Role of nitric oxide on the vasorelaxant effect of atrial natriuretic peptide on rabbit aorta basal tone

Liliana Romano, Alfredo Coviello, Susana Jerez, and María Peral de Bruno

Abstract: The role of nitric oxide (NO) on the vasorelaxant effect of atrial natriuretic peptide (ANP) on the basal tone of rabbit aortic rings conditioned to angiotensin II (Ang II) was studied. ANP aortic relaxation and nitrite release were measured in the presence and absence of endothelium and a NO-synthase inhibitor. Ang II at 10^{-8} M triggered a contractile response, conditioning the vessel to a vasorelaxant effect of ANP (10^{-8} M). This effect was significantly enhanced by endothelium removal, N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M), and methylene blue (10^{-5} M). ANP decrease of basal tone in Ang-II-sensitized aortic rings was improved when a higher concentration of Ang II was used (10^{-6} M). Basal and Ang-II-stimulated nitrite release were measured in stretched (S) and nonstretched (NS) aortic rings. Nitrite release was significantly increased in S rings ($p < 0.001$). L-NAME (10^{-4} M) partially inhibited nitrite release in both basal and Ang-II-stimulated S aortic rings. In NS aortic rings, the NO inhibitor did not inhibit basal nitrite release but blunted the Ang-II-stimulated nitrite level. A significant negative correlation between nitrite release and the ANP vasorelaxant effect on basal tone was dependent on the Ang-II-sensitizing dose. The present results demonstrate that ANP relaxant effects on aortic basal tone are related to NO levels, which are regulated by S- and Ang-II-concentration-dependent NO generation and quenching.

Key words: atrial natriuretic peptide, nitric oxide, vascular reactivity, basal tone, rabbit aorta.

Résumé : On a examiné le rôle joué par le monoxyde d'azote (NO) dans l'effet vasorelaxant du peptide natriurétique auriculaire (ANP) sur le tonus basal d'anneaux aortiques de lapins sensibilisés à l'angiotensine II (Ang II). La relaxation aortique par l'ANP et la libération de nitrites ont été mesurées en présence et en absence d'endothélium et d'un inhibiteur de la NO synthase. L'Ang II (10^{-8} M) a déclenché une réponse contractile conditionnant le vaisseau à un effet vasorelaxant de l'ANP (10^{-8} M). Cet effet a été augmenté de manière significative par le retrait de l'endothélium ainsi que par N^G-nitro-L-arginine méthyl ester (L-NAME, 10^{-4} M) et le bleu de méthylène (10^{-5} M). La diminution du tonus basal par l'ANP dans les anneaux aortiques sensibilisés à l'Ang II a été accrue lorsqu'une plus forte concentration d'Ang II a été utilisée (10^{-6} M). La libération de nitrites stimulée par l'Ang II et basale a été mesurée dans des anneaux aortiques étirés (É) et non étirés (NÉ). La libération de nitrites a été augmentée significativement dans les anneaux É ($p < 0{,}001$). L-NAME (10^{-4} M) a inhibé partiellement la libération basale ainsi que stimulée par l'Ang II dans ces anneaux. Dans les anneaux NÉ, l'inhibiteur de NO n'a pas inhibé la libération basale, mais il a diminué le taux de nitrites stimulés par l'Ang II. Une corrélation négative significative entre la libération de nitrites et l'effet vasorelaxant de l'ANP sur le tonus basal a été fonction de la dose sensibilisant à l'Ang II. Les résultats démontrent que l'effet relaxant de l'ANP sur le tonus basal aortique est lié aux taux de NO qui sont régulés par les muscles É ainsi qu'à la production et à l'inhibition de NO fonction de la concentration d'Ang II.

Mots clés : peptide natriurétique auriculaire, monoxyde d'azote, réactivité vasculaire, tonus basal, aorte de lapin.

[Traduit par la Rédaction]

Introduction

Atrial natriuretic peptide (ANP) displays a strong natriuretic and vasodilator effect. Therefore, it is an important factor in the regulation of arterial blood pressure and body-fluid homeostasis (De Bold et al. 1981; Levin et al. 1998). The vasorelaxant effect of ANP has been studied in vitro in precontracted vascular smooth muscle with several agonists: angiotensin II (Ang II), norepinephrine (NE), and histamine (Garcia et al. 1984). Our findings showed that ANP was able to relax rabbit aorta basal tone when the vessel was previously sensitized with Ang II and allowed to return to baseline levels (Peral de Bruno and Coviello 1992; Peral de Bruno et al. 1992). The ANP effect in precontracted

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vascular smooth muscle is mediated by decreasing intracellular calcium release with agonists (Meisheri et al. 1986) through the formation of cGMP (Waldmann and Munrad 1989) by the guanylyl-cyclase-coupled natriuretic peptide receptors NPR-A and NPR-B (Chinkers et al. 1989; Schulz and Waldmann 1999). It has been recently demonstrated that the vasorelaxant effect of ANP may be the result of calcium-activated K^+ channels (Tanaka et al. 1998). Nitric oxide (NO) is another important vasorelaxant factor regulating vascular tone (Palmer et al. 1987) resulting from NO synthase activation. Whereas ANP and NO mediate their actions by increasing cGMP levels, cGMP acts on soluble guanylyl cyclase (Palmer et al. 1988). Although a large body of literature shows that ANP vasorelaxant action is endothelium independent (Winquist et al. 1984), an interaction between the hypotensive ANP activity and the endothelium has been reported, suggesting that NO may be an ANP intracellular messenger (Costa et al. 2000). However, in our laboratory we demonstrated that the removal of the endothelium or N^G-nitro-L-arginine methyl ester (L-NAME) treatment increased the ANP vasorelaxant effect on aorta basal tone of rats with coarctation hypertension. This may be due to an increase in basal tone resulting from NO removal, which enhances the ANP vasorelaxant effect (Peral de Bruno et al. 1999*b*). It is known that Ang II, while promoting its direct vasoconstrictor effect on vascular smooth muscle, also buffers its response via generation of NO-dependent activation through Ang II type 1 (AT_1) endothelium receptors. NO acts as a counter-regulatory mechanism when basal tone is increased. However, Ang II also releases superoxide anions from vascular adventitial fibroblasts (Pagano et al. 1997). Superoxides in turn quench NO, resulting in increased vascular tone. It is unknown at this time how this mechanism may be involved in regulating the response to ANP and if changes in Ang-II concentration modify ANP-evoked vasodilation through NO levels.

