Abstract—Recently, a new derivative of angiotensin (Ang) II, called “Ang A,” has been discovered to be present in plasma of healthy humans and, in increased concentrations, in end-stage renal failure patients. The objectives of the study were to investigate the blood pressure and renal hemodynamic responses to Ang A in normotensive and hypertensive rats and in genetically modified mice and the binding properties of Ang A to Ang II type 1 (AT1) or Ang II type 2 (AT2) receptors. Intravenous and intrarenal administration of Ang A induced dose-dependent pressor and renal vasoconstrictor responses in normotensive rats, which were blocked by the AT1 receptor antagonist candesartan but were not altered by the AT2 receptor ligands PD123319, CGP42112A, or compound 21. Similar responses were observed after intravenous administration in spontaneously hypertensive rats. Deletion of AT1a receptors in mice almost completely abolished the pressor and renal vasoconstrictor responses to Ang A, indicating that its effects are mediated via AT1a receptors. Ang A was less potent than Ang II in vivo. The in vitro study demonstrated that Ang A is a full agonist for AT1 receptors, with similar affinity for AT1 and AT2 receptors as Ang II. Overall, the responses to Ang A and Ang II were similar. Ang A has no physiological role to modulate the pressor and renal hemodynamic effects of Ang II. (Hypertension. 2011;57:956-964.) ● Online Data Supplement

Key Words: angiotensin A ■ angiotensin II ■ renal blood flow ■ blood pressure ■ AT1 receptors ■ AT2 receptors

Although angiotensin (Ang) II (Asp-Ang-Val-Tyr-Ile-His-Pro-Phe) is the primary effector peptide of the renin-Ang system,1-3 some of the Ang II metabolites, such as Ang III, Ang IV, and Ang-(1-7), are also biologically active.4 Recently, Jankowski et al5 discovered a novel human Ang-derived peptide, Ang A (Ala-Arg-Val-Ile-His-Pro-Phe), in plasma of healthy humans in a concentration of 6.7±4.7 pg/mL (versus 88.0±12.3 pg/mL for Ang II) and in increased concentrations (28.4±11.0 pg/mL for Ang A versus 127.5±29.4 pg/mL for Ang II) in end-stage renal failure patients. Ang A is generated from Ang II by enzymatic decarboxylation of Asp1 in the presence of mononuclear leukocytes and was reported to be a partial agonist with the same affinity for the Ang II type 1 (AT1) receptor as Ang II but with a higher affinity for the Ang II type 2 (AT2) receptor.5 Others reported that both Ang A and Ang II have similar hypertensive activity and a greater proliferative effect of Ang A than Ang II on rat A10 vascular smooth muscle cells.6

Because Ang A was reported to be present in human plasma in a concentration that may have relevant effects, and because it was claimed to have a relatively more pronounced action at the AT2 receptor, thereby counteracting the harmful effects of Ang II,5 we found it of interest to further study the responses to Ang A in more detail. The objectives of the present study were, therefore, to investigate the blood pressure and renal hemodynamic responses to Ang A in normotensive and spontaneously hypertensive rats as compared with Ang II and to determine the receptors involved in these responses. We used genetically modified mice, in which AT1a, AT1b, or AT2 receptor genes were deleted to further determine the receptor subtypes involved in the responses to Ang A. Because another Ang metabolite, Ang IV, was shown to act at the insulin-regulated aminopeptidase (IRAP),7 we

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DOI: 10.1161/HYPERTENSIONAHA.110.161836
also explored whether the genetic deletion of IRAP would modify the responses to Ang A. In parallel in vitro experiments, we studied the binding properties of Ang A and Ang II to the AT₁ or AT₂ receptor in Chinese hamster ovary (CHO) cells expressing recombinant human AT₁ receptors (CHO-hAT₁ cells) or AT₂ receptors (CHO-hAT₂ cells). Finally, the ability of both peptides to stimulate AT₁ receptor–mediated inositol phosphate (IP) accumulation was investigated in CHO-hAT₁ cells.

Materials and Methods

All of the animal procedures were performed in accordance with national and European regulations and were approved by the Vrije Universiteit Brussel Ethical Committee for Animal Experiments of the Faculty of Medicine and Pharmacy. For expanded Methods, please see the online Data Supplement available at http://hyper.ahajournals.org.

In Vivo Experiments in Normotensive and Spontaneously Hypertensive Rats

Animals and Surgical Procedures

Adult male normotensive Wistar Kyoto rats (Iffa Credo, Brussels, Belgium) and adult male spontaneously hypertensive rats (SHRs; Harlan, Horst, The Netherlands) weighing between 275 to 325 g were used in this study. The surgical procedure was described previously¹ and in the supplemental Methods section. Briefly, after intubation of the trachea, the right jugular vein was catheterized for fluid maintenance, drug administration, and supplemental anesthesia. The right carotid artery was cannulated for continuous monitoring of mean arterial pressure (MAP). An ultrasound probe was secured around the left renal artery for measurement of total renal blood flow (RBF). Drugs were administered locally (intrarenal: IR) via a catheter inserted into the lower abdominal aorta and advanced to the origin of the left renal artery.

