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AT_2-antagonist sensitive potentiation of angiotensin II-induced vasoconstrictions by blockade of nitric oxide synthesis in rat renal vasculature

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1 Although the actions of angiotensin II (Ang II) on renal haemodynamics appear to be mediated by activation of the AT_1 receptor subtype, AT_2 binding sites have also been evidenced in the adult kidney vasculature. As NO is known to mask part of the renal effects of vasoconstrictor drugs, we queried whether the Ang II-induced vasoconstrictions could occur via multiple receptor subtypes during inhibition of NO synthesis. We explored the effect of AT_1 and AT_2 receptor (AT-R) antagonists on Ang II-induced pressure increases during NO synthase or soluble guanylyl cyclase inhibition in rat isolated kidneys perfused in the presence of indomethacin at constant flow in a single-pass circuit.

2 In the absence of NO blockade, the AT_1-R antagonist L-158809 (500 nM) antagonized the Ang II-induced vasoconstrictions, while the AT_2-R antagonist PD-123319 (500 nM) had no effect.

3 Perfusing kidneys in the presence of either NO synthase inhibitors, L-NAME (100 μM) or L-NOARG (1 mM), or soluble guanylyl cyclase inhibitor, LY-83583 (10 μM), significantly increased both molar pD_2 (from 9.40 ± 0.25 to 10.36 ± 0.11) and E_{max} values (from 24.9 ± 3.1 to 79.9 ± 4.9 mmHg) of the concentration–response curve for Ang II-induced vasoconstriction.

4 In the presence of L-NAME, 500 nM L158809 abolished the Ang II-induced vasoconstrictions whatever the concentration tested. On the other hand, 500 nM PD-123319 reversed the left shift of the concentration–response curve for Ang II (molar pD_2 value 9.72 ± 0.13) leaving E_{max} value unaffected (91.3 ± 7.6 mmHg).

5 In the presence of L-NAME, the potentiated vasoconstriction induced by 0.1 nM and the augmented vasoconstriction induced by 10 nM Ang II were fully inhibited in a concentration-dependent manner by L-158809 (0.05–500 nM). By contrast, PD-123319 (0.5–500 nM) did not affect the 10 nM Ang II-induced vasoconstriction and concentration-dependently decreased the 0.1 nM Ang II-induced vasoconstriction plateauing at 65% inhibition above 5 nM antagonist.

6 Similar to PD-123319, during NO blockade the AT_2-R antagonist CGP-42112A at 5 nM decreased by 50% the 0.1 nM Ang II-induced vasoconstriction and at 500 nM had no effect on 10 nM Ang II-induced vasoconstriction.

7 In conclusion, the renal Ang II-induced vasoconstriction, which is antagonized only by AT_1-R antagonist in the presence of endogenous NO, becomes sensitive to both AT_1- and AT_2-R antagonists during NO synthesis inhibition. While AT_1-R antagonist inhibited both L-NAME-potentiated and -augmented components of Ang II-induced vasoconstriction, AT_2-R antagonists inhibited only the L-NAME-potentiated component.

Keywords: Isolated perfused kidney; vascular resistance; NO-synthase; guanylyl cyclase; receptors

Introduction

According to the nomenclature proposed earlier by De Gasparo *et al*. (1995), at least two major classes of angiotensin receptor (AT-R) subtypes for angiotensin II (Ang II) have been characterized: type 1 (AT_1), having a high affinity for losartan but a low affinity for PD-123319, and type 2 (AT_2), binding with high affinity PD-123319 or PD-123177 and losartan with low affinity. Although AT_2-R have been detected in large vessels of various species (for reviews see Navar *et al*., 1996; Zhuo *et al*., 1996), AT_1-R are the dominant subtype in the adult kidney vasculature and all of the major actions of Ang II on renal hemodynamics appear to be mediated primarily by activation of the AT_1-R subtype (Navar *et al*., 1996). Moreover, among the two isoforms of the AT_1-R, AT_{1A} and AT_{1B}, AT_{1A} is the predominant isoform in the kidney (Gasc *et al*., 1994; Healy *et al*., 1995). The hypothesis that AT_2-R antagonists affect renal haemodynamics remains elusive. Much of the uncertainty stems from scantiness of AT_2-R compared to AT_1-R, and also because AT_2-R ligands may behave as antagonists of AT_1-R when used at concentrations higher than 1 μM (Brechler *et al*., 1993). For instance, 1 μM of PD-123319 corresponds to approximately 100-fold its K_i for the AT_2-R and 0.01-fold its affinity for the AT_1-R. There is some evidence, however, indicating the involvement of AT_2-R in regulating pressure-induced natriuresis in anaesthetized rats (Lo *et al*., 1995). In addition, the increase of both exogenous and endogenous Ang II has been shown to result in AT_2-R-mediated production of cGMP and AT_1-R-mediated production of prostaglandin E_2 in the renal interstitial fluid of conscious rats (Siragy & Carey, 1996). The question of whether this AT_2-R-mediated change is involved in the control of renal haemodynamics has not been assessed.

