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Atrial Natriuretic Peptide Modifies Arterial Blood Pressure Through Nitric Oxide Pathway in Rats

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***Abstract*—**The aim of the present study was to determine the relationship between the hypotensive effect of the atrial natriuretic peptide (ANP) and the nitric oxide (NO) pathway. N^G-nitro-L-arginine methyl ester bolus (L-NAME, 1 mg/kg) reverted the decrease in mean arterial pressure induced by ANP administration (5 μg/kg bolus and 0.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$ infusion), and the injection of L-NAME before peptide administration suppressed the ANP hypotensive response. To confirm these findings, a histochemical reaction was used to determine NADPH-diaphorase activity (a NO synthase marker) in the endothelium and smooth muscle of aorta and arterioles of the small and large intestine. ANP increased aorta and arteriole endothelium staining after both in vivo administration and in vitro tissue incubation. In both cases, L-NAME prevented the ANP effect on NADPH-diaphorase activity. Tissues incubated with 8-bromoguanosine 3′,5′-cyclic monophosphate mimicked ANP action. In addition, ANP administration increased urinary excretion of NO_x end products. These findings indicate that ANP increases NO synthesis capability and NO production and suggest that the cGMP pathway may be involved. In conclusion, the NO pathway could be an intercellular messenger in the ANP endothelium-dependent vasorelaxation mechanism. **(*Hypertension*. 2000;35:1119-1123.)**

Key Words: natriuretic peptides ■ nitric oxide ■ arterial pressure ■ NADPH diaphorase ■ cyclic GMP ■ vasodilation

Atrial natriuretic peptide (ANP) and nitric oxide (NO), a free radical in the form of a highly diffusible gas, are vasoactive substances that induce hypotension through their vasorelaxant effects.[1-3] Several factors, such as an increase in intracellular cGMP levels, a decrease in cytosolic Ca^{2+} levels, and the activation of calcium-gated potassium channels, are involved in the cellular vasorelaxation mechanism.[4,5] An increase in cGMP results in the activation of cGMP-dependent protein kinases and the phosphorylation of proteins involved in the dephosphorylation of myosin light chains, which is a precondition for smooth muscle relaxation.[6]

Both ANP and NO induce an increase in intracellular cGMP levels, but they do so through different pathways. The cellular effects of ANP are mainly mediated by the guanylyl cyclase-coupled natriuretic receptors, natriuretic peptide receptor (NPR)-A and NPR-B.[7,8] These receptors are expressed on the surface of different types of cells, including renal endothelial cells, both arterial and venous smooth muscle and endothelial cells, etc.[6] These receptors present an intracellular protein kinaselike domain (KLD), a domain with an autoinhibitory function that mediates adenine nucleotide effects on the guanylyl cyclase domain. On binding of ANP to the extracellular domain of the receptor, a conformational change would ensue, allowing binding of ATP to the KLD. This, in turn, would lead to a further conformational change, freeing the guanylyl cyclase domain from the inhibitory constraint of the KLD and enabling cGMP production.[9]

NO is produced by 2 different nitric oxide synthase (NOS) enzyme systems: a constitutive enzyme (cNOS) and an inducible enzyme (iNOS) in vascular smooth muscle and endothelium. iNOS is highly regulated by cytokines and other factors.[10,11] NO binds the heme moiety of soluble guanylyl cyclase, which results in an allosteric modulation of the enzyme and leads to an increase in cGMP levels. This increase in cGMP production, similar to the cGMP produced by ANP through particulate guanylyl cyclase, leads to a decrease in intracellular Ca^{2+} levels through a multiple cascade of the events proposed.[12] On the basis of the above evidence, the aim of the present study was to evaluate potential interactions between ANP and NO in the vascular smooth muscle cells and endothelial cells.

Methods

Animals

Male Wistar rats (weight 250 to 300 g) from the breeding laboratories at Facultad de Farmacia y Bioquímica (Universidad de Buenos Aires, Argentina) were used in each experimental group. Animals were housed in a temperature- and humidity-controlled environment with an automatic light/dark cycle of 12/12 hours, and they were fed

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From the Departamento de Ciencias Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, PROSIVAD-CONICET, Buenos Aires, Argentina.