Systemic and Local IR Administration

Eight groups of normotensive rats were used to evaluate the blood pressure and renal hemodynamic effects of Ang A compared with Ang II after IV or IR administration and to investigate the influence of pretreatment with either the selective AT₁ receptor antagonist candesartan or the AT₂ receptor antagonist PD123319. Responses to the AT₂ receptor agonists CGP42114A and compound 21 were also explored whether the genetic deletion of IRAP would modify the responses to Ang A. In parallel in vitro experiments, we studied the binding properties of Ang A and Ang II to the AT₁ or AT₂ receptor in Chinese hamster ovary (CHO) cells expressing recombinant human AT₁ receptors (CHO-hAT₁ cells) or AT₂ receptors (CHO-hAT₂ cells). Finally, the ability of both peptides to stimulate AT₁ receptor–mediated inositol phosphate (IP) accumulation was investigated in CHO-hAT₁ cells.

Data Analysis

Data were presented as mean±SEM; all of the calculations and graphs were obtained by using GraphPad Prism 4.03 (GraphPad Software, Inc, San Diego, CA). The renal vascular resistance (RVR) or cortical vascular resistance (CVR) was calculated as MAP divided by RBF or CBF, respectively. For statistical evaluation of Ang A–versus Ang II–treated groups, baseline parameters and responses to Ang A and Ang II in SHR versus normotensive rats, and the AT WT versus IRAP WT mice group, Mann-Whitney tests were used. For determination of intragroup differences on changes induced by Ang A or Ang II with versus without pretreatment of candesartan; PD123319, CGP42112A, or compound 21, or for statistical evaluation on the different treatments within SHR, Wilcoxon signed-rank tests were used. For statistical evaluation among groups (knockout mice versus WT mice; Ang A or Ang II with versus without pretreatment of candesartan; or PD123319, CGP42112A, or compound 21), 1-way ANOVA followed by post hoc Duncan multiple comparison tests was used. Two-way ANOVA followed by Bonferroni post hoc test was used for statistical evaluation of the effects in function of time after local administration. A value of P<0.05 was considered statistically significant.

Results

Pressor and Renal Vasoconstrictor Effects of Ang A and Ang II in Normotensive and Hypertensive Rats (Systemic Administration)

The baseline values of MAP, RBF, and RVR were 101±7 mm Hg, 3.9±0.4 mL/min, and 27.3±2.9 mm Hg·min/mL in normotensive rats (n=18) and 150±7 mm Hg, 2.6±0.3 mL/min, and 62.8±7.1 mm Hg·min/mL in SHRs (n=14). As shown in Figure 1, IV administration of Ang A and Ang II in normotensive rats produced dose-dependent decreases in RBF and increases in MAP and RVR.

At higher doses (1 and 10 nmol/kg), Ang A and Ang II both induced nearly maximal RBF responses, whereas at the lower dose (0.1 nmol/kg), the responses to Ang A (RBF: −32.8±3.9%, n=18; RVR: 103.4±27.9%, n=18) were significantly less than those induced by the same dose of Ang II (RBF: −62.8±4.1%, n=18, P<0.001; RVR: 357.6±157.5%, n=18, P<0.01), suggesting that Ang A is less potent than Ang II. No vasodilator response to Ang A or Ang II was detected. Candesartan (IV bolus, 1 mg/kg) alone reduced MAP and increased RBF (−15.6±2.7 mm Hg, 12.3±3.6%, respectively; n=5) and blocked the response to Ang A and Ang II (Figure 1). IV bolus injections of the AT₂ receptor antagonist PD123319 (1 mg/kg) had no effect on MAP (data not shown) and did not alter the pressor and renal vasoconstrictor effects to Ang A and Ang II (Figure 1). In contrast to the observation in SHRs, the AT₂ receptor antagonists compound 21 (30 µg/kg, a dose that was reported to decrease MAP in anesthetized SHRs by Wan et al⁹)
CGP42112A (100 μg/kg) did not change baseline MAP or the pressor and renal vasoconstrictor effects to Ang A and Ang II in our hands (data not shown).

In SHRs also, Ang A and Ang II produced dose-dependent decreases in RBF and increases in MAP and RVR that were abolished by candesartan (1 mg/kg) but not modified by PD123319 (1 mg/kg) pretreatment (Figure 2A). Candesartan (IV bolus, 1 mg/kg, n=4) alone reduced MAP by 32.8±4.0 mm Hg and increased RBF by 30±14% significantly more in SHRs than in normotensive rats (both P<0.05). No blood pressure lowering or vasodilator effect in response to Ang A or II was detected, and none was detected after pretreatment with candesartan as well.

In a separate group of SHRs (n=4, Figure 2B), candesartan (IV bolus, 1 mg/kg, n=4) alone reduced MAP and increased RBF. Subsequent administration of the AT₂ receptor agonist compound 21 (0.01 and 0.05 mg/kg, IV bolus) did not further reduce MAP or further increase RBF, whereas the D₁ receptor agonist fenoldopam (100 nmol/kg), used here as a positive control of the experimental setup, was capable of further decreasing MAP and increasing RBF.

Pressor and Renal Vasoconstrictor Effects of Ang A and Ang II in Normotensive Rats (IR Administration)

The baseline values of MAP, RBF, and RVR were 110±5 mm Hg, 4.1±0.3 mL/min, and 26.1±2.2 mm Hg·min/mL, respectively (n=20). No significant changes in MAP, RBF, or RVR were observed after IR infusion of Ang A or Ang II at a concentration of 0.01 nmol/20 μL/min (data not shown). IR infusion of Ang A (0.1 nmol/20 μL per minute) tended to decrease RBF and significantly decreased RBF and increased MAP and RVR at a higher dose (1 nmol/20 μL per minute); Ang II (0.1 and 1.0 nmol/20 μL per minute) dose-dependently decreased RBF and increased MAP and RVR (Figure 3). Responses to Ang A were less pronounced than those to Ang II at the same dose (0.1 nmol/20 μL per minute), suggesting again that Ang A is less potent than Ang II (Figures 3 and 4).