Increased intracellular calcium has been attributed to NO inhibition of basal tone of aortic hypertensive rats in vitro (Pucci et al. 1994, 1995). This observation has been confirmed in rabbit aorta sensitized with Ang II, in which removal of extracellular calcium significantly reduces basal tone through AT_1 receptors (Peral de Bruno et al. 1999*a*).

The purpose of the present paper was to determine the role of NO in the vasorelaxant effect of ANP in a model of Ang-II-increased rabbit aortic basal tone.

Material and methods

Animal preparation and experimental protocols

Contractility measurement

The thoracic aortas of male and female rabbits (Flanders hybrid) weighing 1.5–2 kg were obtained from a slaughterhouse. The thoracic aorta was rapidly dissected, immersed in Krebs solution and carefully cleaned of connective tissue. Rings of 5 mm length were transversely cut; care was taken to maintain the endothelium intact. They were then fixed in a Lucite chamber to two stainless steel wire holders: one was anchored and the other was connected to an isometric force transducer (UC2, Gould Inc., Rolling Meadows, Ill.) and a recorder (BD 41, Kipp and Zonnen, Hugo Sachs Elektronik, March-Hugstetten, Germany). The chamber contained 7 mL Krebs solution (128 mM NaCl, 4.7 mM KCl, 14.4 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 0.1 mM Na_2–EDTA, and 11.1 mM glucose) in which a 95% O_2 – 5% CO_2 mixture was bubbled at pH 7.2 and 37°C.

The rings in all experiments were equilibrated for 120 min at 2 g of force, which was previously found to be the optimal tension for contraction induced by KCl (100 mM) and Ang II (Peral de Bruno et al. 1992). The experiment began after a stable baseline value was obtained while changing the bathing solution every 15 min; the rings were then challenged with Ang II. Then, the bathing media was changed five times at 10-min intervals. Once the previous baseline level was regained, the recording pen was adjusted at a higher level. After a stable baseline value had been achieved, 10^{-8} M atrial natriuretic peptide (ANP; α-hANP(99–126)) was added. Paired control experiments were performed, taking care to avoid preconditioning of the vessel to biologically active compounds before adding ANP. The endothelium was removed in some experiments by rubbing the luminal surface. NE (10^{-6} M) stimulation was performed in all cases at the end of the experiment. In addition, the presence or absence of functional endothelium was tested by assessing the ability of acetylcholine (10^{-6} M) to relax NE-contracted aortic rings.

Protocol

Experiments were designed to investigate the role of endothelium in the vasorelaxant effect of ANP in Ang-II-sensitized aortic rings. For this purpose, basal tone in rubbed and unrubbed aortic rings was assessed as the difference between baseline levels before and after ANP incubation (10^{-8} M) in the presence or absence of the NO synthase inhibitor L-NAME (10^{-4} M). This was added 15 min after Ang II stimulation and 45 min before ANP, and its effect on basal tone was recorded. This protocol was followed because the response to Ang II in the presence of L-NAME at this concentration was increased (2372 ± 288 (N = 8) vs. 3346 ± 424 mg (N = 5), $p < 0.01$), a fact that, according to previous reports, modified the response to ANP (Peral de Bruno et al. 1992). The sensitizing concentration of Ang II used in these experiments was 10^{-6} M, which was effective in producing a vasorelaxant response to ANP (Peral de Bruno et al. 1999*a*).

These experiments were repeated with 10^{-8} M Ang II and 10^{-4} M L-NAME. Paired controls were performed with L-arginine (10^{-3} M) to assess specific reversibility of NO inhibition.

Methylene blue (a guanylyl cyclase inhibitor) was added (2×10^{-5} M) during the washing period after stimulation with Ang II (10^{-6} M) in unrubbed aortic rings. When a stable baseline value was reached, ANP (10^{-8} M) was added and the vasorelaxant effect was registered.

Calculation of nitrite release

Nitrite was measured with the Griess reaction. The technique, used by other authors (Seyedi et al. 1995; Zhang et al. 1998) in coronary vessels, was adapted in previous work from our laboratory to measure nitrite release in isolated rabbit aorta (Jerez et al. 2001*b*). The Griess reaction, in which NO metabolites are transformed in diazoic-coloured

compounds, is one of the most frequently used assays (Privat et al. 1997) to measure NO production indirectly. Two sets of standard curves were prepared for each experiment. The contents of tubes containing 500 μL of 0, 1, 2.5, 5, 7.5, and 10 μM $NaNO_2$, *N*-(1-naphthyl)ethylenediamine (50 μL of a 0.2% solution), and sulfanilamide (450 μL of a 0.1% solution) were added to each tube containing standard or experimental samples and vortexed for a few seconds. The tubes were kept at room temperature for 5–10 min until a full pink color developed. Absorbance was measured at 540 nm with a spectrophotometer (Metrolab 1000, Buenos Aires, Argentina) that was calibrated to zero with a blank solution. Nitrite absorbance was computed with the use of regression analysis ($y = a + bx$) and converted to a straight line. Only curves with a correlation coefficient >0.95 were used.

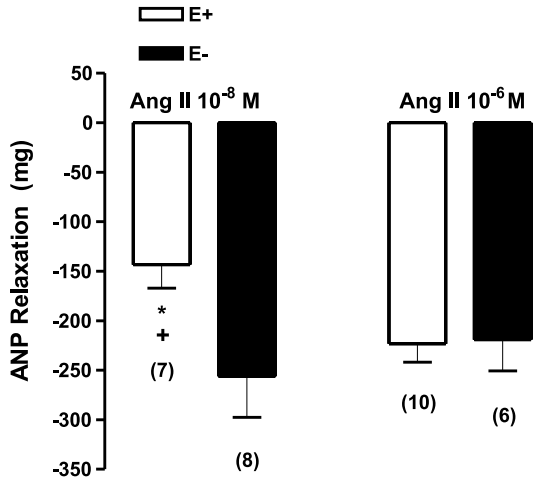
Two experimental designs were followed for nitrite dosage. In the first design, nonstretched (NS) 5-mm-length aortic rings were placed in Eppendorf tubes with 0.5 mL of Krebs solution bubbled with carbogen at 7.2 pH and 37°C. Unrubbed aortic rings were cut in 5-mm-length pieces and placed in tubes with aerated Krebs solution. After an equilibration period of 120 min, the preparation was washed six times each 15 min. This protocol was designed to maintain similar conditions in stretched (S) and NS vessels. We found that repeated washing during equilibration maneuvers induced a significant decrease in nitrite levels (127.2 ± 33.3 and 44 ± 5.9 pmol/mg tissue ($N = 20$, $p < 0.01$) at 15 and 30 min, respectively). However, after 30 min washing, the nitrite levels remained stable until the end of the equilibration period (32.6 ± 4.8 and 28.4 ± 2.5 pmol/mg tissue ($N = 20$, not significant) at 105 and 120 min, respectively). This effect was only observed in NS vessels. After the equilibration period, the rings were divided among eight groups: (*i*) control; (*ii*) 10^{-8} M Ang II; (*iii*) 10^{-7} M Ang II; (*iv*) 10^{-6} M Ang II; (*v*) 10^{-4} M L-NAME; (*vi*) 10^{-4} M L-NAME plus 10^{-8} M Ang II; (*vii*) 10^{-4} M L-NAME plus 10^{-7} M Ang II; and (*viii*) 10^{-4} M L-NAME plus 10^{-6} M Ang II. A sample for nitrite measurement was taken at the end of the equilibration period and the control, Ang II, L-NAME, and Ang II plus L-NAME periods. Two samples were taken after 45 and 60 min washing.

