Angiotensin-(1–7) with Thioether Bridge: An Angiotensin-Converting Enzyme-Resistant, Potent Angiotensin-(1–7) Analog[S]

Leon D. Kluskens,[1] S. Adriaan Nelemans, Rick Rink, Louwe de Vries,
Anita Meter-Arkema,[2] Yong Wang,[3] Thomas Walther,[3] Anneke Kuipers, Gert N. Moll,
and Marijke Haas[4]

BiOMaDe Technology Foundation, Groningen, The Netherlands (L.D.K., R.R., L.d.V., A.M.-A., A.K., G.N.M., M.H.); Department of Molecular Pharmacology, University Centre for Pharmacy, University of Groningen, Groningen, The Netherlands (S.A.N.); and Department of Cardiology and Pneumology, University Hospital Benjamin Franklin, Free University, Berlin, Germany (Y.W., T.W.)

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 resistant 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 2-fold larger than that obtained for natural Ang-(1–7). The Ang-(1–7) receptor antagonist D-Pro[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 cyclized Ang-(1–7) also interacts with the Ang-(1–7) receptor. Taken together, applying a highly innovative enzymatic peptide stabilization method, we generated a stable Ang-(1–7) analog with strongly enhanced therapeutic potential.

Angiotensin (Ang)-(1–7) is an endogenous heptapeptide that plays an important role in the renin-angiotensin system (RAS) (Trask and Ferrario, 2007). Its alleged biological inactivity was first refuted by Schiavone et al. (1988), who demonstrated a dose-related stimulation of arginine vasopressin release by Ang-(1–7) equipotent to angiotensin-II (AngII). Since then, its significance in the complex RAS has been slowly unraveled. Although in some cases, Ang-(1–7) acts similar to AngII (Schiavone et al., 1988; Rodgers et al., 2002, 2006), Ang-(1–7) mainly exerts effects that oppose those of AngII, like vasoconstriction, fibrosis, and proliferation (Santos et al., 2000; Schindler et al., 2007). Discoveries such as the counteractive effect of Ang-(1–7) on the cardiovascular actions of AngII (Roks et al., 1999; Ferrario, 2002) and its ability to attenuate the development of heart failure (Loot et al., 2002) subsequently have supported this concept. It has been demonstrated more recently that Ang-(1–7) also plays a protective role in hepatic diseases such as liver fibrosis (Pereira et al., 2007) and can even inhibit the growth of human lung cancer cells in vivo (Menon et al., 2007). Furthermore, the discovery that the G protein-coupled receptor Mas is involved in Ang-(1–7) signaling has greatly contrib-

ABBREVIATIONS: Ang, angiotensin; RAS, renin-angiotensin system; ACE, angiotensin-converting enzyme; cAng-(1–7), 4–7 thioether-bridged angiotensin-(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.

uted to the elucidation of the heptapeptide's biological role (Santos et al., 2003b).

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 analogs with improved pharmacodynamic properties. Intramolecular thioether bridge formation especially 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 with their linear counterparts (Rew et al., 2002; Tugyi et al., 2005). Thioether bridges are more stable than peptide bonds and disulfide bridges (Tugyi et al., 2005). Cyclization imposes conformational constraints, which probably caused the significantly enhanced receptor interaction of thioether enkephalin (Rew et al., 2002).

Multistep chemical synthesis of thioether-bridged peptides is costly and cumbersome. We recently described a fermentative procedure to stabilize therapeutic peptides by introducing thioether bridges (Kluskens et al., 2005). This procedure involves plasmid-encoded nisin modification enzymes expressed by *Lactococcus lactis:* a dehydratase (nisin dehydratase) enzymatically dehydrates a Ser (or Thr) into a dehydroalanine (or dehydrobutyrine) (Kuipers et al., 2004). The generated dehydroresidues either enzyme (nisin cyclase) catalyzed or spontaneously reacted with a cysteine thiol group, which yielded an intramolecular thioether bridge in the peptide (Kluskens et al., 2005; Rink et al., 2007).

