Microbial production of thioether-stabilized peptides
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Angiotensin-(1-7) with thioether-bridge: an angiotensin-converting enzyme-resistant, potent Angiotensin-(1-7) analogue

Abstract
The in vivo efficacy of many therapeutic peptides is hampered by their rapid proteolytic degradation. Cyclization of these therapeutic peptides is an excellent way to render them more resistance against breakdown. Here we describe the enzymatic introduction of a thioether ring in angiotensin (Ang-(1-7)), a heptapeptide that plays a pivotal role in the renin-angiotensin system and possesses important therapeutic activities. The lactic acid bacterium Lactococcus lactis equipped with the plasmid-based nisin modification machinery was used to produce thioether bridged Ang-(1-7). The resulting cyclized Ang-(1-7) is fully resistant against purified angiotensin-converting enzyme, has significantly increased stability in homogenates of different organs and in plasma derived from pig, and displays a strongly (34-fold) enhanced survival in Sprague Dawley (SD) rats, in vivo. With respect to functional activity, cyclized Ang-(1-7) induces relaxation of precontracted SD rat aorta rings, in vitro. The magnitude of this effect is two-fold larger than that obtained for natural Ang-(1-7). The Ang-(1-7) receptor antagonist D-Pro\textsubscript{7}-Ang-(1-7) which completely inhibits the activity of natural Ang-(1-7) also abolishes the vasodilation by cyclized Ang-(1-7), providing evidence that also cyclized Ang-(1-7) interacts with the Ang-(1-7) receptor. Taken together, applying a highly innovative enzymatic peptide stabilization method we generated a stable Ang-(1-7) analogue with strongly enhanced therapeutic potential.
Chapter 7

Introduction

Angiotensin-(1-7) is an endogenous heptapeptide that plays an important role in the renin-angiotensin system (RAS) (204). Its alleged biological inactivity was first refuted by Schiavone et al., who demonstrated a dose-related stimulation of arginine vasopressin release by Ang-(1-7) equipotent to angiotensin-II (AngII) (184). Since then, its significance in the complex RAS has been slowly unraveled. Whereas in some cases, Ang-(1-7) acts similar to AngII (164, 165, 184), Ang-(1-7) mainly exerts effects which oppose those of AngII, like vasoconstriction, fibrosis and proliferation (175, 185). Discoveries such as the counteractive effect of Ang-(1-7) on the cardiovascular actions of AngII (46, 167) and its ability to attenuate the development of heart failure (112) have subsequently supported this concept. More recently, it has been demonstrated that Ang-(1-7) also plays a protective role in hepatic diseases such as liver fibrosis (150), and can even inhibit the growth of human lung cancer cells in vivo (123). Furthermore, the discovery that the G protein-coupled receptor Mas is involved in Ang-(1-7) signaling has greatly contributed to the elucidation of the heptapeptide’s biological role (177).

Its variety of beneficial actions against a range of diseases would make Ang-(1-7) an ideal therapeutic agent. However, its rapid in vivo catabolism by angiotensin-converting enzyme (ACE) and other proteases has so far reduced its potential as a drug.

Cyclization of therapeutically important peptides has been proven to be a successful method to create more stable peptide analogues with improved pharmacodynamic properties. Especially intramolecular thioether bridge formation is an effective way to protect peptides against proteolytic degradation. Introduction of thioether bridges in peptides has been effectively applied on peptides like somatostatin, enkephalin and an epitope of the herpes simplex virus glycoprotein D. For all these peptides an increased catabolic stability was observed compared to their linear counterparts (155, 205). Thioether bridges are more stable than peptide bonds and disulfide bridges (205). Cyclization imposes conformational constraints, which likely caused the significantly enhanced receptor interaction of thioether enkephalin (155).

