

Mass-Spectrometric Identification of a Novel Angiotensin Peptide in Human Plasma

Vera Jankowski, Raymond Vanholder, Markus van der Giet, Markus Tölle, Sevil Karadogan, Johan Gobom, Jens Furkert, Alexander Oksche, Eberhard Krause, Thi Nguyet Anh Tran, Martin Tepel, Mirjam Schuchardt, Hartmut Schlüter, Annette Wiedon, Michael Beyermann, Michael Bader, Mihail Todiras, Walter Zidek, Joachim Jankowski

Objective—Angiotensin peptides play a central role in cardiovascular physiology and pathology. Among these peptides, angiotensin II (Ang II) has been investigated most intensively. However, further angiotensin peptides such as Ang 1-7, Ang III, and Ang IV also contribute to vascular regulation, and may elicit additional, different, or even opposite effects to Ang II. Here, we describe a novel Ang II-related, strong vasoconstrictive substance in plasma from healthy humans and end-stage renal failure patients.

Methods and Results—Chromatographic purification and structural analysis by matrix-assisted laser desorption/ionisation time-of-flight/time-of-flight (MALDI-TOF/TOF) revealed an angiotensin octapeptide with the sequence Ala-Arg-Val-Tyr-Ile-His-Pro-Phe, which differs from Ang II in Ala¹ instead of Asp¹. Des[Asp¹]-[Ala¹]-Ang II, in the following named Angiotensin A (Ang A), is most likely generated enzymatically. In the presence of mononuclear leukocytes, Ang II is converted to Ang A by decarboxylation of Asp¹. Ang A has the same affinity to the AT₁ receptor as Ang II, but a higher affinity to the AT₂ receptor. In the isolated perfused rat kidney, Ang A revealed a smaller vasoconstrictive effect than Ang II, which was not modified in the presence of the AT₂ receptor antagonist PD 123319, suggesting a lower intrinsic activity at the AT₁ receptor. Ang II and Ang A concentrations in plasma of healthy subjects and end-stage renal failure patients were determined by matrix-assisted laser desorption/ionisation mass-analysis, because conventional enzyme immunoassay for Ang II quantification did not distinguish between Ang II and Ang A. In healthy subjects, Ang A concentrations were less than 20% of the Ang II concentrations, but the ratio Ang A / Ang II was higher in end-stage renal failure patients.

Conclusion—Ang A is a novel human strong vasoconstrictive angiotensin-derived peptide, most likely generated by enzymatic transformation through mononuclear leukocyte-derived aspartate decarboxylase. Plasma Ang A concentration is increased in end-stage renal failure. Because of its stronger agonism at the AT₂ receptor, Ang A may modulate the harmful effects of Ang II. (*Arterioscler Thromb Vasc Biol.* 2007;27:297-302.)

Key Words: mass-spectrometry ■ vasoconstriction ■ angiotensin-peptide ■ human plasma

The octapeptide, angiotensin II (Ang II) is generally accepted to play a central role in the physiology and pathology of vascular regulation. The secular therapeutic success of ACE inhibitors and AT₁ blockers as cardiovascular protective drugs further underscored the role of Ang II in the pathophysiology of atherosclerosis and hypertension. Further peptides of the angiotensin family, including angiotensins III and IV and angiotensin 1-7 are less well characterized.^{1,2} These peptides have additional or antagonizing effects when compared with Ang II.³

Angiotensin 1-7 has been shown to be a vasodilator.⁴ Similarly, angiotensin IV is an agonist to the AT₄ receptor mediating vasodi-

lation.⁵ These various angiotensin peptides may gain growing importance as on ACE inhibition increased amounts of other angiotensin peptides are formed via ACE-independent pathways.⁶

To identify novel vasoconstrictive peptides, we screened chromatographic fractions obtained from human plasma for vasoconstrictive effects using the isolated perfused rat kidney as a bioassay.

Methods

Controls, Patients, and Isolation of Plasma

Details of the characteristics of patients and controls are given in the Table and in the supplemental Methods (available online at <http://>

Original received August 7, 2006; final version accepted November 16, 2006.

From the Charité-Universitätsmedizin Berlin (V.J., M.v.d.G., M.Tölle, S.K., M.Tepel, M.S., H.S., A.W., W.Z., J.J.), Campus Benjamin Franklin Medizinische Klinik IV, Germany; Nephrology Section (R.V.), Department of Internal Medicine, University Hospital, Gent, Belgium; Max Planck Institute for Molecular Genetics (J.G.), Berlin, Germany; Charité-Universitätsmedizin Berlin (J.F., A.O.), Institut für Pharmakologie, Germany; Forschungsinstitut für Molekulare Pharmakologie (E.K., M.Beyermann, M.T.), Berlin, Germany; and Max-Delbrück-Center for Molecular Medicine (M.Bader), Berlin, Germany.

Correspondence to Priv.-Doz. Dr J. Jankowski, Charité Universitätsmedizin Berlin, Campus Benjamin Franklin Med Klinik IV, Hindenburgdamm 30, 12200 Berlin, Germany. E-mail Joachim.Jankowski@charite.de

© 2007 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000253889.09765.5f

Clinical and Biochemical Characteristics of Control Subjects and End-Stage Renal Failure Patients

	Healthy Subjects (n=7)	End-Stage Renal Patients (n=7)	P Value
Age, years	63±7	66±3	n.s.
Sex, m/f	4/3	4/3	n.s.
Blood pressure (systolic/diastolic), mm Hg	117±5/66±3	132±6/74±5	n.s.
Red blood cells, 10 ⁶ /μL	4.3±0.3	3.4±0.3	n.s.
White blood cells, 10 ³ /μL	7.4±0.7	9.9±1.6	n.s.
Platelets, 10 ³ /μL	266±44	250±51	n.s.
Serum creatinine, μmol/L	83.3±5.5	503.3±69.5	<0.01
Blood urea nitrogen, mmol/L	5.5±0.5	18.9±1.4	<0.01

Values are mean±SEM.

atvb.ahajournals.org). We obtained peripheral blood (10 mL) by catheterization of the cubital vein and collected the blood in tubes containing K₂-EDTA (7.2 mg). The blood samples were centrifuged at 300g for 30 minutes at 4°C for isolation of plasma. The resulting plasma was fractionated by chromatography (see supplemental Methods).

Analytic Methods

For analytical purposes, samples were submitted to size-exclusion and reversed phase chromatography and mass-spectrometry (see supplemental Methods).

Measurements of Perfusion in the Isolated Perfused Rat Kidney

The effects of aliquots of lyophilized fractions of the analytic reversed-phase chromatography on vascular tone were evaluated in an isolated rat kidney perfused at a constant flow rate while perfusion pressure was continuously monitored. Details of the preparation and the perfusion procedure are described elsewhere^{7,8} and in the supplemental Methods. The underlying substance of the fraction with a vasoconstrictive effect was identified by matrix-assisted laser desorption/ionisation time-of-flight/time-of-flight (MALDI-TOF/TOF) mass-spectrometry (see supplemental Methods).