Here, we 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 with 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.

Materials and Methods

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

Animals. Specified pathogen-free male Sprague-Dawley (SD) rats (Harlan, Zeist, The Netherlands) weighing 350 to 450 g were used. Before the experiment, the animals were housed together with free access to tap water and solid chow (Harlan) in a temperature- and humidity-controlled room and a 12-/12-h 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 ACE at 37°C over a period of 7 h in a buffer containing 100 mM Tris-HCl, pH 8.3, 300 mM NaCl, and 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 and 20 mM

phosphate buffer, pH 7.4. Analysis and Ang-(1–7) detection in these in vitro studies were carried out as described previously (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 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 previously (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 g. Animals were kept under O$_2$/isoflurane (1.5–2%) anesthesia throughout the study. A 30-min stabilization period with 1 ml/h 0.9% NaCl was followed by a subsequent simultaneous infusion of both 100 μM cAng-(1–7) and 100 μM Ang-(1–7) in 0.9% NaCl at a constant rate of 1 ml/h. Blood (200–300 μl) was sampled via the carotid artery every 30 min for 2 h. 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 previously (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). 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 under 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 (Silva et al., 2007) and D-Pro^7-Ang-(1–7) (Santos et al., 2003a) were applied 10 min before addition of 30 nM PE at a concentration of 0.1 μM. Vasodilation data are represented as percentage 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–10 μM) and 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 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 were done using unpaired Student's t statistics with the exception of the in vivo infusion study in which paired analysis was used. Repeated measures analysis of variance with Bonferroni 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 $p \leq 0.05$. Asterisks 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. Be-
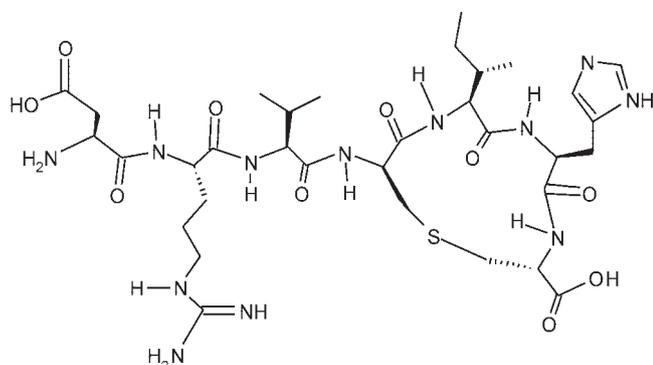
Fig. 1. Hypothetical chemical structure of cAng-(1–7). The thioether bridge from positions 4 to 7 (N terminus on the left) is depicted in the D-L configuration expected on the basis of the DL configuration of the thioether bridges in nisin and other lantibiotics.

cause 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 positions 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. Matrix-assisted laser desorption ionization/time of flight 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 (Kluskens et al., 2005; Rink et al., 2007).

Resistance of cAng-(1–7) against Proteolysis by Purified ACE and Plasma. In the circulation, endogenous Ang-(1–7) is rapidly catabolized, primarily by ACE (Yamada et al., 1998). 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. Although natural Ang-(1–7) was completely degraded within 90 min in a purified ACE solution, equiamounts of cAng-(1–7) were completely resistant against ACE degradation over the full period of 4 h ($p < 0.001$) (Fig. 2A). After 4 h of incubation in plasma, natural Ang-(1–7) was largely degraded (5% remaining), whereas most of the 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 (Santos et al., 2000). The proteolytic stability of cAng-(1–7) was compared with 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 h, whereas more than 80% of the initial amount of cAng-(1–7) remained intact ($p < 0.001$). 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. In addition, 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 with natural Ang-(1–7).

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 SD rats during a continuous intravenous infusion of both peptides. Cyclization resulted in a strong, 34 ± 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). The creatinine level in the plasma was 49.82 ± 1.33 µM at 0 min of infusion and 50.08 ± 1.38 µM at 120 min. Hence, renal functioning was not affected by the infusion.

