BASIC RESEARCH

An orally active formulation of angiotensin-(1-7) produces an antithrombotic effect

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INTRODUCTION AND OBJECTIVE: The heptapeptide angiotensin-(1-7) is a component of the renin-angiotensin system, which promotes many beneficial cardiovascular effects, including antithrombotic activity. We have recently shown that the antithrombotic effect of angiotensin-(1-7) involves receptor Mas-mediated NO-release from platelets. Here, we describe an orally active formulation based on angiotensin-(1-7) inclusion in cyclodextrin [Ang-(1-7)- CyD] as an antithrombotic agent. Cyclodextrins are pharmaceutical tools that are used to enhance drug stability, absorption across biological barriers and gastric protection.

METHOD: To test the antithrombotic effect of Ang-(1-7)-CyD, thrombus formation was induced in the abdominal vena cava of spontaneously hypertensive rats that were pretreated either acutely or chronically with Ang-(1-7)-CyD. Male Mas-knockout and wild-type mice were used to verify the role of the Mas receptor on the effect of Ang-(1-7)-CyD.

RESULTS: Acute or chronic oral treatment with Ang-(1-7)-CyD promoted an antithrombotic effect (measured by thrombus weight; all values are, respectively, untreated vs. treated animals) in spontaneously hypertensive rats (acute: 2.86 $\pm$ 0.43 mg vs. 1.14 $\pm$ 0.40 mg; chronic: 4.27 $\pm$ 1.03 mg vs. 1.39 $\pm$ 0.68 mg). This effect was abolished in Mas-knockout mice (thrombus weight in Mas wild-type: 0.76 $\pm$ 0.10 mg vs. 0.37 $\pm$ 0.02 mg; thrombus weight in Mas-knockout: 0.96 $\pm$ 0.11 mg vs. 0.87 $\pm$ 0.14 mg). Furthermore, the antithrombotic effect of Ang-(1-7)-CyD was associated with an increase in the plasma level of Angiotensin-(1-7).

CONCLUSION: These results show for the first time that the oral formulation Ang-(1-7)-CyD has biological activity and produces a Mas-dependent antithrombotic effect.

KEYWORDS: Angiotensin-(1-7); renin-angiotensin-system; receptor Mas; antithrombotic; cyclodextrin.

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INTRODUCTION

Thrombogenic events such as ischemic stroke, pulmonary embolism, deep venous thrombosis, mesenteric ischemia, and acute coronary syndrome are major complications of some pathological conditions, including hypertension, atherosclerosis and diabetes mellitus.[1] Despite the large number of therapeutic advances that have led to increasingly effective drug treatments, thrombogenic events still are a major cause of morbidity and mortality worldwide,[2] and significant efforts have been made to identify new antithrombotic therapies.

The renin-angiotensin system (RAS) is the major hormonal system regulator of cardiovascular function; it plays a pivotal role in the homeostasis of arterial pressure, cardiac function, hydroelectrolyte balance, and it participates in hemostasis.[3] The vasoactive peptide angiotensin (Ang) II is the main active member of the RAS; some of the most important current therapies for cardiovascular pathologies include blockade of the synthesis and/or activity of Ang II using angiotensin-converting enzyme (ACE) inhibitors[4] or angiotensin receptor blockers (ARBs).[5]

Ang-(1-7) is another biologically active component of the RAS; its actions are often the opposite of those described for Ang II.[6,7] Ang II promotes cell proliferation, vasoconstriction and pro-thrombotic activity, whereas Ang-(1-7) has antiproliferative, vasodilator and antithrombotic actions, suggesting that it has great potential to treat cardiovascular diseases.[8,9] The intrinsic antithrombotic effects of ACE inhibitors and ARBs may be mediated by Ang-(1-7)[10] because these drugs increase the plasma level of

Ang-(1-7).[11] We recently showed that the antithrombotic effect of Ang-(1-7) depends on Mas,[12] the known receptor for Ang-(1-7). [13] Furthermore, we demonstrated that Mas is present on platelets and that the interaction between Ang-(1-7) and Mas the only known recepter on these cells promotes the production of nitric oxide (NO).[12]

Many protein and peptide drugs cannot be administered orally because they are degraded by stomach and intestinal digestive enzymes.[14] Cyclodextrins (CyDs) are pharmaceutical tools that are used to design and evaluate drug formulations that can be used to enhance drug stability, absorption across biological barriers and gastric protection.[14] These compounds are cyclic oligosaccharides that consist of six to eight glucopyranose units that form molecules with polar outer surfaces and an apolar cavity. Their amphiphilic characteristics make them soluble and allow the formation of supramolecular inclusion complexes that are stabilized by non-covalent interactions with a variety of guest molecules.[14] CyDs are neither hydrolyzed nor absorbed in the stomach or small intestine. However, the colon microflora break them into small saccharides, delivering the included molecule to the large intestine, where it may be absorbed.[15] A formulation based on Ang-(1-7) included in CyD [Ang-(1-7)-CyD] was recently characterized; however, its biological action has not been evaluated.[15]

In the present study, we show for the first time that Ang-(1-7)-CyD possesses biological activity and promotes an antithrombotic effect.

