# Vascular Relaxation, Antihypertensive Effect, and Cardioprotection of a Novel Peptide Agonist of the Mas Receptor

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***Abstract***—Mas stimulation with angiotensin (Ang)-(1-7) produces cardioprotective effects and vasorelaxation. Using a computational discovery platform for predicting novel naturally occurring peptides that may activate G protein–coupled receptors, we discovered a novel Mas agonist peptide, CGEN-856S. An endothelium- and NO-dependent vasodilating effect was observed for CGEN-856S in thoracic aorta rings of rats (maximal value for the relaxant effect: 39.99±5.034%), which was similar to that produced by Ang-(1-7) ($10^{-10}$ to $10^{-6}$ mol/L). In addition, the vasodilator activity of this peptide depended on a functional Mas receptor, because it was abolished in aorta rings of Mas-knockout mice. CGEN-856S appears to bind the Mas receptor at the same binding domain as Ang-(1-7), as suggested by the blocking of its vasorelaxant effect with the Ang-(1-7) analogue D-Ala$^7$-Ang-(1-7), and by its competitive inhibition of Ang-(1-7) binding to *Mas*-transfected cells. The effect of CGEN-856S on reperfusion arrhythmias and cardiac function was studied on ischemia reperfusion of isolated rat hearts. We found that picomolar concentration of CGEN-856S (0.04 nmol/L) had an antiarrhythmogenic effect, as demonstrated by a reduction in the incidence and duration of reperfusion arrhythmias. Furthermore, acute infusion of CGEN-856S produced a shallow dose-dependent decrease in mean arterial pressure of conscious spontaneously hypertensive rats. The maximum change during infusion was observed at the highest dose. Strikingly, blood pressure continued to drop in the postinfusion period. The results presented here indicate that the novel Mas agonist, CGEN-856S, might have a therapeutic value, because it induces vasorelaxing, antihypertensive, and cardioprotective effects. **(*Hypertension*. 2010;56:112-120.)**

**Key Words:** Mas1 ■ angiotensin (1-7) ■ hypertension ■ reperfusion arrhythmias

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Angiotensin-(1-7) (Ang-[1-7]) is a biologically active component of the renin-angiotensin system (RAS).[1–3] The identification of angiotensin-converting enzyme (ACE) 2 in 2000,[4] and years later of Mas as a receptor for Ang-(1-7),[5] allowed researchers to build up a new concept of the RAS. In this novel concept, the result of the limited proteolysis processing starting with the hydrolysis of angiotensinogen by renin can be divided in 2 major functional arms, one represented by ACE/angiotensin II (Ang II)/Ang II type 1 ($AT_1$) receptors and the other composed of ACE2/Ang-(1-7)/Mas.[1] These 2 arms have some similar effects in the brain.[6,7] However, in the periphery most of the actions of the ACE2/Ang-(1-7)/Mas oppose many of the deleterious effects elicited by Ang II acting on $AT_1$ receptors.[1,8,9]

Genetic ablation of Mas leads to impairment of many cardiovascular and metabolic functions supporting the concept that the ACE2/Ang-(1-7)/Mas axis has an important physiological and physiopathological role.[10] Effects described for Ang-(1-7) acting through Mas include vasodilation, antiarrhythmogenesis, antifibrogenesis, antithrombogenesis, facilitation of erectile function, and improvement of glicidic and lipidic metabolism.[1,8,10,11]

The growing evidence supporting the beneficial effects of the Mas/Ang-(1-7) pathway prompted us to search for novel peptide ligands for Mas. This was achieved using a computational biology discovery platform, which we developed recently, that uses machine learning algorithms designed to predict novel G protein–coupled receptor (GPCR) peptide ligands cleaved from secreted proteins (extracted from the Swiss-Prot protein database) by convertase proteolysis, as described previously.[12,13] The ligands identified might, therefore, exist endogenously because of naturally occurring

Received March 5, 2010; first decision March 22, 2010; revision accepted April 13, 2010.

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*Hypertension* **is available at http://hyper.ahajournals.org** **DOI: 10.1161/HYPERTENSIONAHA.110.152942**

proteolysis. The predicted peptide ligands were synthesized and screened for activation of 152 GPCRs by calcium flux and cAMP assays.[12] Two novel peptides, designated CGEN-856 and CGEN-857, displayed high specificity for the Mas receptor, eliciting calcium influx in Chinese hamster ovary (CHO) cells overexpressing Mas. Both peptides did not show activation of $AT_1$ or Ang II type 2 ($AT_2$),[12] Ang II receptors that are known to be weakly activated by Ang-(1-7). In this study we report on the vasodilating, cardioprotective, and antihypertensive activities of a derivative of one of these peptides, CGEN-856S.