The vascular endothelium releases a number of vasoactive substances, including the vasodilator NO (Ignarro *et al*., 1987). Tonic constitutive release of endothelial NO plays a major modulatory role in maintaining blood pressure and renal hemodynamics in the baseline state (Baylis *et al*., 1990) and many studies have shown that NO attenuates the renal effects of vasoconstrictor drugs (Adachi *et al*., 1996; Parekh *et al*., 1996). Thus, it has currently been reported (for a review see Navar *et al*., 1996) that blocking NO synthesis potentiates the effect of

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Ang II *in vitro* as well as *in vivo* as long as endogenous Ang II is acutely activated or exogenous Ang II levels are raised by infusion. To date, however, the sensitivity of the vasoconstrictions induced by Ang II to AT_2-R antagonists during blockade of endogenous NO in the renal vasculature has not actually been addressed. As basal constitutive NO release masks part of the vasoconstrictor effects of Ang II, and because both AT_1 and AT_2 binding sites are present in the adult kidney vasculature, it is conceivable that the NO-blunted component of the Ang II-induced vasoconstriction could occur via multiple receptor subtypes. To investigate this hypothesis, the current studies were designed to explore the effect of AT_1-R (L-158809) and AT_2-R (PD-123319 and CGP-42112A) antagonists on Ang II-induced vasoconstrictions during blockade of the NO/cGMP pathway. We chose to conduct these studies in a model of isolated perfused rat kidney which lacks circulating angiotensinogen and Ang II production and allows the exclusion of the effects of any other extrarenal factor.

Methods

Preparation of the isolated rat kidney

Male Wistar rats, weighing 170–220 g (Iffa Credo, l'Arbresle, France), with free access to standard food and water, were anaesthetized by intraperitoneal injection of sodium pentobarbital (65 mg kg^{-1}) and used for the preparation of the isolated perfused kidney as described previously (Helwig *et al*., 1991; Saussine *et al*., 1993). Briefly, after Heparin (1000 units) was injected into the left femoral artery, the right kidney was perfused in an open single-pass circuit through the superior mesenteric artery and started *in situ* without ischaemia immediately after the suprarenal aorta had been tied. Perfusion pressure was continuously monitored with a pressure transducer (Statham P23Db, Statham Laboratories Inc., Hato Rey, Puerto Rico) through the infrarenal aorta. While perfused, the kidney was separated from surrounding connective tissue, excised from animal body and transferred onto a thermostatically-controlled holder. The kidney preparations were rendered non-filtering by tying the ureter to minimize the involvement of tubule-mediated regulatory mechanisms on renovascular tonus. The composition of the basic perfusion solution was described in an earlier paper (Massfelder *et al*., 1996) and consisted of a synthetic Ringer-gelatin-lactate solution used as plasma substitute in hypovolemic patients (Plasmion®: Rhône-Poulenc Rorer Laboratories, Antony, France), supplemented extemporaneously with 1.5 mM Ca^{2+}, 11 mM glucose and 24 mM $NaCO_3^-$. As Ang II-mediated vasoconstriction has also been shown to be enhanced by cyclooxygenase inhibitors (Bugge & Stokke, 1994), kidneys were systematically perfused in the presence of indomethacin (10 μM, Sigma, St Louis, U.S.A.) to obviate the involvement of vasoactive prostaglandins. The perfusate was thermostated at 37°C and was continuously filtered through a 1.2 μM sieve and gassed with 95% O_2-5% CO_2. The pH was 7.39–7.44 in the prewarmed, preoxygenated medium. This medium was designated as 'control perfusate'.

Experimental protocols

The vasoconstrictor responses to Ang II were measured as perfusion pressure changes under constant perfusion flow conditions. The perfusion flows (expressed in ml min^{-1}) were adjusted during a 60 min equilibration period to achieve a common pressure baseline of 89.9 ± 0.2 mmHg ($N = 158$); thereafter, the flow thus adjusted was maintained constant. The resulting vascular resistance at the end of the equilibration period was expressed for 1 g kidney weight (in mmHg min g ml^{-1}) and the left kidney was used as a weight basis for calculation.

As indicated in Table 1, the kidney preparations were perfused with control perfusate or with perfusate containing from the outset till the end of the experiments various drugs: the NO-synthase inhibitors L-NAME or L-NOARG (NG-nitro-L-arginine-methyl-ester and NG-nitro-L-arginine, Sigma, St Louis, U.S.A.), the soluble guanylyl cyclase inhibitor, LY-83583 (Calbiochem, La Jolla, U.S.A.), L-158809 (Merck, Rahway, U.S.A.), an AT_1-R antagonist, and PD-123319 (Research Biochemicals International, Natick, U.S.A.) or CGP-42112A (Neosystem Laboratory, Strasbourg, France), AT_2-R antagonists. L-NAME, L-NOARG and LY83583 have been used at concentrations which have been shown by us and others to be maximal for NO inhibition in the renovascular system (Luo *et al*., 1995; Navar *et al*., 1996; Massfelder *et al*., 1996). For AT_1-R in vascular smooth muscles, the K_i values of AT_1-R antagonists (L-158809) and AT_2-R antagonists (PD-123319 and CGP42112A) have been reported to be $\leqslant 50$ nM and > 0.5 μM, respectively. For AT_2-R, the K_i values of AT_1-R