Potent Dilating Effect of cAng-(1–7) on Precontracted Aorta Rings of SD Rats. Thoracic aortic rings with intact endothelium were precontracted with PE; 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). A 10-fold lower amount of cAng-(1–7) was needed to achieve 10% dilatation [0.3 nM cAng-(1–7)
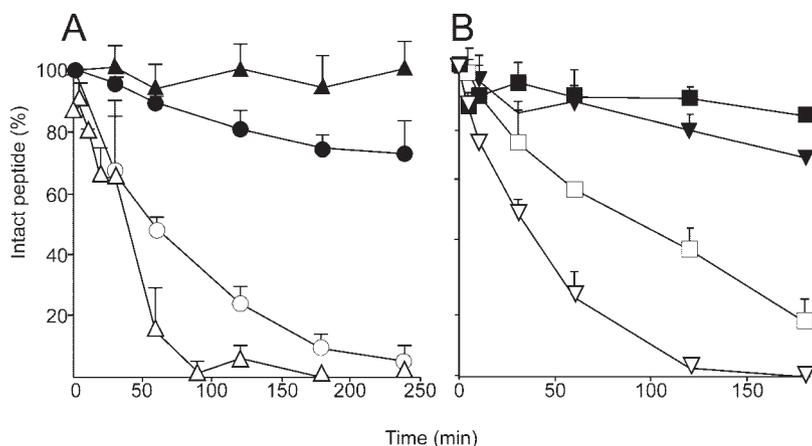

Fig. 2. A and B, enhanced proteolytic resistance of cAng-(1–7). Proteolytic resistance was measured of natural (△, ○, □, ▽) and cyclized Ang-(1–7), (▲, ●, ■, ▼), against ACE (A, △, ▲), plasma (A, ○, ●), liver (B, □, ■), and kidney homogenate (B, ▽, ▼) at pH 7.4. Each point represents the mean ± S.E.M., generated from at least three separate experiments.
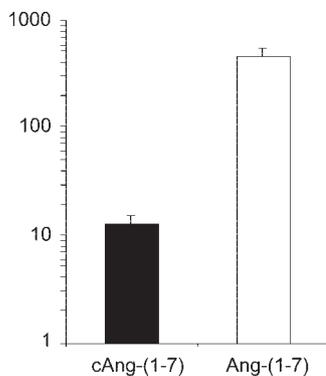
Fig. 3. Long-lasting in vivo survival of cAng-(1–7). Plasma clearance (milliliters per minute) of natural (open bar) and cAng-(1–7) (filled bar) in Sprague-Dawley rats under anesthesia during a constant intravenous infusion of both peptides. Each bar represents the mean ± S.E.M. ($n = 5$).
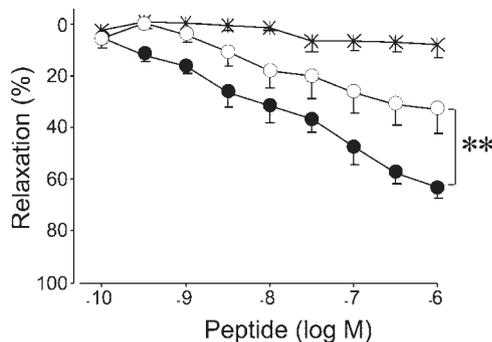

Fig. 4. Enhanced relaxation activity of cAng-(1–7). Relaxation of SD rat aorta rings during cumulative addition of natural (○) or cyclized (●) Ang-(1–7) or in control incubation (×), after precontraction with 0.3 μM phenylephrine, related to a vehicle experiment. Each point represents the mean ± S.E.M. generated from at least seven (natural) and 11 (cyclized) separate experiments.

