2 ± 1.2	259.2 ± 18.3*	227.9 ± 14.5*†	245.8 ± 18.9*	230.2 ± 13.2*†	50.8 ± 16.8*†	16.0 ± 10.9*†
SHR	312.3 ± 23.1*	418.3 ± 24.8‡	361.3 ± 24.2‡§	415.9 ± 27.9‡	258.4 ± 24.1‡§	201.6 ± 19.1‡§	42.1 ± 37.8‡§
Renal medulla							
WKY	79.3 ± 11.8	214.2 ± 12.7*	192.6 ± 10.8*†	212.2 ± 13.9*	189.2 ± 14.8*†	62.7 ± 15.5*†	17.5 ± 15.4*†
SHR	349.5 ± 29.7*	451.7 ± 32.8‡	408.5 ± 33.7‡§	453.4 ± 26.8‡	412.8 ± 34.3‡§	239.8 ± 25.3‡§	55.0 ± 42.9‡§

Values are mean ± SE; *n* = 8 rats/group. Changes in ANP-induced renal nitric oxide synthase (NOS) activity provoked by aminoguanidine [AG; inducible NOS (iNOS) inhibitor, 1 mM], 7-nitroindazole (7-NI; neuronal NOS-specific inhibitor, 10 µM), and/or calmidazolium (Cz; calmodulin antagonist, 1 µM) in SHR and WKY. **P* < 0.01 vs. basal activity WKY. †*P* < 0.01 vs. ANP-induced activity WKY. ‡*P* < 0.01 vs. basal activity SHR. §*P* < 0.01 vs. ANP-induced activity SHR.