In the second experimental design, in vitro simultaneous measurements of vascular reactivity and nitrite release in S rabbit aortic rings were performed, as described in Contractility measurement above.We took 0.5 mL Krebs solution from the bathing solution at different experimental times: during the washing equilibration period each 15 min, immediately after the equilibration period in nonstimulated aortic rings (basal), at Ang II (10^{-6}–10^{-8} M) peak contraction, and at 45–60 min washing in the absence or presence of L-NAME.

Drugs

Atrial natriuretic peptide (α-hANP(99–126)), human angiotensin II ([Asp^1,Ile^5]Ang II), norepinephrine (DL-arterenol), acetylcholine bromide, *p*-aminobenzenesulfonamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, and methylene blue were purchased from Sigma (St. Louis, Mo.). L-NAME was a gift from Dr. Alberto Nasjletti (New York University, Valhalla, N.Y.) and Dr. Howard Lippton (Tulane Medical School, New Orleans, La.), respectively. Pyrogen-free distilled water was used in the preparation of all solutions. Stock ANP solutions were prepared in 0.1 mM acetic acid and kept refrigerated at –15°C.

Fig. 1. ANP effect on basal tone in rabbit aortic rings with (E+) and without (E–) endothelium. The number of experiments is given in parentheses. Data are means ± SE. *, $p < 0.05$ for 10^{-8} M Ang II with E+ vs. 10^{-8} M Ang II with E– (unpaired *t* test); +, $p < 0.05$ for 10^{-8} M Ang II with E+ vs. all groups (ANOVA and Newman–Keuls post-hoc test).



Statistical analysis

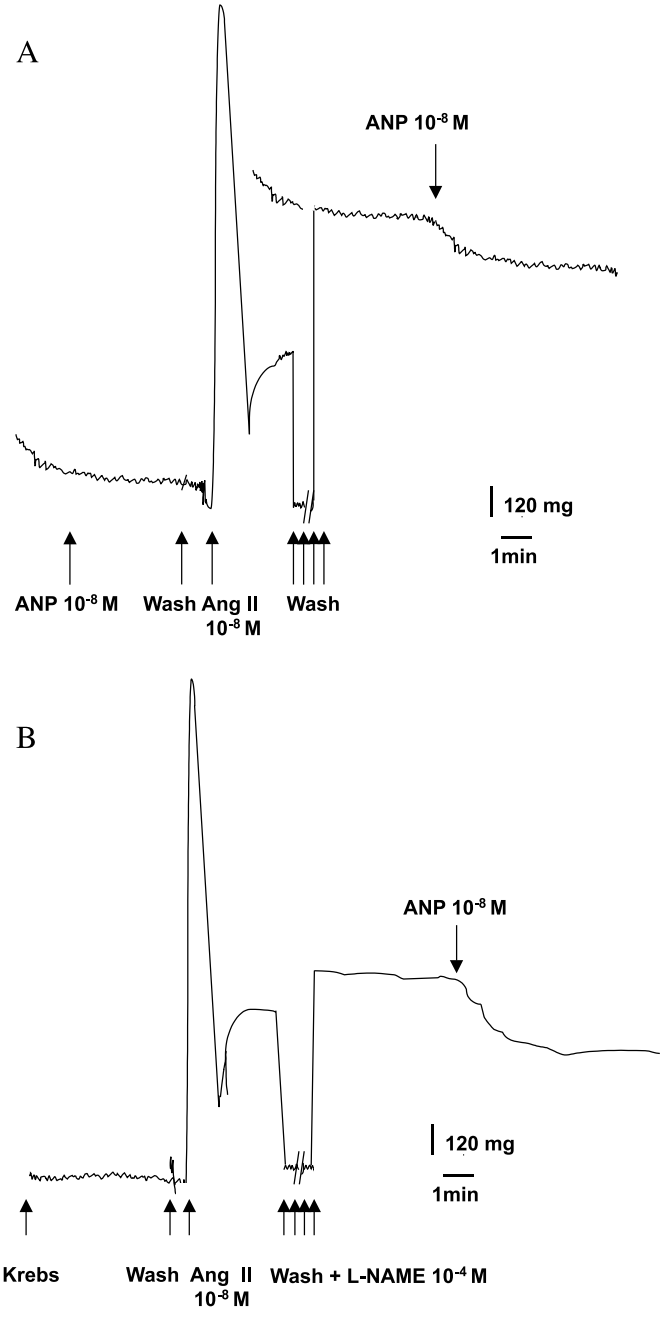
Data for contractility measurements are shown as milligrams of tension, whereas data for nitrite release are expressed in picomoles per milligram of tissue or as a percentage of basal levels. Detailed results are expressed as means ± SE. A Student's *t* test for paired and nonpaired samples was used. In some cases, the data were analyzed by ANOVA, and a Newman–Keuls test was used when appropriate. Results were considered significant when $p < 0.05$.