Table 1 Renal vascular resistance (RVR) and perfusate flow (RPF) in rat isolated kidneys perfused in the absence or presence of L-NAME, L-NOARG, LY-83853, L-158809, PD-123319 and CGP-42112A

Drugs added to perfusate		*Kidney weight* (g)	*RPF* (ml min^{-1} g^{-1})	*RVR* (mmHg min g ml^{-1})
None (control perfusate)	(25)	0.79±0.03	11.1±0.5	8.5±0.3
L-NAME (0.1 mM)	(37)	0.83±0.02	7.8±0.3	12.3±0.6*
L-NOARG (1 mM)	(3)	0.85±0.01	6.4±0.5	14.3±1.1*
LY-83583 (10 μM)	(3)	0.78±0.03	7.3±0.3	12.4±0.5*
L-158809 (0.5 μM)	(6)	0.71±0.02	9.9±0.4	9.1±0.5
PD-123319 (0.5 μM)	(7)	0.66±0.02	10.4±0.6	8.9±0.6
L-NAME (0.1 mM)+L-158809 (50 pM)	(4)	0.78±0.06	6.7±0.2	13.6±0.4*
L-NAME (0.1 mM)+L-158809 (0.5 nM)	(6)	0.85±0.05	7.8±0.8	12.8±0.5*
L-NAME (0.1 mM)+L-158809 (5 nM)	(8)	0.80±0.02	5.9±0.2	15.3±0.4*
L-NAME (0.1 mM)+L-158809 (50 nM)	(3)	0.83±0.01	7.1±0.3	12.6±0.6*
L-NAME (0.1 mM)+L-158809 (0.5 μM)	(9)	0.87±0.01	7.4±0.5	12.9±0.5*
L-NAME (0.1 mM)+PD-123319 (0.3 nM)	(7)	0.84±0.02	6.5±0.5	14.5±1.5*
L-NAME (0.1 mM)+PD-123319 (5 nM)	(3)	0.79±0.10	6.7±0.6	13.5±1.3*
L-NAME (0.1 mM)+PD-123319 (50 nM)	(3)	0.92±0.04	6.1±0.6	15.3±1.6*
L-NAME (0.1 mM)+PD-123319 (0.5 μM)	(24)	0.84±0.03	8.3±0.3	11.1±0.4*
L-NAME (0.1 mM)+CGP-42112A (5 nM)	(4)	0.70±0.03	7.0±0.4	12.6±0.6*
L-NAME (0.1 mM)+CGP-42112A (0.5 μM)	(3)	0.82±0.04	6.1±0.6	14.8±1.7*
LY-83583 (10 μM)+PD-123319 (5 nM)	(3)	0.88±0.01	6.6±0.4	13.8±0.9*

Results are expressed as means±s.e.means. The number of kidney preparations perfused with the indicated perfusate is shown in parentheses. The values of RPF and RVR were measured after the initial 60 min equilibration period and before the administration of Ang II was started. The left kidney was used as a weight basis for calculation of RPF and RVR. *$P<0.05$ compared to control perfusate (none).

and AT_2-R antagonists are >10 μM and <10 nM, respectively (De Gasparo *et al*., 1995). Further, AT_2-R ligands may behave as antagonists of AT_1-R when used at concentrations higher than 1 μM (Brechler *et al*., 1993; De Gasparo *et al*., 1995). Therefore, the concentrations of AT-R antagonists used in the present studies ranged between 0.05 and 500 nM.

After the initial 60 min equilibration period, a single concentration of Ang II (Neosystem Laboratory, Strasbourg, France) or norepinephrine (NE, Sigma, St Louis, U.S.A.) was infused. In some experiments, 10 nM Ang II was infused together with 10 μM acetylcholine (ACH, Sigma, St Louis, U.S.A.), to assess the effect of L-NAME on the vasodilatory capacity of ACH. The vasoactive drugs, dissolved in an aliquot of the perfusion medium, were infused into the perfusion circuit, proximal to the renal artery by means of an automatically pushed syringe, with an infusion rate of 0.5 ml min^{-1}. Pressure values measured during drug infusion were corrected for the 0.5 ml min^{-1} overflow pressures (usually 7–12 mmHg) induced by the push-syringe. The concentration of infused drug was adjusted to obtain the required final concentration in renal artery perfusate, as indicated in the figures. As perfusion pressure reached steady-state conditions within 5 min after starting drug infusion, the drugs were infused over a fixed period of 6 min. As observed in pilot experiments (data not shown), a dose–response curve of Ang II-induced vasoconstrictions in the same kidney preparation could not be carried out as low concentrations of peptide influenced the subsequent responses to higher concentrations of peptide.