METHODS

Animals

The following animals were used: 15-week-old spontaneously hypertensive rats (SHRs) that were bred at the Biological Science Institute (CEBIO, Federal University of Minas Gerais) and *Mas*-knockout ($Mas^{-/-}$) and wild-type ($Mas^{+/+}$) male mice on a pure genetic background C57BL/6 (15 weeks old) that were produced at the Max-Delbrück Center for Molecular Medicine, Berlin and maintained at the transgenic animal facility of the Laboratory of Hypertension, Biological Science Institute, Federal University of Minas Gerais (UFMG). Animals were kept in temperature-controlled rooms with 12 h/12 h light/dark cycles and free access to water and food. The animal care committee from the UFMG, Brazil, approved all experimental protocols.

Thrombus induction in rats

The effects of acute [10 μg/kg, 30 μg/kg or 100 μg/kg of Ang-(1-7) 5 hours before thrombus induction and chronic [30 μg/ kg of Ang-(1-7)-CyD once per day for eight weeks] oral administration of Ang-(1-7)-CyD were tested in SHR. Thrombus formation was induced in the abdominal vena cava by ligature, as described previously.[10] Twelve hours before thrombus induction, the SHRs were deprived of food but had free access to water. For each rat, the abdomen was opened under ketamine/xylazine anesthesia (100 mg/kg of ketamine and 10 mg/kg of xylazine), and the vena cava was carefully separated from the surrounding tissues and ligated tightly with a cotton thread just below the left renal vein. The abdomen was then closed with a double layer of sutures (peritoneum with muscles and skin separately). After two hours, each animal was reanesthetized, the abdomen was reopened, and the vena cava was carefully dissected and inspected for the presence of thrombus. The thrombus was air-dried at 37°C overnight, and its weight was measured.

Thrombus induction in mice

To test the role of Mas on the antithrombotic effect of Ang-(1-7)-CyD, thrombus formation was induced in the abdominal vena cava of $Mas^{-/-}$ and $Mas^{+/+}$ mice with a ferric chloride ($FeCl_3$) solution, as described previously.[12] Mice were deprived of food but had free access to water twelve hours before thrombus induction. Acute oral administration of Ang-(1-7)-CyD [100 μg/kg of Ang-(1-7)] was performed two hours before starting the surgical procedures. After anesthesia (ketamine 100 mg/kg, xylazine 10 mg/kg), the abdomen was opened, and the vena cava was exposed and carefully ligated with a cotton thread just below the left renal vein. A filter paper (2 mm × 5 mm) steeped in a 35% ferric chloride solution was applied below the ligature. After thirty minutes, the thrombus that formed was carefully removed and air-dried at 37°C overnight. The thrombotic response was assessed by measuring the thrombus weight.