Multistep chemical synthesis of thioether-bridged peptides is costly and cumbersome. Recently, we described a fermentative procedure to stabilize therapeutic peptides by introducing thioether bridges (77). This procedure involves plasmid-encoded nisin-modification enzymes expressed by Lactococcus lactis: a dehydratase (NisB) enzymatically dehydrates a Ser (or Thr) into a dehydroalanine (or dehydrobutyrine) (86). The generated dehydroresidues either enzyme (NisC)-catalysed or spontaneously react with a cysteine thiol group, which yields an intramolecular thioether bridge in the peptide (77, 160).
We here present a prime example of the successful biological production of a stable and active thioether-cyclized peptide hormone, cyclized Ang-(1-7) (cAng-(1-7)), by the nisin biosynthesis machinery. We demonstrate via *in vitro* and *in vivo* (rat) assays that the stability of cAng-(1-7) is strongly enhanced compared to its natural counterpart. We furthermore demonstrate that cAng-(1-7) has enhanced relaxing activity on precontracted rat aorta rings via the Ang-(1-7) receptor indicating enhanced receptor interaction.

### Methods

**Chemicals.** Ang-(1-7) and other chemicals were obtained as described (Supplemental Materials and Methods). cAng-(1-7) was designed, produced and purified as described (Supplemental Materials and Methods).

**Animals.** Specified pathogen-free male Sprague Dawley rats (SD) (Harlan, Zeist, The Netherlands), weighing 350–450 gram were used. Prior to the experiment, the animals were housed together with free access of tap water and solid chow (Harlan, Zeist, The Netherlands) in a temperature and humidity controlled room and a 12/12h light/dark cycle. All protocols described were approved by the University of Groningen Committee for Animal Experimentation.

**Ang-(1-7) stability in ACE solution and plasma.** Natural and cyclized Ang-(1-7) (50 µM) was incubated with angiotensin-converting enzyme (ACE) at 37°C over a period of 7 hour in a buffer containing 100 mM Tris-HCl pH 8.3, 300 mM NaCl, 10 µM ZnCl$_2$. ACE was used in final concentrations of 0.03 units/mL. Incubation in plasma occurred at concentrations of 100 µM natural or cAng-(1-7), in 15-fold diluted plasma, 20 mM phosphate buffer, pH 7.4. Analysis and Ang-(1-7) detection in these *in vitro* studies were carried out as described (Supplemental Materials and Methods).

**Ang-(1-7) stability in pig organ homogenates.** Natural and cyclized Ang-(1-7) were incubated with pig homogenate of kidney cortex or liver (0.2 and 0.1 µmol Ang-(1-7)/mg homogenate, respectively) at 37°C. Incubation was performed in 20 mM phosphate buffered at pH 7.4 (cytosolic) and pH 5 (lysosomal) over a period of 3 h. The reaction was stopped by heat incubation (100°C, 5 min). Preparation of organ homogenates, analysis and Ang-(1-7) *in vitro* detection were performed as described (Supplemental Materials and Methods).

**Ang-(1-7) infusion studies in Sprague-Dawley rats.** The *in vivo* kinetics of cAng-(1-7) and natural Ang-(1-7) were determined by continuous intravenous infusion of the peptides in male Sprague-Dawley rats of approximately 400 gram. Animals were kept under O$_2$/Isoflurane (1.5-2%) anaesthesia throughout the study. After a 30 min stabilization period with 0.9 % NaCl 1 mL/h, 100 µM cAng-(1-7) and 100 µM Ang-(1-7) in 0.9 % NaCl was infused with a constant rate of 1 mL/hour. Blood (200-300 µl) was sampled via the carotid artery every 30 min for 2 hours. Creatinine levels in plasma were measured according to Bartels and Böhmer, 1971; n = 6. Further
analysis and Ang-(1-7) detection in the in vivo studies were performed as described (Supplemental Materials and Methods).

To ensure that enzyme saturation was not the determinant of the peptide clearances, the study was repeated once using a similar infusion dose of cAng-(1-7) (100 µM) but a 10-fold higher dose of natural Ang-(1-7) (1 mM). Indeed, the clearances of natural and cAng-(1-7) were not depending on the used dose of natural Ang-(1-7) indicating that enzyme saturation did not play a role.

**Vasodilation on precontracted aorta-rings.** Arterial rings for organ bath experiments were prepared as described in Supplemental Materials and Methods. Before testing the vasodilating effects of the peptides the rings were precontracted to 50% of their maximum contraction level with 30 nM phenylephrine (PE). Natural or cyclized Ang-(1-7) were added cumulatively in a range of 0.1 nM to 1 µM. If appropriate Ang-(1-7) receptor blockers A-779 (191) and D-Pro\(^7\)-Ang-(1-7) (176) were applied 10 min prior to addition of 30 nM PE at a concentration of 0.1 µM.

