Angiotensin Analogues That Selectively Augment the Force of Contraction of the Isolated Heart

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SUMMARY Angiotensin II (Ang II) produces a positive inotropic effect on the heart; however, its usefulness as an inotropic agent is limited because of its inherent vasoconstrictor action. We therefore designed Ang II analogues that are potent, positive inotropic agents with minimal myotropic properties. Replacement of the proline residue in position 7 with alanine reduced the pressor and vascular contractile response to less than 1% of Ang II. In spite of negligible vascular actions, however, [7-alanine]Ang II produced 50% of the inotropic activity of Ang II in the cat papillary muscle. The results of pharmacological evaluation of various position 7-substituted analogues were as follows: 1) Replacement of proline in position 7 of angiotensin I (Ang I) and Ang II with primary amino acids produced cardiac-specific, positive inotropic properties. 2) The selectivity of positive cardiac inotropic activity of position 7-substituted analogues of Ang II was dependent upon the nature of the amino acid in position 1. Replacement of aspartic acid in position 1 with sarcosine increased vasoconstrictor activity, thereby diminishing cardiac selectivity. However, this change did not affect cardiac selectivity in Ang I analogues. 3) Introduction of any type of steric hindrance in position 7 (e.g., replacement of alanine with N-methyl- or α-methylalanine) led to a considerable loss in inotropic activity. In conclusion, contrary to rigid, structural requirements (solution conformation) for the pressor action of Ang II, a less organized structure or a random conformation at the carboxyl terminus appears to favor cardiac-selective contractile response (or positive inotropic response).

KEY WORDS peptide synthesis • solid-phase procedure • rabbit aortic strips • vasoconstrictor activity • coronary perfusion circuit

ANGIOTENSIN II (Ang II) and possibly angiotensin I (Ang I) and angiotensin III (Ang III), have a direct positive inotropic effect on the myocardium that is independent of intact adrenergic ganglia, nerves, or endogenous catecholamine stores and is mediated through an increased calcium current. However, Ang II cannot be used clinically to enhance cardiac contractile force (or positive inotropic response) since it is also a potent vasoconstrictor agent. In the intact mammalian system it constricts the coronary arteries and, because vasoconstriction leads to decreased coronary blood flow and diminished oxygen supply, this produces decreased myocardial contractility. In view of this limitation, we undertook studies to design Ang II analogues that would retain the positive inotropic response of Ang II but not its vasoconstrictor action.

Replacement of the proline residue in position 7 with alanine reduced the pressor and myotropic properties to below 1% of Ang II. However, in spite of negligible vascular actions, [7-alanine]Ang II produced 50% of the inotropic activity of Ang II in the cat papillary muscle. This was the first indication of a dissociation of positive inotropic activity of Ang II from its pressor and vascular contractile action. In continuation of these studies, this paper reports the synthesis and structure-activity relationship with various position 7-substituted analogues of Ang I and Ang II. Pharmacological evaluations of these analogues were carried out by Trachte et al., Cross et al., and Baker and Khosla.

Materials and Methods

Synthesis and Purification of Angiotensin Analogues

All the analogues were synthesized by the solid-phase procedure. The protocol used for the synthesis and purification was similar to the one described by Khosla et al. The homogeneity of the peptides was determined by 1) thin-layer chromatography (TLC) in solvents of
different pH, 2) ionophoresis at pH 2.0 and 8.6, and 3) amino acid analysis.

TLC (cellulose, Merck) was carried out by using the following solvent systems: 1) 1-butanol/acetic acid/water (BAW, 4:1:5); 2) 1-butanol/pyridine/water (BPW, 65:35:35); and 3) 1-butanol/acetic acid/water/pyridine (BAWP, 30:6:24:20). Compounds were detected on the chromatograms with ninhydrin and diazotized sulfanilic acid (Pauly’s reagent).