Results

Vasorelaxant activity of ANP

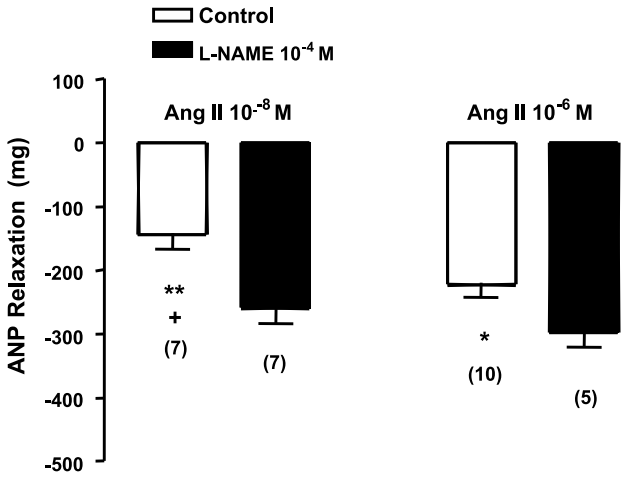
Figure 1 shows the effect of ANP (10^{-8} M) on the basal tone of sensitized (10^{-8} and 10^{-6} M Ang II) rabbit aortic rings with and without endothelium. ANP decreased the isometric tension in unrubbed rings that were previously stimulated with 10^{-8} M Ang II (Fig. 2A). The average of this effect was 145 ± 23 mg ($N = 7$). However, when ANP at the same concentration was added before Ang-II treatment, no relaxing effect of ANP could be obtained (Fig. 2A). The vasodilator effect of ANP in Ang-II-sensitized aortic rings was significantly increased ($p < 0.05$) by endothelium removal (255 ± 41 mg, $N = 8$) (Fig. 1). When the ring was sensitized with a higher concentration of Ang II (10^{-6} M), the ANP effect was significantly increased (245 ± 19 mg, $N = 10$) over the lower Ang-II-sensitizing concentration. However, no difference in the ANP vasorelaxant effect was observed with endothelium removal at either higher or lower concentrations (Fig. 1). Since the Ang II response was similar at 10^{-8} and 10^{-6} M concentrations (2372 ± 288 mg, $N = 8$, and 2333 ± 79 mg, $N = 8$, respectively), the increased response to ANP was associated with higher Ang II doses but not with the magnitude of previous contractile effect. L-NAME (10^{-4} M) introduced no alterations in the basal tone

Fig. 2. (A) Tracing from a typical experiment showing the the lack of effect of ANP on the previously unstimulated basal tone of aortic rabbit rings and its vasorelaxant response in Ang-II-sensitized rabbit aorta. (B) Effect of L-NAME on the basal tone of Ang-II-sensitized rabbit aorta and the further increase in the ANP relaxing effect.



of unrubbed aortic rings (data not shown). However, it improved the vasorelaxant effect of ANP (10^{-8} M) after 1 h washing with Ang-II-sensitizing concentrations (10^{-8} M) (Fig. 2B). Figure 3 shows the effects of different Ang-II-sensitizing concentrations and of NO inhibition by L-NAME on the response of basal tone to ANP (10^{-8} M) unrubbed aortic rings. Treatment with L-NAME (10^{-4} M) significantly increased (81.1%, $p < 0.01$) the vasorelaxant response to ANP in Ang-II-sensitized (10^{-8} M) aortic rings. This inhibitor also

Fig. 3. Effect of L-NAME on the vasorelaxant effect of ANP on the basal tone of unrubbed rabbit aortic rings sensitized with two different concentrations of Ang II. **, $p < 0.01$ for 10^{-8} M Ang II control vs. 10^{-8} M Ang II plus L-NAME (unpaired *t* test); *, $p < 0.05$ for 10^{-6} M Ang II control vs. 10^{-6} M Ang II plus L-NAME (unpaired *t* test); +, $p < 0.05$ for 10^{-6} M Ang II control vs. all groups (ANOVA and Newman–Keuls post-hoc test). The number of experiments is given in parentheses.



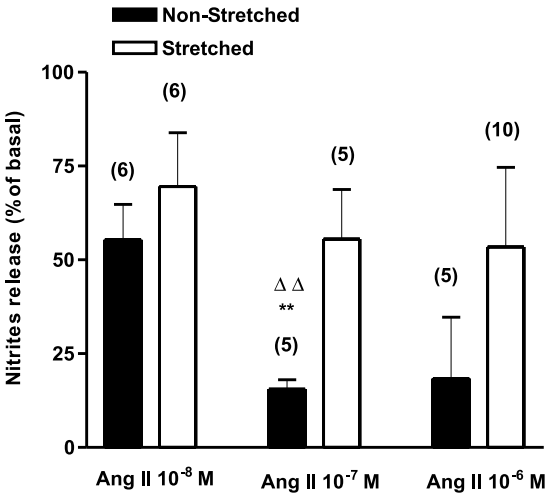
significantly increased the response to ANP in higher Ang-II-sensitizing concentrations (10^{-6} M) (33.2%, $p < 0.05$). No significant difference was found between the vasorelaxant responses to ANP in 10^{-8} and 10^{-6} M Ang II in the presence of L-NAME (Fig. 3). The effects of L-NAME were reversed with pretreatment with L-arginine (10^{-3} M). An increase in the response to 10^{-8} M ANP (28 ± 6%, $N = 5$, $p < 0.05$) was also induced by pretreatment with methylene blue (2×10^{-5} M), through inhibition of soluble guanylyl cyclase.

Levels of nitrite release in S aortic rings were significantly higher than basal ones in NS rings at both the beginning (15 min) and at the end (120 min) of the equilibration period. Mean values were 127.4 ± 33.4 ($N = 20$) and 28.4 ± 2.5 ($N = 20$) pmol/mg tissue versus 274.7 ± 13.7 ($N = 12$) and 254.8 ± 14.8 ($N = 12$) pmol/mg tissue in NS and S rings, respectively ($p < 0.001$, ANOVA and Newman–Keuls post-hoc test). This difference was maintained during the entire equilibration period.

Figure 4 shows the effect of Ang-II nitrite release in unrubbed NS and S aortic rings. Ang II (10^{-8} M) significantly increased nitrite release by 55.3 ± 9.4 ($N = 6$) and 69.5 ± 14.4% ($N = 6$) in both NS and S aortic rings, respectively, over basal levels ($p < 0.01$). No difference in nitrite release induced by 10^{-8} M Ang II was found between NS and S. On the other hand, in S, no additional increase in nitrite release could be obtained with higher Ang-II (10^{-7} and 10^{-6} M) concentrations. In NS rabbit aorta, 10^{-7} M Ang II significantly decreased ($p < 0.01$) nitrite release as compared with lower concentrations of Ang II (Fig. 4). This difference could not be obtained with 10^{-6} M Ang II.

Basal nitrite release was partially and significantly inhibited by 10^{-4} M L-NAME in S rabbit aortic rings (505 ± 58 pmol/mg tissue ($N = 10$) in control vs. 257 ± 30 pmol/mg tissue ($N = 8$) with L-NAME, $p < 0.01$, Fig. 5A). In NS vessels, L-NAME at this concentration had no effect on basal

Fig. 4. Nitrites released by three different Ang-II concentrations in stretched and nonstretched aortic rings. **, $p < 0.01$ for nonstretched rings with 10^{-7} M Ang II vs. stretched rings with 10^{-7} M Ang II; ΔΔ, $p < 0.01$ for nonstretched rings with 10^{-8} M Ang II vs. nonstretched rings with 10^{-7} M Ang II (ANOVA and Newman–Keuls post-hoc test). The number of experiments is given in parentheses.