Vasodilation data are represented as % relaxation of 30 nM PE contraction. The vasodilating effect of the peptides was washed-out, followed by a full concentration-response curve of PE (0.1 nM to 10 µM), wash-out (two times) and again by establishing tonus by 30 nM PE to test the endothelium responsiveness of the rings. All preparations were sensitive to acetylcholine (10 µM) reducing this tonus by 51 ± 3 %.

**ACE activity.** The inhibitory effect of cAng-(1-7), natural Ang-(1-7), Ang-(1-5) and AngI (Ang-(1-10)) on the activity of ACE was spectrophotometrically examined in rat plasma using hippuryl-L-histidyl-leucine (HHL) as a substrate (Cushman and Cheung, 1971). As a control, the specific ACE inhibitor captopril was used.

**Statistical analysis and calculation.** All data were presented as mean ± S.E.M.. Statistical comparison of individual data was done using unpaired Student’s t-statistics except for the in vivo infusion study in which paired analysis was used. Repeated measures ANOVA with Bonferoni for post-hoc test was used to compare the curves of proteolytic stability, concentration-response curves of aorta ring dilatation and ACE activity. Differences were considered significant at \( p \leq 0.05 \). Asterixes indicate: *, \( p < 0.05 \); **, \( p < 0.01 \) and ***, \( p < 0.001 \). Plasma clearance of Ang-(1-7) was calculated by dividing the infusion rate by the plasma concentration of the peptide.

**Results**

**Introduction of a thioether bridge in Ang-(1-7) linking amino acids 4 to 7.** The heptapeptide Ang-(1-7) consists of the residues H-Asp\(^1\)-Arg\(^2\)-Val\(^3\)-Tyr\(^4\)-Ile\(^5\)-His\(^6\)-Pro\(^7\)-OH. As the introduction of a thioether bridge requires the presence of a dehydratable residue (e.g. Ser) and a thiol-containing Cys, both corresponding amino acids were introduced at position 4 and 7, respectively, by site-directed mutagenesis. Figure 1 shows the chemical structure of the generated thioether-cyclized peptide Ang-(1-7).
The thioether bridge was introduced by a fermentative method using *L. lactis* as a host organism containing the nisin modification enzymes. Maldi-TOF mass spectrometry confirmed successful dehydration (-18 Da mass shift) and the presence of a thioether bridge (no mass shift of +25 Da after incubation with 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate), as explained previously in detail (77, 160).

![Figure 1. Hypothetical chemical structure of cAng-(1-7). The thioether bridge from position 4 to 7 (N-terminus on the left) is depicted in the D-L configuration expected on the basis of the DL configuration of the thioether bridges in nisin and other lantibiotics.](image)

**Resistance of cAng-(1-7) against proteolysis by purified angiotensin-converting enzyme (ACE) and plasma.** In the circulation, endogenous Ang-(1-7) is rapidly catabolized, primarily by ACE (228). In the present study, cAng-(1-7) and natural Ang-(1-7) were incubated with purified ACE or plasma and the amount of remaining intact peptide was determined. The study showed that cyclization of Ang-(1-7) clearly resulted in enhanced proteolytic resistance. Whereas natural Ang-(1-7) was completely degraded within 90 min in a purified ACE solution, equi-amounts of cAng-(1-7) were completely resistant against ACE degradation over the full period of 4 hours (*p* < 0.001) (Fig. 2A). After 4 hours incubation in plasma, natural Ang-(1-7) was largely degraded (5% remaining), whereas most of cAng-(1-7) remained intact (75% remaining) (*p* < 0.001) (Fig. 2A).

