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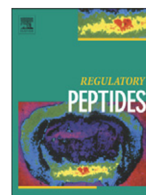

Biological and conformational evaluation of angiotensin II lactam bridge containing analogues

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ABSTRACT

Angiotensin II (AII) is the active octapeptide product of the renin enzymatic cascade, which is responsible for sustaining blood pressure. In an attempt to establish the AII-receptor-bound conformation of this octapeptide, we designed conformationally constrained analogues by scanning the entire AII sequence with an i-(i + 2) and i-(i + 3) lactam bridge consisting of an Asp-$(Xaa)_n$-Lys scaffold. Most analogues presented low agonistic activity when compared to AII in the different bioassays tested. The exceptions are cyclo(0-1a) [Asp^0, endo-(Lys^{1a})]-AII (**1**) and [Asp^0, endo-(Lys^{1a})]-AII (**2**), both of which showed activity similar to AII. Based on peptide **1** and the analogue cyclo(3–5)[Sar^1, Asp^3, Lys^5]-AII characterized by Matsoukas et al., we analyzed the agonistic and antagonistic activities, respectively, through a new monocyclic peptide series synthesized by using the following combinations of residues as bridgehead elements for the lactam bond formation: D- or L-Asp combined with D- or L-Lys or L-Glu combined with L-Orn. Six analogues showed an approximately 20% increase in biological activity when compared with peptide (**1**) and were equipotent to AII. In contrast, six analogues presented antagonistic activity. These results suggest that the position of the lactam bridge is more important than the bridge length or chirality for recognition of and binding to the angiotensin II AT1-receptor.
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1. Introduction

Angiotensin II (AII) is a natural peptide produced in the blood as a result of two different enzymatic hydrolyses that takes place in the renal hypertension system [1]. Kidney juxtaglomerular cells [2], in certain circumstances, liberate an enzyme called renin that acts on the full protein substrate angiotensinogen to liberate the angiotensin I peptide [3]. This decapeptide is hydrolyzed by the angiotensin-converting enzyme to generate the peptide AII [4,5]. The renin–angiotensin system plays an important role in the regulation of blood pressure. AII, the hormone responsible for this physiological function, interacts with several tissues, including the adrenal cortex, myocardial tissue, vascular flat muscle [6], the kidneys and the brain. The effects of AII include modulating the liberation of aldosterone from the adrenal cortex adrenal [7], the heart rate control [8], vascular tonus [9], glomerular filtration and the pituitary secretion of the vasopressin [10,11]. AII also acts in the outlying central nervous system of mice [12]. These functions of AII can be altered in vascular diseases such as hypertension and arteriosclerosis, promoting the growth and hypertrophy of the flat and heart muscles [13]. An understanding of the AII-receptor interaction with the AII peptide is crucial for the control of the renin–angiotensin system; however, the greatest difficulty in achieving this goal is establishing the bioactive conformation of AII. Over many decades, the conformation of AII has been studied using different approaches, such as CD studies [14], Raman spectroscopy [15] and NMR [16–18]. Structure-activity relationship studies focusing on AII have demonstrated the importance of the amino acid residues Tyr^4, His^6 and Phe^8 for biological

Abbreviations: AAA, Amino acid analysis; ACN, Acetonitrile; AII, angiotensin II; BOP, (Benzotriazol-1-yl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate; BSA, bovine serum albumin; CD, Circular Dichroism; CE, Capillary Electrophoresis; CHO, Chinese hamster ovary; DCM, Dichloromethane; DIC, N,N′-Diisopropylcarbodiimide; DIPEA, N,N-Diisopropylethylamine; DMEM, Dulbecco's modified Eagle's medium; DMF, Dimethylformamide; DMS, Dimethyl sulfide; DMSO, Dimethyl sulfoxide; FAR-UV, Far-Ultraviolet; FFT, Fast Fourier Transform; Fmoc, Fluorenylmethyloxycarbonyl; HF, Hydrogen fluoride; HOBt, N-Hydroxybenzotriazole; IP, intraperitoneal; LC/ESI-MS, Liquid Chromatography/Electrospray Ionization Mass Spectrometry; MeOH, Methanol; NMP, N-methylpyrrolidone; OFm, Fluorenylmethyloxycarbonyl ester; RP-HPLC, Reverse-phase High-Performance Liquid Chromatography; Sar, Sarcosine; SDS, Sodium dodecyl sulfate; t-Boc, tert-Butoxycarbonyl; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEAP, Triethylammonium phosphate; TFA, Trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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activity [19,20]. The antagonistic activity of AII, characterized by slow receptor desensitization rates, can be obtained with an amino acid replacement. This antagonistic behavior is highly dependent on the nature of the amino acid in position 8 [21] and by aliphatic amino acids this peptide (Ile, Ala and Thr). AII competitive antagonists are also obtained by modifying the hydroxyl group of the Tyr^4 residue [22].