Preparation and Incubation of Mononuclear Leukocytes With Ang II and Angiotensinogen

Mononuclear leukocytes were obtained from healthy subjects according to established techniques⁹ (see supplemental Methods). To test whether Ang A is synthesized out of Ang II in the presence of mononuclear leukocytes 10⁻⁶ mmol/L AT-II and angiotensinogen, respectively, were added to the supernatant. After 0, 0.2, 0.5, 1, and 3 hours, aliquots of the supernatant were analyzed by MALDI mass spectrometry. The incubation with Ang II was repeated with thermally denatured mononuclear leukocytes. For this, mononuclear leukocytes were heated at 100°C for 10 minutes in a reaction vial.

Cell Culture, Transfection Method, and Membrane Preparation for Receptor Binding Studies

Details of the cell culture are given in the supplemental Methods. HEK 293 cells expressing either the AT₁- or AT₂-receptor protein were grown on 100-mm Petri dishes, washed twice with 5 mL of PBS (137 mmol/L, NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH₂PO₄, 8.0 mmol/L Na₂HPO₄, pH 7.4), harvested with a rubber policeman, and centrifuged at 400g for 10 minutes. For receptor binding studies, HEK 293 membranes were prepared, as described the supplemental Methods.

¹²⁵I-Sar¹,Ile⁸-Ang II Displacement Binding Analysis

Membranes (5 μg) were incubated in a final volume of 200 μL of Tris/BAME buffer containing 1 μmol/L ¹²⁵I-Sar¹,Ile⁸-Ang II alone or

increasing concentrations of unlabeled ligand (1×10⁻¹² to 1×10⁻⁶ mol/L) for 2 hours at 25°C at 300 rpm in a shaking water bath (supplemental Methods). The values were used for calculations of the K_i values of unlabeled ligands (see supplemental Methods).

Ca²⁺-Fluorescent Image Plate Reader Assay

Calcium was measured in VSMCs as previously published.¹⁰ The method is described in the supplemental Methods.

Quantification of Ang and Ang II

Ang A and Ang II was quantified by a MALDI-MS based method as recently described.¹¹ The supernatants of mononuclear leukocytes after incubation with Ang II were directly quantified (see supplemental Methods).

Quantification of Ang II in Presence and Absence of Ang A by Enzyme Immunoassay

The “angiotensin II enzyme immunoassay kit” of SpiBio (Société de Pharmacologie et d’Immunologie, Massy, France) was used to test the cross-reactivity between Ang A and Ang II with a conventional enzymatic immunoassay used for Ang II-determinations (see supplemental Methods).

Impact of Ang A on Blood Pressure in Angiotensin II Receptor AT1A Knockout Mice

In these experiments, wild-type mice were compared with angiotensin II receptor AT1A KO mice, which had been bred as previously described by Ito et al¹² (see supplemental Methods).

Results

Human plasma was first fractionated by size-exclusion chromatography. A typical size-exclusion chromatogram is shown in Figure 1A. Each fraction obtained from the size-exclusion step was tested for vasoactivity in the isolated perfused rat kidney. One of the fractions showing a strong vasoconstrictive effect is labeled by an arrow in Figure 1A. This fraction was chromatographed by reversed-phase chromatography (Figure 1B). The reversed-phase chromatography allowed on the one hand to further fractionate the eluate, and on the other hand to desalt the eluate of the size-exclusion chromatography.

The fractions obtained from each reversed phase chromatography elution step were tested for vasoconstrictive properties in the isolated perfused rat kidney. The vasoconstrictive fraction detected using the isolated perfused kidney is again indicated by an arrow in Figure 1B. The insert of Figure 1B shows the vasoconstrictive effect of the labeled fraction.

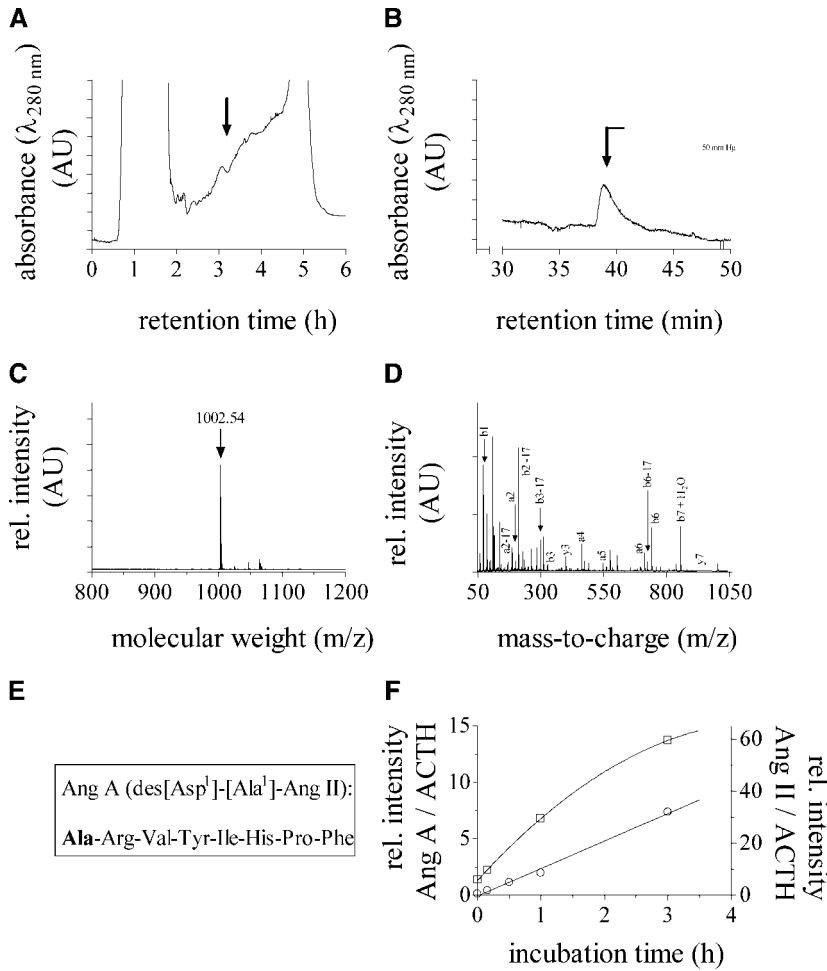


Figure 1. A, Size-exclusion chromatogram of human plasma (conditions: Sephacryl S-100 high-resolution, Pharmacia BioTech, Sweden; column dimension: 1000×16 mm; eluent: 0.9% NaCl in water; flow rate: 1.0 mL/min; abscissa: retention time [h]; ordinate: UV-absorption at 280 nm [arbitrary units]). The arrow indicates a fraction with strong vasoconstrictive properties on testing in the isolated perfused rat kidney. B, Reversed phase chromatography of the size-exclusion fraction labeled by an arrow in A using an analytical reversed-phase high performance liquid chromatographic column (conditions: Chromolith Performance [100×4.6 mm ID], Merck, Darmstadt, Germany; eluent A: 0.1% trifluoroacetic acid in water; eluent B: 0.1% trifluoroacetic acid in water-acetonitrile [20:80, v/v-%]; gradient: 0 to 2 minutes: 0% eluent B, 2 to 32 minutes: 0% to 75% B, 32 to 32.5 minutes: 75% to 100% eluent B; 32.5 to 33.5 minutes: 100% B; flow rate: 2.0 mL/min; abscissa: retention time [min]; ordinate: UV-absorption at 280 nm [arbitrary units]). The inset shows the effect of the labeled fraction on the perfusion pressure of the isolated perfused rat kidney. C, MALDI-TOF mass spectrum of the vasoconstrictive substance isolated by RP-HPLC. The peak with a m/z ratio of 1002.54 (calculated m/z 1002.55) corresponds to peptide sequence Ala-Arg-Val-Tyr-Ile-His-Pro-Phe. D, MALDI-TOF/TOF-MS/MS spectrum of the peak with m/z 1002.54 shown in C. Relevant ions are labeled according to the accepted nomenclature. Both the b ions and the y ions confirm the amino acid sequence of des [Asp¹]-[Ala¹]-Ang II (Ang A). E, Amino acid sequence of Ang A (bold: Difference from the amino acid sequence of angiotensin II). F, Quantification of Ang A (○) and Ang II (□) in supernatants from mononuclear leukocytes after incubation with Ang II (relative to internal standard ACTH).