Saline (0.9%), PD123319 (0.5 mg/kg, IR bolus; +5.0 μg/min per kilogram, IR infusion), or compound 21 (0.1 μg/kg), per se, had no effect on MAP and RBF (data not shown) or on the effects of Ang A and Ang II (Figure 4).

Figure 1. Responses of mean arterial pressure (MAP), renal blood flow (RBF), and renal vascular resistance (RVR) in normotensive rats to IV bolus injection of angiotensin (Ang) A (left) alone (C, 0.01, 0.10, 1.00, and 10.00 nmol/kg), with PD123319 (○), or with candesartan (▼), and to IV bolus injection of Ang II (right) alone (●, 0.01, 0.10, 1.00, and 10.00 nmol/kg), with PD123319 (▲) or with candesartan (▼). Values are expressed as mean±SEM (n=5 to 18). *P<0.05, **P<0.01, ***P<0.001, Ang A alone-treated group vs Ang II alone-treated group at a corresponding dose. #P<0.05, ##P<0.01, Ang peptide with vs without candesartan.
Candesartan (5 μg/kg, IR bolus) alone decreased MAP and increased RBF (data not shown) and blocked the effects of Ang A and Ang II (Figure 4).

Pressor and Renal Vasoconstrictor Effects of Ang A and Ang II in Various Receptor-Deleted Mice

The baseline values of MAP, CBF, and CVR in AT WT mice were 72.3 ± 4.6 mm Hg, 74.4 ± 10.1 perfusion units, and 1.05 ± 0.12 mm Hg/perfusion units, respectively (n = 7). In AT1a (-/-) mice, baseline MAP (n = 5; P < 0.001), CBF (n = 5; P < 0.05), and CVR (n = 5; P < 0.05) were all significantly lower than in AT WT mice. AT2 (-/-) mice had significantly higher baseline MAP (n = 5; P < 0.05) than AT WT groups; baseline CBF (n = 6) was comparable, and, accordingly, CVR (n = 5) tended to be higher, although this difference did not reach statistical significance. AT1b (-/-) and IRAP (-/-) mice had comparable baseline MAP, CBF, and CVR as their corresponding WT controls. Because no differences in response to Ang A and Ang II between AT WT and IRAP WT mice (data not shown) were observed, data from these 2 groups of WT mice were merged as “WT mice” in Figure 5.

In WT mice, IV Ang A and Ang II dose-dependently increased MAP and CVR and reduced CBF, and no vasodilator responses were observed. Vasodilator responses were also not observed with lower doses in preliminary pilot dose-finding experiments or after AT1 receptor blockade (data not shown).

Deletion of AT1a receptors almost completely abolished the pressor and renal vasoconstrictor responses to Ang A and Ang II, whereas vasoconstrictor responses to endothelin 1 were not different from WT mice (data not shown). Small,
nonsignificant reductions in CBF were observed at the highest doses of Ang II (1 nmol/kg) and Ang A (10 nmol/kg) only. We succeeded in reassessing these responses to the highest doses of Ang II (1 nmol/kg) and Ang A (10 nmol/kg) after pretreatment with only. We succeeded in reassessing these responses to the highest doses of Ang II (1 nmol/kg) and Ang A (10 nmol/kg) after pretreatment with

As shown in Figure 5, Ang A (1 nmol/kg) decreased CBF and increased CVR significantly more in AT2 (−/−) mice than in WT mice (CBF: −76.4±3.0% versus −56.3±6.0%, P<0.05; CVR: 550.7±87.9% versus 276.0±65.4%, P<0.05). CBF and CVR responses to Ang II (0.01 nmol/kg) were also significantly greater in AT2 (−/−) than in WT mice (CBF: −37.2±3.1% versus −8.1±3.4%, P<0.05; CVR: 98.2±18.7% versus 19.5±8.1%, P<0.01). At a dose of 0.1 nmol/kg, the pressor response to Ang II was also higher in AT2 (−/−) mice than in WT animals (35.0±3.1 versus 15.6±2.8 mm Hg; P<0.05). These data suggest a counter-regulatory effect of the AT2 receptor. The responses to Ang A and Ang II in ATib (−/−) and IRAP (−/−) mice did not differ from those observed in WT mice.

All changes of MAP, CBF, and CVR in response to Ang II and Ang A in WT, ATib (−/−), AT2 (−/−), and IRAP (−/−) mice were abolished by AT1 receptor blockade with candesartan (1 mg/kg; Figure 6). Notably, no hypotensive or vasodilator effects in response to Ang II and Ang A injection were observed, not even after AT1 receptor blockade. In WT, ATib (−/−), AT2 (−/−), and IRAP (−/−) mice, candesartan alone decreased MAP and increased the CBF. The effects of candesartan alone were not statistically different among the WT and (−/−) groups, although candesartan tended to decrease MAP and increase CBF more in AT2 (−/−) mice than in WT mice (MAP: −28.8±2.4 mm Hg versus −20.4±6.0 mm Hg, P=0.25; CBF: 38.6±16.8% versus 26.8±11.5%, n=4, P=0.25).