versus 3 nM natural Ang-(1–7)], whereas 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)]. In addition, the maximal degree of vasodilation that was obtained was almost doubled with cAng-(1–7) compared with 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 prechallenge level with a wash-out half-life of 2.1 ± 0.3 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-Ala⁷-Ang-(1–7) (A-779) and D-Pro⁷-Ang-(1–7) at 0.1 μM (Fig. 5). In line with recent findings on aorta rings of Sprague-Dawley rats (Silva et al., 2007), natural Ang-(1–7) dilation was abolished by D-Pro⁷-Ang-(1–7) and insensitive for A-779 antagonism (Fig. 5A). cAng-(1–7)-induced dilation was fully blocked by D-Pro⁷-Ang-(1–7), providing evidence for the involvement of the Ang-
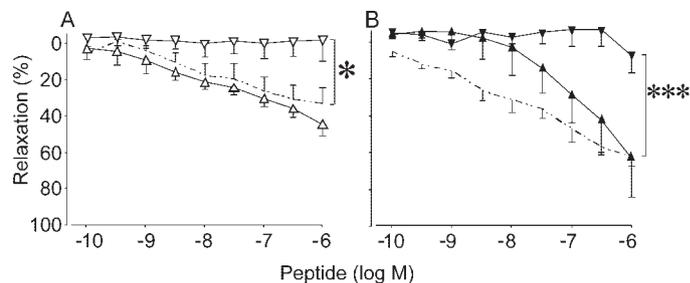

Fig. 5. Effects of Ang-(1–7)-receptor antagonists. Vasodilating effect of natural (A) and cyclized (B) Ang-(1–7) in the presence of Ang-(1–7)-receptor antagonists A-779 or D-Pro⁷-Ang-(1–7) (0.1 μM) compared with control (control curves are similar to the ones in Fig. 3). A, interrupted line, Ang-(1–7); △, Ang-(1–7) + A-779; ▽, Ang-(1–7) + D-Pro⁷-Ang-(1–7). B, interrupted line, cAng-(1–7); ▲, cAng-(1–7) + A-779; ▼, cAng-(1–7) + D-Pro⁷-Ang-(1–7). Each point in the inhibition experiment represents the mean ± S.E.M. generated from at least six [D-Pro⁷-Ang-(1–7)] and eight (A-779) separate experiments.

(1–7) receptor in the vasorelaxant properties of cAng-(1–7). It is interesting that at low concentrations of cAng-(1–7), its activity was also antagonized by A-779 (Fig. 5B). 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 (Deddish et al., 1998) 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 because of the cyclization.