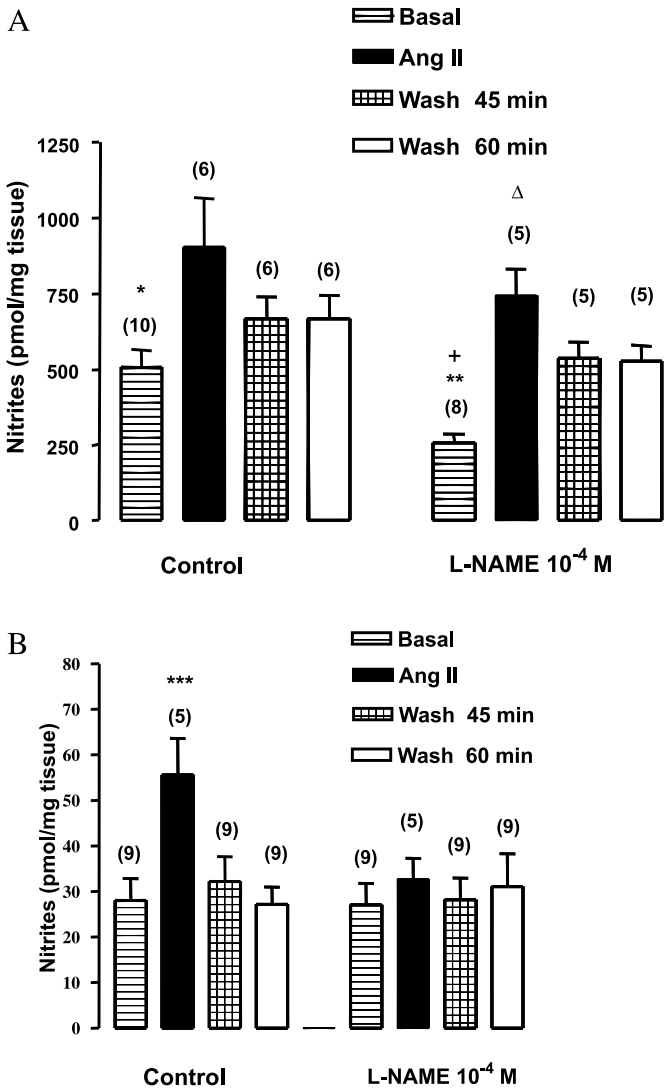
nitrites levels (28.0 ± 4.8 pmol/mg tissue (N = 9) in control vs. 32.7 ± 4.6 pmol/mg tissue (N = 5) with L-NAME, not significant, Fig. 5B). In nitrite release induced by 10^{-8} M Ang II, L-NAME was effective in both S and NS aortic rings (Fig. 5A and 5B). In S aortic rings, the nitrite level stimulated by 10^{-6} M Ang II was 775 ± 107 pmol/mg tissue (N = 10), and L-NAME significantly decreased this value to 580 ± 75 pmol/mg tissue (N = 8) ($p < 0.05$, unpaired t-test).

In NS aortic rings stimulated by 10^{-6} M Ang-II, no differences were found whether in the presence or absence of L-NAME (10^{-4} M). The values obtained were 35.3 ± 1.5 (N = 7) and 33.2 ± 10.5 pmol/mg tissue (N = 6) (not significant, unpaired t-test) in the control and L-NAME groups, respectively. A similar response to L-NAME was obtained with a lower Ang-II (10^{-7} M) concentration. The values were 34.5 ± 4.6 (N = 5) and 32.7 ± 4.3 (N = 5) pmol/mg tissue (not significant, unpaired t-test) in the control and L-NAME groups, respectively. Lack of total inhibition of NO release by L-NAME in basal conditions has been previously observed (Seyedi et al. 1995; Jerez et al. 2001*b*), a fact that has no explanation at present.

Figure 5 also shows the results of the Ang-II-sensitization experiments performed on S and NS aortic rings to test the effect of washing on nitrite release. In unrubbed S aortic rings, after 10^{-8} M Ang II stimulation, nitrite release did not return to basal levels after 45 and 60 min washing (Fig. 5A, left bars). In the presence of L-NAME, nitrite levels decreased to control levels after 45 and 60 min washing (Fig. 5A right bars). However, in unrubbed NS aortic rings stimulated by 10^{-8} M Ang II, nitrite release returned to previous basal levels after washing in the presence or absence of L-NAME (Fig. 5B, right and left bars, respectively).

Figure 6 shows a significant negative correlation ($p < 0.02$) between nitrite levels and the ANP vasorelaxant effect in unrubbed S aortic rings sensitized by 10^{-8} M Ang II. This correlation disappeared at higher Ang-II concentrations (10^{-6} M).

