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Angiotensin II cyclic analogs as tools to investigate AT_1R biased signaling mechanisms

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ABSTRACT

G protein coupled receptors (GPCRs) produce pleiotropic effects by their capacity to engage numerous signaling pathways once activated. Functional selectivity (also called biased signaling), where specific compounds can bring GPCRs to adopt conformations that enable selective receptor coupling to distinct signaling pathways, continues to be significantly investigated. However, an important but often overlooked aspect of functional selectivity is the capability of ligands such as angiotensin II (AngII) to adopt specific conformations that may preferentially bind to selective GPCRs structures. Understanding both receptor and ligand conformation is of the utmost importance for the design of new drugs targeting GPCRs. In this study, we examined the properties of AngII cyclic analogs to impart biased agonism on the angiotensin type 1 receptor (AT_1R). Positions 3 and 5 of AngII were substituted for cysteine and homocysteine residues ([$Sar^1Hcy^{3,5}$]AngII, [$Sar^1Cys^3Hcy^5$]AngII and [$Sar^1Cys^{3,5}$]AngII) and the resulting analogs were evaluated for their capacity to activate the Gq/11, G12, Gi2, Gi3, Gz, ERK and β-arrestin (βarr) signaling pathways *via* AT_1R. Interestingly, [$Sar^1Hcy^{3,5}$]AngII exhibited potency and full efficacy on all pathways tested with the exception of the Gq pathway. Molecular dynamic simulations showed that the energy barrier associated with the insertion of residue Phe^8 of AngII within the hydrophobic core of AT_1R, associated with Gq/11 activation, is increased with [$Sar^1Hcy^{3,5}$]AngII. These results suggest that constraining the movements of molecular determinants within a given ligand by introducing cyclic structures may lead to the generation of novel ligands providing more efficient biased agonism.

1. Introduction

The octapeptide hormone angiotensin II (AngII) is the active component of the renin-angiotensin system, responsible for controlling blood pressure and water retention via smooth muscle contraction and ion transport. It exerts a wide variety of physiological effects, including vascular contraction, aldosterone secretion, neuronal activation, and cardiovascular cell growth and proliferation. Virtually all the known physiological effects of AngII are produced through the activation of the AT_1 receptor (AT_1R), which belongs to the G protein-coupled receptor (GPCR) superfamily [1] and whose structure in complex with a selective AT_1R antagonist has recently been elucidated [2].

The AT_1R interacts with Gq/11 leading to the activation of phospholipase C (PLC), in turn leading to the formation of diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3). IP_3 binds to the IP_3 receptor on the endoplasmic reticulum, whereupon Ca^{2+} is released into the cytosol. Together, Ca^{2+} and DAG allow the activation of protein kinase C (PKC) [3]. Also, AT_1R interacts with G12, thereby activating RhoA and ROCK, via RhoGEF regulation, leading to cytoskeleton reorganization [4]. Additionally, reports have demonstrated that AT_1R interacts with Gi, thereby leading to an inhibition of cAMP production [5–7]. The AT_1R can also activate the ERK1/2 kinase pathway mediated by PKC (G protein-dependent) or by EGFR transactivation, which is G protein-independent [8,9]. Following receptor activation, G protein-coupled receptor kinases (GRKs) phosphorylate the AT_1R, facilitating β-arrestin (βarr) recruitment and terminating G protein signaling [10]. βarrs are involved in the desensitization and internalization of GPCRs [11] but also serve as scaffolds for further GPCR signaling to the MAPK pathway [12].

Biased signaling is the ability of a ligand to stabilize a receptor under a particular conformation that promotes activation of specific signaling pathways over others [13,14]. The therapeutic potential of functional selectivity is increasingly exploited for the design of new drugs since some signaling pathways produce beneficial effects while others can have harmful consequences. For example, activation of the Gq/11 pathway by AngII may cause adverse effects to the failing heart

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by increasing blood pressure, while βarr recruitment can produce a beneficial effect by promoting cardiomyocyte growth, thus improving heart performance [15,16]. The therapeutic potential of AT_1R signaling via the G12 and Gi pathways has yet to be evaluated.

Based on extensive photolabeling experiments [17], we have demonstrated that the conformation of AngII is highly dynamic even when interacting with the AT_1R. We have also proposed an integrative model of this complex and unveiled structural and dynamical determinants that favor Gq/11 or βarr signaling. Of note, we have shown that Gq/11 signaling is promoted by the opening of a hydrophobic core just above the so-called major H-Bond network (MHN) or sodium binding site [18], notably by the insertion in this core of the C-terminal Phe^8 residue of AngII. In addition, the perturbation of the MHN was shown to modulate the signaling outcome. Our model of the dynamical AngII-AT_1R complex and molecular dynamics simulations also suggest that the backbone of AngII can adopt multiple conformations.

We have recently shown that changes at positions 4 and 8 of AngII can lead to biased signaling of the AT_1R [19]. To further pursue our understanding of the molecular and dynamical basis of functional selectivity, we asked how constraining the AngII backbone and thus limiting its dynamical behavior would impact the signaling pathways of the AT_1R. Therefore, we synthesized AngII analogs that were substituted at positions 3 and 5 with either cysteine or homocysteine (Hcy) and cyclised through the formation of a disulfide bond. Using this strategy, we synthesized [$Sar^1Hcy^{3,5}$]AngII, [$Sar^1Cys^3Hcy^5$]AngII and [$Sar^1Cys^{3,5}$]AngII. This cyclisation scheme through the oxidation of the sulfhydryl group has the advantage of limiting the changes in physiochemical properties and bulkiness of the cycle. Note that previous work has shown that cyclisation through these side-chains of AngII led to compounds that can still bind and elicit Gq/11 activation [20,21]. However, the impact on the biased signaling has not been characterized. Here, we investigate the impact of restraining the conformation of AngII on the binding and functional selectivity of the AT_1R by measuring the Gq, G12, Gi2, Gi3, Gz, ERK and βarr signaling pathways. We then performed molecular dynamics simulations to evaluate the impact of AngII cyclic analogs on the conformational landscape of the AT_1R.

