Angiotensin IV Is a Potent Agonist for Constitutive Active Human AT₁ Receptors

DISTINCT ROLES OF THE N- AND C-TERMINAL RESIDUES OF ANGIOTENSIN II DURING AT₁ RECEPTOR ACTIVATION

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The octapeptide hormone, angiotensin II (Ang II), exerts its major physiological effects by activating AT₁ receptors. In vivo Ang II is degraded to bioactive peptides, including Ang III (angiotensin-(2–8)) and Ang IV (angiotensin-(3–8)). These peptides stimulate inositol phosphate generation in human AT₁ receptor expressing CHO-K1 cells, but the potency of Ang IV is very low. Substitution of Asn₁¹¹ with glycine, which is known to cause constitutive receptor activity by disrupting its interaction with the seventh transmembrane helix (TM VII), selectively increased the potency of Ang IV (900-fold) and angiotensin-(4–8), and leads to partial agonism of angiotensin-(5–8). Consistent with the need for the interaction between Arg² of Ang II and Ang III with Asp²⁸¹, substitution of this residue with alanine (D281A) decreased the peptide’s potency without affecting that of Ang IV. All effects of the D281A mutation were superseded by the N111G mutation. The increased affinity of Ang IV to the N111G mutant was also demonstrated by binding studies. A model is proposed in which the Arg²-Asp²⁸¹ interaction causes a conformational change in TM VII of the receptor, which, similar to the N111G mutation, eliminates the constraining intramolecular interaction between Asn₁¹¹ and TM VII. The receptor adopts a more relaxed conformation, allowing the binding of the C-terminal five residues of Ang II that switches this “preactivated” receptor into the fully active conformation.

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1 The abbreviations used are: Ang II, angiotensin II (DRVYIHPF); Ang III, angiotensin III; Ang IV, angiotensin IV; DiVal Ang IV (VYVYIHPF); GPCR, a G-protein-coupled receptor; IP, inositol phosphate; R, inactive receptor conformation; R*, preactivated receptor conformation; R″, activated receptor conformation; DMEM, Dulbecco’s modified Eagle’s medium.
angiotensin peptide ligands (14). In the present study we have investigated the interaction between different angiotensin peptides and wild type, partially activated (N111G) and Asp281 mutant (D281A) AT₁ receptors to study the role of Asp¹ and Arg² during receptor activation. It is shown that Arg² is only implied during the initial step of agonist-induced activation of the receptor and that Ang IV is a full and potent agonist for the N111G constitutive active receptor.

EXPERIMENTAL PROCEDURES

Mutagenesis of Human AT₁ Receptor DNA, Cell Culture, and Transient Transfection—The human AT₁ receptor gene, cloned in the mammalian expression vector pcDNA3 (Invitrogen), was used for expression and mutagenesis. The mutation of Asn¹¹¹ to Gly, Asp²⁸¹ to Ala, or their combination was created by the polymerase chain reaction method using the Mutagene kit (Bio-Rad). The DNA sequence of mutated receptor was confirmed by dideoxynucleotide sequencing (Amersham Biosciences). The wild type and the mutated receptors were transiently expressed in CHO-K1 cells and cultured as described previously (15).

Measurement of Inositol Phosphate Accumulation—Transfected cells were labeled for 20 h with 1 µCi/ml myo-[³²P]inositol (Amersham Biosciences). The labeled cells were washed twice with DMEM medium and incubated with this medium containing 10 mM LiCl for 15 min. Then agonists were added, and the incubation was continued for another 15 min at 37 °C. At the end of incubation, the medium was removed, and the total soluble inositol phosphate (IP) was extracted from the cells as described previously (15). The amount of [³²P]IP eluted from the column was counted and a concentration-response curve generated using non-linear regression analysis. DiVal Ang IV was synthesized by Dr. G. Munske (Washington State University).