**Resistance of cAng-(1-7) to proteolysis in pig organ homogenates.** Besides ACE, other proteases are able to cleave Ang-(1-7) into smaller fragments (175). The proteolytic stability of cAng-(1-7) was compared to natural Ang-(1-7) using homogenates of pig-kidney and -liver, organs rich of a wide range of proteases. In kidney homogenate at pH 7.4, natural Ang-(1-7) was fully degraded within 3 hours, whereas more than 80% of the initial amount of cAng-(1-7) remained intact (*p* <
Similar results were obtained when both peptides were incubated with liver homogenate ($p = 0.002$) (Fig. 2B). ACE-inhibition by lisinopril did not affect the degradation rate of Ang-(1-7) in kidney and liver homogenate indicating that other proteases than ACE are responsible for the degradation in these organs. Also at lysosomal pH 5.0, the degradation of cAng-(1-7) was slower than of natural Ang-(1-7) (data not shown). These data consistently demonstrate strongly enhanced resistance of cAng-(1-7) against proteolytic degradation in protease-rich organ homogenates compared to natural Ang-(1-7).

**Figure 2AB. Enhanced proteolytic resistance of cAng-(1-7).** Proteolytic resistance was measured of natural (Δ, ○, □, ▼) and cyclized Ang-(1-7), (▲, ●, ■, ▼), against ACE (A: Δ, ▲), plasma (A: ○, ●), liver (B: □, ■) and kidney homogenate (B: ▼, ▼) at pH 7.4. Each point represents the mean ± S.E.M., generated from at least 3 separate experiments.

*Long lasting in vivo survival of cAng-(1-7).* The *in vivo* survival of cAng-(1-7) relative to natural Ang-(1-7) was determined by analyzing the drug concentration in plasma of anesthetized Sprague-Dawley (SD) rats during a continuous intravenous infusion of both peptides. Cyclization resulted in a strong, $34 \pm 2$ -fold, reduction of plasma clearance ($P = 0.01$) (Fig. 3). Hence the high resistance of cAng-(1-7) against proteolytic degradation, as shown *in vitro*, apparently results in a large increase in survival rate of the drug *in vivo*. This makes cAng-(1-7) much more attractive for therapeutic application than the natural Ang-(1-7). Creatinine level in the plasma was $49.82 \pm 1.33$ µM at 0 minutes of infusion, and $50.08 \pm 1.38$ µM at 120 min. Hence renal functioning was not affected by the infusion.
Moreover, the observed vasodilating effects by cAng(1-7) of a thioether bridge in Ang-(1-7) does not impede its vasodilative properties. Furthermore, the observed vasodilating effects by cAng-(1-7) occurred at a lower concentration than by natural Ang-(1-7).

**Potent dilating effect of cAng-(1-7) on precontracted aorta rings of SD rats.** Thoracic aortic rings with intact endothelium were precontracted with phenylephrine (PE) and subsequently relaxation of the established muscle tone was measured with cumulative additions of cAng-(1-7) or natural Ang-(1-7) (Fig. 4). Both natural and cAng-(1-7) displayed a significant vasodilating effect, indicating that the introduction of a thioether bridge in Ang-(1-7) does not impede its vasodilative properties. Moreover, the observed vasodilating effects by cAng-(1-7) occurred at a lower concentration than by natural Ang-(1-7).

**Figure 3. Long lasting in vivo survival of cAng-(1-7).** Plasma clearance (ml/min) of natural (open bar) and cAng-(1-7) (filled bar) in Sprague Dawley rats under anesthesia during a constant intravenous infusion of both peptides. Each bar represents the mean ± S.E.M. (n = 5).

**Figure 4. Enhanced relaxation activity of cAng-(1-7).** Relaxation of SD rat aorta rings during cumulative addition of natural (○) or cyclized Ang-(1-7) (●) or in control incubation (×), after precontraction with 0.3 µM phenylephrine, related to a vehicle experiment. Each point represents the mean ± S.E.M. generated from at least seven (natural) and eleven (cyclized) separate experiments.
A 10-fold lower amount of cAng-(1-7) was needed to achieve 10% dilatation (0.3 nM cAng-(1-7) versus 3 nM natural Ang-(1-7)) while even 100-fold less cAng-(1-7) was required to dilate the precontracted aortic rings for 40% (0.03 µM cAng-(1-7) and 3 µM natural Ang-(1-7)). Additionally, the maximal degree of vasodilation that was obtained was almost doubled with cAng-(1-7) compared to natural Ang-(1-7). At the highest concentration tested, 1 µM, cAng-(1-7) caused relaxation of 63 ± 5% whereas 1 µM natural Ang-(1-7) resulted in 33 ± 9% relaxation, only (P < 0.01).