IV bolus injections of the AT2 receptor antagonist PD123319 (1 mg/kg) in WT mice had no effect on MAP or CBF and did not alter the pressor and renal vasoconstrictor effects of Ang A and Ang II (Figure S1, available in the online Data Supplement). The AT1 receptor agonist compound 21 (0.6 µg/kg, n=3; 6 and 60 µg/kg, n=2) also had no
In Vitro Experiments
Radioligand binding experiments revealed that both Ang II and Ang A concentration-dependently inhibited the binding of \[^{3}H\]valsartan to CHO-hAT\(_1\) cells and that the corresponding inhibition constant value of Ang A was not significantly different from that of Ang II (Table). Also, in CHO-hAT\(_2\) cells, the competition binding curves of both peptides indicated that the inhibition constant values were not significantly different (Table).

In CHO-hAT\(_1\) cells, Ang A and Ang II caused a concentration-dependent increase of IP accumulation with comparable EC\(_{50}\) values for Ang A and Ang II (Table). The maximal effect of Ang A was similar to that produced by Ang II (94±3%).

Discussion
The principal findings of the present study are that the novel peptide Ang A, similar to Ang II, produced pressor and renal vasoconstrictor effects in rats and mice mediated via the AT\(_{1a}\) receptor, that it has similar affinity to AT\(_1\) and AT\(_2\) receptors as Ang II, and that it acts as a full agonist at AT\(_1\) receptors. No evidence for AT\(_2\) receptor–mediated vasodilator responses was found.

In line with previous observations in mice and in isolated perfused rat kidney,\(^{5,6}\) IV bolus injections and IR infusions of Ang A dose-dependently increased MAP and decreased RBF in rats through AT\(_1\) receptor stimulation. Ang A was less potent than Ang II but induced at higher doses maximal reductions in RBF similar to Ang II, which is difficult to reconcile with the suggestion of Jankowski et al\(^6\) that Ang A “is the first metabolite of the renin-angiotensin system acting as a partial agonist.”

It has been suggested that AT\(_2\) receptor stimulation has hypotensive and vasodilator effects, opposing AT\(_1\)-mediated pressor and vasoconstrictor effects.\(^1\) Jankowski et al\(^6\) reported Ang A to bind to AT\(_2\) receptors with higher affinity than Ang II and proposed that “Ang A may modulate the harmful effects of Ang II.” In the present experiments in normotensive rats, AT\(_1\) receptor blockade increased RBF, indicating a tonic influence of endogenous Ang II via AT\(_1\) receptors on the renal circulation, but did not unmask a putative AT\(_2\) receptor–dependent renal vasodilator response to Ang A or to Ang II. The AT\(_2\) receptor antagonist PD123319 did not enhance the pressor and renal vasoconstrictor responses to both peptides as could be expected if AT\(_2\) receptor stimulation would counteract the AT\(_1\)-mediated effects. Moreover, selective AT\(_2\) receptor agonists did not modify baseline pressure or RBF, nor did they change the responses induced by both Angs. The absence of an AT\(_2\) receptor–mediated vasodilator response to Ang may be explained by a lack of significant expression of AT\(_2\) receptors in normotensive rats, which is in line with previous studies showing unmasking of AT\(_2\) receptor–mediated effects by AT\(_1\) receptor blockade in SHRs but not in normotensive rats.\(^{11,12}\)

In SHRs, we observed AT\(_1\) receptor–dependent pressor and renal vascular responses to Ang A and Ang II and significantly greater increases in RBF after AT\(_1\) receptor blockade than in normotensive rats, indicating a greater tonic influence of endogenous Ang II via AT\(_1\) receptors on the renal circulation, but did again not unmask a putative AT\(_2\) receptor–dependent renal vasodilator response to Ang A or to Ang II. Moreover, PD123319 did not enhance the pressor and renal vasoconstrictor responses in SHRs, and compound 21 again did not modify baseline MAP or RBF or the responses induced by both Angs. In another study,\(^{13}\) compound 21 also did not decrease blood pressure when administered alone in SHRs, at doses ranging from 50 to 1000 ng/kg per minute, suggesting that AT\(_1\) receptor blockade was needed to unmask AT\(_2\) receptor–mediated vasodilatation in SHRs, as had been reported previously by Li and Widdop.\(^{12}\) In stroke-prone SHRs, compound 21 (10 mg/kg per day) given orally had no effect on blood pressure, although it delayed the occurrence of brain damage and prolonged survival and delayed the time to proteinuria development.\(^{14}\) Taken together, studies of the blood pressure–lowering effect of AT\(_2\) receptor stimulation with compound 21 in SHRs have yielded conflicting results, and a depressor effect was only observed in certain experimental conditions and for a limited dose range. In our hands, a putative AT\(_2\) receptor–mediated vasodilator effect was not unmasked by candesartan. It could be argued that the reduction in vascular tone by candesartan could make it difficult to detect a further reduction in vascular tone by AT\(_2\) receptor
However, we demonstrated that dopamine D1 receptor stimulation by fenoldopam clearly further reduced blood pressure and RVR under these conditions, showing that non-AT2 receptor–mediated vasodilatation remained detectable.