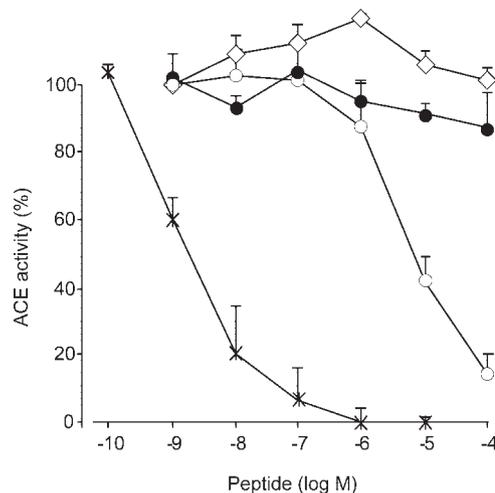**Fig. 6.** cAng-(1–7) has no or little ACE inhibiting activity. Inhibitory effect of natural Ang-(1–7) (○), Ang-(1–5) (◇), cAng-(1–7) (●), and captopril (×) on the activity of ACE. Each point represents the mean ± S.E.M. generated from three separate experiments. The curve of cAng-(1–7) is significantly 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 (Yamada et al., 1998) 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 (Rodgers et al., 2006) and two Ang-(1–7) antagonists with a D-Ala (A-779) or D-Pro at position 7 (Santos et al., 1994, 2003a), the effect that exchanging residues has on the peptide's functionality is not known. Here, we clearly demonstrated that Ang-(1–7) with a thioether bridge between positions 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 positions four and seven of the peptide, this major catabolic pathway was blocked for the thioether Ang-(1–7) analog. Ring introduction also reduced peptide catabolism by other enzymes than ACE as indicated by the enhanced stability in liver and kidney homogenates. Because 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. 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. Because 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 (Vickers et al., 2002), treatments have lately been focusing on increasing the ACE2 concentration. Techniques such as vector-mediated overexpression of ACE2 in spontaneously hypertensive rat models demonstrated a protective effect of ACE2 on high blood pressure and cardiac pathophysiology induced by hypertension (Díez-Freire et al., 2006). Until now, administration of exogenous ACE2 is hampered by suboptimal solubility and activity. Very recently, promising effects in spontaneously hypertensive rats have been shown using small-molecule ACE2 activators (Hernández Prada et al., 2008). It is unfortunate that 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) (Gurley and Coffman, 2008). Exogenous Ang-(1–7) induced attractive effects in vivo (Benter et al., 1995; Kucharewicz et al., 2002; Loot et al., 2002; Rodgers et al., 2002, 2006; Langeveld et al., 2005; Menon et al., 2007), whereas no signs of toxicity were observed in phase I/II clinical studies using Ang-(1–7) for bone marrow protection during anticancer therapy (Rodgers et al., 2006). 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 (Santos et al., 2005). Ang-(1–7) fused to a furin-cleavable protein in transgenic rats (Santos et al., 2004) 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 (Santos and Ferreira, 2006), the value of this compound for therapeutic application is yet unknown because 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 analog 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 positions four to seven enhances the activity and because 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 (Iusuf et al., 2008), 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-Pro7-Ang-(1–7). This suggests that both compounds are full Mas receptor antagonists (Santos et al., 2003a,b). However, in cerebral arteries of the canine brain, endothelium-dependent relaxation by Ang-(1–7) was not blocked by A-779 (Feterik et al., 2000). In addition, in another study, Ang-(1–7) activity was not prevented by A-779 (Silva et al., 2007). In precontracted aorta rings of SD rats, the Ang-(1–7)-induced vasodilation seemed to be mediated via an A-779-insensitive but D-Pro7-Ang-(1–7)-sensitive receptor (Silva et al., 2007).

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-Pro7-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 et al. (2007), 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 4 orders of magnitude, which is much broader than the 2 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[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 (Deddish et al., 1998). 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 (Patchett and Cordes, 1985). 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 suggested 2 decades ago as a tool to incorporate unnatural amino acids in therapeutic peptides (Schnell et al., 1988), 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 (Rink et al., 2007). 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 because of better receptor interaction compared with its natural counterpart. This makes cAng-(1–7) highly interesting for therapeutic purposes.

References

Bartels H and Böhmer M (1971) Micro-determination of creatinine. *Clin Chim Acta* **32**:81–85.

Benter IF, Ferrario CM, Morris M, and Diz DI (1995) Antihypertensive actions of angiotensin-(1–7) in spontaneously hypertensive rats. *Am J Physiol* **269**:H313–H319.

Cushman DW and Cheung HS (1971) Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem Pharmacol* **20**:1637–1648.

Deddish PA, Marcic B, Jackman HL, Wang HZ, Skidgel RA, and Erdös EG (1998) N-domain-specific substrate and C-domain inhibitors of angiotensin-converting enzyme: angiotensin-(1–7) and keto-ACE. *Hypertension* **31**:912–917.

Díez-Freire C, Vázquez J, Correa de Adjounian MF, Ferrari MF, Yuan L, Silver X, Torres R, and Raizada MK (2006) ACE2 gene transfer attenuates hypertension-linked pathophysiological changes in the SHR. *Physiol Genomics* **27**:12–19.

Ferrario CM (2002) Angiotensin I, angiotensin II and their biologically active peptides. *J Hypertens* **20**:805–807.

Feterik K, Smith L, and Katusic ZS (2000) Angiotensin-(1–7) causes endothelium-dependent relaxation in canine middle cerebral artery. *Brain Res* **873**:75–82.