Radioligand Binding Assays—Competition binding experiments were carried out at 37 °C for 1 h on intact adherent cells as described previously (16) using 1.5 nM [³H]candesartan (for the wild type and D281A mutant receptor) or 5 nM [³H]candesartan (for the N111G and N111G/D281A mutant receptor). The equilibrium dissociation rate constant of [³H]candesartan was determined by non-regression analysis using GraphPad Prism (San Diego, CA) of saturation binding curves (at a concentration range between 0.15 and 15 nM) for the N111G and N111G/D281A receptors or of association and dissociation curves for the wild type and D281A receptors (at a radioligand concentration of 1.5 nM). [³H]Candesartan (17 Ci/mmol) was obtained from AstraZeneca. Nonspecific binding was determined in the presence of 1 µM unlabeled candesartan. Monodinated [²²⁵]IAng IV was a kind gift of Prof. G. Slegers (Radiopharmacy Department, University of Ghent). The [²²⁵]IAng IV binding assay was carried out similarly, and non-specific binding was determined in the presence of 1 µM unlabeled Ang IV.

RESULTS

Wild type human AT₁ receptors and mutant receptors, in which Asn¹¹¹ was replaced by glycine (N111G), Asp²⁸¹ was replaced by alanine (D281A), or in which both replacements were combined, were transiently expressed in CHO-K1 cells. Their function was assessed by measuring the inositol phosphate accumulation. The higher basal level of inositol phosphates in cells with the N111G and the double mutated receptor agrees with previous findings (7, 10, 13). As shown in Fig 1 and Table I, Ang II and its N-terminal amino acid-deleted fragment Ang III were almost equipotent agonists for the wild type receptor. Ang IV (deletion of two N-terminal amino acids) and DiVal Ang IV were full agonists but with much weaker potency. On the other hand the C-terminal pentapeptide angiotensin-(4–8) was barely active and the tetrapeptide angiotensin-(5–8) was completely inactive at concentrations up to 100 µM. The N111G substitution only minimally affected the binding affinity of Ang II and Ang III, but it produced a dramatic increase in the potency of Ang IV (900-fold) and of DiVal Ang IV (1500-fold) (Table I). Concentration-response curves of these four investigated agonists were now alike for the N111G-mutated AT₁ receptor. The potency of angiotensin-(4–8) also increased dramatically, and it became a full agonist with a somewhat lower potency (EC₅₀ = 28.3 nM) compared with Ang II. On the other hand, angiotensin-(5–8), which lacks the important Tyr⁴ residue, was only a very weak and partial agonist for the N111G-mutated receptor (Fig. 1). In parallel, the binding affinities of Ang II, Ang III, Ang IV, and DiVal Ang IV were investigated by competition binding experiments with the highly potent and AT₁ receptor-selective antagonist

| Inositol phosphate production, EC₅₀ | Wild type | N111G | N111G/D281A | D281A |
|-----------------------------------|----------|-------|-------------|-------|
| EC₅₀ (nM)                         |          |       |             |       |
| Ang II                            | 1.55 ± 0.32 | 1.20 ± 0.28 | 2.29 ± 0.28 | 52 ± 20 |
| Ang III                           | 3.43 ± 1.05 | 3.78 ± 1.14 | 2.13 ± 0.11 | 228 ± 44 |
| Ang IV                            | 1244 ± 170 | 1.39 ± 0.25 | 1.51 ± 0.43 | 2590 ± 835 |
| DiVal Ang IV                      | 4162 ± 708 | 2.64 ± 0.56 | 1.47 ± 0.52 | 8352 ± 604 |

| [³H]Candesartan competition binding, Kᵣ (nM) |
|---------------------------------------------|
| Ang II                                      | 13.9 ± 3.2 | 1.04 ± 0.25 | 1.44 ± 0.96 | >1000 |
| Ang III                                     | 18.2 ± 0.9 | 3.38 ± 0.64 | 3.07 ± 0.25 | >1000 |
| Ang IV                                      | >1000      | 14.5 ± 3.4  | 11.3 ± 3.4  | >1000 |
| DiVal Ang IV                                | >1000      | 41.4 ± 5.5  | 94.2 ± 20.9 | >1000 |
| Candesartan                                 | 0.055 ± 0.007 | 5.58 ± 0.80 | 5.40 ± 0.04 | 0.18 ± 0.09 |