We also used transgenic mice lacking AT1a, AT1b, and AT2 receptors or IRAP to discriminate between the AT1 receptor subtypes, AT1a and AT1b, to further define a possible role of AT2 receptors and to explore whether deletion of the IRAP enzyme modifies the responses to Ang A. The results confirm the observations in rats and unequivocally establish a role for AT1a receptors in mediating the pressor and renal vasoconstrictor effects of Ang A in mice. Deletion of the AT1a receptor nearly completely abolished the pressor and renal vasoconstrictor responses to Ang II and Ang A. Because these AT1a (−/−) mice responded normally to endothelin, it can be ruled out that the lack of vasoconstrictor response to Ang A and Ang II was attributed to a general impairment in vasoactive properties.

The pressor and renal responses to Ang II and Ang A observed in AT1b (−/−) and IRAP (−/−) mice were similar to those of WT mice and were also abolished by candesartan, suggesting that AT1b and IRAP receptors are not involved in the renal hemodynamic responses to these peptides. As previously reported, AT2 (−/−) mice had a higher baseline MAP and a more pronounced pressor and renal vascular response to lower doses of Ang A and Ang II, which could be in line with the hypothesis that AT2 receptors may antagonize the function of AT1 receptors in the regulation of blood pressure and vascular tone in WT mice. However, this is difficult to reconcile with the fact that compound 21 did not reduce blood pressure and renal vascular tone in WT mice and that responses to Ang A and Ang II were not enhanced by PD123319. An alternative explanation for the more pronounced effects in AT2 (−/−) mice could be an increase in AT1 receptor expression in vascular smooth muscle cells in AT2 receptor–deficient mice, as reported by Tanaka et al.

Our in vitro data suggest that the in vivo observation in normotensive rats and mice of a lower potency of Ang A than Ang II as a renal vasoconstrictor might be explained by pharmacokinetic reasons, such as a shorter half-life because of an enhanced sensitivity to aminopeptidases. Indeed, our in vitro binding data on human AT1 receptors expressed in CHO-K1 cells showed that Ang A has a similar binding properties as Ang II.
affinity as compared with Ang II, which is in agreement with the in vitro data from Jankowski et al.\textsuperscript{5} These authors also concluded that Ang A is a partial agonist, although they reported that the maximal calcium release in vascular smooth muscle cells obtained with receptor-saturating concentrations of both peptides was comparable.\textsuperscript{5} Our in vitro experiments with AT1-mediated IP accumulation showed that both peptides are capable of producing a similar maximal effect, indicating that Ang A is a full AT1 receptor agonist, which is in line with our in vivo observations that it produces similar maximal RBF responses as Ang II. These results are not surprising, because Ang III, in which the N-terminal aspartate residue is deleted, also has, compared with Ang II, a similar binding affinity to AT1 receptors and equal efficacy in producing AT1-mediated responses, such as IP accumulation in CHO-hAT1 cells,\textsuperscript{17} as well as aldosterone production in adrenal glomerulosa cells.\textsuperscript{18} Our data are also consistent with conclusions by Khosla et al\textsuperscript{19} that the amino terminus of Ang II exhibits little structural specificity for activity and can be altered without major changes in pressor or myotrophic activity.

We studied the direct interaction of Ang A compared with Ang II with the AT2 receptor by radioligand binding of \[^{125}\text{I}\\text{Sar}1-\text{Ile}8\text{Ang II} \] to CHO-hAT2 cells. These experiments revealed that both peptides displayed similar inhibition constant values for this receptor. This observation is in

\begin{table}
\centering
\caption{EC\textsubscript{50} and/or Inhibition Constant Values of Ang II and Ang A in CHO-hAT\textsubscript{1} Cells and CHO-hAT\textsubscript{2} Cells}
\begin{tabular}{lccc}
\hline
Cells & Measure & Ang II & Ang A \\
\hline
CHO-hAT\textsubscript{1} cells & \(K_i\) nM & 0.9 \pm 0.2 (n=5) & 1.6 \pm 0.5 (n=5) \\
CHO-hAT\textsubscript{1} cells & EC\textsubscript{50} nM & 6.3 \pm 3.2 (n=5) & 6.7 \pm 2.4 (n=5) \\
CHO-hAT\textsubscript{2} cells & \(K_i\) nM & 1.6 \pm 0.38 (n=7) & 2.3 \pm 0.5 (n=7) \\
\hline
\end{tabular}
\end{table}

\(K_i\) indicates inhibition constant; Ang, angiotensin; CHO-hAT\textsubscript{1}, Chinese hamster ovary cells expressing recombinant human Ang II type 1 receptors; CHO-hAT\textsubscript{2}, Chinese hamster ovary cells expressing recombinant human Ang II type 2 receptors. Binding and functional data of Ang II and Ang A were determined in CHO-hAT\textsubscript{1} and CHO-hAT\textsubscript{2} cells. The \(K_i\) is related to the binding affinity and the EC\textsubscript{50} is related to inositol phosphate accumulation in the functional study. In the binding study,\textsuperscript{18} valsartan and \[^{125}\text{I}\\text{Sar}1-\text{Ile}8\text{Ang II} \] were used as radioligands in CHO-hAT\textsubscript{1} cells and in CHO-hAT\textsubscript{2} cells, respectively. In the functional study, the accumulation of \[^{3}\text{H}\\text{inositol phosphate}\] was measured in CHO-hAT\textsubscript{1} cells. All of the values correspond with mean \pm SEM of \textquotedblleft n\textquotedblright independent experiments performed in triplicate.
contrast with the data of Jankowski et al., who reported Ang A to have a higher affinity than Ang II on membranes of HEK293 cells transiently transfected with AT2 receptors. The reason for this discrepancy is unclear but may be because of different experimental conditions. Although the binding experiments of Jankowski et al. were carried out in cell membranes at 25°C with $^{[125]I}$-[Sar1-Ile8]Ang II in HEK293 cells, in the present study, radioligand binding was measured in intact cells at 37°C in CHO-K1 cells.