Ionophoresis was carried out on filter paper strips (2043A, Schleicher & Schuell) in a Beckman electrophoresis cell (Model R, Series D, Beckman Instruments, Fullerton, CA, USA) at 400 V using 0.25 M acetic acid (pH 2) or Beckman barbiturate buffer B2 (pH 8.6). Histidine, assigned the mobility of 1, was run simultaneously as a reference compound, and E(His) indicates the electrophoretic mobility of the peptide relative to the mobility of histidine.

Free peptides were hydrolyzed in a mixture of 6 N HCl and 90% aqueous phenol (100:0.1) at 110°C for 16 hours in sealed tubes under N₂. Amino acid analyses were performed on a Model MM-100 Glenco (Houston, TX, USA) amino acid analyzer using o-phthalaldehyde, or on a Waters automated HPLC system (Waters Associates, Milford, MA, USA) using precolumn derivatization (phenylisothiocyanate) followed by reverse-phase separation on a C₁₈ column.

[7-Alanine]Ang II

Side-chain functional protecting groups employed for tert- butyloxycarbonyl histidine were either benzylmethyl or benzoxymethyl. Coupling of each protected amino acid was carried out through N,N'-dipropylcarbodiimide in the presence of 1 M excess of 1-hydroxybenzotriazole or through the preformed active ester of 1-hydroxybenzotriazole. Residual, uncoupled amino groups were blocked by acetylation with 5% 1-acetylimidazole in methylene chloride containing 1% dipropylethylamine for 30 minutes. Cleavage of the peptide from the polymer was carried out with HBr/CF₃COOH for 10 minutes under ice-cold conditions. In spite of all these precautions, yields of this peptide (and also of some other position 7-substituted analogues) were generally low (10-15%) due to the presence of several impurities in the crude product, presumably degradation products. Even after careful purification on several chromatographic columns, traces of impurities may still be present. It is not clear whether these impurities were formed during the synthesis or during the cleavage process.

TLC (cellulose) Rf values were as follows: 0.60 (BAW), 0.36 (BPW), 0.67 (BAWP); E(His) was 0.65 (pH 2). Amino acid analysis gave the ratio in the acid hydrolysate: Asp, 1.04; Arg, 1.16; Val, 1.17; Tyr, 1.08; lle, 1.0; His, 1.12; Ala, 1.16; Phe, 0.96.

[1-Sarcosine, 7-Alanine]Ang I

TLC (cellulose) Rf values were as follows: 0.43 (BAW), 0.50 (BPW), 0.56 (BPW 65:35:65); E(His) was 0.88 (pH 2). Amino acid analysis gave the ratio in the hydrolysate: Sar, 1.06; Arg, 1.01; Val, 1.20; Tyr, 1.0; lle, 1.31; His, 1.97; Ala, 0.99; Phe, 0.88; Leu, 1.11.

[1-Sarcosine, 7-α-Methylalanine]Ang I

TLC (Cellulose) Rf values were as follows: 0.56 (BAW), 0.42 (BPW), 0.74 (BAWP); E(His) values were 0.92 (pH 2.6), 0.90 (pH 8.6). Amino acid analysis gave the ratio in the acid hydrolysate: Sar, 0.85; Arg, 1.05; Val, 1.0; Tyr, 1.10; lle + Leu, 2.11; His, 2.0; α-MeAla, 0.93; Phe, 1.13.

[1-Sarcosine, 5-Valine, 7-N-Methylalanine]Ang I

TLC (cellulose) Rf values were as follows: 0.39 (BAW), 0.48 (BPW), 0.74 (BAWP); E(His) values were 0.91 (pH 2.0), 0.79 (pH 8.6). Amino acid analysis gave the ratio in the acid hydrolysate: Sar + N-MeAla, 1.42; Arg, 1.0; Val, 1.89; Tyr, 1.07; His, 1.97; Phe, 1.06; Leu, 1.28.