![Graph showing concentration-response curves of angiotensin-(4–8) and angiotensin-(5–8) for wild type and mutant AT₁ receptors.](Image)
Insight into the molecular mechanism by which Ang II activates the AT₁ receptor has been gained by structure-activity relationship studies with peptide analogs as well as receptor mutants. In this context Arg² as well as the aromatic residues Tyr⁴ and Phe⁸ of Ang II have essential roles in the binding and receptor activation (12, 17, 18). Measuring inositol phosphate production, this mutation caused by the absence of Arg², because Ang III (which is only different from Ang IV in this residue) is a fully potent agonist. On the other hand Asp¹ of Ang II is not involved in this process, since Ang III displays the same characteristics both for the wild type and the mutant receptor. This observation is in agreement with previous findings indicating that Asp¹ of Ang II is not required for agonism and only weakly interacts with amino acid residues such as His¹⁸³ of the receptor (14).

Noda et al. (13) have proposed that full activation of the AT₁ receptor is preceded by the formation of a preactivated state. It was also suggested that the N111G mutation mimics the preactivated state of the receptor, because it impedes the interaction of Asn¹¹¹ with TM VII that stabilizes the inactive conformation and, as a consequence, produces constitutive activation (13). To explain our findings and previous data we propose that Arg² of Ang II plays an important role in this preactivation process or, in other words, that Arg² participates in the destabilization of the receptor’s inactive conformation. This preactivation goes along with the opening of a transmembrane pocket, which provides optimal binding of the C-terminal amino acids. Furthermore, our model shows that once the receptor is in this preactivated state, the five C-terminal amino acids contain the essential information required for full receptor activation. Indeed, Ang IV is a full agonist, and its low potency suggests that receptor preactivation can occur spontaneously even in the absence of Arg².

To further evaluate the role of Arg² of Ang II, mutant AT₁ receptors were created based on the assumption that this amino acid interacts with Asp²⁸¹ located at the extracellular end of TM VII of the receptor (14). Both our functional and binding studies on Asp²⁸¹ to alanine mutated receptor are in agreement with this proposal and previous findings. When measuring inositol phosphate production, this mutation caused a 33-fold and 66-fold increase of the EC₅₀ of Ang II and Ang III, respectively, and their potency was also markedly increased for the N111G-mutated but not in wild type AT₁ receptor-expressing cells (Fig. 2).

The impact of the D281A mutation on binding and function of the angiotensin related peptides was examined. The concentration-response curves for Ang IV and DiVal Ang IV were only modestly affected by the D281A substitution (Fig. 1). [³H]Candesartan binding (Table I) was also only marginally affected by this mutation. On the other hand, the concentration-response curves of the Ang II and Ang III were shifted to the right by 33- and 66-fold, respectively, and their potency was also markedly reduced in competition binding experiments. The effects of the D281A substitution could be completely reversed by the N111G mutation. The functional properties of the angiotensin peptides as well as their binding affinities were the same in N111G/D281A when compared with the single N111G substitution.

**DISCUSSION**

Insight into the molecular mechanism by which Ang II activates the AT₁ receptor has been gained by structure-activity relationship studies with peptide analogs as well as receptor mutants. In this context Arg² as well as the aromatic residues Tyr⁴ and Phe⁸ of Ang II have essential roles in the binding and receptor activation (12, 17, 18). Measuring inositol phosphate production in human AT₁ receptor-expressing CHO-K1 cells (16). The weak potency of Ang IV and angiotensin-(4–8) to preactivate the wild type receptor is caused by the absence of Arg², because Ang III (which is only different from Ang IV in this residue) is a fully potent agonist. On the other hand Asp¹ of Ang II is not involved in this process, since Ang III displays the same characteristics both for the wild type and the mutant receptor. This observation is in agreement with previous findings indicating that Asp¹ of Ang II is not required for agonism and only weakly interacts with amino acid residues such as His¹⁸³ of the receptor (14).