We have also made great effort to measure the plasma levels of Ang A in rats. We possess a well-validated nano liquid chromatography-tandem mass spectrometry method for the determination of Ang II and Ang IV in microdialysis samples. This method, which has limits of quantification for these Angs in the low picomolar range, did not allow us to detect Ang A in plasma of rats (data not shown), suggesting that the endogenous plasma levels of Ang A in rats are very low compared with plasma concentrations of Ang II. This is in line with Jankowski et al., who reported lower plasma concentrations for Ang A versus Ang II in healthy subjects and end-stage renal failure patients.

**Perspectives**

The present study demonstrates that Ang A, a recently discovered novel human Ang-derived peptide, where only the N-terminal amino acid is different from Ang II, displays similar in vitro and in vivo properties as Ang II. The present study indicates that, in contrast to the previous report by Jankowski et al., Ang A is also a full agonist and that it does not have greater affinity for AT2 receptors than Ang II, which is in agreement with the hypothesis that the N-terminal aspartate residue of Ang II does not play a key role in binding to and stimulating the AT2 receptors. These findings, together with our observations that the plasma Ang A levels are below the detection limit of our sensitive nano liquid chromatography-tandem mass spectrometry assay in normotensive rats, do not provide evidence for a physiological role of Ang A as a naturally occurring peptide counteracting the Ang II-mediated vasoconstrictor effects.

**Sources of Funding**

This research was supported by a grant from the Vrije Universiteit Brussel Research Council. P.V. is holder of a Vrije Universiteit Brussel research fellowship.

**Disclosures**

None.

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Pressor and Renal Hemodynamic Effects of the Novel Angiotensin A Peptide Are Angiotensin II Type 1A Receptor Dependent
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*Hypertension*. 2011;57:956-964; originally published online April 4, 2011; doi: 10.1161/HYPERTENSIONAHA.110.161836

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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PRESSOR AND RENAL HEMODYNAMIC EFFECTS OF THE NOVEL ANGIOTENSIN A PEPTIDE ARE AT$_{1a}$ RECEPTOR DEPENDENT

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Short title: Ang A and AT$_1$/AT$_2$ receptors

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Expanded Methods

Animals were maintained on a normal diet with free access to tap water, at room temperature and a 12h light-dark cycle.

In vivo experiments in normotensive and hypertensive rats

Animals and surgical procedures

Adult male normotensive Wistar Kyoto rats (Ifa Credo, Brussels, Belgium) and adult male spontaneously hypertensive rats (SHR, Harlan, the Netherlands) weighing between 275-325g were anesthetized with an intraperitoneal (i.p.) injection of 60 mg/kg sodium pentobarbital (Nembutal®) (Ceva Sante Animal, Brussels, Belgium) and fixed in the supine position on a heating pat kept at 37°C. The surgical procedure was a modification of the methods previously described1,2. Briefly, following intubation of the trachea, the right jugular vein was catheterized for fluid maintenance, drug administration and supplemental anesthesia. The right carotid artery was cannulated for continuous monitoring of Mean arterial pressor (MAP) with a pressure transducer (HP Hewlett Packard, Boebingen, Germany). An ultrasound probe was secured around the left renal artery and connected with a transonic flow meter (type T 106, Transonic Systems Inc., NY, USA) for measuring total renal blood flow. For local (intrarenal, i.r.) administration of drugs, a catheter was inserted into the lower abdominal aorta and the catheter tip was advanced to the origin of the left renal artery. This catheter was connected to a 1 ml syringe that was driven by an infusion pump (model MD-1001, Bioanalytical systems Inc. USA). A 0.9% saline solution (20µl/min) was then infused into left renal artery from this catheter, and this catheter was also used for local infusion of drugs. The experimental protocol commenced after a 1h equilibration period following abdominal surgery. First the baseline values were recorded for 15 min before administration of compounds. During local administration, anesthesia was maintained with 0.8-1µl/min intravenous (i.v.) infusion of sodium pentobarbital.

Systemic administration in normotensive rats

Three groups of normotensive rats (n=4-5, per group) received i.v. bolus injections of increasing doses of Ang A and Ang II (each, 0.01 to 10 nmol/kg) in a random order. Subsequently, an i.v. bolus injection of either the selective AT1 receptor antagonist candesartan (1 mg/kg, group 1) or the AT2 receptor agonists CGP42112A (100 µg/kg, group 2) or compound 21 (30 µg/kg, group 3) was administered. Fifteen min later, all animals (group 1-3) received again i.v. bolus injections of increasing doses of Ang A and Ang II.

The fourth group of normotensive rats (n=5) received i.v. bolus injections of increasing doses of Ang A and Ang II (each, 0.01 to 10 nmol/kg) in a random order. Subsequently, an i.v. bolus injection of the AT2 receptor antagonist PD123319 (1 mg/kg) was administered. Since PD123319 has a short pharmacokinetic half life (22 min), we calculated that an additional i.v. bolus injection of 0.26 mg/kg PD123319 every 10 min would be necessary to maintain a steady
state concentration of the AT$_2$ receptor antagonist. Therefore, we next co-administered every 10 min the i.v. bolus injections of increasing doses of Ang A (0.01 to 10 nmol/kg) and Ang II (0.01 to 10 nmol/kg) with an i.v. bolus injection of 0.26 mg/kg PD123319.