Correspondence to María de los Angeles Costa, Departamento de Ciencias Biológicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junin 956, 7 mo (1113), Buenos Aires, Argentina. E-mail mcosta@huemul.ffyb.uba.ar

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a standard rat chow (Nutrimentos Purina) and tap water ad libitum until experiments were performed.

Experimental Design

Animals were used in compliance with the guidelines of the "Position of the American Heart Association on Research Animal Use."

Effect of ANP on Mean Arterial Pressure and Nitrate+Nitrite Urinary Excretion

Rats were anesthetized with urethane (1g/kg body wt, IP), and their jugular vein, carotid artery, and urinary bladder were cannulated with polyethylene catheters for drug administration, mean arterial pressure (MAP) recording, and urine collection, respectively. On completion of surgery, a saline infusion (0.05 mL/min) was started and maintained for 45 minutes in all the protocols to ensure stability of hemodynamics and renal parameters. In addition, different protocols were performed.

Protocol 1

Six control and 6 experimental animals were injected with saline (1 mL/kg) or ANP (5 μg/kg, 1 mL/kg) and then infused for 90 minutes with saline (0.0393 mL/min) or ANP (0.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$), respectively. Both control and experimental animals received N^G-nitro-L-arginine methyl ester (L-NAME) 1 mg/kg 30 minutes after the beginning of the infusion. MAP was measured at 0, 30, and 90 minutes.

Protocol 2

Twelve rats were injected with L-NAME (1 mg/kg), and after 30 minutes they were injected with saline (0.1 mL/kg) or ANP (5 μg/kg, 1 mL/kg) and infused with saline or ANP (0.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$), respectively, for 60 minutes. MAP was measured at 0, 30, and 90 minutes.

Protocol 3

Seven animals were infused 60 minutes with saline (control period) and then received an ANP bolus (5 μg/kg) and an infusion of ANP (0.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$). MAP was monitored during the whole procedure. Urine was collected at 60 (control period), 70, 80, and 90 minutes.

Nitrate+Nitrite Measurement

The concentration of NO_x-derived end products (nitrite and nitrate) in urine samples was determined according to the procedure described by Verdon et al.[13]

Effect of ANP on NADPH Diaphorase Activity

Protocol 4

Twelve animals were divided into 3 groups: Control, ANP, and L-NAME+ANP. Control and ANP rats were infused with saline or ANP (0.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$), respectively, for 30 minutes, at a rate of 0.0393 mL/min. An L-NAME bolus (1 mg/kg) was administered to the L-NAME+ANP group 30 minutes before ANP infusion. After infusion, 150 mm of jejunum-ileum, 30 mm of distal colon, and 30 mm of aorta were removed under surgical conditions, after which, the rats were disposed. To eliminate any blood and intestinal contents, the removed tissue was carefully washed with Kreb's solution (mmol/L): NaCl 115.8, KCl 3.8, $CaCl_2$ 1.2, $MgSO_4$ 1.1, KH_2PO_4 1.2, $NaHCO_3$ 25.4, EDTA 0.002, and glucose 10, bubbled with 95% O_2 and 5% CO_2 to pH 7.4. Finally, the organs were cut into ≈10-mm segments.

Protocol 5

Sixteen rats were anesthetized with ether, and ≈150 mm of jejunum-ileum, 30 mm of distal colon, and 30 mm of aorta were removed under surgical conditions. Samples were obtained and processed as described in protocol 4. Segments of small intestine, colon, and aorta were divided into 4 groups: Control, ANP, L-NAME+ANP, and cGMP.

TABLE 1. Effects of ANP Administration on MAP in L-NAME–Treated Rats, and Effects of L-NAME on MAP in ANP-Treated Rats

	PAM (mm Hg)		
	0 Minutes	30 Minutes	90 Minutes
Protocol 1			
Control (saline)	107±9	108±9	125±9*
Experimental (ANP)	110±10	88±6*†	129±7*
Protocol 2			
Control (saline)	106±7	141±9*	141±9*
Experimental (ANP)	105±6	138±6*	156±7*

Protocol 1: After 30 minutes, L-NAME was injected. Protocol 2: After 0 minutes, L-NAME was injected. After 30 minutes, ANP or saline was administered. Values represent the mean±SE. **P*<0.01 vs basal, †*P*<0.01 vs control (n=6 for each group). Data were analyzed by ANOVA, followed by Bonferroni's test.