Mass-spectrometric analysis of the underlying substance by MALDI-TOF revealed a mass peak at m/z 1002.54 (Figure 1C) corresponding to the peptide Ala-Arg-Val-Tyr-Ile-His-Pro-Phe, as confirmed by tandem MS (MALDI-TOF/TOF). In particular, the presence of alanine in the N-terminal position was obtained from the N-terminal b ions as shown in Figure 1D. The difference in the identified amino acid sequence compared with that of angiotensin II is given in Figure 1E in bold.

No DNA sequence code for des[Asp¹]-[Ala¹]-Ang II, which in the following will be named Angiotensin A (where A stands for alanine) or Ang A, was found in data-bases covering the human genome. Therefore, we addressed the question, whether Ang A may arise from Ang II by decarboxylation of Asp. Although a number of tissues may be likely candidates for this transformation, we first tested human mononuclear leukocytes. During the incubation of lipopolysaccharide (LPS)-activated mononuclear leukocytes in the presence of Ang II a significant increase in Ang A levels in the supernatant was detected. The amount of Ang A and Ang II in the supernatant is quantified in Figure 1F (relative to internal standard corticotropin [ACTH]). The synthesis and release of Ang II by mononuclear leukocytes was recently described.¹¹ LPS-stimulation enhanced the Ang II conversion, but mononuclear leukocytes also converted Ang II without LPS-stimulation (rel. mass-signal intensity

[AU] 0.03±0.01 versus 0.007±0.009; n=3). No increase in Ang A levels were detected with intact mononuclear leukocytes in the absence of Ang II nor when a denatured mononuclear leukocyte preparation was used (data not shown). During incubation of LPS-activated mononuclear leukocytes in the presence of renin substrate or angiotensinogen, significant increases in Ang A, Ang II, and Ang I levels in the supernatant, but no mass-signal of a modified des[Asp¹]-[Ala¹]-Ang I were detected (data not shown). Therefore, a modified angiotensinogen is to be excluded as a source of Ang A.

These experiments suggest that Ang A is not derived from a precursor protein like angiotensinogen nor modified angiotensinogen, but is generated from Ang II by enzymatic decarboxylation involving mononuclear leukocytes. A non-enzymatic decarboxylation was excluded by the finding that after heat-denaturing Ang A synthesis is abolished.

After isolation of Ang A from human plasma, we analyzed the vasoconstrictive action of this novel peptide in the isolated perfused rat kidney model. In this model, Ang A caused a dose-dependent vasoconstriction, which was 90% of the maximal effect induced by Ang II (Figure 2A). The effect induced by Ang A was abolished in the presence of the angiotensin-receptor antagonist AT₁ EXP 3174. The EC₅₀ value (mol/L) of Ang A induced vasoconstriction ([4.43±1.95]×10⁻⁷) was an order of magnitude lower than

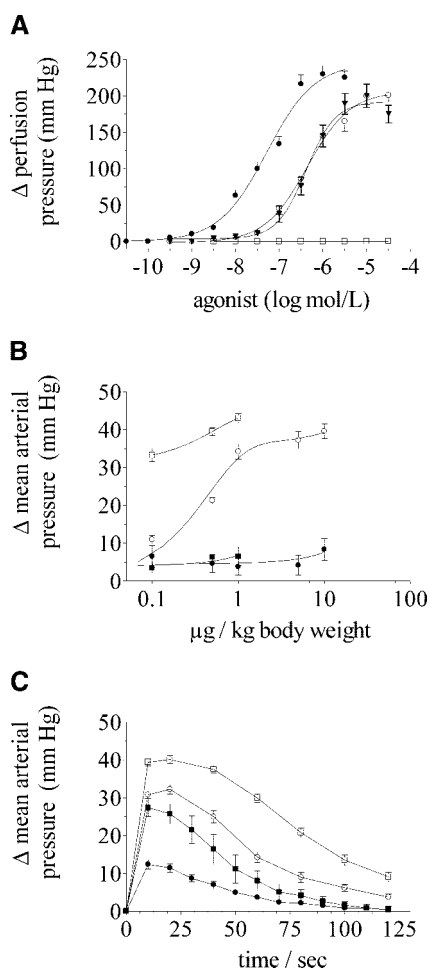


Figure 2. A, Change in perfusion pressure in the isolated perfused rat kidney induced by Ang A (○), Ang II (●), and Ang A in the presence of angiotensin-receptor antagonist AT₁ EXP 3174 (□) or in the presence of angiotensin-receptor antagonist AT₂ PD 123319 3174 (itriaf) (abscissa: agonist [log mol/L]; ordinate: change in perfusion pressure [mm Hg]). Means±SEM, n=6. B, Rise in mean arterial blood pressure in Angiotensin II receptor AT_{1A} knockout (filled symbols) mice and in wild-type mice (open symbols) after injection of Ang A (●/○) and Ang II (■/□). Maximal increase was detected 10 to 40 sec after injection. (abscissa: concentration of agonist [μg/kg body weight]; ordinate: change in mean arterial pressure [mm Hg]). Means±SEM, n=6. C, Alteration of mean arterial blood pressure in wild-type mice after injection of Ang A (○/●) and Ang II (□/■) (open symbols: 100 ng/kg body weight; filled symbols: 1 μg/kg body weight; abscissa: time after injection concentration of agonist [sec]; ordinate: change in mean arterial pressure [mm Hg]). Means±SEM, n=6.

that of Ang II-mediated vasoconstriction ($[5.20 \pm 2.52] \times 10^{-8}$). The AT₂ receptor antagonist PD123319 had no significant effect on the vasoconstriction induced by Ang A.

Thus, Ang A revealed a lower vasoconstrictive potency and efficacy than Ang II, suggesting that it acts only as a partial agonist. Accordingly, Ang A was less active than Ang II in an in vivo assay (Figure 2B). Increasing concentrations of the peptides were injected into the femoral vein of wild-type and Ang II AT_{1A} receptor-deficient mice, and the blood pressure changes were monitored by a femoral artery catheter. Both peptides induced a strong hypertensive response, but Ang A only at ≈ 10 times higher concentrations

than Ang II. Both peptides signal via the AT_{1A} receptor, because the blood pressure response was blunted in knockout animals (Figure 2B). The kinetics of response to Ang A and Ang II application were comparable in this set-up (Figure 2C).