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**Fig. 2. Specific binding of [¹²⁵I]Ang IV to wild type and N111G mutant AT₁ receptors.** CHO-K1 cells expressing wild type (filled squares) or N111G (filled triangles) mutated human AT₁ receptors were incubated with 0.5 nM [¹²⁵I]Ang IV at 37 °C for the indicated periods of time. Specific binding was calculated by subtracting nonspecific binding in the presence of 1 μM unlabeled Ang IV.
and, likewise, a dramatic reduction in their affinity in competition binding studies. As expected, the D281A mutation had only marginal effect on the potency of Ang IV and DiVal Ang IV. Our proposal that the Arg2–Asp281 interaction is only necessary during receptor preactivation is further supported by findings with a receptor in which the N111G mutation is combined with the D281A mutation. Compared with the single D281A mutation, this double mutation rescues the impaired binding affinity and potency of Ang II and Ang III. It also produces a dramatic increase in the affinity and potency of Ang IV compared with the wild type or D281A mutant receptor. The N111G/D281A double mutant receptor not only displays the same agonist binding characteristics as the constitutively active N111G mutant, it also increases basal inositol phosphates production (data not shown), which is characteristic for the preactivated state of the receptor. Taken together, preactivation of the receptor by Ang II may involve the disruption of the Asn111–TM VII interaction along with the binding of Arg2 of the peptide to Asp281 to stabilize the new receptor conformation. Because the Asn111–TM VII interaction is already disrupted by the N111G mutation, this receptor mutant does not require Arg2 to adopt the preactivated state. Asn111 is likely to play a dual role. It stabilizes the inactive conformation of the receptor by interacting with TM VII, and it also interacts with Tyr4 of the ligand during the process of receptor preactivation. When the receptor is preactivated this latter interaction is probably no longer required to obtain full receptor activation, since Noda et al. (13) demonstrated that [Sar4, His4, Ile8]Ang II (where Sar indicates sarcosine) cannot activate the wild type receptor while being an agonist of the N111G mutant receptor.

Altered antagonist binding is characteristic of constitutive active AT1 receptors (7, 9, 10, 20). It was shown previously that the N111G mutant receptor has decreased affinity to losartan, a surmountable non-peptide antagonist of the AT1 receptor (9). Our data show that compared with the wild type AT1 receptor the N111G mutant receptor also has 100-fold lower affinity for the insurmountable non-peptide antagonist candesartan. The reduced affinity of non-peptide antagonists to this mutant receptor suggests that, while receptor preactivation causes optimal alignment of the binding pocket for the C-terminal residues of Ang II, it also causes misalignment of the residues involved in the binding of non-peptide AT1 receptor blockers. Since non-peptide AT1 receptor blockers have much higher affinity to the inactive conformation of the receptor, they could act as inverse agonists irrespectively of their surmountable/insurmountable character.

In conclusion, our data show that the two N-terminal amino acid residues of Ang II are no longer required for its high affinity binding to the N111G mutated AT1 receptor (i.e. the receptor in a preactivated state) and that Val3 plays only a minor role in this process. Based on these findings, we propose a model of AT1 receptor activation that pinpoints the role of Arg2 of Ang II in the preactivation process, i.e. it stabilizes a conformation of the receptor that is optimal for binding of the C-terminal five residues of Ang II (Fig. 3). These C-terminal residues are then sufficient to produce full receptor activation. Furthermore, whereas Asn111 stabilizes the inactive conformation of the receptor by interacting with TM VII, it is also likely that it interacts with Tyr4 of the ligand during the preactivation process of the receptor (13).

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