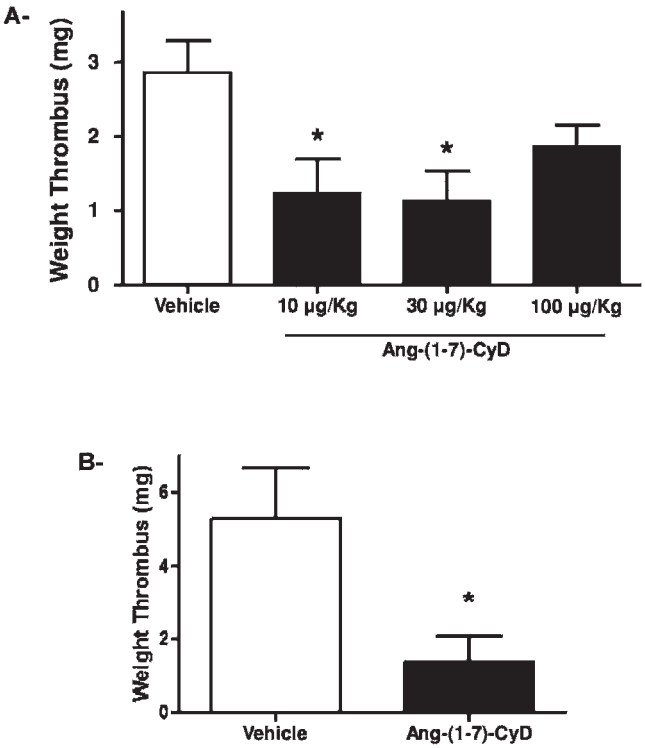

Figure 1 - Ang-(1-7)-CyD promoted antithrombotic effects in SHRs. A) Acute treatment with Ang-(1-7)-CyD (five hours before thrombus induction) inhibited thrombus formation when administered in doses of the equivalent of 10 μg/kg or 30 μg/kg of Ang-(1-7). B) Chronic administration of Ang-(1-7)-CyD [equivalent of 30 μg/kg of Ang-(1-7) per day] over the course of eight weeks promoted a strong inhibition of thrombus formation in SHRs. *$p<0.05$ significantly different from the respective control group treated with CyD (one-way ANOVA for panel A and unpaired Student's t-test for panel B). Each column represents the mean ± SEM from 7-10 experiments.

Radioimunnoassay

Wild type mice underwent acute oral administration of Ang-(1-7)-CyD [100 μg/kg of Ang-(1-7)] two hours prior to blood collection. Blood samples were obtained by decapitation and collected in polypropylene tubes containing 1 mM p-hydroxymercury benzoate, 30 mM 1, 10-phenanthroline, 1 mM PMSF, 1 mM pepstatin A, and 7.5% EDTA (100 μl/ml of blood).[16] The samples were pooled in groups of four to improve the sensitivity of the assay. After centrifugation, plasma samples were frozen and stored at -80℃.

The samples were extracted onto a Bond-Elut phenyl-silica cartridge (Varian, USA). The columns were pre-activated by sequential washes with 10 ml of 99.9% acetonitrile/0.1% heptafluorobutyric acid (HFBA) and 10 ml of 0.1% HFBA. Sequential washes with 10 ml of 99.9% acetonitrile/0.1% HFBA, 10 ml of 0.1% HFBA, 3 ml of 0.1% HFBA containing 0.1% bovine serum albumin (BSA), 10 ml of 10% acetonitrile/0.1% HFBA and 3 ml of 0.1 % HFBA were used to activate the columns. After sample application, the columns were washed with 20 ml of 0.1% HFBA and 3 ml of 20% acetonitrile/0.1% HFBA. The adsorbed peptides were eluted with 3 ml of 99.9% acetonitrile/0.1% HFBA into polypropylene tubes rinsed with 0.1% BSA.[13] After evaporation, angiotensin peptide levels were measured by a radioimmunoassay (RIA), as described previously.[17]

Statistical analysis

The results are expressed as mean ± SEM. Statistical analyses for SHR thrombus formation after chronic treatment, mouse thrombus formation, and plasma Ang-(1-7) level measurements were performed using the unpaired Student's t-test. The statistical analysis for SHR thrombus formation following acute treatment was performed by one-way ANOVA followed by a Dunnett post-test.

RESULTS

Acute and prolonged treatment with Ang-(1-7)-CyD promotes an antithrombotic effect in SHR

As shown in Figure 1A, acute treatment with Ang-(1-7)-CyD, equivalent to 10 μg or 30 μg of Ang-(1-7), promoted a significant antithrombotic effect in SHRs. The maximal effect occurred at a dose of 30 μg of Ang-(1-7); this dose promoted a 60% inhibition of thrombus formation (2.86 ± 0.43 mg vs. 1.14 ± 0.40 mg, n = 9 and n = 10, respectively; this and all subsequent comparisons refer to untreated vs. treated). As observed in other dose-response studies with other Ang-(1-7) effects,[10,12] a higher dose of Ang-(1-7), equivalent to 100 μg of Ang-(1-7), did not significantly change the thrombus weight in SHRs. In addition, we evaluated whether chronic treatment with this formulation could result in desensitization of the antithrombotic