After the cumulative addition of cAng-(1-7) and natural Ang-(1-7), aortic rings were put back into the PE solution only, to determine the wash-out rate of the induced dilation by the Ang-(1-7). With both peptides, the contraction was rapidly back to pre-challenge level with a wash-out half-life of 2.1 ± 0.3 min and 1.7 ± 0.3 min of cAng-(1-7) and natural Ang-(1-7) respectively (n=6, not significantly different). This is a strong indication that the vasodilating effect of cAng-(1-7) is a result of ligand/receptor interaction.

The involvement of the Ang-(1-7) receptor on the vasodilating properties of cAng-(1-7) was examined using the Ang-(1-7) receptor antagonists D-Ala^7^-Ang-(1-7) (A-779) and D-Pro^7^-Ang-(1-7) at 0.1 µM (Fig. 5). In line with recent findings on aorta-rings of Sprague-Dawley rats (191), natural Ang-(1-7) dilation was abolished by D-Pro^7^-Ang-(1-7) and insensitive for A-779 antagonism (Fig. 5A). cAng-(1-7)-induced dilation was fully blocked by D-Pro^7^-Ang-(1-7), providing evidence for the involvement of the Ang-(1-7) receptor in the vasorelaxant properties of cAng-(1-7). Interestingly, at low concentrations of cAng-(1-7), its activity was also antagonized by A-779 (Fig. 5B).

Figure 5. Effects of Ang-(1-7)-receptor-antagonists. Vasodilating effect of natural (A) and cyclized (B) Ang-(1-7) in the presence of Ang-(1-7)-receptor-antagonists A-779 or D-Pro^7^-Ang-(1-7) (0.1 µM) compared to control (control curves are similar to the ones in figure 3). A: interrupted line: Ang-(1-7); △: Ang-(1-7) + A-779; ◀: Ang-(1-7) + D-Pro^7^-Ang-(1-7). B: interrupted line: cAng-(1-7); ▲: cAng-(1-7) + A-779; ▼: cAng-(1-7) + D-Pro^7^-Ang-(1-7). Each point in the inhibition experiment represents the mean ± S.E.M. generated from at least six (D-Pro^7^-Ang-(1-7)) and eight (A-779) separate experiments.
Experiments on the *in vivo* vasodilating effect of cAng-(1-7) and its *in vivo* receptor specificity are under way.

*No effect of cAng-(1-7) on ACE activity.* Besides being a substrate of the N-terminal part of ACE for cleavage into Ang-(1-5), at high concentrations natural Ang-(1-7) inhibits ACE activity (32) by direct binding to the C-terminal domain of ACE. The inhibitory effect of cAng-(1-7) on the activity of ACE was analyzed using hippuryl-histidyl-leucine as a substrate. Figure 6 shows that natural Ang-(1-7) inhibits the activity of ACE at concentrations in the micromolar range, with an IC$_{50}$ around 10 µM. The breakdown product of natural Ang-(1-7), Ang-(1-5), at concentrations up to 0.1 mM did not induce ACE inhibition whereas the ACE-inhibitor captopril was very effective (EC$_{50}$ = 1 nM). In case of cAng-(1-7), ACE activity was not inhibited. Even at a concentration 10-fold higher than the IC$_{50}$ for natural Ang-(1-7), ACE activity was unaffected. These data demonstrate that cAng-(1-7) has largely or completely lost its capacity to act as an ACE inhibitor due to the cyclization.

**Figure 6. cAng-(1-7) has no or little ACE inhibiting activity.** Inhibitory effect of natural Ang-(1-7) (○), Ang-(1-5) (◊), cAng-(1-7) (●), and captopril (×) on the activity of ACE. Each point represents the mean ± S.E.M. generated from 3 separate experiments. The curve of cAng-(1-7) is significantly different from that of natural Ang-(1-7) (p < 0.05).