To elucidate whether the lower potency of the vasoconstriction induced by Ang A could be attributed to a lower affinity of Ang A to the AT₁ receptor than that of Ang II, and/or to a higher affinity to the AT₂ receptor, binding of Ang A and Ang II to AT₁ and AT₂ receptors expressed in human embryonic kidney (HEK) 293 cells was studied. We characterized the receptor binding of both peptides by displacement of the radioligand [¹²⁵I]-Sar¹Ile⁸ angiotensin II by increasing concentrations of unlabeled Ang II and Ang A (Figure 3A and 3B). IC₅₀-values of the rat AT₁-receptor (Figure 3A) and the rat AT₂-receptor (Figure 3B) were $[-\log IC_{50}]$ 9.54 ± 0.04 and 9.92 ± 0.04 for Ang A, and $[-\log IC_{50}]$ 9.49 ± 0.06 and 9.59 ± 0.04 for Ang II. Whereas no significant difference in the affinity of AT₁ receptors for Ang A and Ang II was observed (Figure 3A), the affinity of AT₂ receptors for Ang A was significantly higher than for Ang II (Figure 3B). VSMCs stimulated with Ang II or Ang A showed a dose-dependent increase in cytosolic calcium with a significant difference within their EC₅₀ values ($[-\log mol/L]$ Ang II: 7.7 ± 0.1 and Ang A: 7.0 ± 0.1 , $P < 0.05$, $n = 5$ independent experiments; Figure 3C). In the presence of EXP3174 (1 μmol/L) cytosolic calcium increase by half-maximal agonistic concentration of Ang II (10 nmol/L) or Ang A (100 nmol/L) was completely inhibited (Figure 3D). These data suggest that the lower potency of Ang A to induce vasoconstriction is caused neither by a lower affinity to AT₁ receptors nor by higher activity at the AT₂ receptor, but due to a lower intrinsic activity at the AT₁ receptor.

Next we quantified the Ang A/Ang II ratio in human plasma by MALDI mass spectrometry. As shown in Figure 3E, the Ang A/Ang II ratio was higher in end-stage renal failure patients than in healthy subjects. Measurements in seven healthy subjects revealed an Ang A concentration of 6.7 ± 4.7 pg/mL, which is $9.7 \pm 3.3\%$ (calculated for each patient; min.: 2.1%; max.: 25.2%) of the Ang II concentration found in the same subjects (88.0 ± 12.3 pg/mL). The Ang A concentration was increased in plasma of end-stage renal failure patients ($n = 7$) to 28.4 ± 11.0 pg/mL; this corresponds to $30.9 \pm 9.1\%$ (calculated for each patient; min.: 5.1%; max.: 73.6%; $P < 0.05$) of the Ang II concentration found in the same patients (127.5 ± 29.4 pg/mL).

A conventional enzyme immunoassay for Ang II quantification did not distinguish between Ang II and Ang A (Figure 3F).

Discussion

Ang A is a novel angiotensin peptide, which is present in human plasma; it is derived from Ang II by enzymatic decarboxylation, emphasizing the physiological importance of Ang A. A yet unknown decarboxylase present in human mononuclear cells is one potential cellular source of Ang A. At present, the nature of this decarboxylase, its substrate specificity, and its tissue distribution are unknown. It remains

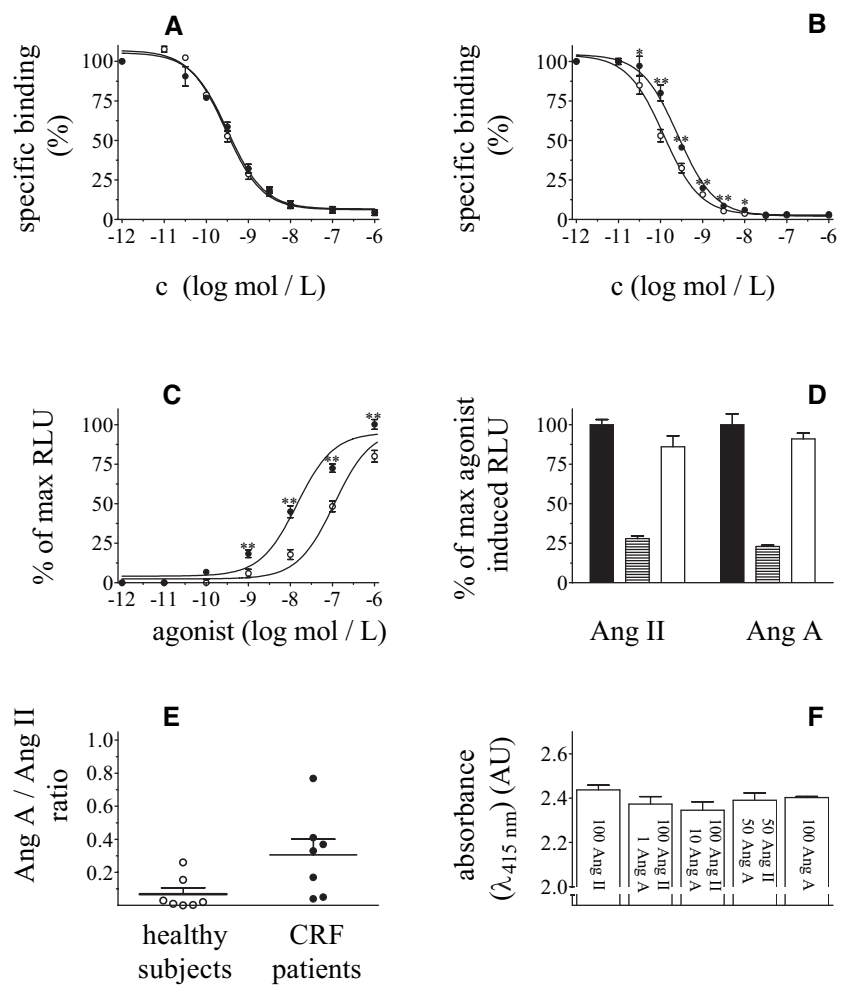


Figure 3. A, Displacement of [¹²⁵I] Sar¹Ile⁸ angiotensin II by Ang A (○) and Ang II (●) in HEK 293 cells transiently transfected with AT₁-receptors. Means ± SEM, n=5 in duplicate. There was no significant difference between Ang A and Ang II. B, Displacement of [¹²⁵I] Sar¹Ile⁸ angiotensin II by Ang A (○) and Ang II (●) in HEK 293 cells transiently transfected with AT₂ receptors. Means ± SEM, n=5 in duplicate; *P<0.05; **P<0.01, Ang A versus Ang II. C, VSMCs were loaded with Fluo4-AM for 60 minutes and stimulated with increasing concentration of Ang A (○) or Ang II (●) for 5 minutes. The peak values in fluorescence emission obtained for the different agonist concentrations were normalized to the maximal response. (ordinate: relative light units; %; Means ± SEM, n=5 in triplicates; **P<0.01, Ang A versus Ang II). D, VSMCs were loaded with Fluo4-AM for 30 minutes and stimulated with half-maximal agonistic concentration of Ang II (10 nmol/L) or Ang A (100 nmol/L) for 5 minutes in the absence (■) of presence of EXP 3174 (1 μmol/L)³ or PD123319 (1 μmol/L; □). Both Ang II or Ang A induced calcium increase in these cells. The peak values in fluorescence emission obtained were normalized to the responses without agonists. Means ± SEM, n=4 in triplicates. E, Mass-spectrometry-based determination of Ang A/Ang II ratio in plasma of healthy control subjects (○; right y-axis) and end-stage renal failure patients (●; left y-axis) (mean ± SEM, n=7, single values and means; P<0.05). F, Quantification of synthetic Ang A and synthetic Ang II by enzyme immunoassay (concentrations used in the assay are given in pmol/L in the corresponding bars; each n=5).

to be shown whether other cells or tissues may also contribute to Ang A formation.