Analysis of data

A computerized data acquisition system continuously collected pressure and flow values at 1 Hz throughout the experiment. Consecutive blocks of 15 data points were averaged to obtain four measurements per min of flow and pressure. All reported values are expressed as means$\pm$s.e.means. ANOVA was performed on the absolute values of the various parameters. Differences were considered significant for $P<0.05$. If the ANOVA detected a significant effect, comparisons among the means were based on the Student–Newman–Keuls test. The concentration–response curves were quantified by calculating molar pD_2 values ($=-\log EC_{50}$) and maximal effects E_{max} by a non-linear curve fit (SigmaStat®, Jandel, Erkrath, Germany) using the following equation:

$$E = E_{max}/(1 + (EC_{50}/C)), \text{with concentration C and effect E.}$$

Results

Effect of perfusate composition on renal vascular resistance

The various pharmacological tools used to block the NO/cGMP pathway or the AT-R, either alone or in combination, were present in the perfusate throughout the experiments. The effects of the various perfusates on renal vascular resistance are shown in Table 1. The NO-synthase inhibitors, L-NAME (100 μM) and L-NOARG (1 mM), and the guanylyl cyclase inhibitor LY-83585 (10 μM), produced a comparable 1.5 to 1.7-fold increase in renal vascular resistance. On the other hand, AT-R antagonists did not affect renal vascular resistance, whether the antagonists were selective for the AT_1 or the AT_2-R or whether they were used in the presence or absence of inhibitors of the NO/cGMP pathway.

Effects of NO-synthase and soluble guanylyl cyclase inhibitors on Ang II-induced vasoconstriction

Figure 1A shows averaged data of the change in perfusion pressure in response to a low Ang II concentration (0.1 nM) in kidneys perfused under control conditions or in the presence of L-NAME (0.1 mM). Under control conditions, low Ang II had virtually no effect on the perfusion pressure. By contrast, 0.1 nM Ang II induced marked vasoconstrictions in the presence of L-NAME. All the vasoconstrictor effects of Ang II reported in the following have been analysed by the maximum increases in perfusion pressure reached during the 6 min infusion period.

Concentration-dependent renal vasoconstrictions induced by Ang II in the absence or presence of L-NAME (0.1 mM) are shown in Figure 1B. In the absence of L-NAME, Ang II (0.1, 1 and 10 nM) induced increases in perfusion pressure ranging between 2.5 ± 2.5 and 23.1 ± 1.9 mmHg (molar $pD_2=9.4\pm0.25$ and $E_{max}=24.9\pm3.1$ mmHg). In the presence of L-NAME, the concentration–response curve was shifted to the left (molar $pD_2=10.36\pm0.11$, $P<0.05$) and thereby revealed marked vasoconstrictor responses to Ang II concentrations below 1 nM (59.5 ± 7.1 mmHg at 0.1 nM). Furthermore, L-NAME increased the E_{max} value to 79.9 ± 4.9 mmHg ($P<0.05$). Thus, at 1 and 10 nM Ang II, L-NAME augmented

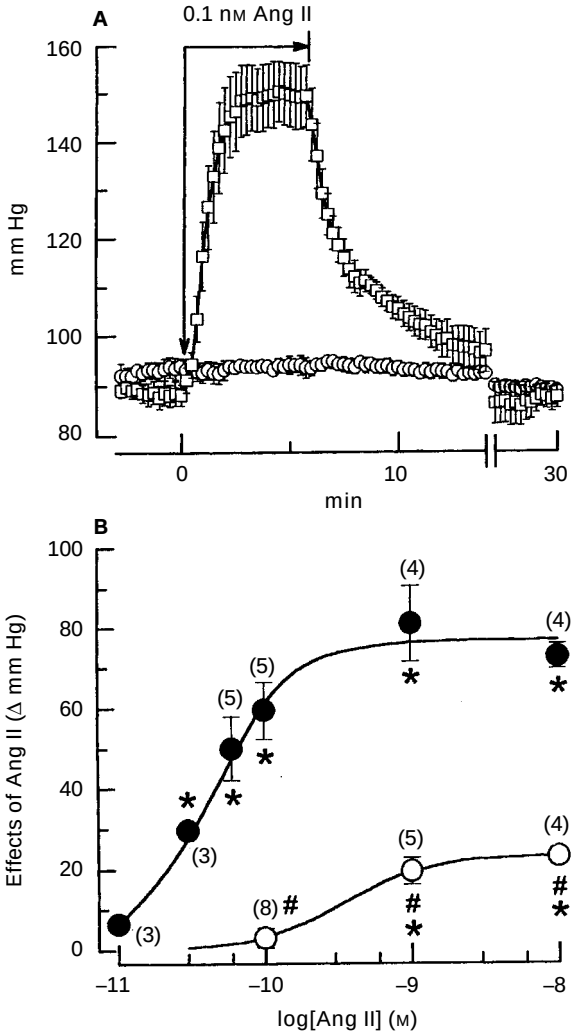

Figure 1 (A) Representative recordings of computer-acquired changes in perfusion pressure showing the vasoconstrictor effect of 0.1 nM of Ang II in isolated kidneys perfused at constant flow with control perfusate (circles, $N=8$) or with perfusate containing 0.1 mM of L-NAME (□, $N=5$). Values shown are means$\pm$s.e.means. (B) Vasoconstrictions induced by increasing concentrations of Ang II in kidneys perfused with control perfusate (○) or with perfusate containing 0.1 mM of L-NAME (●). For each Ang II concentration, a different set of kidneys was mounted (N in parentheses) using the protocol depicted in panel (A). $^*P<0.05$ vs baseline pressure. $^{\#}P<0.05$ vs L-NAME perfusate. The dose–response curves were fitted as in the Methods. Results are presented as increases in perfusion pressure and are given as means$\pm$s.e.means.

the vasoconstrictor responses from 19.6±3.3 to 81.1±9.5 mmHg and from 23.1±1.9 to 72.9±3.1 mmHg, respectively.