## Methods

### Animals

Male Wistar rats and spontaneously hypertensive rats (SHRs) weighing 240 to 300 g were obtained from the animal facility of the Biological Sciences Institute, Federal University of Minas Gerais. Mas-knockout FVB/N ($Mas^{-/-}$) mice and FVB/N ($Mas^{+/+}$) mice were obtained from the transgenic animal facilities of the Laboratory of Hypertension, Federal University of Minas Gerais. All of the experimental protocols were performed in accordance with the guidelines for the human use of laboratory animals of our institute and approved by local authorities.

### Discovery of Novel Mas Agonistic Peptides

Novel Mas-agonistic peptides were discovered using a new computational discovery platform aimed at discovering novel GPCR ligands encrypted in the human proteome.[12] Predicted peptides were screened for activation of 152 selected GPCRs using calcium flux assays, as described previously.[12] Two peptides, P61 and P33, were shown to induce calcium flux in *Mas*-transfected CHO cells.[12] These peptides, designated CGEN-856 and CGEN-857 (amino acid sequence FLGY*C*IYLNRKRRGDPAFKRRLRD and SM*C*HRWSRAVLFPAAHRP, respectively), have no significant homology to angiotensins, to known GPCR ligands, or to each other.[12] Both peptides contain a cysteine, and, thus, 3 forms of each peptide were synthesized, a monomer where the sulfhydryl moiety of the cysteine was protected with the acetamidomethyl group to avoid dimerization and a monomer in which the cysteine was substituted with serine or valine to create the monomeric forms CGEN-856S and CGEN-857V, respectively. In addition, a dimer via S-S bond was synthesized to create the dimeric forms CGEN-856D and CGEN-857D. The monomeric peptides and their dimeric forms elicited calcium flux in CHO cells cotransfected with the Mas receptor and G$\alpha$16 (Reference 12 and data not shown). The monomeric and dimeric forms of CGEN-856 and CGEN-857 also elicited vasodilative properties in an aortic ring assay (data not shown).

Further evaluation of the forms that seemed most active indicated additional Mas-agonistic activities in several experimental models. In this study, we describe the vasodilating, cardioprotective, and antihypertensive activities of one peptide, CGEN-856S.

### Rat and Mouse Aortic Ring Preparation and Mounting

Rat and mouse aortic ring preparations and mounting are described in the online Data Supplement, available at http://hyper.ahajournals.org.

### Isolated Heart Preparation

Wistar rats were decapitated 10 to 15 minutes after IP injection of 400 IU of heparin. The thorax was opened, and the heart was carefully dissected and perfused with Krebs-Ringer solution (KRS) containing (in millimoles per liter): NaCl 118.4, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 2H_2O$ 2.5, glucose 11.7, and $NaHCO_3$ 26.5. The perfusion fluid was maintained at 37±1°C, with a pressure of 65 mm Hg and constant oxygenation (5% $CO_2$-95% $O_2$). A force transducer was attached through a heart clip to the apex of the ventricles to record the contractile force (tension, grams) on a computer, by a data-acquisition system (Biopac System). A diastolic tension of 1.0±0.2 g was applied to the hearts. Electric activity was recorded with an ECG (Nihon Kohden) with the aid of 2 cotton wicks placed directly on the surface of the right atrium and left ventricle. Coronary flow was measured every 5 minutes by collecting and determining the volume of heart effluent during a 1-minute interval.

The hearts were perfused for an initial 15-minute period with KRS. After this period, the hearts were perfused for an additional 20 minutes (equilibration period) with KRS (control; n=14) or KRS containing CGEN-856S (0.008 nmol/L; n=6), CGEN-856S (0.04 nmol/L; n=6), or CGEN-856S (0.2 nmol/L; n=6). To compare the effect of CGEN-856S with Ang-(1-7) on reperfusion arrhythmias, hearts were perfused with Ang-(1-7) (0.2 nmol/L; n=6) in the same setup condition. After the equilibration period, the left anterior descending coronary artery was ligated by the method described by Lubbe et al[14] beneath the left auricular appendage together with the adjacent veins. The ligature was released after 15 minutes, and reperfusion with KRS (in the absence or presence of various doses of CGEN-856S or Ang-[1-7], as described above) was performed for an additional 30 minutes. Cardiac arrhythmias were defined as the presence of ventricular tachycardia and/or ventricular fibrillation after the ligature of the coronary artery was released. To obtain a quantitative measurement, the arrhythmias were graded arbitrarily by their duration as follows: the occurrence of cardiac arrhythmias for 0 to 3 minutes was assigned the factor 2; 3 to 6 minutes was assigned the factor 4; 6 to 10 minutes was assigned the factor 6; 10 to 15 minutes was assigned the factor 8; 15 to 20 minutes was assigned the factor 10; 20 to 25 minutes was assigned the factor 11; and 25 to 30 minutes was assigned the factor 12. A value of 0 to 12 was, thus, obtained in each experiment and is denoted as arrhythmia severity index (ASI).[15] An arrhythmia with a 30-minute duration was considered irreversible.