In an attempt to characterize the bioactive conformation of this hormone, several cyclic analogues have been created by the incorporation of disulfide or lactam bridges and tested for their activity [23–25]. Among them, the following bridged monocyclic disulfides demonstrated high affinity for the AT1 receptor: c[$Cys^{3,5}$]-AII, c[Cys^3, Hcy^5]-AII, c[Hcy^3, Cys^5]-AII and c[$Hcy^{3,5}$]-AII [23,26–31]. In addition, cyclic analogues were created by inserting a lactam bridge in the same region of the molecule, which generated relevant antagonistic activity [27].

In this work, we performed a systematic AII structure–activity relationship study using conformationally restricted analogues created via the incorporation of a lactam bridge. Peptides were designed by scanning the entire AII sequence with i-(i+2) and i-(i+3) lactam bridges, consisting of an Asp-$(Xaa)_n$-Lys scaffold. All the amino acids residues important for the biological activity of AII (i.e., His, Phe side chains, the hydroxyl group of Tyr, and the C-terminal carboxylic group) were left unchanged. The effect of lactamization was also evaluated by testing the corresponding linear analogues. The agonistic and antagonistic effects of the peptides were compared to AII using Chinese hamster ovary (CHO) cells transfected with the AT1-receptor and then grown in suspension on a Cytosensor microphysiometer. Furthermore, the contractile responses of isolated guinea-pig ileum and rat-uterus preparations as well as the arterial pressure alterations induced by the peptides were also evaluated. The conformational behaviors of the compounds were analyzed using circular dichroism (CD).

2. Experimental procedures

2.1. Peptide synthesis, purification and characterization

AII and its analogues were synthesized using a common protocol for manual solid-phase methodology and the tert-butoxycarbonyl (t-Boc) strategy [32,33]. Chloromethylated resin [34] with a degree of substitution varying from 0.5 to 0.8 mmol/g was employed. The N^{α}-terminal protecting group was removed with 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) in the presence of 2% anisole for 20 min. Couplings were carried-out using a 2.5-fold excess of 1,3-diisopropylcarbodiimide/N-hydroxybenzotriazole (DIC/HOBt) in DCM-dimethylformamide (DMF) (1:1, v/v) and were monitored using the Kaiser ninhydrin test [35]. Recouplings for 1 h were performed when needed using a 2.5-fold excess of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) in the presence of N,N-diisopropylethylamine (DIPEA) in DCM/N-methylpyrrolidone (NMP) (1:1, v/v) [36]. Acetylations were performed using 50% acetic anhydride in DMF for 15 min, when required. Orthogonal protection of the aspartic acid side chain residues with OFm (Fluorenylmethyloxycarbonyl ester) and the lysine residues with Fmoc (9-fluorenylmethyloxycarbonyl) were employed. Side-chain to side-chain cyclization (lactamization) was performed with the peptide still attached to the resin [37]. After washes with DCM and DMF, the OFm and Fmoc groups were removed by treating the peptide with 20% piperidine in DMF [38,39].