A bolus injection of 0.9% saline solution was always given as a control before the application of the lowest dose of each compound. The order in which Ang A and Ang II were administered was randomized, but each peptide was always administered at increasing doses. The doses of Ang A were selected based on a previous study in rats$^{3,4}$. At least 5-min elapsed between the recovery of a response and the next injection of an Ang peptide. This experimental set-up is capable to detect renal vasodilator responses as we showed in earlier experiments with the D$_1$ receptor agonist fenoldopam$^4$.

**Systemic administration in hypertensive rats**

Three groups of SHR (n=4-6, per group) received an intravenous (i.v.) bolus injection of saline (group 5), the selective AT$_1$ receptor antagonist candesartan (1 mg/kg, group 6) or the AT$_2$ receptor agonist compound 21$^5$ (0.05 mg/kg, group 7). The doses of compound 21 were based on a previous study$^4$ wherein compound 21 reduced blood pressure in SHR when administered i.v. in the dose range 0.008-0.05 mg/kg. Fifteen min later, all groups received i.v. bolus injections of increasing doses of Ang A and Ang II (each, 0.001 to 10 nmol/kg) in a random order. Group 8 of SHR (n=5) received an i.v. bolus injection of the AT$_2$ receptor antagonist PD123319 (1 mg/kg), followed with an i.v. bolus injection of 0.26 mg/kg PD123319 in every 10 min until the end of the experiment. Fifteen min later the first injection of PD123319, the animals received i.v. bolus injections of increasing doses of Ang A and Ang II (0.01 to 10 nmol/kg).

Group 9 of SHR (n=4) received an i.v. bolus injection of saline, candesartan (1 mg/kg), compound 21 (0.01, 0.05 mg/kg) and the D$_1$ receptor agonist fenoldopam (100 nmol/kg), consecutively. The interval time between each injection was 15 min.

**Local i.r. artery administration**

Twenty rats were randomly divided into 4 groups (group 5-8, n=5, per group) and received respectively an i.r. bolus injection (60 µl) of either vehicle (saline, group 5), candesartan (5 µg/kg, group 6), compound 21 (0.1µg/kg, group 7) or PD122319 (0.5 mg/kg, group 8). This was followed by a continuous i.r. infusion of vehicle (saline, 20 µl/min) for groups 5-7, or by a continuous i.r. infusion of PD123319 (5 µg/min/kg) for group 8 until the end of the experiment. 15 minutes after the i.r. bolus injection of vehicle, candesartan, compound 21 or PD122319, all animals co-received an i.r. infusion of increasing concentrations of Ang A and Ang II (each, 0, 0.1, 1 nmol/20µl/min) for 10 min. The order in which the two Ang were administered was randomized, but each Ang was administered at increasing doses. The selection of the doses was based on the results of the previous experiments in rats$^{3,5}$. At least 30-min elapsed between the recovery of a response and the next injection of Ang II or Ang A.
In vivo experiments in mice

Animals and surgical procedures

We used adult (8-9-month old) homozygous male AT1a (-/-), AT1b (-/-) and AT2 (-/-) mice and their corresponding wild type (AT WT) mice which were bred on a 129xC57BL/6 background from breeding stocks of T. Walther at the Charité (Campus Benjamin Franklin, Berlin, Germany). The mice were held in the unit at least one month before being transferred to the experimentation rooms. The parent heterozygous IRAP (+/-) mice on a C57BL/6 genetic background were from the breeding stocks of S.Y. Chai at Howard Florey Institute (University of Melbourne, Parkville, Victoria, Australia) and were bred in the animal facility of the Vrije Universiteit Brussel. The generated offspring IRAP (-/-) mice and their corresponding wild type (IRAP WT) (8-month old) were used for this study. The genotypes were determined by PCR as described in a previous study. A group of mice (Iffa Credo, Brussels, Belgium) based on a C57BL/6 genetic background (6-month old) was also used to study the effects of Ang A and Ang II with and without the AT2 receptor antagonist. All the mice were anesthetized with an intraperitoneal (i.p.) injection of 100 mg/kg sodium pentobarbital (Nembutal®) (Ceva Sante Animal, Brussels, Belgium) and fixed in the supine position on a heating pad kept at 37°C. The surgical procedure was a modification of the methods previously described. Briefly, the left femoral artery was cannulated for continuous monitoring of MAP with a pressure transducer (HP Hewlett Packard, Boebingen, Germany). Another catheter was inserted into the right jugular vein for fluid maintenance and drug administration. The mice were then turned to be fixed in the prone position. A dorsal incision was made to expose the left kidney, and the kidney was then covered with saline-soaked cotton. Subsequently, the 0.8 x 30 mm needle probe (Vasamed, Eden Prairie, USA) of the laser Doppler flow meter (model BPM 403A; TSI incorporated, St. Paul, Minnesota, USA) was placed on the posterior lateral surface of the left kidney to measure relative change of superficial renal cortical blood flow (CFB) and was fixed by the use of a micromanipulator. The experimental protocol of systemic administration commenced after 30 minutes equilibration period following abdominal surgery. The baseline values of MAP, CBF and cortical vascular resistance (CVR) were recorded for 10 min before administration of compounds.