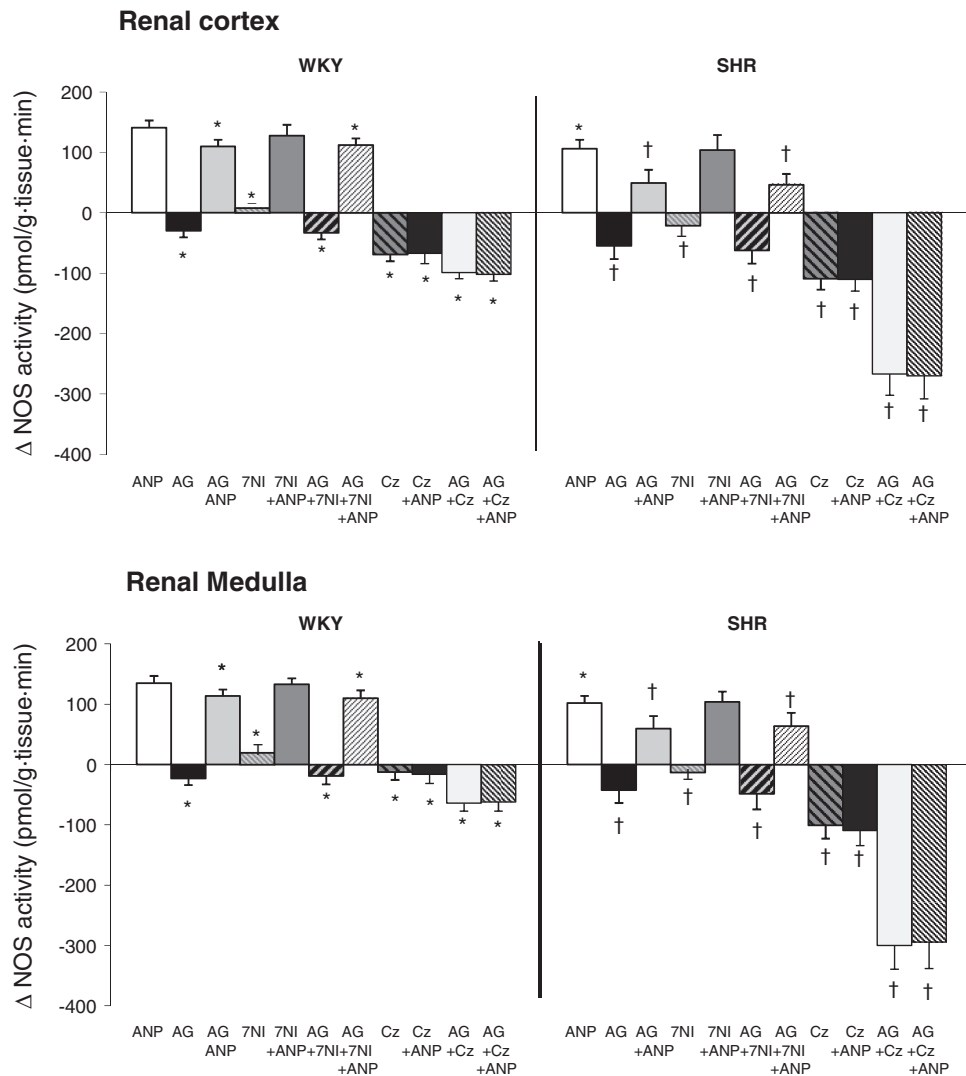


Fig. 6. NOS isoforms involved in ANP-induced renal NOS activity. Changes in renal NOS activity provoked by ANP (1 μ M), AG (iNOS inhibitor, 1 mM), 7-NI (nNOS-specific inhibitor, 10 μ M), or Cz (calmodulin antagonist, 1 μ M) in SHR and WKY. Data are expressed as delta (Δ) between different treatments and basal NOS activity. Values are means $\pm$ SE; $n = 8$ rats/group. * $P < 0.01$ vs. ANP-induced activity in WKY. † $P < 0.01$ vs. ANP-induced activity in SHR.

DISCUSSION

As demonstrated previously, our results confirmed that high arterial blood pressure in SHR is associated with a significant increase in urinary excretion of NO metabolites, indicating that the L-arginine/NO pathway is upregulated in this model of hypertension. In this regard, in the present study we have demonstrated that basal NOS activity in the kidney of hypertensive animals is higher compared with the activity observed

in renal tissues from normotensive ones. In addition, iNOS blockade and Ca-calmodulin inhibition reduced basal NOS activity in both groups. These results demonstrate that both inducible and endothelial isoforms are involved in basal renal NOS activity in SHR and WKY.

With respect to the activity and expression of the NOS isoforms in SHR kidneys, the results are controversial. Vaziri et al. (33) have demonstrated that 12-wk-old SHR present an elevation of renal NOS activity coupled with significant increases in renal iNOS and eNOS expression. In accordance with these findings, Nava et al. (12) have shown an augmented expression of renal eNOS and iNOS in 20-wk-old SHR compared with WKY. Conversely, Kumar et al. (17, 18) have shown that nNOS protein levels in the renal cortex are diminished in SHR relative to WKY and that there is a decrease in abundance of eNOS protein in the inner medulla of SHR kidneys (17, 18). With regard to these findings, our results have shown that the three isoforms of the enzyme are expressed in the renal cortex and medulla in both groups of animals. Accordingly, tissues from hypertensive rats showed greater protein levels of the three isoforms than levels present in normotensive ones. These results indicate that the upregulation of NOS isoforms in renal tissues may play an important role in

Table 3. Effect of iNOS blockade with aminoguanidine on basal or ANP-induced NOS activity in *in vitro* studies in SHR and WKY renal cortex and medulla

	Δ [ANP-Basal]	Δ [AG-Basal]	Δ [(ANP + AG)-ANP]
Renal cortex			
WKY	141.5 $\pm$ 14.9	-29.7 $\pm$ 3.9	-31.1 $\pm$ 4.8
SHR	106.1 $\pm$ 7.1*	-54.6 $\pm$ 3.1†	-56.7 $\pm$ 2.3‡
Renal medulla			
WKY	135.7 $\pm$ 11.8	-22.9 $\pm$ 4.8	-21.4 $\pm$ 6.3
SHR	102.5 $\pm$ 9.7*	-42.5 $\pm$ 7.7†	-42.5 $\pm$ 9.1‡

Values are mean $\pm$ SE, $n = 8$ rats/group. AG, 1 mM; Δ NOS activity, pmol·g.tissue⁻¹·min⁻¹. * $P < 0.01$ vs. Δ [ANP-Basal] WKY. † $P < 0.01$ vs. Δ [AG-Basal] WKY. ‡ $P < 0.01$ vs. Δ [(ANP+AG)-ANP] WKY.