### Acute Blood Pressure Measurement

Experiments were performed in 12- to 14-week–old male Wistar rats and SHRs. Twenty-four hours before the experiments, polyethylene catheters were implanted into the abdominal aorta for mean arterial pressure (MAP) and heart rate (HR) measurements and in the femoral vein for intravenous infusions. Different doses of CGEN-856S (0.3, 3.0, 30.0 and 300.0 ng/kg per minute; n=7 and 8 for SHR and Wistar rats, respectively) or saline (n=8 for both groups) were infused (30 minutes for each dose). After the drug infusion, blood pressure and HR were monitored for an additional 60-minute period.

### ACE Activity Assay

Measurement of ACE activity is described in the online Data Supplement.

### Radioligand Binding Competition Assay at Human $AT_1$ and $AT_2$ Receptors

Radioligand binding competition assay at human $AT_1$ and $AT_2$ receptors is described in the online Data Supplement.

### Fluorescent Binding on Mas-Transfected Cells

Binding of CGEN-856S in *Mas*-transfected cells was performed using a method described previously.[16,17] CHO cells were stably transfected with rat *Mas* cDNA driven by a cytomegalovirus promoter and selected by neomycin.[5,18] Fluorescent Ang-(1-7) (at $10^{-8}$ mol/L) was incubated in 6-well plates for 60 minutes at 4°C in 1.5 mL of serum-free medium (DMEM) supplemented with 0.2% BSA, 0.005% bacitracin, 0.1 mol/L of PMSF, and 0.5 mol/L of orthophenanthrolene with *Mas*-transfected CHO cells in the presence or absence of Ang-(1-7) or CGEN-856S (both at $10^{-7}$ and $10^{-6}$ mol/L). After 2 washes with ice-cold serum-free DMEM, the slides were mounted for evaluation by confocal microscopy. Nonspecific binding was determined in the presence of $10^{-5}$ mol/L of Ang-(1-7). Relative fluorescence measurements were performed on a Zeiss LSM 510 META laser scanning confocal microscope excited at 488 nm with an argon-ion laser (oil-immersion objective lens: ×63).

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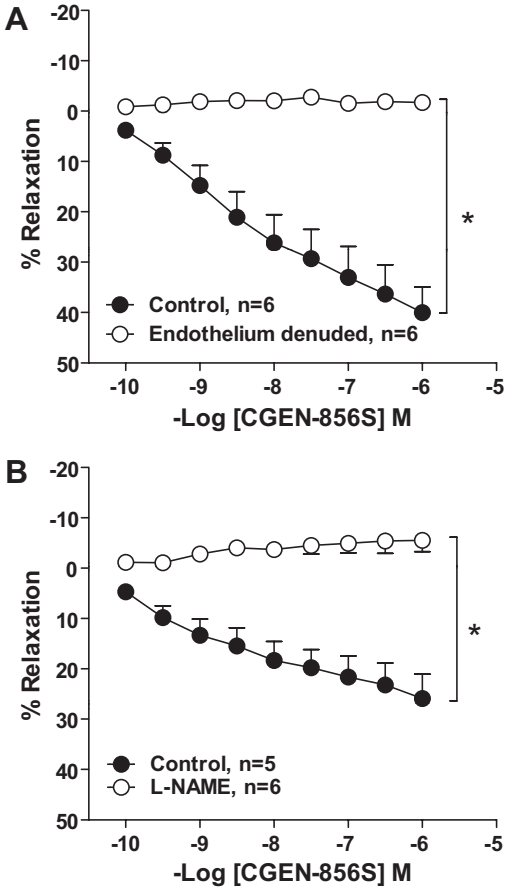


**Figure 1.** Vasodilator effect of CGEN-856S. A, Aortic rings from Wistar rats containing (control) or lacking functional endothelium. B, Aortic rings from Wistar rats in the absence or presence of L-NAME (100 $\mu$mol/L). Each point represents the mean±SEM generated from 5 to 6 separated experiments; *$P<0.05$ (2-way ANOVA followed by Bonferroni test).

## Statistical Analysis

Data are reported as mean±SEM. Statistical analysis was performed by the 1-way ANOVA followed by the Newman-Keuls test or by 2-way ANOVA followed by the Bonferroni test. The vasodilator effects were expressed as the percentage decrease of the maximal contraction induced by phenylephrine. Experimental values were considered statistically significant when $P<0.05$.