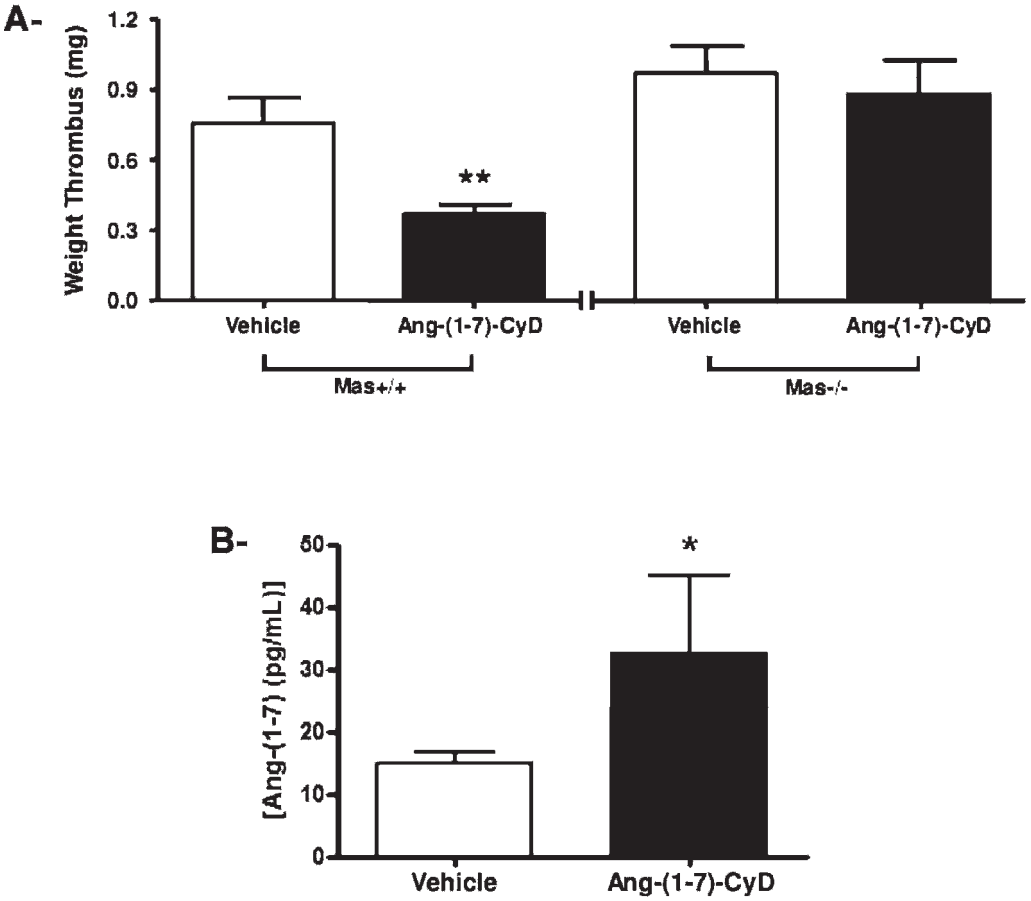

Figure 2 - The antithrombotic effect of Ang-(1-7)-CyD is dependent on Mas. A) Ang-(1-7)-CyD promoted a reduction in $FeCl_3$ solution-induced thrombus weight in *Mas*$^{+/+}$ mice; this effect was absent in *Mas*$^{-/-}$ mice. B) Oral administration of Ang-(1-7)-CyD [equivalent of 100 μg of Ang-(1-7)] increases the plasma level of Ang-(1-7) in C57Bl/6 mice. *$p<0.05$ and **$p<0.01$ significantly different from the control group treated with CyD (unpaired Student's t-test). Each column represents the mean ± SEM from 6-8 experiments.

effect. Strikingly, prolonged oral treatment with Ang-(1-7)-CyD promoted a potent antithrombotic effect in SHRs (67% inhibition of thrombus formation, 4.27 ± 1.03 mg vs. 1.39 mg ± 0.68 mg, n = 10 and n = 7, respectively) (Figure 1B). No difference was observed between the treatments with CyD alone, water or Ang-(1-7) alone (data not shown).

The antithrombotic effect of Ang-(1-7)-CyD is dependent on Mas

Based on the antithrombotic effect of Ang-(1-7)-CyD observed in SHRs, we next evaluated the role of the Mas receptor on this activity using a Mas-knocked out mouse. The oral formulation Ang-(1-7)-CyD [equivalent of 100 μg of Ang-(1-7)] inhibited thrombus formation in $Mas^{+/+}$ mice. However, this effect was abolished in $Mas^{-/-}$ mice ($Mas^{+/+}$: 0.76 ± 0.10 mg vs. 0.37 ± 0.02 mg, n = 6 and n = 7, respectively; $Mas^{-/-}$: 0.97 ± 0.11 mg vs. 0.87 ± 0.14 mg; n = 6 and n = 6, respectively, Figure 2A). No difference was observed among groups treated with CyD, water or Ang-(1-7) in $Mas^{+/+}$ mice (data not shown).