[1-Sarcosine, 5-Valine, 7-Sarcosine]Ang I

TLC (cellulose) Rf values were as follows: 0.3 (BAW), 0.36 (BPW), 0.69 (BAWP); E(His) values were 0.92 (pH 2.5), 0.80 (pH 8.6). Amino acid analysis gave the ratio in the acid hydrolysate: Sar, 2.27; Arg, 1.0; Val, 1.85; Tyr, 1.13; His, 2.09; Phe, 1.0; Leu, 1.3.

Positive Inotropic Effects

Trachte and co-workers measured the inotropic effect in rabbit left atrial tissues. The analogues were added in cumulative doses. Converting enzyme inhibition was carried out with teprotide.

Cross et al. measured inotropic effects in the left ventricle of the isolated perfused cat heart by observing the increases in pressure generated. Analogues were administered as a single bolus injection into the coronary perfusion circuit. The response to each dose was plotted as a percentage of the maximum pressure increase achieved in that preparation with increasing doses of Ang II. Converting enzyme inhibition was carried out by a bolus injection of captopril (10⁻⁴ mol) administered 1 minute before the dose of test analogue.

Baker and Khosla measured inotropic activity in point-stimulated rabbit left atrial tissues. The atria were prepared by the method of Blumberg et al.

Pressor and Vasoconstrictor Activities

Trachte and co-workers measured vasoconstrictor activity from cumulative dose-dependent responses on helical rabbit aortic strips. Baker and Khosla used rabbit aortic rings for this study. Cross et al. measured pressor response in conscious sheep by direct puncture of the carotid artery, which had been isolated in a loop of skin at a previous operation. Rat pressor assays
Results

Angiotensin II increased contractile force of the isolated rabbit left atria in a dose-dependent manner (ED_{50}, 30 nM). [1-Sarcosine]Ang II was 10-fold more potent than Ang II. Converting enzyme inhibition (teprotide, 10 μg/ml) had no effect on contractile response to either Ang II or [1-sarcosine]Ang II.

In the presence of teprotide, the inotropic potency of [7-alanine]Ang II and [1-sarcosine, 7-alanine]Ang II was equal to that of Ang II and [1-sarcosine]Ang II, respectively. [1-Sarcosine, 7-alanine]Ang II produced 12.97% of the myotropic activity of Ang II on rabbit aortic strips (ED_{50}, 25.1 ± 6.3 ng/ml, means ± SE), which increased 30-fold in the presence of teprotide. In the rat pressor assays this peptide produced 0.55 ± 0.02% (n = 28) of the pressor activity of Ang II; however, after converting enzyme inhibition, the pressor activity increased to 25.05 ± 1.79% (n = 12).

The inotropic potency of Ang I in atrial tissue was significantly less than that of Ang II (ED_{50}, 100 nM), and the response was attenuated by teprotide. Similar results were obtained in the perfused cat heart. [1-Sarcosine]Ang I, [1-sarcosine, 5-valine]Ang I, [1-sarcosine, 7-α-methylalanine]Ang I, [1-sarcosine, 5-valine, 7-N-methylalanine]Ang I, and [1-sarcosine, 5-valine, 7-sarcosine]Ang I possess, respectively, 200, 200, 33, 100, and 200% of the inotropic activity of Ang I (perfused cat heart); 200, >200, 100, 200, and 100% of thepressor activity of Ang I (sheep); 93.4 ± 5.3 (n = 11), 53.14 ± 1.86 (n = 20), 3.69 ± 0.91 (n = 9), —, 8.39 ± 0.8% (n = 16) of the pressor activity of Ang II (rat pressor assays). The myotropic activity of [1-Sarcosine]Ang I, and [1-sarcosine, 5-valine, 7-valine]Ang I on rabbit aortic strips was 41.23% (ED_{50}, 14.26 ± 3.47 ng/ml) and 0% of that of Ang II, respectively. Both the inotropic activity and the pressor activity of these analogues were either abolished or reduced by 50 to 60% in the presence of captopril.