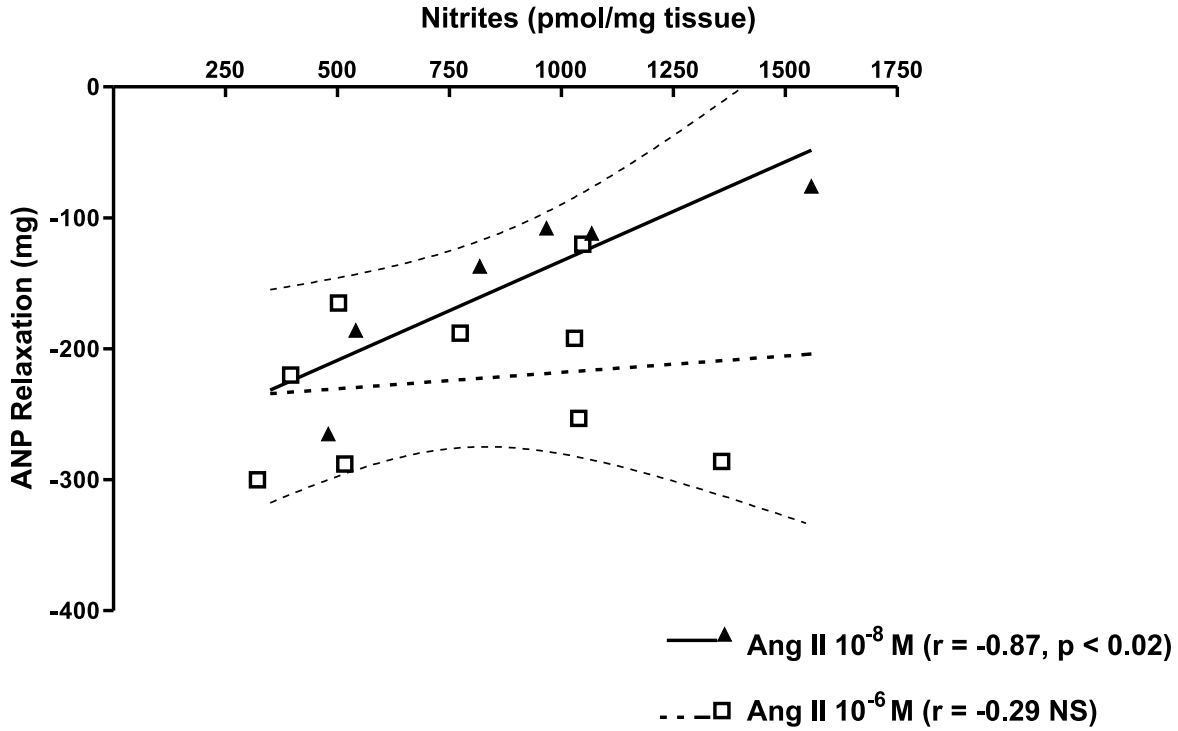
Fig. 5. Effect of L-NAME and washing on basal and Ang-II-stimulated (10^{-8} M) nitrite contents in stretched (A) and nonstretched (B) unrubbed rabbit aortic rings. (A) *, $p < 0.01$ for basal vs. Ang II and washing in the control groups (ANOVA and Newman–Keuls post-hoc test); **, $p < 0.01$ for basal vs. Ang II and washing in the L-NAME groups (ANOVA and Newman–Keuls post-hoc test); +, $p < 0.05$ for basal in the control group vs. basal in the L-NAME group (unpaired t test); Δ, $p < 0.05$ for Ang II in the control group vs. Ang II in the L-NAME group (unpaired t test). (B) ***, $p < 0.01$ for Ang II in the control group vs. Ang II in the L-NAME group (unpaired t test). The number of experiments is given in parentheses.



Discussion

Atrial natriuretic peptide reduced basal tension of noncontracted isolated rabbit aorta after full recovery from a previous Ang-II challenge. This effect was absent in rabbit thoracic aorta not previously exposed to Ang II (Peral de Bruno et al. 1992). This paper confirms a role for the endothelium in this action. Mechanical removal of endothelium, as well as NO synthase or guanylyl cyclase blockade, improved

Fig. 6. Correlation between nitrite release by Ang II and the vasorelaxant action of 10^{-8} M ANP in stretched, unrubbed aortic rings sensitized with 10^{-8} and 10^{-7} M Ang II. The thinner and thicker dotted lines indicate SE for 10^{-6} and 10^{-8} M Ang II, respectively. There was a significant negative correlation only with the lower concentration of Ang II.



the ANP vasorelaxant effect. In previous work carried out in coarctation-hypertensive rat aortic rings, an increase in the ANP response was observed when endothelium was removed (Peral de Bruno et al. 1999*b*). The response to ANP in the presence or absence of endothelium was dependent on the previous Ang-II sensitization dose. Enhancement was only achieved by endothelial removal and a 10^{-8} M Ang II concentration; this fact is in agreement with results obtained with maximal nitrite release at this dose (Fig. 6). At a higher Ang-II concentration (10^{-6} M), a maximal relaxant response to ANP was obtained, and no further increase in ANP response by endothelial removal was achieved. The lack of differences between the response to ANP in arteries with and without endothelium in tissues sensitized with a maximal dose of Ang II (10^{-6} M) suggests that the NO effect is almost partially suppressed by oxidative stress induced by Ang-II-stimulated superoxide anion formation. This is in agreement with previous finding in the literature (Pagano et al. 1997, 1998), in which it has been demonstrated that Ang II enhanced a constitutively active, phagocyte-like NADPH oxidase from adventitial fibroblasts in rabbit aortic rings. On the other hand, there may be a decrease in the counter-regulatory effect of NO on the basal tone when endothelium is removed, a fact that may explain the increase in the ANP vasorelaxant effect in this condition. Furthermore, in our laboratory we reported that Ang-II-induced nitrite release in NS rabbit aortic rings is decreased at supramaximal Ang-II concentrations (Jerez et al. 2001*b*). This effect has been accounted for by an Ang-II action on oxidative stress that increases superoxide anion through NADH/NADPH oxidase activation (Griendling et al. 1994; Rajagopalan et al. 1996). The lack of differences found between the ANP vasorelaxant effect in supramaximal-Ang-II-sensitized rubbed and unrubbed aorta is in agreement with previous reports of other hormones like Ang II. In our laboratory (Jerez et al. 2001*a*) we demonstrated in rabbit aorta that contractile response to Ang II remains unchanged by the presence or absence of endothelium. This finding was also described by Saye et al. (1984). These results may be explained if Ang II releases similar relaxing and contractile factors from endothelium. However, this differs from the observations of Zhang et al. (1994), who reported that the Ang-II response is increased by endothelial disruption.

The ANP vasorelaxant effect enhancement in Ang-II-sensitized arteries in the absence of NO cannot be explained by an ANP- and NO-pathway interaction, as suggested for findings in rat vessels, which support the view of a stimulating action of ANP in endothelium and smooth muscle NO synthase (Costa et al. 2000). The fact that L-NAME (a nitric oxide synthase inhibitor) was unable to suppress the ANP vasorelaxant effect may suggest that ANP-induced NO synthesis might not be involved. The vasorelaxant effect of ANP persists in the absence of endothelium and, furthermore, it is enhanced. Our results are in agreement with reports elsewhere (Meisheri et al. 1986; Winquist et al. 1984) indicating a direct effect of ANP through the particulate guanylyl cyclase family (Waldmann and Munrad 1989; Schulz and Waldmann 1999). In regard to the present study, ANP might trigger an increased and persistent response after soluble guanylyl cyclase inhibition with methylene blue. The results presented here provide evidence that a transient exposure to Ang II induces changes in vascular smooth muscle that might be enhanced in the absence of NO. Such changes would lead to increased vascular tone, which, in

turn, would condition an increased ANP effect. These effects might be mediated by increased calcium permeability, which may increase intracellular free calcium and, consequently, basal tone (Peral de Bruno et al. 1999*a*). An increased Ang-II-dependent tone has been described in coarctation-induced hypertensive rats (Pucci et al. 1994, 1995; Dellipizzi et al. 1997; Peral de Bruno et al. 1999*b*).