The Ang A/Ang II ratio in plasma of healthy subjects was below 0.2 in all, but this ratio increased to up to 0.7 in patients with stage 5 chronic kidney disease. The increased Ang A/Ang II ratio in patients with stage 5 chronic kidney disease may either indicate an increased activity of decarboxylase in human mononuclear cells from patients with chronic kidney disease stage 5, a reduced enzymatic degradation of Ang A in these patients, or renal excretion. An increase of the Ang A half-life is not unlikely, because modifications of half life of low molecular proteins or peptides in chronic renal failure patients are described frequently (eg.^{13,14}). Presently it is unknown whether the increased Ang A/Ang II ratio in plasma may be related to well-known uremic complications in patients with chronic kidney disease stage 5. Conventional enzyme immunoassays do not distinguish between Ang II and Ang A; these assays quantify the sum of Ang II and Ang A.

Interestingly, the affinity of Ang A to the AT₁ receptor is nearly equal to that of Ang II. However, the potency to induce vasoconstriction is lower than that of Ang II. Thus, Ang A is a less potent, and only partial agonist at the AT₁ receptor. This potential partial agonism will be the more effective in subjects with an increased Ang A/Ang II ratio. It is notable that Ang III, which lacks the amino-terminal aspartate resi-

due, has a similar affinity to the AT₁ receptor as Ang A. In contrast to Ang A, for Ang III the same biological activity as for Ang II has been demonstrated.¹⁵ In addition, Ang II and Ang III cause a similar vascular effect when injected intracerebroventricularly or directly into certain brain regions.^{16,17} In the case of Ang II the increase in blood pressure can be inhibited by a selective inhibitor of aminopeptidase A (APA), which mediates the generation of Ang III by the cleavage of the N-terminal aspartate residue of Ang II.¹⁸ These data suggest that the vasoconstrictive effect of Ang II is mediated by Ang III. Whether Ang III provokes an increase in blood pressure and release of plasma arginine-vasopressin (arginine vasopressin) through the activation of AT₁ and/or AT₂ receptors or a yet unidentified effector molecule is still not clear.¹⁹ In the case of Ang A, the processing to Ang III could be impaired, because the APA has a preference for N-terminal acidic residues such as aspartate or glutamate. Therefore, it is intriguing to speculate that decarboxylation of aspartate in Ang II may prevent or delay Ang III formation.

Interestingly, the affinity of Ang A to the AT₂ receptor is higher than that of Ang II. Whether the intrinsic activity of Ang A at the AT₂ receptor also translates into an increase in intrinsic activity requires a suitable model to study AT₂-mediated signaling events. For example, the mechanisms by which the AT₂ receptor mediates vasodilation and which

signaling pathways are involved remain obscure. Several authors favor an indirect mechanism, such as stimulation of the B₂ bradykinin receptor, possibly via an intracellular acidification and resulting in an increased NO and cGMP production.^{20,21} Furthermore, there are reports suggesting that phosphotyrosine activity is increased after AT₂ receptor stimulation.^{22–24} Further elucidation of AT₂ receptor signaling potentially will help to study the downstream effects of Ang A binding to AT₂ receptor. Our data demonstrate that Ang-A represents a new metabolite of Ang II system, which is the first one acting as a partial agonist. Further studies will be required to analyze whether this new peptide reveals altered metabolism to Ang III and Ang IV peptides, thereby adding to the complexity of the regulation of the cardiovascular system.

Acknowledgments

We thank Brigitte Egbers for technical assistance.

Disclosures

None.