**Discussion**

Ang-(1-7) has a variety of interesting properties for therapeutic purposes. However, the very rapid breakdown in the circulation (228) makes the natural peptide unsuitable as a drug. Therefore, we aimed at introducing a thioether bridge that stabilizes the peptide without causing loss of activity. The role of the Ang-(1-7) residues in receptor binding and activity has not been elucidated. Except for Norleucine3 (165) and two Ang-(1-7) antagonists with a D-Ala (A-779) or D-Pro at
position 7 (174, 176) the effect that exchanging residues has on the peptide’s functionality is not known. We here clearly demonstrated that Ang-(1-7) with a thioether bridge between position four and seven has strongly increased stability and significantly increased activity.

In vivo, circulating Ang-(1-7) is rapidly inactivated by lung ACE, which cleaves off two amino acids at the C-terminus, resulting in Ang-(1-5). By introducing a thioether bridge between position four and seven of the peptide, this major catabolic pathway was blocked for the thioether Ang-(1-7) analogue. Ring introduction also reduced peptide catabolism by other enzymes than ACE as indicated by the enhanced stability in liver and kidney homogenates. Since the extent of catabolism largely determines the in vivo clearance and thus the exposure time of the body to the peptide a major improvement was expected by cyclization of the peptide. Indeed, the body clearance of cAng-(1-7) was more than 30-fold lower than of natural Ang-(1-7).

Several studies are known that aim at enhancement of the endogenous levels of Ang-(1-7) for therapeutic benefit. Since it was established that Ang-(1-7) is mainly degraded by ACE, approaches to increase Ang-(1-7) levels have been focused on inhibiting this protease, often in combination with blockers for the AngII receptor AT$_1$. However, ACE inhibition also results in lower AngII levels and, subsequently, less available substrate for generation of Ang-(1-7). With the discovery of ACE2, a second important protease in the RAS that generates Ang-(1-7) from AngI via Ang-(1-9) and from AngII directly (218), treatments have lately been focusing on increasing the ACE2 concentration. Techniques such as vector-mediated overexpression of ACE2 in SHR rat models demonstrated a protective effect of ACE2 on high blood pressure and cardiac pathophysiology induced by hypertension (41). Until now, administration of exogenous ACE2 is hampered by suboptimal solubility and activity. Very recently, promising effects in SHR rats have been shown using small-molecule ACE2 activators (58). Unfortunately, the contribution of ACE2 to cardiovascular physiology and disease also lies in depletion of the AngII pool rather than the synthesis of Ang-(1-7) (54). Exogenous Ang-(1-7)-induced attractive effects in vivo (85, 6, 112, 95, 123, 164, 165) while no signs of toxicity were observed in phase I/II clinical studies using Ang-(1-7) for bone marrow protection during anticancer therapy (165). However, the rapid degradation of the natural peptide makes it necessary to use high doses. A small but significant bioavailability of oral Ang-(1-7) seems possible using liposomes or cyclodextrin for encapsulation (179). Ang-(1-7) fused to a furin-cleavable protein in transgenic rats (178) provides a good analytical alternative, but will be more complicated when patient administration is desired. Only one Ang-(1-7) agonist, the nonpeptide AVE 0991, has been described. However, as reviewed (180), the value of this compound for therapeutic application is yet unknown, since only few of the benefits of Ang-(1-7) have been established for AVE 0991 too and toxicity data are not available. In general, all therapeutic Ang-(1-7) shortcomings listed above indicate
that a stable and functional analogue of Ang-(1-7) itself is the ideal compound for cardiovascular therapy.

The proteolytic resistant cAng-(1-7) presented in the present study proved to be a highly active vasodilator on the isolated aorta ring of the SD rat. Both the affinity and maximal effect of cAng-(1-7) were shown to be higher than that of its natural counterpart, despite the fact that the introduction of a thioether bridge entails a drastic change of the C-terminal part of Ang-(1-7). Given the fact that the introduction of a thioether bridge from position four to seven enhances the activity and since known antagonists of Ang-(1-7) all contain a mutation at position seven, it is evident that not only the amino acids at those positions, but also the established conformation plays a crucial role in the activity. It is known that peptide cyclization may result in a more constrained entity with reduced conformational freedom, which may confer a higher receptor affinity and/or stronger activity. The enhanced effect of cAng-(1-7) points into this direction.