Table 4. Changes in ANP-induced renal NOS activity provoked by the inhibition or blockade of NPR-A/B, Gi_{1-2} and/or PKG in SHR and WKY

	Basal	ANP	cANP	ANP			
				A	KT-5823	PTx	A+PTx
Renal cortex							
WKY	118.2 ± 1.2	259.2 ± 18.3*	188.8 ± 16.5*†	195.7 ± 19.3*†	205.1 ± 15.1*†	201.3 ± 18.8*†	108.0 ± 11.9†
SHR	312.3 ± 23.1*	417.7 ± 20.8†‡	361.3 ± 22.3‡	362.8 ± 22.9‡	362.4 ± 24.8‡	369.2 ± 18.2‡	307.1 ± 20.8‡
Renal medulla							
WKY	78.7 ± 11.8	214.2 ± 14.7*	176.1 ± 16.8*†	169.2 ± 11.9*†	176.3 ± 15.8*†	179.1 ± 13.5*†	85.5 ± 12.3†
SHR	349.5 ± 29.7*	451.7 ± 13.8†‡	400.5 ± 17.7‡§	405.4 ± 18.0‡§	409.3 ± 22.5‡§	404.2 ± 21.2‡§	335.2 ± 27.8‡§

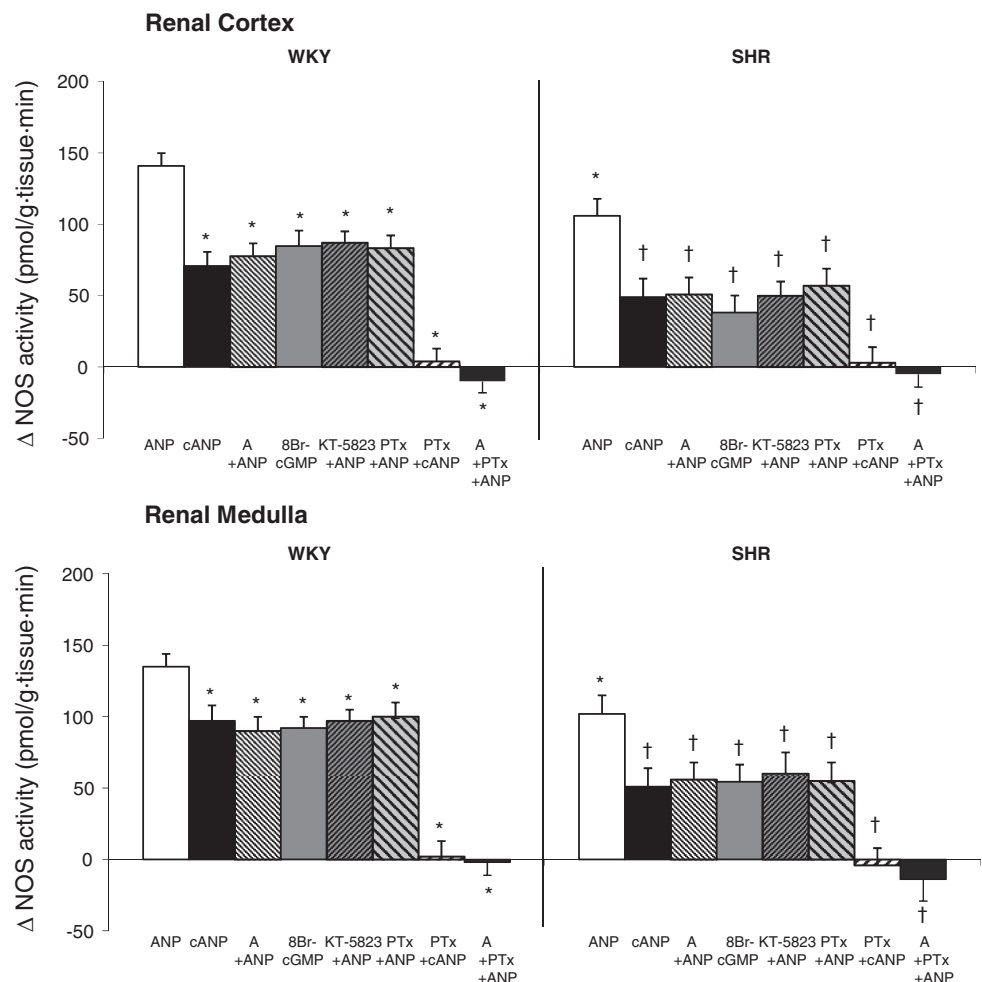
Values are mean ± SE, $n = 8$ rats/group. Changes in ANP-induced renal NOS activity provoked by cANP (4–23), cANP (4–23), NPR-C receptor selective agonist, 1 μ M; anantin (A; NPR-A/B receptor antagonist, 100 nM); KT-5823 (cGMP-dependent protein kinase G inhibitor, 2 μ M), or pertussis toxin (PTx; Gi_{1-2} protein inhibitor, 800 ng/ml) in SHR and WKY. * $P < 0.01$ vs. basal activity WKY. † $P < 0.01$ vs. ANP-induced activity WKY. ‡ $P < 0.01$ vs. basal activity SHR. § $P < 0.01$ vs. ANP-induced activity SHR.