References

- Handa RK, Ferrario CM, Strandhoy JW. Renal actions of angiotensin-(1–7): in vivo and in vitro studies. *Am J Physiol.* 1996;270:F141–F147.
- Lochard N, Thibault G, Silversides DW, Touyz RM, Reudelhuber TL. Chronic production of angiotensin IV in the brain leads to hypertension that is reversible with an angiotensin II AT₁ receptor antagonist. *Circ Res.* 2004;94:1451–1457.
- Petrescu G, Gurzu B, Slatineanu SM, Costuleanu M, Costuleanu N. [Interactions between angiotensin-(1–7) and angiotensin II in isolated rat portal vein]. *Rev Med Chir Soc Med Nat Iasi.* 2003;107:102–107.
- Ren Y, Garvin JL, Carretero OA. Vasodilator action of angiotensin-(1–7) on isolated rabbit afferent arterioles. *Hypertension.* 2002;39:799–802.
- de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev.* 2000;52:415–472.
- Ferrario CM, Chappell MC, Dean RH, Iyer SN. Novel angiotensin peptides regulate blood pressure, endothelial function, and natriuresis. *J Am Soc Nephrol.* 1998;9:1716–1722.
- Jankowski V, Tölle M, Vanholder R, Schönfelder G, van der Giet M, Henning L, Schlüter H, Paul M, Zidek W, Jankowski J. Identification of uridine adenosine tetraphosphate (Up₄A) as an endothelium-derived vasoconstrictive factor. *Nature Medicine.* 2005;11:223–227.
- van der Giet M, Khattab M, Borgel J, Schlüter H, Zidek W. Differential effects of diadenosine phosphates on purinoceptors in the rat isolated perfused kidney. *Br J Pharmacol.* 1997;120:1453–1460.
- Tepel M, Schlotmann R, Barenbrock M, Kisters K, Klaus T, Spieker C, Walter M, Meyer C, Bretzel RG, Zidek W. Lymphocytic Na(+)-H+ exchange increases after an oral glucose challenge. *Circ Res.* 1995;77:1024–1029.
- Giebing G, Tölle M, Jürgensen J, Eichhorst J, Furkert J, Beyermann M, Neuschäfer-Rube F, Rosenthal W, Zidek W, van der Giet M, Oksche A. Arrestin-independent internalization and recycling of the urotensin receptor contribute to long-lasting urotensin II-mediated vasoconstriction. *Circ Res.* 2005;97:707–715.
- Jankowski V, Vanholder R, van der Giet M, Henning L, Tölle M, Schönfelder G, Krakow A, Karadogan S, Gustavsson N, Gobom J, Webb J, Lehrach H, Giebing G, Schlüter H, Hilgers KF, Zidek W, Jankowski J. Detection of angiotensin II in supernatants of stimulated mononuclear leukocytes by MALDI-TOF-TOF-mass analysis. *Hypertension.* 2005;46:591–597.
- Ito M, Oliverio MI, Mannon PJ, Best CF, Maeda N, Smithies O, Coffman TM. Regulation of blood pressure by the type 1A angiotensin II receptor gene. *Proc Natl Acad Sci U S A.* 1995;92:3521–3525.
- Baumelou A, Singlas E, Petitclerc T, Desmichels D, Jacobs C, Soria J. Pharmacokinetics of a low molecular weight heparin (reviparine) in hemodialyzed patients. *Nephron.* 1994;68:202–206.
- Follea G, Laville M, Pozet N, Dechavanne M. Pharmacokinetic studies of standard heparin and low molecular weight heparin in patients with chronic renal failure. *Haemostasis.* 1986;16:147–151.
- Rebas E, Lachowicz-Ochedalska A. The effect of angiotensin III on protein tyrosine kinase activity in rat pituitary. *Regul Pept.* 2005;130:14–18.
- Wright JW, Morseth SL, Abhold RH, Harding JW. Pressor action and dipsogenicity induced by angiotensin II and III in rats. *Am J Physiol.* 1985;249:R514–R521.
- Zini S, Fournie-Zaluski MC, Chauvel E, Roques BP, Corvol P, Llorens-Cortes C. Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: predominant role of angiotensin III in the control of vasopressin release. *Proc Natl Acad Sci U S A.* 1996;93:11968–11973.
- Rozenfeld R, Reaux A, Iturriz X, Fassot C, Fournie-Zaluski MC, David C, Maigret B, Roques BP, Corvol P, Llorens-Cortes C. Aminopeptidase A, generating one of the main effector peptides of the brain renin-angiotensin system, angiotensin III, plays a key role in central control of blood pressure. *Proc West Pharmacol Soc.* 2003;46:39–44.
- Yang CR, Phillips MI, Renaud LP. Angiotensin II receptor activation depolarizes rat supraoptic neurons in vitro. *Am J Physiol.* 1992;263:R1333–R1338.
- Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S, Miyazaki M, Nozawa Y, Ozono R, Nakagawa K, Miwa T, Kawada N, Mori Y, Shibasaki Y, Tanaka Y, Fujiyama S, Koyama Y, Fujiyama A, Takahashi H, Iwasaka T. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. *J Clin Invest.* 1999;104:925–935.
- Gohlke P, Pees C, Unger T. AT₂ receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism. *Hypertension.* 1998;31:349–355.
- Tsuzuki S, Matoba T, Eguchi S, Inagami T. Angiotensin II type 2 receptor inhibits cell proliferation and activates tyrosine phosphatase. *Hypertension.* 1996;28:916–918.
- Bedeck K, Elbaz N, Sutren M, Masson M, Susini C, Strosberg AD, Nahmias C. Angiotensin II type 2 receptors mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase. *Biochem J.* 1997;325:449–454.
- Horiuchi M, Hayashida W, Akishita M, Tamura K, Daviet L, Lehtonen JY, Dzau VJ. Stimulation of different subtypes of angiotensin II receptors, AT₁ and AT₂ receptors, regulates STAT activation by negative crosstalk. *Circ Res.* 1999;84:876–882.

Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

Mass-Spectrometric Identification of a Novel Angiotensin Peptide in Human Plasma

Vera Jankowski, Raymond Vanholder, Markus van der Giet, Markus Tölle, Sevil Karadogan, Johan Gobom, Jens Furkert, Alexander Oksche, Eberhard Krause, Thi Nguyet Anh Tran, Martin Tepel, Mirjam Schuchardt, Hartmut Schlüter, Annette Wiedon, Michael Beyermann, Michael Bader, Mihail Todiras, Walter Zidek and Joachim Jankowski

Arterioscler Thromb Vasc Biol. 2007;27:297-302; originally published online November 30, 2006;

doi: 10.1161/01.ATV.0000253889.09765.5f

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2006 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://atvb.ahajournals.org/content/27/2/297>

Data Supplement (unedited) at:

<http://atvb.ahajournals.org/content/suppl/2006/11/30/01.ATV.0000253889.09765.5f.DC1.html>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:

<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:

<http://atvb.ahajournals.org/subscriptions/>

SUPPLEMENTARY METHODS

CHEMICALS

HPLC water (gradient grade) and acetonitrile were purchased from Merck (Darmstadt, Germany), all other substances from Sigma-Aldrich (Munich, Germany).

CONTROLS AND PATIENTS

Details of the characteristics of patients and controls are given in **Table 1**. A total of 7 patients with stage 5 chronic kidney disease (4 males, 3 females; mean age 66 ± 3 years) who were on regular hemodialysis were investigated. Written informed consent was obtained from each patient and ethical approval by the local ethics committee was obtained for the study. The cause of chronic kidney disease stage 5 was diabetic nephropathy in two cases, nephrosclerosis in 2 cases, polycystic kidney disease in one case and unknown in two cases. One patient was on angiotensin converting enzyme inhibitors, two on β -blockers, two on calcium channel blockers, and four on erythropoietin therapy. None of the patients was a smoker.

Mean duration of hemodialysis at inclusion was 27 ± 11 months. All patients were routinely dialyzed for 4 hours three times weekly using biocompatible membranes with no dialyzer reuse. Dialyses were performed using standardized techniques with bicarbonate-based dialysates and controlled ultrafiltration rate. Blood flow rates were 250 to 300 mL/min, dialysate flow rates were 500 mL/min, dialysate conductivity was 135 mS. Mean kt/V (the amount of plasma cleared of urea divided by the urea distribution volume) was 1.1 ± 0.1 in single pool. Patient dry body weight was defined as the body weight below, which a normal albuminemic patient experiences hypotension or muscle cramps and postural hypotension is clinically manifest. The mean dry weight was 73 ± 5 kg. Blood pressure was measured predialysis with a sphygmomanometer after 10 minutes of recumbency. Phases I and V of the

Korotkoff sounds were taken as the systolic and diastolic blood pressure, respectively. Mean systolic blood pressure was 132 ± 6 mmHg and mean diastolic blood pressure was 74 ± 5 mmHg. Heart rate was 74 ± 5 mmHg.

SIZE-EXCLUSION CHROMATOGRAPHY

We directly fractionated the human plasma by size-exclusion-chromatography. For this, we equilibrated the size-exclusion chromatography gel (Sephacryl S-100 High Resolution; 1000 x 16 mm, S100 HR, Pharmacia BioTech, Uppsala, Sweden) with 0.9% NaCl in water. We loaded human plasma onto the column to elute it with 0.9% NaCl in water at a flow rate of 1 mL/min. We monitored the elution with a UV-detector at 280 nm.

ANALYTICAL REVERSED-PHASE CHROMATOGRAPHY

We loaded the eluate onto a monolithic reversed phase chromatography column (ChromolithTM Performance; 100 x 4.6 mm I.D., Merck, Darmstadt, Germany). We used 0.1% trifluoroacetic acid (TFA) in water as an equilibration buffer (flow rate: 2 mL/min). We eluted the retained substances using 0.1% trifluoroacetic acid (TFA) in water-acetonitrile (20:80, v/v-%) and the following gradient: 0-2 min: 0% eluent B, 2-32 min: 0-75% B, 32-32.5 min: 75- 100% eluent B; 32.5-33.5 min: 100% B; flow rate: 2.0 mL/min. We monitored the elution with a UV-detector at 280 nm.