As reviewed (64), multiple Ang-(1-7) receptors seem to exist. Several studies have shown that Ang-(1-7) mainly acts via the Mas receptor and that its activity can be fully prevented by A-779 and D-Pro\textsuperscript{7}-Ang-(1-7). This suggests that both compounds are full Mas receptor antagonists (176b). However, in cerebral arteries of the canine brain, endothelium-dependent relaxation by Ang-(1-7) was not blocked by A-779 (47). Also in another study Ang-(1-7) activity was not prevented by A-779 (191). In precontracted aorta rings of SD rats, the Ang-(1-7) induced vasodilation appeared mediated via an A-779-insensitive but D-Pro\textsuperscript{7}-Ang-(1-7)-sensitive receptor (191).

In the present study, we compared the effect of the two antagonists on the vasodilation of both natural and cAng-(1-7) in precontracted aorta-rings of SD rats. The Ang-(1-7) receptor antagonist A-779 did not prevent the vasodilation of natural Ang-(1-7) and only partially the vasodilation of cAng-(1-7). On the other hand, the antagonist D-Pro\textsuperscript{7}-Ang-(1-7) completely prevented the vasodilation by both peptides. These results indicate that after cyclization, the peptide maintained the receptor profile of the natural Ang-(1-7).

Like the dilatation curve of natural Ang-(1-7) in SD-rat aorta rings shown by Silva and co-workers, the curves of natural and cyclic Ang-(1-7) in the present study are not the result of interaction with a single receptor or a single binding state, indicated by the lack of steepness of the curve. The observed cAng-(1-7)-induced relaxation spans a concentration range of at least four orders of magnitude, which is much broader than the two orders expected for basic agonist-receptor interaction. Together with the observed antagonism by A-779 at low cAng-(1-7) concentrations these results suggest interaction with two receptor binding sites or receptor subtypes, one inducible at low concentrations of cAng-(1-7), sensitive to both A-779 and D-Pro\textsuperscript{7}-Ang-(1-7) and another one inducible with natural Ang-(1-7) and high
concentrations of cAng-(1-7), sensitive to D-Pro\(^7\)-Ang-(1-7) but not to A-779. Further investigations are needed to warrant a final conclusion.

Natural Ang-(1-7) serves as a cleavable substrate for the N-terminal-domain of ACE and as an inhibitor of cleavage by the C-terminal domain of ACE, which cleaves Angiotensin-I (32). We demonstrated that in contrast to natural Ang-(1-7), cAng-(1-7) in the micromolar range has no inhibitory effect on activity of the C-terminal part of ACE. Many C-domain ACE inhibitors contain a C-terminal proline, which is thought to be an important residue in the interaction of ACE inhibitors with the enzyme (145). In cAng-(1-7), this residue was replaced by a thioether bridged amino acid, thereby apparently reducing its affinity for the binding site. This lack of ACE-inhibitory activity of cAng-(1-7) indicates that a more specific therapeutic agent is obtained by the ring introduction.

Although already two decades ago suggested as a tool to incorporate unnatural amino acids in therapeutic peptides (183), the use of the lantibiotic enzymes has now, for the first time, resulted in the production of an active and stable therapeutic agent. Recent developments show that multiple thioether bridges can be incorporated in designed nonlantibiotic peptides (160). Therefore, the ever growing knowledge on the lantibiotic modification enzymes will only expand the possibilities to use them in stabilizing peptides with interesting biological properties.

The cyclized Ang-(1-7) described in the present study combines strongly enhanced proteolytic resistance with improved activity apparently due to better receptor interaction compared to its natural counterpart. This makes cAng-(1-7) highly interesting for therapeutic purposes.

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**Footnotes**

Abbreviations used: Ang, angiotensin; RAS, renin-angiotensin system; ACE, angiotensin-converting enzyme; cAng-(1–7), 4–7 thioether-bridgedangiotensin-(1–7); SD, Sprague-Dawley; PE, phenylephrine; A-779, D-Ala7-Ang-(1–7); AVE 0991, 5-formyl-4-methoxy-2-phenyl1-[[4-[2-ethylaminocarbonylsulfonamido)-5-isobutyl-3-thienyl]-phenyl]-methyl]-imidazole.

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