# Results

## CGEN-856S Induced NO- and Mas-Dependant Vasorelaxation in Isolated Aortic Rings

CGEN-856S produced a concentration-dependent vasodilator effect in endothelium-containing aortic rings precontracted with phenylephrine, which was abolished in endothelium-denuded vessels (Figure 1A). Maximal value for the relaxant effect of CGEN-856S in vessels with endothelium was 39.990±5.034%. In the presence of the NO synthase inhibitor $N^G$-nitro-L-arginine methyl ester (L-NAME), the vasodilator effect of CGEN-856S was also abolished, indicating the participation of NO in this effect (Figure 1B).

To further ascertain whether the vasorelaxation produced in aortic rings by CGEN-856S was because of a specific effect on the Mas receptor, we initially used the Ang-(1-7)/Mas antagonist D-Ala$^7$-Ang-(1-7), named A-779. As shown in Figure 2A, the vasorelaxation produced by CGEN-856S was inhibited by the Ang-(1-7) antagonist. We next used thoracic aortic rings taken from Mas-knockout FVB/N ($Mas^{-/-}$) and $Mas^{+/+}$ FVB/N mice. As shown in Figure 2B, CGEN-856S induced vasorelaxation in aortic rings from $Mas^{+/+}$ mice, whereas this effect was absent in aortic rings from $Mas^{-/-}$ mice.

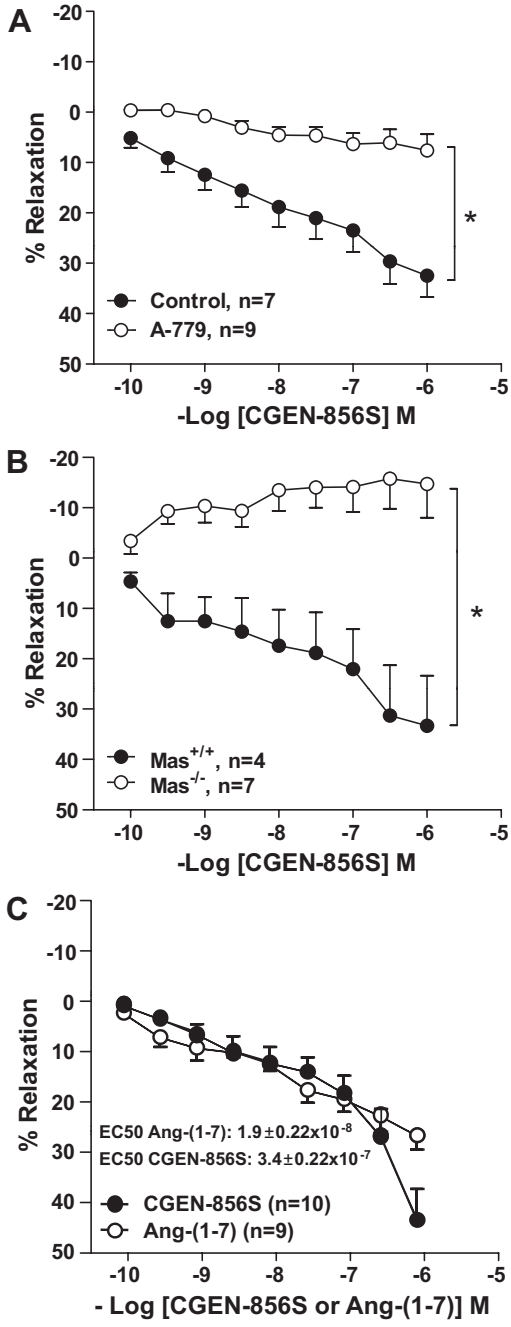


**Figure 2.** Vasodilator effect of CGEN-856S on inhibition or absence of functional Mas. A, Inhibition of the vasodilator effect of CGEN-856S in aorta rings of Wistar rats in the absence or presence of the Ang-(1-7) antagonist A-779 ($10^{-6}$ mol/L). B, Vasodilator effect of CGEN-856S in aorta rings taken from $Mas^{+/+}$ or $Mas^{-/-}$ mice. C, Vasodilator effect of CGEN-856S and Ang-(1-7) in aorta rings of Wistar rats containing functional endothelium. Each point represents the mean±SEM generated from 4 to 10 separate experiments; *$P<0.05$ (2-way ANOVA followed by Bonferroni test).