Control group segments were incubated for 10 minutes at 37°C in Krebs solution, whereas ANP group segments were incubated for 10 minutes at 37°C in Krebs supplemented with 0.5 μmol/L ANP. Segments from the L-NAME+ANP group were first incubated for 5 minutes at 37°C in Krebs containing 0.1 mmol/L L-NAME and then for 10 minutes more adding 0.5 μmol/L ANP. Finally, segments from the cGMP group were incubated for 10 minutes at 37°C in Krebs supplemented with 0.1 mmol/L 8-bromoguanosine 3′, 5′-cyclic monophosphate (8-Br-cGMP) (stable analogue of cGMP).

Histochemistry

All tissues were processed by the NADPH-diaphorese (NADPH-d) histochemical method according to Rothe et al.[14] This technique is used as an histochemical marker of isozyme-independent NOS, because it has been demonstrated that NADPH-d activity is inhibited by preincubation with diphenyleneiodonium, a potent inhibitor of NOS.[15–17] Observation, optical density (OD) measurement, and photography were performed on an Axiophot microscope (Zeiss). In the absence of β-NADPH, cells were unstained, although the substitution of β-NADPH for β-NADH resulted in the nonspecific staining of entire sections (data not shown). Valtschanoff et al[18] reported that nitroblue tetrazolium contains a monoformazan impurity that gives a diffuse purple background. To test this effect, a number of sections were incubated in acetone dimethyl sulfoxide for 10 minutes before mounting; because no differences were observed in stained sections, this step was omitted.

In all cases, special care was taken to fix and process control and experimental tissues simultaneously. To avoid technical variations in tissue staining, the time and the temperature of incubation with the reaction mixture was carefully controlled, and samples were randomly processed.

Computed-Image Analysis

NADPH-d–stained cells from the different groups were measured by a computed image analyzer (Kontron-ZEISS VIDAS). The mean of each OD value resulted from measuring OD in different tissue areas of the same section, and different sections of the same organ. Each set of OD measurements (control and experimental groups) was performed blindly and under similar conditions of light, gain, offset, and magnification.

Statistical Analysis

Data are presented as the mean±SE. Differences between control and experimental data were evaluated using 1-way ANOVA followed by Bonferroni's test. A *P* value of <0.05 was considered a significant difference.

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TABLE 2. Effects of ANP on Nitrate+Nitrite Excretion (Protocol 3)

Experimental Condition	Basal	ANP		
Time, minutes	0	10	20	30
Mean±SE, nmol/min per 100 g	0.08±0.03	0.40±0.11*	0.21±0.05	0.22±0.04

Data were analyzed by repeated-measures ANOVA, after being transformed to logarithms, because variances were significantly different ($P<0.05$ by Bartlett's test for equal variances). *$P<0.001$ vs basal (n=7).

Results

Table 1 (protocol 1) shows that ANP administration induced a hypotensive effect that was either reverted or suppressed when NO synthesis was inhibited by the injection of L-NAME. Moreover, L-NAME increased MAP in control and ANP-treated rats (Table 1, protocol 2). On the other hand, ANP induced a significant rise of NO_x end product excretion with a simultaneous decrease in MAP (basal=87.0±3.2; 10 minutes=70.7±3.5; 30 minutes=69.2±2.1; n=6, $P<0.01$ versus basal) (Table 2).

An increase in cell staining indicated enhanced NADPH-d activity in the endothelium of aorta and intestinal arterioles from ANP-treated rats. L-NAME blunted this ANP effect. No changes were observed in smooth muscle staining (Table 3, protocol 4).

When aorta and intestinal arterioles were incubated in vitro with ANP, an increase in endothelium staining was observed. In both tissues, preincubation with L-NAME suppressed the ANP effect. Incubation of the tissues with 8-Br-cGMP enhanced aorta and arteriole endothelium staining (Table 3, protocol 5). Photomicrographs showing these effects are presented in Figure 1.