Inotropic activity of [1-sarcosine, 7-alanine]Ang I in perfused cat heart was between 10 and 33% of that of Ang I, which was increased twofold in the presence of captopril. Similarly, in atrial tissue the inotropic activity of this peptide, in the presence of teprotide, was almost equal to that of Ang I. In the rat pressor assays it produced 0.27 ± 0.017% (n = 13) of the pressor activity of Ang II. The pressor response (0.5- or 1-μg dose) was reduced but not totally abolished after treatment with captopril. It produced 0.71 ± 0.11% (n = 11) of the myotropic activity of Ang II in rabbit aortic strips (ED_{50}, 277.96 ± 14.5 ng/ml).

In the point-stimulated rabbit left atrial tissues, Ang I increased developed force in a concentration-dependent manner. The converting enzyme inhibitor enalaprilat decreased the potency of Ang I in a concentration-dependent manner with maximum effects at 1 × 10^{-7} M. Although the cumulative concentration-response curve for [1-sarcosine, 7-alanine]Ang I indicates that it is a weak inotropic agent, enalaprilat (1 × 10^{-7} M) did not have a significant effect on its cumulative concentration-response curve. In contrast, the cumulative concentration-response curves for [1-sarcosine, 7-α-methylalanine]Ang I, [1-sarcosine, 5-valine, 7-N-methyl]Ang I, and [1-sarcosine, 5-valine, 7-sarcosine]Ang I were significantly shifted to the right.

Contractile activity determined in rabbit aortic rings indicated that, in the presence of enalaprilat, cumulative concentration-response curve for [1-sarcosine, 7-alanine]Ang I was not shifted significantly to the right, but the curves for all other Ang I analogues were shifted to the right. Substitution of aspartic acid in position 1 by sarcosine did not enhance vascular potency, as was observed with the corresponding Ang II analogues.

Discussion

The fold at the carboxyl terminus of Ang II in solvents such as trifluoroethanol is dependent upon the presence of proline residue in position 7, and is stabilized by a hydrogen bond between the NH of phenylalanine (position 8) and carbonyl of histidine (position 6) to form a seven-member ring. This structure appears to be necessary for the pressor and myotropic actions of Ang II. In addition, proline residue in position 7 protects Ang II against enzymatic degradation by converting enzyme. Therefore, replacement of proline with a noncyclic aliphatic amino acid (e.g., alanine) would be expected to disrupt this highly organized structure, and should result in loss of vascular activity and ease of enzymatic degradation with converting enzyme. This indeed was the case; [7-alanine]Ang II produced negligible pressor and vasoconstrictor properties. However, this analogue has been found to potentiate cardiac inotropic properties in the perfused heart, and addition of a converting enzyme inhibitor enhanced its cardiac stimulant potency to a level equal to that of Ang II. Chain length can be extended to the decapeptide stage (e.g., in [1-sarcosine, 7-alanine]Ang I) with considerable retention of inotropic activity. Replacement of aspartic acid (position 1) with sarcosine attenuated cardiac selectivity in Ang II analogues but not in Ang I analogues. Inotropic activity decreased, while vasoconstrictor activity increased when alanine in position 7 was replaced with α-methylalanine, N-methylalanine, or sarcosine.

Thus, substitution of proline (position 7) with alanine in Ang II appears to decrease the vascular action of the molecule, thereby enhancing its cardiac inotropic properties. Furthermore, contrary to the rigid structural requirements for the pressor action of Ang II, a less organized or a random conformation at the carboxyl terminus appears to favor cardiac selectivity. Of particular interest is the analogue [1-sarcosine, 7-alanine]Ang I. It possesses a high degree of specificity for cardiac receptors. In addition, its inotropic action is potentiated by converting enzyme inhibition. A likely explanation is that this analogue is more effective as an inotropic agent when it is not being converted into
the corresponding octapeptide by the converting enzyme.

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