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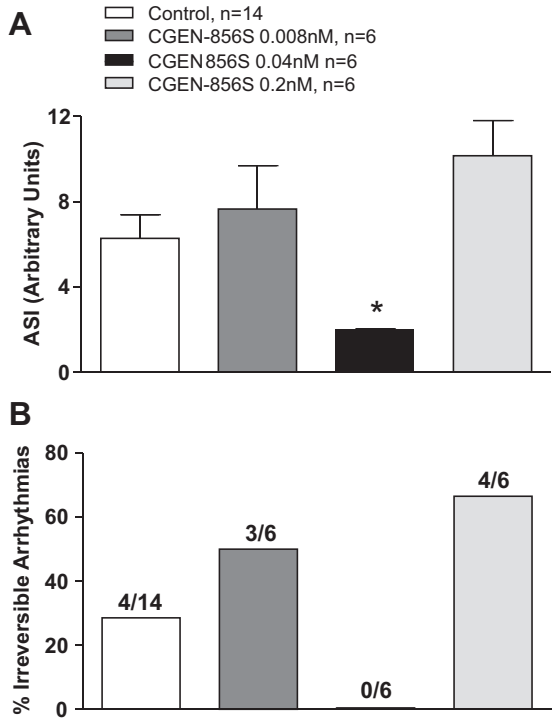


**Figure 3.** Effect of CGEN-856S on ischemia/reperfusion arrhythmias in isolated hearts. Arrhythmias were produced by 15-minute occlusion of the left anterior descending coronary artery in isolated rat hearts, followed by reperfusion. The hearts were perfused with KRS in the absence or presence of CGEN-856S (at 0.008, 0.040, or 0.200 nmol/L). A, Averaged ASI. B, Percentage of irreversible reperfusion arrhythmias. Numbers above the bars indicate the incidence of irreversible arrhythmias (>30 minutes) during the reperfusion period. *$P<0.05$ vs control group (1-way ANOVA followed by Newman-Keuls test).

As shown in Figure 2C, the vasorelaxation produced by CGEN-856S resembled that produced by Ang-(1-7); however, the $EC_{50}$ for Ang-(1-7) was lower ($EC_{50}=1.89\times10^{-8}\pm0.22$ mol/L for Ang-(1-7) versus $3.4\times10^{-7}\pm0.22$ mol/L for CGEN-856S), whereas the maximal value for the relaxant effect was higher for CGEN-856S (24.40% for Ang-[1-7] versus 54.62% for CGEN-856S).

## CGEN-856S Decreased Ischemia/Reperfusion Arrhythmias in Isolated Hearts

The antiarrhythmogenic effect of CGEN-856S was evaluated in isolated rat hearts. We initially tested CGEN-856S at a concentration (0.2 nmol/L), which has been described previously as antiarrhythmogenic for Ang-(1-7).[19] At this concentration, CGEN-856S produced no significant effect in the duration and incidence of reperfusion arrhythmias (ASI: 10.17±1.64 versus 6.28±1.11 for the control group; $P=0.068$). However, at a lower concentration, 0.04 nmol/L, CGEN-856S decreased the duration of arrhythmias (ASI: 2.000±0.002 versus 6.280±1.110 for the control group; Figure 3A) and abolished the occurrence of irreversible arrhythmias (Figure 3B), as observed for Ang-(1-7) at 0.2 nmol/L (Figure S1, in the online Data Supplement). At a lower concentration, 0.008 nmol/L, the peptide had no effect on the arrhythmias (Figure 3A and 3B).

CGEN-856S produced additional effects in the isolated hearts at its effective antiarrhythmic concentration. It increased the postischemic systolic tension in comparison with the KRS perfused hearts and showed a slight increase in the coronary flow at 5 minutes of reperfusion (Figure 4A and 4E, respectively). CGEN-856S also prevented the increase of diastolic tension observed in untreated hearts during the reperfusion period (Figure 4B). A slight but significant effect on postischemic −dT/dt was also observed (189.00±1.72 versus 164.50±3.50 g/s, for the control group; Figure 4D). No significant effect was observed on the +dT/dt (Figure 4C) and baseline or postischemic HR (Figure 4F).

## CGEN-856S Decreased MAP in SHRs

In keeping with its vasorelaxing effect on rat aortic rings, acute infusion of CGEN-856S in conscious SHRs produced a dose-dependent decrease in MAP (Figure 5A). The reduction in MAP started to be significant at a dose of 3 ng/kg per minute (maximum change: −5.9±2.3 mm Hg; Figure 5A). The maximum change in MAP during infusion was observed at the highest dose (300 ng/kg per minute; Δ: −9.7±3.7 mm Hg). Strikingly, the blood pressure continued to drop in the postinfusion period. The maximum change was observed at the end of the observation period (60 minutes postinfusion; Δ: −16.6±2.3 mm Hg). Vehicle infusion in SHRs did not alter MAP (Figure 5A). A small and transient but significant reduction in HR was induced by CGEN-856S in SHRs (Figure S2A). However, no clear dose-related effect was observed for the HR changes.