Additional experiments were performed to document that the potentiating effect of L-NAME was due to its inhibitory action on NO production. The results are shown in Figure 2. We first asked whether another NO synthase inhibitor, L-NOARG (1 nM) (Moncada *et al*., 1991) and an inhibitor of soluble guanylyl cyclase, LY-83583 (10 μM) (Luo *et al*., 1995) are able to mimic the effect of 0.1 mM L-NAME on 0.1 nM Ang II-induced vasoconstriction. L-NOARG and LY-83583 comparably potentiated vasoconstrictions in response to 0.1 nM Ang II. As NO has been reported to attenuate the action of the major renal vasoconstrictors, we checked that L-NAME was able to enhance the vasoconstrictor effect of NE. Thus, the response to 3 μM NE was also markedly enhanced by L-NAME (22.2±4.5 vs 115.1±4.1 mmHg, $P<0.05$). We finally made sure that the concentration of L-NAME used was able to block ACH-induced vasodilation. ACH at 10 μM reduced by 66% the vasoconstriction induced by 10 nM Ang II in kidneys perfused with control perfusate (23.1±1.9 vs 7.8±2.0 mmHg, $P<0.05$). Adding 0.1 mM L-NAME to the perfusate not only augmented the vasoconstriction induced by 10 nM Ang II, but also abolished the ACH-induced vasodilation (72.9±3.1 vs 63.8±4.3 mmHg, $P>0.05$). Taken together, these results support the conclusion that blockade of the NO/cGMP pathway by perfusing isolated rat kidneys in the presence of 0.1 mM L-NAME potentiated vasoconstrictions in response to low subnanomolar concentrations of Ang II and augmented the vasoconstrictions elicited by higher nanomolar concentrations of Ang II.

Effects of AT-R antagonists on Ang II vasoconstrictions during NO-synthase blockade

On the basis of the known selectivity of AT-R antagonists (De Gasparo *et al*., 1995), we first examined the effect of a high concentration (0.5 μM) of L-158809 and PD-123319 on Ang II-induced vasoconstrictions. In control-perfused kidneys, PD-123319 had no measurable effect on the vasoconstrictions elicited by 1 nM ($N=4$) or 10 nM ($N=3$) Ang II, whereas L-158809 abolished the vasoconstrictions induced by both 1nM ($N=3$) and 10 nM ($N=3$) Ang II (data not shown). L-158809 also abolished the vasoconstrictions induced by 0.1, 1 and 10 nM Ang II in L-NAME-perfused kidneys (Figure 3). The effect of PD-123319 was examined on the full concentration–response curve of Ang II-induced vasoconstrictions in L-NAME-perfused kidneys (Figure 3). PD-123319 significantly shifted the curve to the right, resulting in comparable pD_2 values for Ang II vasoconstrictions in absence or presence of L-NAME+PD-123319 (9.40±0.25 vs 9.72±0.13, $P>0.05$). On the other hand, the augmentation of the Ang II maximal effect by L-NAME was not significantly affected by PD-123319 (E_{max} values: 91.3±7.6 vs 79.9±4.9 mmHg).

Next, the concentration-related inhibitory effects of PD-123319 and L-158809 were examined on the L-NAME-potentiated and L-NAME-augmented vasoconstrictions produced by 0.1 nM and 10 nM Ang II, respectively (Figure 4A and B). Both PD-123319 and L-158809 inhibited in a concentration-dependent manner the L-NAME-potentiated vasoconstrictions induced by 0.1 nM Ang II (Figure 4A). The inhibition produced by L-158809 plateaued at 90–100% for a concentration of 0.5 nM. On the other hand, the inhibition induced by PD-123319 plateaued at 60–65% for a concentration of 5 nM, indicating that the 0.5 μM of PD-123319 used in Figure 3 caused a maximal right shift of the concentration–response curve of Ang II vasoconstrictions in the presence of L-NAME. As expected, the inhibitory potency of L-158809 on 10 nM Ang II (Figure 4B), was shifted by about two log units to the right compared to 0.1 nM Ang II. Importantly, a molar ratio of PD-123319 to Ang II of only 50, which maximally inhibited the vasoconstriction induced by 0.1 nM Ang II, had no effect on the vasoconstriction induced by 10 nM Ang II. Taken together, during NO blockade by L-NAME, the vasoconstrictions induced by the full range of Ang II concentrations were completely antagonized by the AT_1-R antagonist. However, the AT_2-R antagonist acted by inhibiting solely the L-NAME potentiation of Ang II-induced vasoconstrictions.