Ang-(1-7)-CyD promotes an increase in the plasma level of Ang-(1-7)

We then verified whether oral treatment with Ang-(1-7)-CyD could increase the plasma level of Ang-(1-7). Using wild type mice, we observed that this formulation promoted a significant increase in the plasma level of Ang-(1-7) (7.79 ± 0.40 pg/mL vs. 19.52 ± 7.38 pg/mL, n = 8 and n = 6, respectively) (Figure 2B). Treatment with vehicles did not promote any change in the plasma level of Ang-(1-7) (water: 7.73 ± 0.70 pg/mL; CyD: 7.79 ± 0.40 pg/mL; Ang-(1-7): 7.80 ± 1.14 pg/mL).

DISCUSSION

This is the first study to demonstrate the biological activity of the oral formulation Ang-(1-7)-CyD. Furthermore, we observed that the potent antithrombotic activity of this formulation is dependent on Mas and involves an increase in the plasma level of Ang-(1-7).

Recently, significant efforts have been made to identify new drug treatments for thrombogenic diseases because thrombogenic events remain a major cause of morbidity and mortality worldwide.[2] Ang-(1-7) is a peptide that promotes many beneficial cardiovascular effects, counter-regulating many of the actions induced by Ang II.[6,7] Its effects include vasodilation,[18,19] improvements in post-ischemic contractile function,[20,21] antifibrotic activity,[22] improvements in endothelial function[23,24] and antithrombotic activity.[10,14] All of these effects suggest that Ang-(1-7) is a potential target for treating cardiovascular disease.

In the present study, we showed that oral administration of Ang-(1-7)-CyD promotes an antithrombotic effect that was associated with an increase in the plasma concentration of Ang-(1-7). Furthermore, the antithrombotic effect of Ang-(1-7)-CyD was abolished in $Mas^{-/-}$ mice. We recently demonstrated that Mas is present on platelets and that the interaction between Ang-(1-7) and Mas on platelets stimulates the production of NO,[12] which is the major antiplatelet agent. Moreover, Ang-(1-7), via Mas, promotes NO and prostacyclin[24] production from endothelial cells, which are important antithrombotic agents in the hemostatic process.[25,26] Thus, the antithrombotic effect of Ang-(1-7) may be due to its interaction with Mas in both platelets and endothelial cells.[12] Together, these findings suggest that the antithrombotic effect of Ang-(1-7)-CyD is due to the increase in plasma Ang-(1-7) levels and consequent activation of Mas on platelets and endothelial cells.

Acute treatment with Ang-(1-7)-CyD, in doses equivalent to 10 or 30 μg/kg of Ang-(1-7), promoted a potent antithrombotic effect in SHRs. On the other hand, a higher dose of Ang-(1-7)-CyD [equivalent to 100 μg/kg of Ang-(1-7)] did not significantly inhibit thrombus formation. This finding is in accordance with previous studies showing a bell-shaped dose-response curve for this heptapeptide. For instance, using an intravenous infusion of Ang-(1-7), Kucharewicz et al.[10] observed that the antithrombotic effect of Ang-(1-7) was abolished at higher doses. We observed the same phenomenon when evaluating the antithrombotic effect of Ang-(1-7) in mice.[12] We evaluated whether chronic treatment with this formulation could produce desensitization of the antithrombotic effect. No evidence for that was obtained; instead, daily treatment with Ang-(1-7)-CyD over the course of eight weeks produced an even greater antithrombotic effect than that of acute treatment in SHR (60% and 67% thrombus weight inhibition for acute and prolonged treatment, respectively).

Inclusion compounds such as Ang-(1-7)-CyD are commonly used by the pharmaceutical industry because they promote the enhancement of drug stability, absorption across biological barriers and gastric protection against digestive enzyme degradation.[14] The formulation based on the inclusion complex formed between Ang-(1-7) and CyD was developed to allow oral administration of Ang-(1-7) and was recently characterized by Lula et al.,[15] although its biological action had not been evaluated previously. We have shown that Ang-(1-7)-CyD can increase the plasma level of Ang-(1-7) when administered orally (Figure 2B), and its action is dependent on Mas (Figure 2A). However, the exact mechanism leading to the Ang-(1-7)-CyD-induced increase in plasma Ang-(1-7) levels is unknown. One hypothesis is that the inclusion compound is not degraded until it reaches the colon and that the colon microflora break CyD into small saccharides, delivering the peptide, which could then be absorbed.[15]

In summary, our study shows that oral administration of Ang-(1-7)-CyD produces a clear antithrombotic effect, and this effect can be explained by an increase in the plasma level of Ang-(1-7) and consequent activation of Mas.

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