CGEN-856S also produced a decrease in the MAP of normotensive Wistar rats (Figure 5B); however, the magnitude of the MAP change was considerably smaller than in SHRs. During CGEN-856S infusion in Wistar rats the change in MAP observed at the highest dose (300 ng/kg per minute) was −6.2±2.3 mm Hg. The maximum reduction in MAP was also observed in the postinfusion period (Δ: −8.1±1.6 mm Hg). In Wistar rats there was no significant effect of CGEN-856S on HR (Figure S2B).

To address whether the effect of CGEN-856S could be because of interference with ACE activity or binding to Ang II receptors, we next tested the effect of the peptide on rat ACE activity and on the binding of Ang II to $AT_1$ and $AT_2$ receptors. CGEN-856S did not inhibit rat ACE activity at concentrations $\leq10^{-5}$ mol/L, in contrast to the inhibition observed for Ang-(1-7) (≈60% inhibition at $10^{-5}$ mol/L; $IC_{50}=2.58\times10^{-6}$ mol/L; Figure S3). In the same assay conditions, captopril, a known ACE inhibitor, essentially abolished ACE activity at $10^{-5}$ mol/L ($IC_{50}=2.51\times10^{-8}$ mol/L; Figure S3). As observed for ACE activity, no evidence was obtained for significant binding of CGEN-856S to the $AT_1$ receptor (Figure S4A), whereas it displayed a 1000-fold lower affinity for the $AT_2$ receptor than Ang II ($IC_{50}=4957.2$ versus 4.1 nmol/L for Ang II; Figure S4B). In contrast, CGEN-856S efficiently displaced the binding of fluorescent Ang-(1-7) to CHO *Mas*-transfected cells to a similar extent to that observed for Ang-(1-7) (Figure 6).

# Discussion

In this study we tested the vascular and cardiac effects of a novel Mas agonist peptide, CGEN-856S, identified using a

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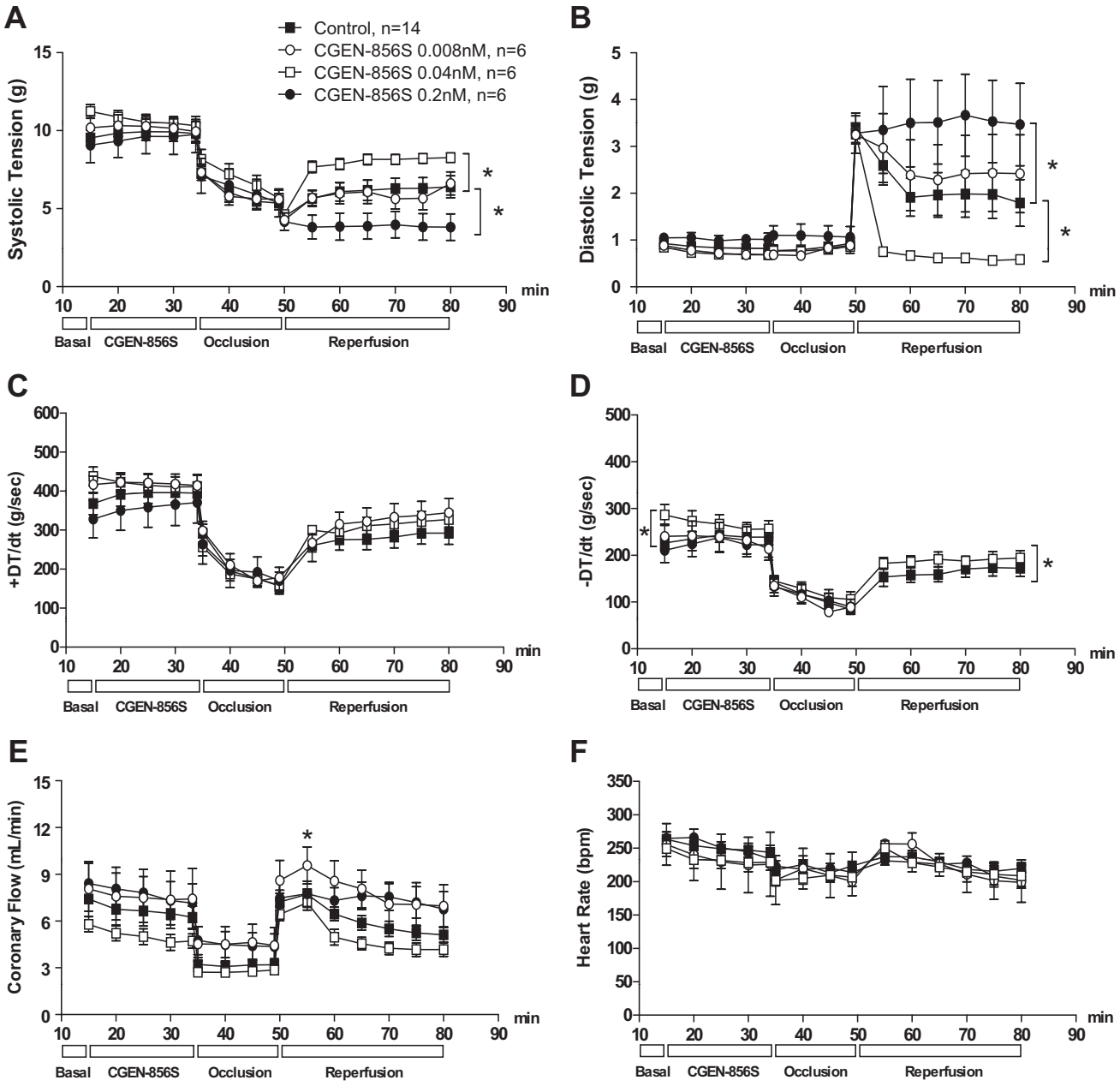