the compensatory mechanism in response to elevation of systolic blood pressure during the development of hypertension in SHR. Our data reinforce other authors' results demonstrating that development of hypertension is not due to a primary impairment of NO production in SHR (33).

Nevertheless, we cannot disregard the possibility that the increment in oxidative stress induced by the increase in peroxynitrite production may also be involved in maintenance of the high levels of blood pressure in this model of hypertension (14, 16).

Our in vivo studies showed that ANP treatment reduced MAP in both normotensive and hypertensive animals. NOS blockade blunted the ANP hypotensive effect in both groups, but L-NAME treatment had a more marked impact on normotensive ones. These results confirm our previous findings indicating that the NO system is involved in the hypotensive effect induced by ANP and that this response is impaired in hypertensive animals (6, 9). Thus our results suggest that ANP's hypotensive effects involve different mechanisms in SHR and in WKY, indicating that the NO system plays a more

Fig. 7. NPR and signaling pathways involved in ANP-induced renal NOS activity. Changes in renal NOS activity provoked by ANP (1 μ M), cANP (4–23; NPR-C receptor selective agonist, 1 μ M); anantin (A; NPR-A/B receptor antagonist, 100 nM); 8-Br-cGMP (stable analog of cGMP, 100 μ M); KT-5823 (cGMP-dependent PKG inhibitor, 2 μ M), or PTx (Gi_{1-2} protein inhibitor, 800 ng/ml) in SHR and WKY. Data are expressed as delta (Δ) between different treatments and basal NOS activity. Values are means ± SE; $n = 8$ rats/group. * $P < 0.01$ vs. ANP-induced activity in WKY. † $P < 0.01$ vs. ANP-induced activity in SHR.



important role in ANP hypotensive effect in WKY than in SHR. Moreover, increased NPR-A expression, associated with cGMP overproduction, have been observed in this model of hypertension (19, 21, 24). According to these findings and our results in SHR, ANP could induce vasorelaxation via activation of the particulate guanylyl cyclase-coupled NPR-A receptor, increasing cGMP production through a mechanism that involves neither NO production nor soluble guanylyl cyclase activity.

In the present study, we have also shown that acute infusion with ANP increased NOS activity in the renal medulla and cortex in both groups. However, in agreement with our previous results in normotensive animals, the increase in renal NOS activity would not be associated with an increase in the protein expression of NOS isoforms in this model of hypertension. This indicates that ANP exerts a positive effect on NOS activity, without modifying expression of the enzyme.