The lactamization reaction was performed using a 3.0-fold excess of (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) to the peptide concentration in the presence of excess DIPEA in 20% dimethyl sulfoxide (DMSO) dissolved in NMP [37] and the reaction progress was monitored using the Kaiser ninhydrin test. Dry-protected peptidyl-resin was exposed to anhydrous HF in the presence of 5% anisole and 5% dimethyl sulfide (DMS) for 75 min at 0 °C. Excess HF and scavengers were eliminated under high vacuum. The crude peptides were precipitated with anhydrous diethyl ether, separated from ether-soluble reaction components by filtration, extracted from the resin with 5% acetic acid in water and lyophilized. Crude lyophilized peptides were then purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) in two steps: triethylammonium phosphate (TEAP) at pH 2.25 and 0.1% TFA on a Waters Associates system (Model Prep 4000). Briefly, the peptides were loaded onto a Vydac C_{18} (25 mm × 250 mm, 15 μm particle size, 300 Å pore size) column at a flow rate of 10.0 mL/min and eluted using linear gradients (slope 0.33% B/min) of TEAP (pH = 2.25)/Acetonitrile (ACN) with detection at 220 nm. Selected fractions were collected and converted to the TFA salt by loading on a preparative column as described above and eluting with a linear gradient (slope 0.33% B/min) of solvents A (0.1% TFA in water) and solvent B (75% ACN in solvent A), at a flow rate of 10.0 mL/min. Selected fractions containing the purified peptide were pooled and lyophilized.

Purified peptides were characterized by liquid-chromatography electrospray-ionization mass spectrometry (LC/ESI-MS), capillary electrophoresis (CE) (Tables S1 and S2 in the Supplementary material), and amino acid analysis (AAA; data not shown).

LC/ESI-MS data were obtained on a Micromass instrument, model ZMD, coupled to a Waters Alliance system (model 2690) and photodiode array detector (model 996), using a Phenomenex Gemini C_{18} column (2.0 mm × 150 mm, 3.0 μm particle size, 110 Å pore size). Solvent A was 0.1% TFA in water, and solvent B was 60% ACN in solvent A. The gradient was 5–95% B for 30 min with a λ_{range} of 190–300 nm. Mass measurements were performed in a positive mode with the following conditions: mass range between 500 and 2000 *m/z*; nitrogen gas flow of 4.1 L/h; capillary, 2.3 kV; cone voltage of 32 V; extractor, 8 V; source heater, 100 °C; solvent heater, 400 °C; ion energy, 1.0 V; and a multiplier of 800 V.

Capillary electrophoresis profiles were obtained using a Waters System, model CIA (Capillary Ion Analyzer), by hydrostatic injection in 25 s, utilizing phosphate buffer at pH 2.50, λ = 214 nm, a silica capillary (75 μm × 60 cm), voltage of 20 kV at a temperature of 30 °C.

Amino acid analysis of the peptides previously hydrolyzed in 6 M HCl at 110 °C for 72 h were performed by ion-exchange chromatography in a Biochrom 20 Plus amino acid analyzer, using the three-buffer system under standard conditions recommended by the manufacturer. The molar ratios of the amino acids were established by considering the concentration unitary of the closest amino acid of the average for all the residues.

2.2. Circular dichroism studies

Far-UV (190–250 nm) CD spectra were recorded at 20 °C using a 1 mm path length quartz cell in a Jasco J180 spectropolarimeter. All spectra were recorded after an accumulation of four runs. The scan rate was 50 nm/min for all measurements with bandwidths of 0.5 nm. All peptides were measured in the following four solutions: water (pH = 7.4), 20 mM sodium dodecyl sulfate (SDS), 50% 2,2,2-trifluoroethanol (TFE) in water, and 60% methanol (MeOH) in water. The CD spectra for the SDS, TFE and MeOH solutions were subtracted, and a Fourier transform filter (FFT) was applied to minimize background effects.

2.3. Cell culture and cell transfection

Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium from GIBCO-BRL. DMEM was supplemented with 10% fetal calf serum and 100 UI/mL of gentamycin and maintained at 37 °C in a humidified 5% CO_2 atmosphere. An expression plasmid containing the wild-type rat AT1 receptor was permanently transfected into CHO cells using the Lipofectin reagent (Life Technologies), as described by the manufacturer. Cells

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