Gurley SB and Coffman TM (2008) Angiotensin-converting enzyme 2 gene targeting studies in mice: mixed messages. *Exp Physiol* **93**:538–542.

Hernández Prada JA, Ferreira AJ, Katovich MJ, Shenoy V, Qi Y, Santos RA, Castellano RK, Lampkins AJ, Gubala V, Ostrov DA, et al. (2008) Structure-based identification of small-molecule angiotensin-converting enzyme 2 activators as novel antihypertensive agents. *Hypertension* **51**:1312–1317.

Iusuf D, Henning RH, van Gilst WH, and Roks AJ (2008) Angiotensin-(1–7): pharmacological properties and pharmacotherapeutic perspectives. *Eur J Pharmacol* **585**:303–312.

Kluskens LD, Kuipers A, Rink R, de Boef E, Fekken S, Driessen AJ, Kuipers OP, and Moll GN (2005) Post-translational modification of therapeutic peptides by NisB, the dehydratase of the lantibiotic nisin. *Biochemistry* **44**:12827–12834.

Kucharewicz I, Pawlak R, Matys T, Pawlak D, and Buczko W (2002) Antithrombotic

effect of captopril and losartan is mediated by angiotensin-(1–7). *Hypertension* **40**:774–779.

Kuipers A, de Boef E, Rink R, Fekken S, Kluskens LD, Driessen AJ, Leenhouts K, Kuipers OP, and Moll GN (2004) NisT, the transporter of the lantibiotic nisin, can transport fully modified, dehydrated, and unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides. *J Biol Chem* **279**:22176–22182.

Langeveld B, van Gilst WH, Tio RA, Zijlstra F, and Roks AJ (2005) Angiotensin-(1–7) attenuates neointimal formation after stent implantation in the rat. *Hypertension* **45**:138–141.

Loot AE, Roks AJ, Henning RH, Tio RA, Suurmeijer AJ, Boomsma F, and van Gilst WH (2002) Angiotensin-(1–7) attenuates the development of heart failure after myocardial infarction in rats. *Circulation* **105**:1548–1550.

Menon J, Soto-Pantoja DR, Callahan MF, Cline JM, Ferrario CM, Tallant EA, and Gallagher PE (2007) Angiotensin-(1–7) inhibits growth of human lung adenocarcinoma xenografts in nude mice through a reduction in cyclooxygenase-2. *Cancer Res* **67**:2809–2815.

Patchett AA and Cordes EH (1985) The design and properties of N-carboxyalkyl-dipeptide inhibitors of angiotensin-converting enzyme. *Adv Enzymol Relat Areas Mol Biol* **57**:1–84.

Pereira RM, Dos Santos RA, Teixeira MM, Leite VH, Costa LP, da Costa Dias FL, Barcelos LS, Collares GB, and Simões e Silva AC (2007) The renin-angiotensin system in a rat model of hepatic fibrosis: evidence for a protective role of angiotensin-(1–7). *J Hepatol* **46**:674–681.

Rew Y, Malkmus S, Svensson C, Yaksh TL, Chung NN, Schiller PW, Cassel JA, DeHaven RN, Taulane JP, and Goodman M (2002) Synthesis and biological activities of cyclic lanthionine enkephalin analogues: delta-opioid receptor selective ligands. *J Med Chem* **45**:3746–3754.

Rink R, Kluskens LD, Kuipers A, Driessen AJ, Kuipers OP, and Moll GN (2007) NisC, the cyclase of the lantibiotic nisin, can catalyze cyclization of designed nonlantibiotic peptides. *Biochemistry* **46**:13179–13189.

Rodgers KE, Oliver J, and diZerega GS (2006) Phase I/II dose escalation study of angiotensin 1–7 [A(1–7)] administered before and after chemotherapy in patients with newly diagnosed breast cancer. *Cancer Chemother Pharmacol* **57**:559–568.