CGP-42112A, another antagonist selective for AT_2-R (De Gasparo *et al*., 1995), at a molar ratio of antagonist to agonist of 50, consistently decreased by about 50% the vasoconstriction induced by 0.1 nM Ang II and had no effect on the vasoconstriction induced by 10 nM Ang II (Figure 5). As was the

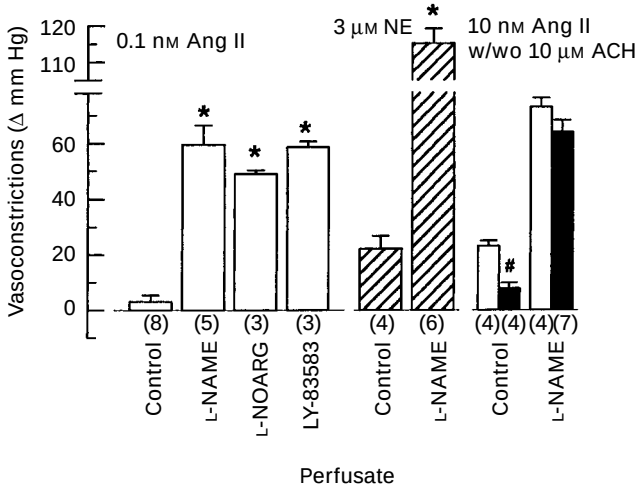

Figure 2 Potentiation of the Ang II- and NE-induced vasoconstrictions and inhibition of ACH-induced vasodilatation in isolated kidneys perfused with control perfusate or with perfusate containing inhibitors of the NO/cGMP pathway as indicated. (□) Vasoconstrictions induced by 0.1 or 10 nM Ang II; (▨) vasoconstrictions induced by 3 μM NE; (■) vasoconstrictions induced by 10 nM Ang II in the presence of 10 μM ACH. *$P<0.05$ vs control perfusate. #$P<0.05$ vs 10 nM Ang II alone. Values are means±s.e.means for the number of kidney preparations indicated in parentheses.

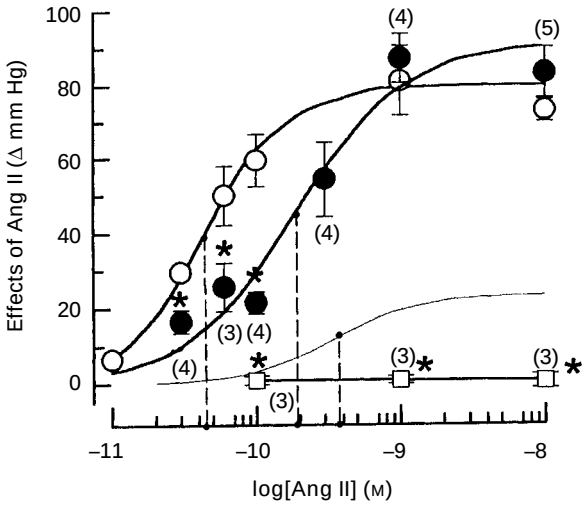

Figure 3 Concentration–response curves for Ang II-induced vasoconstrictions in isolated kidneys perfused with perfusate containing 0.1 mM L-NAME in the absence of antagonists (○) or in the presence of the AT_1-R antagonist L-158809 (0.5 μM; □) or of the AT_2-R antagonist PD-123319 (0.5 μM; ●). The hairline reproduces the concentration–response curve obtained in the absence of L-NAME. The dose–response curves were fitted as stated in Methods. (- - -) EC_{50} values. Values are means±s.e.means for the number of kidney preparations indicated in parentheses. Asterisks indicate significant inhibitions ($P<0.05$) caused by AT-R antagonists.

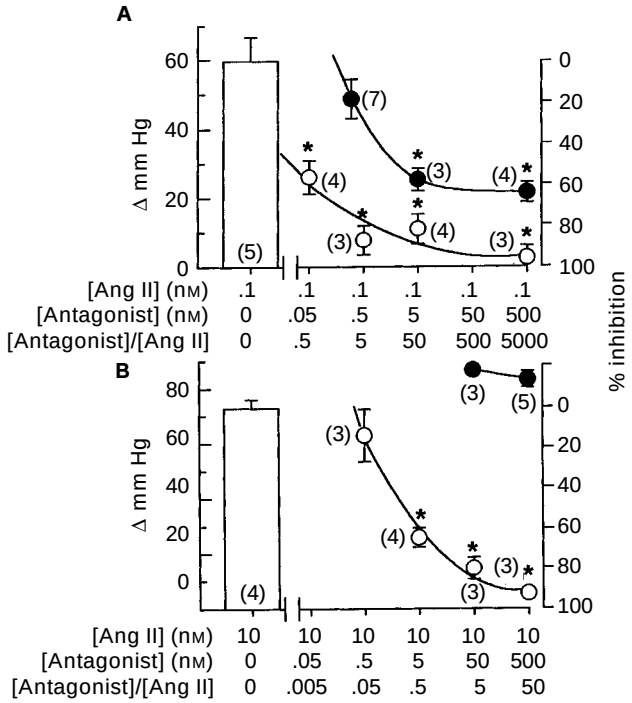

Figure 4 Vasoconstrictions induced by 0.1 nM (A) and 10 nM (B) of Ang II in kidneys perfused with perfusate containing 0.1 mM L-NAME, in the absence (□) or presence of increasing concentrations of PD-123319 (●) or L-158809 (○). Values are means ± s.e.means for the number of kidney preparations indicated in parentheses. Asterisks indicate significant inhibitions ($P<0.05$) caused by AT-R antagonists.