**Figure 4.** Time course of systolic tension (A), diastolic tension (B), rate of tension rise (+dT/dt; C), rate of tension fall (−dT/dt; D), coronary flow (E), and HR (F) in isolated perfused rat hearts during basal, left anterior descending coronary artery occlusion, and reperfusion periods. Isolated hearts were perfused with KRS in the absence or presence of CGEN-856S (at 0.008, 0.040, or 0.200 nmol/L). *$P<0.05$ compared with control group (2-way ANOVA followed by Bonferroni test).

computational biology discovery platform.[12] The amino acid sequence of CGEN-856S is unrelated to that of angiotensins. The data obtained for this peptide clearly validate this in silico strategy for identifying GPCR ligands. Furthermore, the results obtained with this novel Mas agonist peptide resembled those reported previously with Ang-(1-7)[1,8,19–21] and another putative small molecule Mas agonist, AVE 0991,[22] reinforcing the growing evidence for an important role of Mas in cardiovascular function.

CGEN-856S produced a vasodilating effect in thoracic aortic rings of mice and rats that resembled that produced by Ang-(1-7). The vasorelaxation produced by CGEN-856S was absent in endothelium-denuded vessels and was abolished by treatment with the NO synthase inhibitor L-NAME. These characteristics of the vasorelaxation induced by CGEN-856S are similar to those described for Ang-(1-7) and indicate the involvement of endothelium-derived NO in the response. Indeed, several lines of evidence linked Mas to the generation of NO in the vasculature and other tissues.[21,23,24] As shown previously for Ang-(1-7), the vasodilating effect of CGEN-856S was mediated by Mas, as demonstrated by its abolishment in Mas-knockout aortic rings and inhibition by the Ang-(1-7) antagonist A-779. It should be noted that similar vasodilating effects were observed also for the monomeric form of CGEN-857 and for the dimeric forms of CGEN-856 and CGEN-857 (data not shown).

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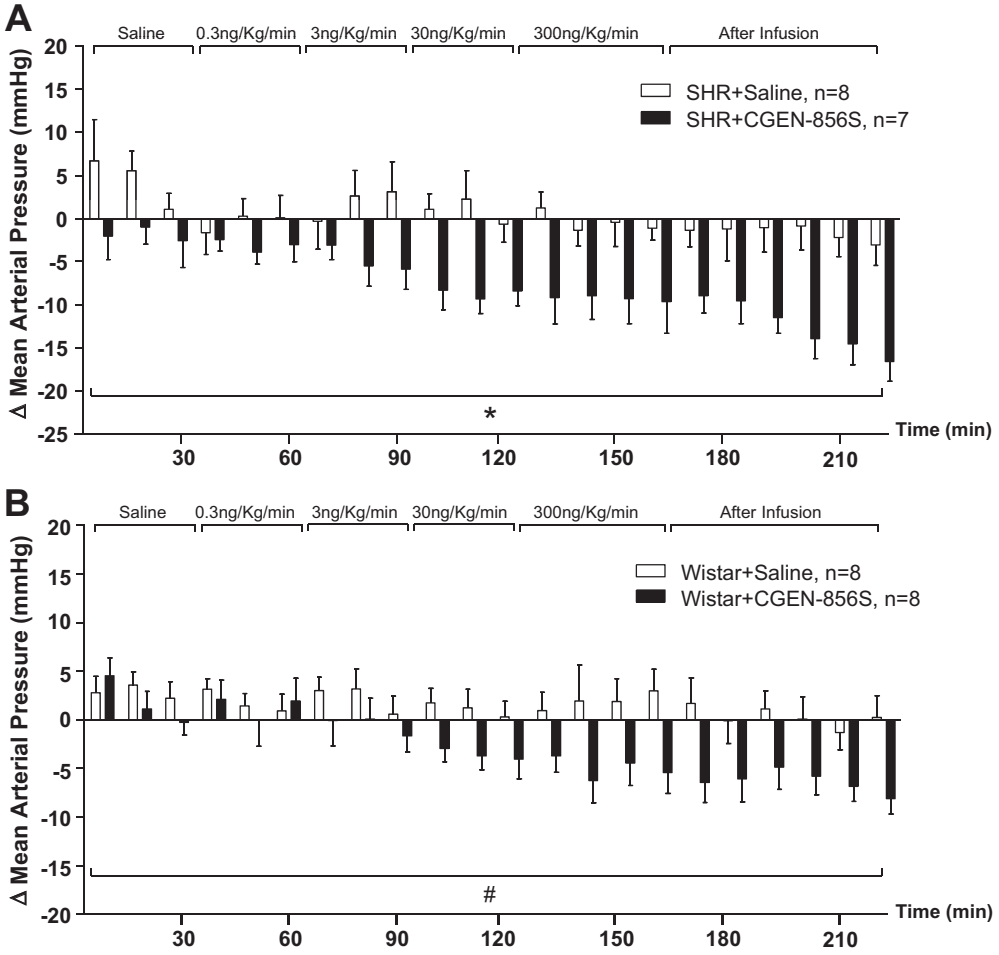