MEASUREMENTS OF PERFUSION PRESSURE IN THE ISOLATED PERFUSED RAT KIDNEY

The kidney was excised and immediately mounted into the perfusion system. Briefly, the isolated rat kidney was perfused by a peristaltic pump in a single-pass system with a solution containing 115 mmol/L NaCl, 4.6 mmol/L KCl, 1 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 1.2 mmol/L NaH₂PO₄, 22 mmol/L NaHCO₃, 49 mmol/L glucose and 35 g of gelatine/L

(Haemaccel-Behringwerke, Marburg, Germany), and equilibrated with 95% O₂/5% CO₂. The perfusion medium and the kidney were kept constantly at 37°C. Perfusion pressure was continuously monitored by a transducer (Gould P23, Oxnard, CA, USA) connected to a bridge amplifier (Hugo Sachs, March-Hugstetten, Germany).

Vasoconstrictor responses of the isolated perfused rat kidney to the fractions to be tested were assessed after an equilibration period at basal tone of 30 min. When considered relevant the angiotensin-receptor antagonist EXP 3174 (1 µmol/L; AT₁) or PD 123319 (1 µmol/L, AT₂) was added to the perfusate 30 min before challenge with the fractions to be tested.

IDENTIFICATION OF THE ISOLATED PEPTIDE BY MASS SPECTROMETRY

Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) measurements were performed using a Voyager-DE STR BioSpectrometry Workstation mass spectrometer (Perspective Biosystems, Framingham, MA, USA). One mL of the peptide solution was mixed with 1 mL of α -cyano-4-hydroxycinnamic acid matrix solution consisting of 10 mg of matrix dissolved in 1 mL of 0.3% TFA in acetonitrile/water (50:50, v/v-%). From the resulting mixture 1 mL was applied to the sample plate. Samples were air-dried at ambient temperature (24°C). Measurements were performed in the reflectron mode at an acceleration voltage of 20 kV, 70% grid voltage and a delay of 150 ns. Each spectrum represented the accumulation of 250 laser shots. Three spectra of one spot measured at different positions were averaged. MS/MS fragment spectra were recorded using a matrix-assisted laser desorption/ionisation time-of-flight / time-of-flight (MALDI-TOF/TOF) instrument (4700 Proteomics analyzer, Applied Biosystems, Framingham, MA, USA) equipped with an Nd:YAG laser (355 nm) operating at a frequency of 200 Hz. Air was used as the collision gas. Spectra were obtained by accumulation of up to 20,000 consecutive laser shots. Fragment data were analysed using the Data Explorer software (version V5.1; Applied Biosystems, Darmstadt, Germany).

PREPARATION OF MONONUCLEAR LEUKOCYTES

Briefly, 400 mL heparinized blood was drawn by venipuncture from the antecubital vein and centrifuged at 240 g for 15 min. After removing the supernatant, mononuclear leukocytes were isolated by layering 5 mL diluted blood (50:50 v/v-% with isotonic NaCl) on 3 mL Histopaque (Sigma-Aldrich, Munich, Germany; 5:6 w/v-% Ficoll; density 1.077 g/mL) and centrifugation at 240 g for 20 min. The mononuclear leukocyte interphase was carefully aspirated, washed three times in isotonic NaCl by centrifugation at 400 g for 5 min, and resuspended in Hanks' balanced salt solution containing (in mmol/L): NaCl, 136; KCl, 5.4; KH_2PO_4 , 0.44; Na_2HPO_4 , 0.34; CaCl_2 , 1; D-glucose, 5.6; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 10; pH 7.4. [Mononuclear leukocytes were incubated in the absence and presence of 10 \$\mu\text{g}\$ lipopolysaccharide \(LPS Serotype 0111:B4 from E. coli\).](#) After incubation for 60 minutes, mononuclear leukocytes were centrifuged to collect the supernatant.

BINDING STUDIES

All binding studies were performed as displacement studies. For binding studies of Ang A with AT_1 - and AT_2 -receptors, HEK 293 cells were transiently transfected with human AT_1 - and AT_2 -receptors. cDNA encoding the human AT_1 (pcDNA3. AT_1)- and AT_2 (pcDNA3. AT_2)-receptor was kindly provided by Tadashi Inagami (Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee, USA).

CELL CULTURE AND TRANSFECTION METHODS

HEK 293 cells were cultured at 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$). Cells were grown on poly-L-lysine-coated plastic material to improve adherence. For a 15-mm-diameter well of a 24-well plate, 5×10^4 HEK 293 cells were transfected with 250

ng of plasmid DNA and 2 mL of Lipofectamin according to the supplier's recommendations. After removal of the transfection reagent, cells were incubated for 48 h.

MEMBRANE PREPARATION FOR RECEPTOR BINDING STUDIES

The pellet was resuspended in Tris-BAME buffer (50 mmol/L Tris, 0.15 mmol/L bacitracin, 0.0015% aprotinin, 10 mmol/L MgCl₂, 2 mmol/L EGTA, pH 7.3), and the suspension was homogenized with a glass/Teflon homogenizer (10 strokes), and centrifuged at 26,000 g for 30 min. The pellet was rehomogenized in Tris-BAME and aliquots of the resulting suspension were stored at -70°C until use.

¹²⁵I-SAR1,ILE⁸-ANG II DISPLACEMENT BINDING ANALYSIS

The samples were then transferred onto GF/C filters (Whatman International Ltd., Maidstone, UK), pretreated with 0.1 (w/v-%) polyethylenimine and washed rapidly twice with PBS using a Brandel cell harvester. Filters were finally transferred into 5 mL vials and radioactivity was determined in a liquid scintillation counter. Data were analysed with RadLig software 4.0 (Cambridge, UK), and graphs were generated with Prism Software 2.02 (GraphPad, San Diego, CA). Saturation analysis yielded K_D values of 1 µmol/L and 100 nmol/L for the AT₁- and AT₂-receptor.

CALCULATION OF DISPLACEMENT CURVES

For AT-receptor displacement studies, non-specific binding was accounted for by determining the number of counts measured in the presence of 1 µmol/L Sar¹, Ile⁸ Ang II. The values obtained were deduced from the total counts to calculate specific binding. Basal values (dpm, mean ± S.E.M., n=12 – 15) of 100% binding were 2670 ± 157 (AT₁-receptor) and 6015 ± 213 (AT₂-receptor). Specific binding in the sole presence of radioligands was normalized to

100%, and the remaining data were corrected to this figure. IC₅₀-values were calculated using a non-linear curve fitting function (Prism™, Graph Pad, San Diego, CA. USA).

Ca²⁺- FLUORESCENT IMAGE PLATE READER (FLIPR) ASSAY

VSMCs were loaded with 1 μM FLUO4-/AM and 0.04% Pluronic F-127 (both from Molecular Probes, Carlsbad, Ca, USA) in HBSS with 20 mmol/L HEPES and 2.5 mmol/L probenecid. After loading, cells were washed twice with HBS by an automated plate washer (Denley Cellwash, Labsystems, Helsinki, Finland) and transferred to the FLIPR (Mithras LB940, Berthold, Bad Wildungen, Germany). About 50,000 cells were in each well. 10 μl of agonists were delivered to the wells containing 100 μl of HBS. Fluorescence emissions from the wells were monitored at an emission wavelength of 515 nm and after excitation with 488 nm. Fluorescence data were collected 60 s before and 5–10 min after stimulation, and analysed off-line.