Rodgers KE, Xiong S, and diZerega GS (2002) Accelerated recovery from irradiation injury by angiotensin peptides. *Cancer Chemother Pharmacol* **49**:403–411.

Roks AJ, van Geel PP, Pinto YM, Buikema H, Henning RH, de Zeeuw D, and van Gilst WH (1999) Angiotensin-(1–7) is a modulator of the human renin angiotensin system. *Hypertension* **34**:296–301.

Santos RA, Campagnole-Santos MJ, and Andrade SP (2000) Angiotensin-(1–7): an update. *Regul Pept* **91**:45–62.

Santos RA, Campagnole-Santos MJ, Baracho NC, Fontes MA, Silva LC, Neves LA, Oliveira DR, Caligiorne SM, Rodrigues AR, and Gropen Júnior C (1994) Characterization of a new angiotensin antagonist selective for angiotensin-(1–7): evidence that the actions of angiotensin-(1–7) are mediated by specific angiotensin receptors. *Brain Res Bull* **35**:293–298.

Santos RA, Ferreira AJ, Nadu AP, Braga AN, de Almeida AP, Campagnole-Santos MJ, Baltatu O, Iliescu R, Reudelhuber TL, and Bader M (2004) Expression of an angiotensin-(1–7)-producing fusion protein produces cardioprotective effects in rats. *Physiol Genomics* **17**:292–299.

Santos RA and Ferreira AJ (2006) Pharmacological effects of AVE 0991, a nonpeptide angiotensin-(1–7) receptor agonist. *Cardiovasc Drug Rev* **24**:239–246.

Santos RA, Frézard F, and Ferreira AJ (2005) Angiotensin-(1–7): blood, heart, and blood vessels. *Curr Med Chem Cardiovasc Hematol Agents* **3**:383–391.

Santos RA, Haibara AS, Campagnole-Santos MJ, Simões e Silva AC, Paula RD, Pinheiro SV, Leite MF, Lemos VS, Silva DM, Guerra MT, et al. (2003a) Characterization of a new selective antagonist for angiotensin-(1–7), D-pro7-angiotensin-(1–7). *Hypertension* **41**:737–743.

Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, et al. (2003b) Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci U S A* **100**:8258–8263.

Schiavone MT, Santos RA, Brosnihan KB, Khosla MC, and Ferrario CM (1988) Release of vasopressin from the rat hypothalamo-neurohypophysial system by angiotensin-(1–7) heptapeptide. *Proc Natl Acad Sci U S A* **85**:4095–4098.

Schindler C, Bramlage P, Kirch W, and Ferrario CM (2007) Role of the vasodilator peptide angiotensin-(1–7) in cardiovascular drug therapy. *Vasc Health Risk Manag* **3**:125–137.

Schnell N, Entian KD, Schneider U, Götz F, Zähner H, Kellner R, and Jung G (1988) Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. *Nature* **333**:276–278.

Silva DM, Vianna HR, Cortes SF, Campagnole-Santos MJ, Santos RA, and Lemos VS (2007) Evidence for a new angiotensin-(1–7) receptor subtype in the aorta of Sprague-Dawley rats. *Peptides* **28**:702–707.

Trask AJ and Ferrario CM (2007) Angiotensin-(1–7): pharmacology and new perspectives in cardiovascular treatments. *Cardiovasc Drug Rev* **25**:162–174.

Tugyi R, Mezö G, Fellinger E, Andreu D, and Hudecz F (2005) The effect of cyclization on the enzymatic degradation of herpes simplex virus glycoprotein D derived epitope peptide. *J Pept Sci* **11**:642–649.

Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, et al. (2002) Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* **277**: 14838–14843.

Yamada K, Iyer SN, Chappell MC, Ganten D, and Ferrario CM (1998) Converting enzyme determines plasma clearance of angiotensin-(1–7). *Hypertension* **32**:496–502.

Address correspondence to: Gert N. Moll, BiOMaDe, Nijenborgh 4, 9747 AG Groningen, The Netherlands. E-mail: Moll@biomade.nl