The effects of Ang A and Ang II in KO mice and their corresponding wild type

AT1a (-/-), AT1b (-/-), AT2 (-/-) mice and their corresponding wild type (AT WT) mice, IRAP (-/-) mice and their corresponding wild type (IRAP WT) mice all received an i.v. bolus injection of saline (65 µl) at the beginning of each experiment. Subsequently, Ang A (0.1, 1, 10 nmol/kg) and Ang II (0.01, 0.1, 1 nmol/kg) were delivered to the mice as i.v. bolus injections via jugular vein catheters. The doses used were selected based on the previous study in rats and on our previous experiments investigating Ang II effects in mice. The order in which the different peptides were administered was randomized. However, both peptides were always administered at increasing doses. At least 5-min elapsed between the recovery of a response and the next injection of Ang A or Ang II. Candesartan (1 mg/kg i.v.) was injected in each mouse following the administration of the different doses of both angiotensins and this was followed by the i.v.
injection of the highest dose of Ang A (10 nmol/kg) and Ang II (1 nmol/kg). The vasoconstrictor response to an i.v. bolus injection of endothelin-1 (2 pmol/kg) at the end of each experiment showed there was no loss of vascular reactivity in any mice of this study.

The effects of Ang A and Ang II with and without the AT_2 receptor antagonist

In a separate group of mice (C57BL/6 genetic background, no gene-interruption, n=4), animals received i.v. bolus injections of increasing doses of Ang A (0.1 to 10 nmol/kg) and Ang II (0.01 to 1 nmol/kg) in a random order. Subsequently, an i.v. bolus injection of the AT_2 receptor antagonist PD123319 (1 mg/kg) was administered. Due to the short pharmacokinetic half life of PD123319 as described before, we next co-administered every 10 min the i.v. bolus injections of increasing doses of Ang A (0.1 to 10 nmol/kg) and Ang II (0.01 to 1 nmol/kg) with an i.v. bolus injection of 0.26 mg/kg PD123319.

In vitro experiments

Receptor binding assays

Ang II and Ang A competition binding experiments were carried out on CHO-hAT1 and hAT2 transfected CHO cells that were cultured in 24 well plates until confluent. Before the binding experiments, the cells were washed 3 times with Dulbecco’s Modified Essential Medium (DMEM, 0.5 ml/well) and the incubations were carried out in a final volume of 0.5 ml/well. The binding of Ang II and Ang A to AT_1 receptors was determined as described previously. Briefly CHO-hAT1 cells were incubated for 40 min at 37°C with 3 nM [^3H]-valsartan and increasing concentrations of unlabelled peptides. Specific radioligand binding was determined by subtracting non-specific binding (in the presence of 1 µM candesartan) from total binding. The binding of Ang II and Ang A to AT_2 receptors was performed similarly. After washing, CHO-K1 cells transfected with the hAT_2 receptor were incubated for 45 min at 37°C with 0.25 nM [^125I]-[Sar1-Ile8]AngII and increasing peptide concentrations. Non-specific binding was determined in the presence 10 µM of unlabeled Ang II. After all incubations, the cells were washed 3 times with cold phosphate buffered saline (PBS) (4°C). The cell bound radioactivity in each well was subsequently solubilised with 500 µl NaOH (0.2 M) and counted for 3 min in a liquid scintillation counter after addition of 3 ml scintillation liquid (Optiphase Hisafe, PerkinElmer) for [^3H]-valsartan or in a gamma counter for [^125I]-[Sar1-Ile8]AngII. The Ki values of the peptides were calculated from the equation Ki=IC_{50}/(1+[L]/K_D)^1 in which [L] is the final radioligand concentration and K_D its equilibrium dissociation constant i.e. 1.66 nM for [^3H]-valsartan and 0.233 nM for [^125I]-[Sar1-Ile8]AngII. The IC_{50} values refer to half-maximal inhibition of the specific radioligand binding and were calculated by non-linear regression analysis of the competition binding curves using GraphPad Prism 5.

IP accumulation

AT_1 receptor signaling was studied in CHO-hAT1 cells. The cells were incubated for 10 minutes at 37 °C in DMEM containing 10 mM LiCl with increasing concentrations of Ang II or Ang A and the accumulation of [^3H]-labelled inositol phosphates was determined as described
previously. The EC$_{50}$ values were calculated by non-linear regression analysis of the concentration-response curves using GraphPad Prism 5.

Drugs

Ang A was synthesized by Aneta Lukaszuk (Department of Chemistry, Vrije Universiteit Brussel, Brussels, Belgium). Ang II was purchased from Neosystem (Strasbourg, France). Ang A and Ang II were dissolved in 0.9% NaCl. CGP42112A, PD123319 and fenoldopam were purchased from Sigma-Aldrich Co. (St. Louis, USA). Candesartan was supplied by Dr. Anders Ljunggren (AstraZeneca, Mölndal, Sweden). Compound 21 was provided by Vicore Pharma AB (Göteborg, Sweden). $[^{3}H]$Valsarotan was provided by Novartis (Basel, Switzerland). $[^{125}I]$[Sar1-Ile8]AngII is from PerkinElmer, Belgium.

References


11. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol.* 1973;22:3099-3108.

Figure S1: In mice, maximal effects of MAP, CBF and CVR in mice to i.v. bolus injections of Ang II (0.01, 0.1, 1 nmol/kg) with and without PD123319 (1 mg/kg); and maximal effects to i.v. bolus injection of Ang A (0.1, 1, 10 nmol/kg) with and without PD123319 (1 mg/kg). Values are expressed as mean±SEM (n=4).