Additionally, we have also shown that ANP increased renal NOS activity in *in vivo* and *in vitro* experiments in both groups, suggesting that NOS stimulation induced by ANP infusion is independent of the renal hemodynamic changes induced by this peptide.

When the NOS isoform involved in ANP's renal effects was analyzed, we observed that ANP-induced NOS stimulation was blocked by the antagonist of calmodulin and that the inhibition of either iNOS or nNOS induced no changes in the interaction between both systems in SHR and WKY. The present findings indicate that the isoform involved in this mechanism in the kidney is eNOS Ca-calmodulin dependent.

Our results show that the specific NPR-C receptor agonist cANP (4–23) increased NOS activity in the renal medulla and cortex in both groups of animals. On the other hand, the blockade of NPR-A/NPR-B blunted the effect of ANP on renal NOS. These results suggest that NPR-A/NPR-B and/or NPR-C participates in the interaction between ANP and the NOS pathway in SHR and WKY kidneys. In the present study, 8-Br-cGMP partially mimicked the effect of ANP on renal NOS activity, and the inhibition of PKG blunted ANP-induced NOS activation, indicating participation of the cGMP/PKG pathway. 8-Br-cGMP stimulated NOS activity to a lesser degree than ANP, probably due to the fact that this cGMP analog has relatively low permeability across the plasma membrane.

Studies performed in SHR have shown an increased affinity and expression of NPR-A in the kidney and an impaired particulate guanylate cyclase activity (19, 32, 35). In view of our results, we cannot determine which pathway, NPR A/B and/or NPR-C, is altered in SHR, leading to a lower NOS response to ANP.

With regard to NPR-C, our results show that ANP-induced renal NOS stimulation involves G_i protein. Furthermore, Martin et al. (21) have shown greater expression of NPR-C in SHR kidneys compared with WKY (21). Our studies show that, when the interaction involved the NPR-C receptor and the G_i protein pathway, the NOS response to ANP in hypertensive animals was impaired in all tissues studied. In view of this, we could speculate that this receptor or any of its pathway steps might be altered in this model of hypertension.

Moreover, we cannot dismiss the possibility that the NOS response to ANP in SHR, a model in which basal activity is enhanced compared with normotensive animals, may be the

upper limit of the enzyme response. This fact can also explain the impaired response to ANP observed in this model of hypertension.

Conclusion

Our results have shown that the renal effects of ANP would be mediated, at least in part, by interaction with the NO system in this genetic model of hypertension, as shown previously with regard to the cardiovascular effects of the peptide in SHR (6). In addition, we have demonstrated that ANP infusion augmented renal NOS activity in SHR, without modifying its expression. We postulate that ANP induces renal eNOS stimulation, interacting with the NPR-C receptor and activating a pathway that involves G_{i-2} protein and Ca-calmodulin. On the other hand, ANP would also interact with NPR-A/B in the kidney, enhancing cGMP formation via particulate guanylyl cyclase, which in turns activates PKG.

The impaired NO system response to ANP in hypertensive animals, involving alterations in both pathways through NPR-A/B or NPR-C, could participate in maintenance of the high levels of arterial blood pressure in this genetic model of hypertension.

ANP is a hypotensive and natriuretic factor involved in regulation of function, vascular tone, and salt and water balance. This important physiological mechanism has not yet found a role in the pharmacological treatments of arterial hypertension and associated pathologies. Explaining the molecular mechanisms in models of arterial hypertension and their relationship to other regulating systems, like the NO system, would contribute to the development of new therapeutic strategies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: R.E., C.C., L.S., and M.R. performed experiments; R.E., C.C., L.S., and M.R. analyzed data; R.E., C.C., L.S., and M.M.L. interpreted results of experiments; R.E., C.A., and M.A.C. edited and revised manuscript; R.E., C.A., and M.A.C. approved final version of manuscript; C.C. prepared figures; M.M.L. and M.A.C. drafted manuscript; C.A. and M.A.C. provided conception and design of research.

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