2. Materials and methods

2.1. Materials

Culture media, trypsin, FBS, penicillin, and streptomycin were from Wisent (St-Bruno, Qc, Canada). OPTI-MEM was from Invitrogen Canada Inc. (Burlington, ON). Polyethyleneimine (PEI) was from Polysciences (Warrington, PA). Go6983 and PD168393 were from EMD Millipore (Missisauga, ON). ^{125}I-AngII (specific radioactivity ~1000 Ci/mmol) was prepared with Iodo-GEN® (Perbio Science, Erembodegem, Belgium) as reported previously [22].

2.2. Peptide synthesis

Peptides were synthesized by manual solid-phase peptide synthesis using Fmoc-protected strategy on Wang resin (Fmoc-protected amino acids and resin were purchased from Novabiochem, Missisauga, ON). Peptides were cleaved with 95% TFA adding EDT and TLS as scavengers. The crude peptides were then cyclized in 2 M $(NH_4)_2CO_3$, pH 6.5, under constant agitation for 4 h at room temperature. Peptides were then purified on a preparative HPLC mounted with a C_{18} column and using a 10–35% gradient of acetonitrile containing 0.05% TFA. Fractions were analysed on an analytical HPLC mounted with a C_{18} column and using a 5–95% acetonitrile gradient containing 0.05% TFA. Pure fractions were pooled, lyophilized, and stored at −20 °C in a dry environment until further use. The pure peptides were characterized on UPLC-MS and showed purity > 95%. Structure of the compounds and UPLC-MS spectra are available through Figshare at https://doi.org//10.6084/m9.figshare.6108440.

2.3. Constructs

The cDNA clone for the human AT_1R was kindly provided by Dr. Sylvain Meloche (University of Montréal). The AT_1R-GFP10 construct was built by inserting the GFP10 sequence at the C-terminus of the AT_1R, joined by a linker sequence (GSAGT) using the In-Fusion® PCR cloning system (Clontech Laboratories, Mountain View, CA) as recommended by the manufacturer. The RLucII-βarr1, RLucII-βarr2, Gα12-RLucII, Gαi2-RlucII, Gαi3-RlucII, Gz-RlucII, Gβ1, Gγ1-GFP10 and Gγ2-GFP10 constructs were kindly provided by Dr. Michel Bouvier (University of Montréal). All constructs were confirmed by automated DNA sequencing.

2.4. Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were maintained in DMEM medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. HEK293 cells stably expressing the AT_1R were maintained in medium containing 0.5 mg/mL G418. For the βarr recruitment assays, HEK293 cells (3×10^6 cells) were transiently transfected with 8700 ng of AT_1R-GFP10 and either 300 ng of RlucII-βarr1 or 300 ng of RlucII-βarr2 using linear polyethylenimine (1 mg/ml) (PEI:DNA ratio 4:1). For G12 activation assays, HEK293 cells (3×10^6 cells) were transiently cotransfected with the following constructs: 3000 ng of AT_1R, 600 ng Gα12-RLucII, 3000 ng Gγ1-GFP10 and 1800 ng Gβ1, using linear polyethylenimine (PEI:DNA ratio 4:1). For Gi2 and Gi3 activation assays, HEK293 cells (3×10^6 cells) were transiently cotransfected with the following constructs: 3000 ng of AT_1R, 600 ng Gαi2-RLucII or Gαi3-RLucII, 3000 ng Gγ2-GFP10 and 3000 ng Gβ1, using linear polyethylenimine (PEI:DNA ratio 3:1). For Gz activation assays, HEK293 cells (3×10^6 cells) were transiently cotransfected with the following constructs: 3000 ng of AT_1R, 600 ng Gz-RLucII, 3000 ng Gγ1-GFP10 and 3000 ng Gβ1, using linear polyethylenimine (PEI:DNA ratio 3:1).

2.5. Binding experiments

HEK293 cells stably expressing the AT_1R were washed once with PBS and submitted to one freeze–thaw cycle. These broken cells were then gently scraped into washing buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$), centrifuged at 2500 × g for 15 min at 4 °C, and resuspended in binding buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% bovine serum albumin, 0.01% bacitracin). Dose displacement experiments were done by incubating broken cells (20–40 μg of protein) for 1 h at room temperature with 0.8 nM ^{125}I-AngII as tracer and increasing concentrations of AngII or analogs. Bound radioactivity was separated from free ligand by filtration through GF/C filters presoaked for at least 3 h in binding buffer. Receptor-bound radioactivity was evaluated by γ counting. Results are presented as means ± S.D. The K_i values in the displacement studies were determined from the IC_{50} values using the Cheng-Prusoff equation.

2.6. Measuring inositol-1 phosphate production

We used the IP-One assay (Cisbio Bioassays, Bedford, MA) to measure inositol 1-phosphate (IP_1) levels. Necessary dilutions of each analog were prepared in stimulation buffer (10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH 7.4). HEK293 cells stably expressing the AT_1R were washed with PBS at room temperature, then trypsinized and distributed at 15,000 cells/well (7 μl) in a white 384-well plate in stimulation buffer. Cells were stimulated at 37 °C for 30 min with increasing concentrations of AngII or analogues. Cells were then lysed with the lysis buffer containing 3 μl of IP_1 coupled to the d2 dye. After addition of 3 μl of anti-IP_1 cryptate terbium conjugate, cells were incubated for 1 h at room