Discussion

On the basis of studies performed by means of endothelium denudation, several authors have postulated that ANP-induced vasodilation was endothelium independent.[19,20] However, present results indicate that an acute inhibition of NO synthesis reverts the hypotensive effect induced by ANP. On the other hand, injection of L-NAME before peptide administration suppresses the ANP hypotensive response, suggesting that an endothelium-derived relaxing factor (ie, NO is involved in the vasodilator effect of ANP). Moreover, the increase in the urinary excretion of NO_x end products and the simultaneous decrease in MAP confirm the hypothesis above mentioned.

Furthermore, research was performed to determine whether ANP modifies NOS activity. By means of histochemical reaction of NADPH-d, which is known to indicate NOS, endothelium and vascular smooth muscle have been identified, microscopically, as being capable of synthesizing NO.[21,22] There is evidence that NOS inhibition by L-arginine analogues also inhibits the NOS-associated catalytic NADPH-d activity.[23-25]

The induction of NADPH-d activity by ANP in the endothelium of conductance and resistance vascular beds, and the fact that L-NAME (a NOS inhibitor) completely suppresses this effect, indicate that ANP enhances NO synthesis capability. Moreover, the same results were obtained in rat enterocytes.[26] On the basis of these studies, ANP-induced NO synthesis would involve the L-arginine–NO pathway.[27]

ANP and NO physiological actions are known to be mainly mediated by cGMP.[8,12,28] Given that 8-Br-cGMP mimicked the effect of ANP on NADPH-d activity both in aorta and in arteriole endothelium, guanylyl cyclase-coupled natriuretic receptors, ie, NPR-A and/or NPR-B, could be involved in this ANP action.

However, NPR-C participation in this ANP action cannot be discarded, because NO release stimulation by ANP through NPR-C receptor has been reported in human proximal renal tubular cells.[29,30] NPR-C or clearance receptors have a short cytoplasmatic tail, whose functions include both a Gi-dependent inhibition of adenylate cyclase and the hydrolysis of phosphoinositides by activation of phospho-

TABLE 3. Effects of ANP on NADPH-d Activity

	OD		
Protocol 4	Control	ANP	L-NAME+ANP
Endothelium			
Aorta	0.083±0.006	0.130±0.012*	0.081±0.005†
Arterioles	0.219±0.006	0.253±0.005*	0.225±0.006†

	OD			
Protocol 5	Control	ANP	cGMP	L-NAME+ANP
Endothelium				
Aorta	0.265±0.007	0.318±0.009‡	0.353±0.017‡	0.270±0.010§
Arterioles	0.102±0.009	0.143±0.006‡	0.264±0.004‡§	0.079±0.010§

Values represent the mean±SE. Protocol 4: *$P<0.01$ vs control, †$P<0.001$ vs ANP (n=10 for each group). Protocol 5: ‡$P<0.01$ vs control, §$P<0.001$ vs ANP (n=17 for each group). Data were analyzed by ANOVA, followed by Bonferroni's test.