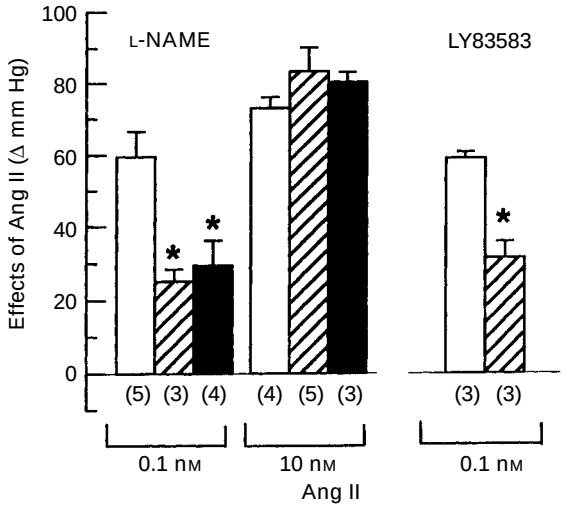

Figure 5 Comparison of the effect of PD-123319 (▨) and CGP-42112A (■) on the vasoconstrictions induced by 0.1 and 10 nM Ang II (□), in kidneys perfused in the presence of 0.1 mM L-NAME or 10 μM LY-83583. The molar ratios of antagonist to agonist were always adjusted to 50 whatever the concentration of Ang II tested, i.e. 5 nM and 0.5 μM antagonist in kidneys receiving 0.1 nM and 10 nM Ang II, respectively. Values are means ± s.e.means for the number of kidney preparations indicated in parentheses. Asterisks indicate significant inhibitions ($P<0.05$) caused by AT-R antagonists.

case with L-NAME, PD-123319 (5 nM) also inhibited 47% of the 0.1 nM Ang II-induced vasoconstriction revealed by LY-83583 (Figure 5).

Discussion

In the present study, we asked whether the renal vasoconstriction induced by Ang II could be sensitive to AT_2-R antagonists during blockade of NO synthesis. We used the isolated rat kidney rendered non-filtering to minimize the involvement of tubule-mediated regulatory mechanisms on renovascular tonus. As cyclooxygenase-derived vasodilatory prostaglandins might obscure NO-mediated responses (Sigmon *et al*., 1992), the kidneys were processed in the presence of indomethacin. The major finding was that Ang II-induced vasoconstrictions in these kidney preparations became sensitive to AT_2-R antagonists in the absence of endogenous NO.

Blockade of the NO/cGMP pathway increases basal vascular resistance

Under basal conditions, L-NAME and L-NOARG strongly increased the vascular resistance and LY-83583 mimicked the effect of NO-synthase inhibitors. Thus, a tonic release of NO contributed to the basal relaxed state and exerted a direct vasodilator effect in the *in vitro* perfused kidney. This finding was consistent with those reported by most of the authors using the isolated perfused rat kidney (Radermacher *et al*., 1990; Gardes *et al*., 1994; Bryant *et al*., 1995; Kaufmann *et al*., 1995; Stephan *et al*., 1995). None of the AT-R antagonists used, under any experimental conditions, was able to significantly affect basal vascular resistance. This observation indicated that endogenous vasoconstrictor Ang II was virtually absent from isolated kidney.

Blockade of the NO/cGMP pathway enhances vasoconstrictor responses to Ang II

While tonic release of NO in the basal relaxed state exerts a direct vasodilatory effect, it has often been stated that suppression of endogenous NO amplifies any vasoconstrictor that is active in the renovascular system (Baylis *et al*., 1990; Adachi *et al*., 1996; Baylis & Qiu, 1996; Parekh *et al*., 1996). The present *in vitro* results were in excellent agreement with this concept: both Ang II- and NE-induced vasoconstrictions were found to be markedly increased during perfusion in the presence of 0.1 mM L-NAME as compared to the vasoconstrictions induced in control-perfused kidneys. Furthermore, the present results demonstrated that NO synthesis inhibition augmented as well as potentiated the Ang II-induced vasoconstrictions. That 0.1 mM L-NAME inhibited endogenous NO production in our experiments has been documented by several other observations. First, L-NAME increased RVR as discussed above, second, it abolished ACH-induced vasodilatation and, finally, L-NOARG, another NO-synthase inhibitor (Moncada *et al*., 1991) and LY-83853, an inhibitor of soluble guanylyl cyclase (Luo *et al*., 1995), comparable to L-NAME potentiated the vasoconstriction induced by 0.1 nM Ang II.

Both inhibition of basal and agonist-triggered NO release could be responsible for enhancement of Ang II-induced vasoconstrictions by L-NAME. Ang II-induced NO-release presumably involves shear stress-dependent mechanisms or AT-R directly coupled to NO release. The shear stress-dependent mechanism has been shown to be triggered by vasoconstriction and associated with an increase in endothelial cytosolic calcium (Busse *et al*., 1993; Juncos *et al*., 1995, 1996). More recent studies performed on extrarenal vascular beds, strongly suggested that endothelial cells are carrying AT-R and that Ang II directly caused the release of vasodilatory endothelium-derived NO (Rowe & Nasjeletti, 1983; Scheuer & Perrone, 1993; Boulanger *et al*., 1995). Thus, in the rat carotid artery, stimulation of AT_1-R caused the release of NO, which in turn inhibited the AT_1-R-mediated contractions. There is some uncertainty with regard to the exact mechanisms by which endogenous NO blockade amplifies Ang II-induced vasoconstriction. Besides the suppression of the attenuating effect of vasodilator NO on the renal action of any vasoconstrictor drug, the inhibition of NO pro-