Comparative results between simultaneous measurements of vascular relaxation and nitrite concentration in vascular tissues are infrequent in the literature. On the other hand, smooth muscle relaxation, as well as NO measurement, has been studied under different experimental conditions. Ang-II effects on vascular NO release have been studied principally through the inhibition of NO synthase in renal preglomerular vessels (Ito et al. 1991; Zhang et al. 1995), isolated arteries from hypertensive rats (Ferrer et al. 2001), and rabbit aorta (Zhang et al. 1994). It has also been studied in Ang-II-induced urinary nitrate and (or) nitrite excretion (Deng et al. 1996) and NO synthase III mRNA expression (Hennington et al. 1998), and by directly measuring nitrites in isolated vessels like dog coronary arteries (Privat et al. 1997), bovine aortic endothelial cells (Wiemer et al. 1993), and rabbit aorta (Jerez et al. 2001*b*). Simultaneous measurements of relaxation and NO release in rat mesenteric arteries have provided evidence that the endothelium-dependent vasodilator acetylcholine is associated with endogenous NO release in the precontracted vessel (Simonsen et al. 1999). However, no correlation has been found between the increase in NO concentration and the isometric force measurement in the noncontracted vessel (resting basal tone). Our findings show differences in Ang-II-stimulated nitrite release between S and NS aortic rings. Submitting the aortic ring to mechanical stretching increases nitrite release and blunts the decrease of nitrite release after 1 h washing. Increased nitrites during stretching may provide a better explanation for the potentiation of the relaxant response to ANP when NO is inhibited by L-NAME. At present, we are unable to explain the mechanisms by which stretching may influence nitrite production in our preparation. Nevertheless, stretching has been reported to increase cytosolic calcium in canine cerebral artery cells by opening dihydropyridine-sensitive Ca^{2+} channels and releasing Ca^{2+} from intracellular stores (Tanaka et al. 1994). Ruiz-Velasco et al. (1996) reported a similar observation in A7r5 vascular smooth muscle cells. Similarly, endothelial cells submitted to stretching also increase intracellular calcium through smooth muscle stretch-activated ion channels and by releasing calcium from intracellular stores (Naruse et al. 1998; Lee et al. 1999). However, stretching-activated channels in endothelial cells are not blocked by dihydropyridines. It is well known that increased calcium activates NO synthase through the calcium–calmodulin complex (Furchgott 1983). This may explain increased nitrite production in stretched aortic rings. Our findings support the view that stretching may influence nitrite production in previously Ang-II-sensitized rings. This finding may be due to the observation that, in this condition, a calcium-AT_1-dependent mechanism is involved in rabbit aorta (Peral et al. 1999*a*). In conclusion, the present results support an endothelial role in ANP vasorelaxant action by means of a counter-regulatory mechanism. Nitric oxide release in the presence of functional endothelium decreases Ang-II-induced basal tone. Thus, NO inhibition increases basal tone and the vasorelaxant effect of ANP. If Ang-II–NO interactions are well known (lower concentrations succeed in buffering its vasoconstrictor action through NO vascular release, whereas in higher ones oxidative stress predominates), in the present work we demonstrate for the first time that these Ang-II-dependent mechanisms are conditioning vessel reactivity to an important regulator of vascular tone, like ANP. This would have importance in the our understanding of additional mechanisms of blood pressure regulation that are available when endothelial dysfunction has been established; furthermore, it would indicate an additive action of ANP in conditions like Ang-II-dependent hypertension when basal tone is increased.

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References

Chinkers, M., Garbers, D.L., Chang, M.S., Lowe, D.G., Chin, H., Goeddel, D.V., and Schulz, S. 1989. A membrane from guanylate cyclase is an atrial natriuretic peptide receptor. Nature (Lond.), **338**: 78–83.

Costa, M.A., Gonzalez Bosc, L.V., Majowicz, M.P., Vidal, N.A., Balaszczuk, A.M., and Arranz, C.T. 2000. Atrial natriuretic peptide modifies arterial blood pressure through nitric oxide pathway in rats. Hypertension, **35**: 1119–1123.

De Bold, A.J., Borenstein, H.B., Veress, A.T., and Sonnenberg, H.A. 1981. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. Life Sci. **28**: 89–94.

Dellipizzi, A., Pucci, M.L., Monsy, A.Y., Deseyn, K., and Nasjletti, A. 1997. Contribution of the prostanoids to calcium-dependent basal tone in the aorta from rats with aortic coarctation-induced hypertension: relationship to nitric oxide. J. Pharmacol. Exp. Ther. **283**: 75–81.

Deng, X., Welch, W.J., and Wilcox, C.S. 1996. Role of nitric oxide in short-term and prolonged effects of angiotensin II on renal hemodynamics. Hypertension, **27**: 1173–1179.

Ferrer, M., Alonso, M.J., Salaices, M., Marín, J., and Balfagón, G. 2001. Angiotensin II increases neurogenic nitric oxide metabolism in mesenteric arteries from hypertensive rats. Life Sci. **68**: 1169–1179.

Furchgott, R.F. 1983. Role of endothelium in the responses of vascular smooth muscle. Circ. Res. **53**: 557–573.

Garcia, R., Thibault, G., Genest, J., and Cantin, M. 1984. Effect of a purified atrial natriuretic factor on rat and rabbit strips and vascular beds. Am. J. Physiol. **247**: R34–R39.

Griendling, K.K., Minieri, C.A., Ollerenshaw, J.D., and Alexander, R.W. 1994. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ. Res. **74**: 1141–1148.

Hennington, B.S., Zhang, H., Miller, M.T., Granger, J.P., and Reckelhoff, J.F. 1998. Angiotensin II stimulates synthesis of en-

dothelial nitric oxide synthase. Hypertension, **31**(Part 2): 283–288.

Ito, S., Johnson, C.S., and Carretero, O.A. 1991. Modulation of angiotensin II-induced vasoconstriction by endothelium-derived relaxing factor in the isolated microperfused rabbit afferent arteriole. J. Clin. Invest. **87**: 1656–1663.

Jerez, S., Peral de Bruno, M., and Coviello, A. 2001*a*. Endothelium-dependent desensitization to angiotensin II in rabbit aorta: mechanisms involved. Can. J. Physiol. Pharmacol. **79**: 481–489.

Jerez, S., Peral de Bruno, M., and Coviello, A. 2001*b*. Efecto de antagonistas de los receptores de angiotensina II y α_2 adrenérgicos sobre la liberación de oxido nítrico (ON) inducida por angiotensina. Medicina, **61**: 431–436.

Lee, J., Ishiara, A., Oxford, G., Johnson, B., and Jacobson, K. 1999. Regulation of cell movement is mediated by stretch-activated calcium channels. Nature (Lond.), **6742**: 382–386.