SYNTHESIS OF THE IDENTIFIED PEPTIDE

The identified peptide was synthesized automatically by the solid-phase method using standard Fmoc chemistry in a continuous flow mode. TentaGel S Random-Access Memory (RAM) resin 0.21 mmol/g was used for peptide amide synthesis. TentaGel S p-hydroxybenzoic acid (PHB) resin (Rapp Polymere, Tuebingen, Germany) was used for the synthesis of the free acid of urocortin, o-benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), n,n-diisopropylethylamine (DIEA) (condition: coupling 20 min, deblocking with 20% piperidine in N,N-dimethyl formamide (DMF) for 15 min, final cleavage with 95% TFA/5% water for 3 h). Purification of crude peptide was carried out by preparative HPLC on PolyEncap A300 (10 μm particle size, 250 mm x 20 mm, Bischoff Analysentechnik GmbH, Leonberg, Germany) in water with increasing concentrations of acetonitrile (ACN) as mobile phase. An eluent gradient of 5-70 (v/v-%) ACN/water (0.1%

TFA) over 70 min with a flow rate of 10 mL/min was used. The purified peptide was lyophilized and characterized by MALDI mass spectroscopy as described above using α -cyano-4-hydroxycinnamic acid and sinapinic acid as matrix and this procedure resulted in the same $[M + H]^+$ mass peaks as the genuine product after its direct isolation ¹.

QUANTIFICATION OF ANG A AND ANG II

The adrenocorticotrophic hormone (ACTH) fragment 18-39 (Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe; 10 μ g per sample) was added as internal standard. Hereby local differences in the peptide concentration on the MALDI spot were neutralized. The lyophilised fractions of the reversed-phase chromatography of the plasma were resuspended in 10 μ L 0.1% TFA before quantification. The supernatants of mononuclear leukocytes after incubation with Ang II were directly quantified. 1 μ L of each fraction was prepared on a prestructured MALDI sample support (MTP AnchorChipTM 400/384, Bruker-Daltonics, Bremen, Germany) using the α -4-hydroxycinnamic acid affinity sample preparation method ². The mass spectrometric measurements were performed on a Bruker Reflex III instrument (Bruker-Daltonics, Bremen, Germany). The instrument was equipped with a nitrogen laser (VSL-337 ND, Laser Science, USA), emitting at 337 nm. The laser beam was focused to a typical diameter of 50 μ m at an angle of 45° to the surface of a target. Samples were checked microscopically. On average, 200 single-shot spectra were accumulated to improve the signal-to-noise ratio.

QUANTIFICATION OF ANG II IN PRESENCE AND ABSENCE OF ANG A BY ENZYME IMMUNOASSAY

The kit was used according to the manufacturer's protocol ³. Briefly, each well of the plate containing immobilised monoclonal anti-Angiotensin II (mAb3 131) ³ was washed five times with a washing buffer (0.01 mol/L phosphate with 0.05% Tween 20; pH 7.4). Different

aqueous mixtures of Ang A and Ang II (100 μ l; 1-100 pg/mL) were distributed over the wells. After an incubation time of 1 h, glutaraldehyde (50 μ l; 0.5 vol-% in 0.1 mol/L phosphate buffer; pH 7.5) was added and the plate was incubated for 5 min at room temperature. Next, borane trimethylamine (50 μ l in 2 mol/L HCl/MeOH (50/50 vol-%) was added for 5 min. After washing five times, an anti-angiotensin II IgG tracer (Société de Pharmacologie et Immunologie, BIO, Massy Cedex, France) (100 μ l; 5 EU/mL) was added to the plate. The plate was incubated overnight at +4°C. After washing five times, 200 μ l Ellman's reagent (0.75 mmol/L acetylthiocholine iodide, 14.5 mmol/L NaCl, 25 mmol/L, 5,5'-dithiobis(2-nitro) benzoic acid, 0.1 mol/L phosphate buffer, pH 7.4) was added. The plate was read at 414 nm after 15 min using an automatic microplate reader (Microplate-Reader 3550, BioRad, Munich, Germany).

IMPACT OF ANG A ON BLOOD PRESSURE IN ANGIOTENSIN II RECEPTOR AT1A KNOCKOUT MICE

All animal experiments were performed according to the institutional guidelines of the Max-Delbrück-Center for Molecular Medicine (Berlin, Germany) and were approved by the local authorities. Mice were anaesthetized with Ketavet (Ketaminhydrochlorid) 100 mg/kg + Rompun (Xylazinehydrochlorid) 10 mg/kg. Applying aseptic techniques, catheters (4 to 5 cm length, 0.25 mm inner diameter, and 0.40 mm outer diameter) were placed in the femoral artery for the measurement of arterial pressure and in the femoral vein for infusions with the aid of a dissecting microscope. The catheters were filled with sterile 0.9% NaCl solution containing heparin (10 U/ml) and tunneled subcutaneously, exteriorized at the back of the neck, and sutured in place between the scapulae. The mice were allowed to recover for 72 h before the experiment was started. During the recovery period 24-48 h after surgery, catheters were flushed with heparinized saline solution (3-5 μ l/gbw).

On the day of the experiment, mice were tested in a conscious, unrestrained state in their home cages. Sixty minutes after the arterial catheter had been coupled with a 23-gauge stainless steel pin to a 25-cm piece of Portex polyethylene tubing (PE-50; 0.58 mm inner diameter, 0.96 mm outer diameter) to a MLT 1050/D pressure transducer (AD Instruments, Colorado Springs, USA), baseline blood pressure and heart rate were measured continuously for 1 hour after the coupling. All hemodynamic data were collected and analyzed on a computer using Chart software (Version 5; Powerlab; Colorado Springs, USA).

Angiotensin II and Ang A were dissolved in a 0.9% NaCl solution and injected in bolus via the venous line in a volume of 1 μ l/gbw. Before each experimental infusion, the response to vehicle was tested.

STATISTICS

All data are given as means \pm S.E.M. of the individual values. For the displacement experiments, Friedman's test was used to compare means of controls with the binding observed in the presence of displacing substances.

REFERENCES

1. Beyermann M, Fechner K, Furkert J, Krause E, Bienert M. A single-point slight alteration set as a tool for structure-activity relationship studies of ovine corticotropin releasing factor. *J Med Chem.* 1996;39:3324-3330.
2. Gobom J, Schuerenberg M, Mueller M, Theiss D, Lehrach H, Nordhoff E. Alpha-cyano-4-hydroxycinnamic acid affinity sample preparation. A protocol for MALDI-MS peptide analysis in proteomics. *Anal Chem.* 2001;73:434-438.
3. Volland H, Pradelles P, Ronco P, Azizi M, Simon D, Creminon C, Grassi J. A solid-phase immobilized epitope immunoassay (SPIE-IA) permitting very sensitive and specific measurement of angiotensin II in plasma. *J Immunol Methods.* 1999;228:37-47.