duction could also enable the release of other endogenous vasoconstrictors in response to Ang II. For instance, there are several reports demonstrating that Ang II stimulates endothelin-1 release from endothelial and mesangial cells (Emori *et al*., 1991; Imai *et al*., 1992; Kohno *et al*., 1992). Moreover, it has been documented that the potentiation by L-NAME of the effect of endogenous endothelium-derived endothelin, could be an important contributor to the pressure effect of NO synthesis inhibition (Ito *et al*., 1991; Nafrialdi *et al*., 1994; Qiu *et al*., 1995). Clearly, further studies are needed to elucidate which of these observations are valid in the present study.

Ang II-induced vasoconstrictions are sensitive to both AT_1 and AT_2-R antagonists during NO-synthesis blockade

In control-perfused kidneys, the vasoconstrictor responses produced by Ang II were selectively mediated by AT_1-R as they were inhibited by L-158809 but not by PD-123319. In L-NAME-perfused kidneys, both the potentiated (<1 nM Ang II) and the augmented ($\geqslant 1$ nM Ang II) vasoconstrictions could be abolished by L-158809. This is consistent with the concept that AT_1-R antagonists blunt most, if not all of the Ang II-mediated effects in the glomerulovascular system *in vitro* and *in vivo*, whether NO was inhibited or not (for a review see Navar *et al*., 1996). In the presence of endogenous NO, only two studies have reported that a small part of the Ang II-induced vasoconstriction was sensitive to AT_2-R antagonists (Chatziantoniou & Arendshorst, 1993; Hayashi *et al*., 1993). It is intriguing, however, that none of the previous studies addressed the specific question of whether the Ang II-induced vasoconstrictions during NO blockade were sensitive to AT_2 antagonists.

The major conclusion reached by the present study was that in the absence of endogenous NO only the potentiated component of Ang II-induced vasoconstrictions was inhibited by AT_2-R antagonists. The L-NAME-augmented component was not affected by AT_2-R antagonists. In support of this, L-NAME increased the pD_2 value for Ang II vasoconstrictions by about one order of magnitude, an effect reversed by AT_2-R antagonist without altering E_{max} values. This reversal of potentiation did not reflect competitive inhibition as PD-123319 was unable to abolish the vasoconstrictions induced by 0.1 nM Ang II and had no effect on the vasoconstriction induced by 10 nM Ang II.

In concentration-dependent studies, the inhibitory potency of PD-123319 on 0.1 nM Ang II-induced vasoconstriction was in the subnanomolar range which is considered to be subtype selective inhibition. It might be argued that L-158809 was more potent than PD-123319 in antagonizing the L-NAME-potentiated vasoconstrictions (Figure 4A). However, the affinity of tetrahydroimidazopyridines (represented by PD-123319 and PD-123177) for human vascular smooth muscles which express only AT_1-R, has been shown to be 10 000 fold lower than that of losartan derivatives such as DuP-753 (Bottari *et al*., 1993). Conversely, the affinity of PD-123319 for human myometrium which expresses only AT_2-R, has been shown to be 10 000 fold higher than that of DuP-753. Therefore, the effect of AT_2-R antagonists reported herein are unlikely to be caused by non-specific actions of PD-123319 and CGP-42112A. The major question raised by the present findings relates to the mechanism of AT_2 antagonists-sensitive potentiation of Ang II-induced vasoconstrictions during NO blockade. One possibility would be that AT_2-R potentiate the AT_1-R-mediated vasoconstrictions via interactions of signalling pathways. Alternatively, unidentified AT-R exhibiting dual sensitivity to AT-R antagonists could be involved. Additional receptor subtypes exist in both renal and extrarenal tissues and there may be variations in the structure of both AT_1-R and AT_2-R subtypes (for reviews see Botarri *et al*., 1993; Chansel *et al*., 1993).

In conclusion, although the renal vasoconstrictions produced by Ang II were primarily mediated by the AT_1-R subtype, the present study revealed that the renal vasoconstrictions masked by the concomitant release of NO exhibited a complex behavior vis-à-vis AT_2-R antagonists. The new finding is that the L-NAME-potentiated vasoconstrictions induced by subnanomolar physiological concentrations of Ang II in the *in vitro* perfused rat kidney were sensitive to AT_2-R antagonists. The present results might be relevant in pathophysiological situations where the synthesis of NO is defective. Thus, total renal vascular resistance, afferent arteriolar resistance and afferent arteriole vasoconstriction in response to a number of drugs, including Ang II, are abnormally elevated in essential hypertension (De Leeuw & Birkenhäger, 1983; Kimura *et al*., 1991; Ruilope *et al*., 1994). More recent studies suggested that these abnormalities were due to a defect in the intrarenal synthesis of NO (Gomez-Alamillo *et al*., 1996). Whether the renal vasoconstrictions induced by Ang II in hypertensive patients are indeed mediated by both AT_1-R and AT_2-R will need further studies.

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