Levin, E.R., Gardner, D.G., and Samson, W.K. 1998. Natriuretic peptides. N. Engl. J. Med. **339**: 321–328.

Meisheri, K.D., Taylor, C.J., and Saneii, H. 1986. Synthetic atrial peptide inhibits intracellular calcium release in smooth muscle. Am. J. Physiol. **25**: C171–C174.

Naruse, K., Yamada, T., and Sokabe, M. 1998. Involvement of SA channels in orienting response of cultured endothelial cells to cyclic stretch. Am. J. Physiol. **274**: H1532–H1538.

Pagano, P.J., Clark, J.K., Cifuentes-Pagano, M.E., Clark, S.M., Callis, G.M., and Quinn, M.T. 1997. Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. Proc. Natl. Acad. Sci. U.S.A. **94**: 14 483 – 14 488.

Pagano, P.J., Chanock, S.J., Siwik, D.A., Colucci, W.S., and Clark, J.K. 1998. Angiotensin II Induces $p67^{phox}$ mRNA expresión and NADPH oxidase superoxide generation in rabbit aortic adventitial fibroblasts. Hypertension, **32**: 331–337.

Palmer, R.M., Ferridge, A.G., and Moncada, S. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature (Lond.), **327**: 524–526.

Palmer, R.M., Ashton, D.S., and Moncada, S. 1988. Endothelial cells synthesize nitric oxide from L-arginine. Nature (Lond.), **333**: 664–666.

Peral de Bruno, M., and Coviello, A. 1992. Effects of atrial natriuretic peptide and toad heart extract on isolated toad *Bufo arenarum* aortic rings. Gen. Comp. Endocrinol. **88**: 424–433.

Peral de Bruno, M., Coviello, A., Meyer, M., and Forssmann, W.G. 1992. Relaxation of non-contracted smooth muscle by atrial natriuretic peptide. Clin. Exp. Hypertens. Part A Theory Pract. **14**: 1125–1139.

Peral de Bruno, M., Romano, L., and Coviello, A. 1999*a*. Losartan reduces the vasorelaxant effect of atrial natriuretic peptide on basal tone in aorta. Clin. Exp. Hypertens. Part A Theory Pract. **21**: 1257–1271.

Peral de Bruno, M., Romano, L., Proto, M., and Coviello, A. 1999*b*. Atrial natriuretic peptide relaxes arterial basal tone induced by coarctation hypertension. Peptides, **20**: 485–491.

Privat, C., Lantoine, F., Bedioui, F., Millanvoye van Brussel, E.R., Devynck, J., and Devynck, M.A. 1997. Nitric oxide production by endothelial cells: comparison of three methods of quantification. Life Sci. **61**: 1193–1202.

Pucci, M.L., Miller, K.B., Dick, L.B., Guan, H., Lin, L., and Nasjletti, A. 1994. Vascular responsiveness to nitric oxide synthesis inhibition in hypertensive rats. Hypertension, **23**: 744–751.

Pucci, M.L., Tong, X., Miller, K.B., Guan, H., Lin, L., and Nasjletti, A. 1995. Calcium- and protein kinase C-dependent basal tone in the aorta of hypertensive rats. Hypertension, **25**: 752–757.

Rajagopalan, S., Kurz, S., Münzel, T., Tarpey, M., Freeman, B.A., Griedling, K.K., and Harrison, D.G. 1996. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. J. Clin. Invest. **97**: 1916–1923.

Ruiz-Velasco, V., Mayer, M.B., and Hymel, L.J. 1996. Dihydropyridine-sensitive Ca^{2+} influx modulated by stretch in A7r5 vascular smooth muscle cells. Eur. J. Pharmacol. **296**: 327–334.

Saye, J.A., Singer, H.A., and Peach, M.J. 1984. Role of endothelium in conversion of angiotensin I to angiotensin II in rabbit aorta. Hypertension, **6**: 216–221.

Schulz, S., and Waldmann, S.A. 1999. The guanylyl cyclase of natriuretic peptide receptors. Vitam. Horm. **57**: 123–151.

Seyedi, N., Xu, X.B., Nasjletti, A., and Hintze, T.H. 1995. Coronary kinin generation mediates nitric oxide release after angiotensin receptor stimulation. Hypertension, **26**: 164–170.

Simonsen, U., Wadsworth, R.M., Buus, N.H., and Mulvany, M.J. 1999. In vitro simultaneous measurements of relaxation and nitric oxide concentration in rat superior mesenteric artery. J. Physiol. **516**: 271–282.

Tanaka, Y., Hata, S., Ishiro, H., Ishii, K., and Nakayama, K. 1994. Stretching releases Ca^{2+} from intracellular storage sites in canine cerebral arteries. Can. J. Physiol. Pharmacol. **72**: 19–24.

Tanaka, I., Aida, M., Tanaka, H., Shigenobu, K., and Toro, L. 1998. Involvement of maxi-K (Ca) channel activation in atrial natriuretic peptide-induced vasorelaxation. Naunyn-Schmiedeberg's Arch. Pharmacol. **35**: 705–708.

Waldmann, S.A., and Munrad, F. 1989. Atrial natriuretic peptide receptors and second messengers. Bioassays, **10**: 16–19.

Wiemer, G., Scholkens, B.A., Wagner, A., Heitsch, H., and Linz, W. 1993. The possible role of angiotensin II subtype AT_2 receptors in endothelial cells and isolated ischemic rat hearts. J. Hypertens. **5**: S234–S235.

Winquist, R.J., Faison, E.P., Waldman, S.A., Schwartz, K., Murad, F., and Rapoport, R.M. 1984. Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. Proc. Natl. Acad. Sci. U.S.A. **81**: 7761–7764.

Zhang, J., Van Meel, J.C.A., Pfaffendorf, M., Zhang, J.S., and Van Zwieten, P.A. 1994. Endothelium-dependent, nitric oxide-mediated inhibition of angiotensin II-induced contractions in rabbit aorta. Eur. J. Pharmacol. **262**: 247–253.

Zhang, J., Pfaffendorf, M., Zhang, J.S., and Van Zwieten, P.A. 1995. Influence of the vascular endothelium on angiotensin II-induced contractions in rabbit renal artery. Fundam. Clin. Pharmacol. **9**: 25–29.

Zhang, X., Nasjletti, A., Xu, X., and Hintze, T.H. 1998. Neutral endopeptidase and angiotensin-converting enzyme inhibitors increase nitric oxide production in isolated canine coronary microvessels by a kinin-dependent mechanism. J. Cardiovasc. Pharmacol. **31**: 623–629.