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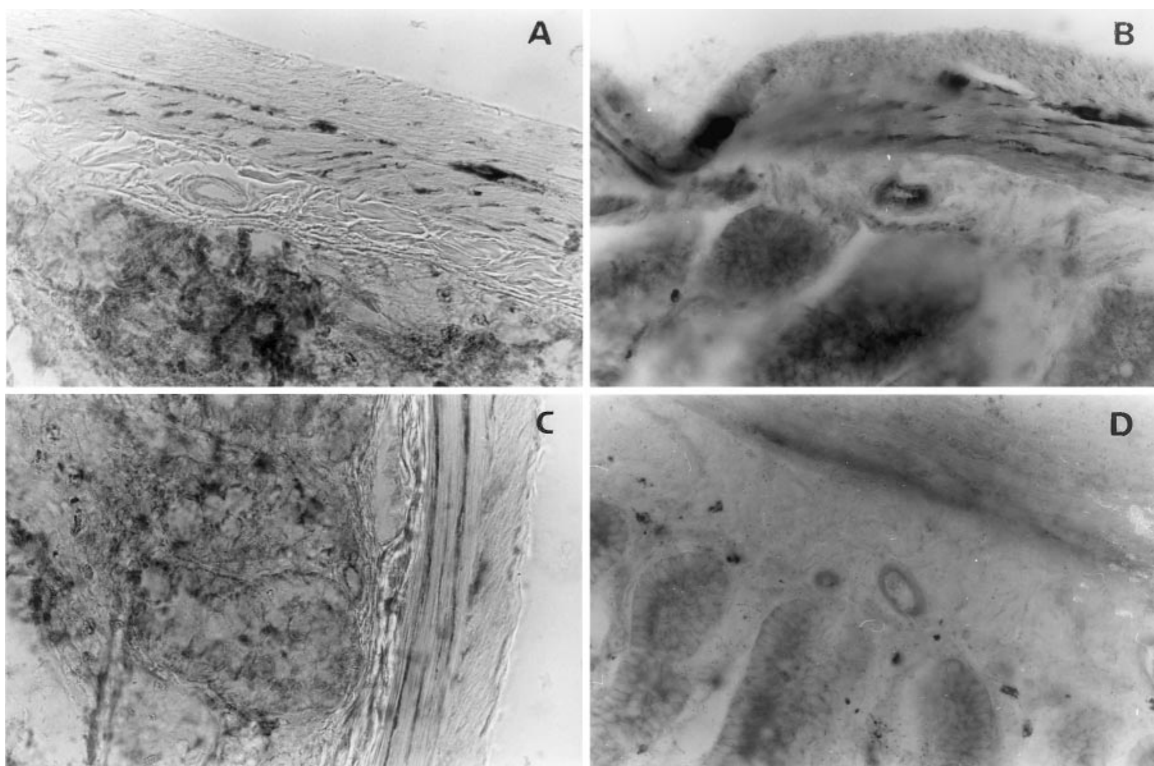

Photomicrographs of intestinal arteriole NADPH-d (+) from (a) control, (b) ANP, (c) cGMP, and (c) L-NAME+ANP in vitro–treated tissues. Note the intensity of endothelium staining from ANP and cGMP-treated tissues compared with control. All images at the same magnification. Scale bar=30 μm.

lipase C, which leads to an enhancement of intracellular Ca^{2+} levels able to regulate Ca^{2+}/calmodulin-dependent NOS.[31-34]

Murthy et al demonstrated that ANP and cANP-(4-23), a selective NPR-C ligand, initiated identical signaling cascades in vascular smooth muscle, consisting of Ca^{2+} influx, activation of eNOS via G_{i1} and G_{i2} proteins, stimulation of cGMP formation, and muscle relaxation.[35,36] In addition, Khurana et al suggested that activation of G-proteins stimulates guanylyl cyclase activity of NPR-A.[37] Therefore, G-proteins could provide cross-talks mechanisms between ANP and NO pathways in endothelial cells.

Other studies have shown that both ANP, via cGMP elevation, and 8-Br-cGMP alone, enhance NO synthesis under basal and agonist-stimulated conditions in cultured rat cardiac myocytes and vascular smooth muscle cells.[38-40] Certainly, Yamamoto et al[38] and Marumo et al[39] showed that activation of cGMP-protein kinase by ANP via cGMP upregulates cytokine-induced iNOS expression after 24-hour incubation in the cells mentioned above. However, on the basis of the present results, this mechanism should be ruled out, because de novo synthesis of the enzyme is unlikely to occur within this brief experimental period.

On the other hand, it has been further demonstrated that ANP activates cGMP-protein kinases inducing endothelial protein phosphorylation.[6,7,9] Moreover, it is generally accepted that protein phosphorylation activates cNOS in shear stress.[41] Then, ANP could probably activate cNOS in a way similar to shear stress, but the G-protein-related mechanism cannot be discarded.

The enhancement of NO production mediated by ANP could explain the presence of ANP receptors in endothelial cells in spite of the fact that they are also expressed in the smooth muscle, suggesting that NO could act as an intercellular messenger for ANP. This hypothesis is supported by the fact that no effect of ANP on NADPH-d activity was found in smooth muscle cells.

In summary, the present study provides evidence that ANP induces a hypotensive effect by enhancement of NOS activity in the vascular endothelium and NO production through the L-arginine-NO pathway and that cGMP may be involved in this hypotensive effect. The cGMP-signaling pathway has been postulated as an important regulator of cardiovascular and renal physiology,[42] the present findings strengthen this hypothesis by associating ANP with the regulation of blood pressure.

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