**Figure 5.** Changes in MAP produced by acute infusion of graded doses of CGEN-856S (0.3, 3.0, 30.0, and 300.0 ng/kg per minute) in conscious hypertensive rats, SHRs (A), and in conscious Wistar rats (B). Baseline values for SHRs: 147.3±1.2 mm Hg for the CGEN-856S group and 141.0±3.9 mm Hg for the control group; baseline values for Wistar rats: 97.4±6.3 mm Hg for the CGEN-856S group and 97.6±6.2 mm Hg for the control group. Bars represent values obtained every 10 minutes of infusion. *$P$<0.05 SHR+CGEN-856S vs SHR+saline; #$P$<0.05 Wistar+CGEN-856S vs Wistar+saline (2-way ANOVA followed by Bonferroni test).

Ang-(1-7) and CGEN-856S appear to bind the Mas receptor at the same binding domains, as suggested by the observation that the vasorelaxant effect of CGEN-856S can be blocked with the Ang-(1-7) analogue D-Ala$^7$-Ang-(1-7), A-779, and by the effective displacement of the fluorescent Ang-(1-7) binding to CHO *Mas*-transfected cells.

As observed before for Ang-(1-7) and AVE 0991,[25,26] the vasodilating effect of the CGEN-856S peptide in rat aortic rings was endothelium dependent and involved NO production, as evidenced by the blockade of endothelial NO synthase with L-NAME. However, unlike Ang-(1-7), CGEN-856S showed induction of calcium flux in *Mas*-transfected cells.[12] It has been described previously that Ang-(1-7), through activation of the Mas receptor, releases NO from cultured human aortic endothelial cells by the $Ca^{++}$-independent signaling cascade phosphatidylinositol 3-kinase/AKt.[21] Whether the phosphatidylinositol 3-kinase/AKt pathway is also a key component of the signaling cascades stimulated by CGEN-856S remains to be clarified. The initial events triggering the intracellular pathways involved in the effect of Ang-(1-7) may differ from those triggered by CGEN-856S because of a different interaction with the receptor (biased agonism).[27,28] Despite these possible differences, our data comparing Ang-(1-7) with CGEN-856S in aortic rings showed a similar pattern of relaxation for the 2 peptides. Similarly, acute infusion of CGEN-856S in SHRs produced an antihypertensive effect equivalent to that observed with Ang-(1-7).[29]

We also examined the antiarrhythmogenic effect of CGEN-856S in an ischemia/reperfusion model of isolated hearts. As shown before for Ang-(1-7),[19] a low concentration of CGEN-856S (0.04 nmol/L) reduced the incidence and duration of cardiac arrhythmias in isolated rat hearts. In addition, as reported previously for Ang-(1-7),[30] CGEN-856S, at its effective antiarrhythmic concentration, improved myocardial function by increasing the postischemic systolic tension and coronary flow after reperfusion, without changing HR. The absence of effect of CGEN-856S at a higher concentration on the reperfusion arrhythmias is similar to previous reports concerning Ang-(1-7). Neves et al[31] previously reported that Ang-(1-7) at 27 nmol/L produced a significant enhancement of reperfusion arrhythmias in isolated rat hearts. However, Ferreira et al[19] and our current data (Figure S1) showed that, at a lower concentration of 0.2

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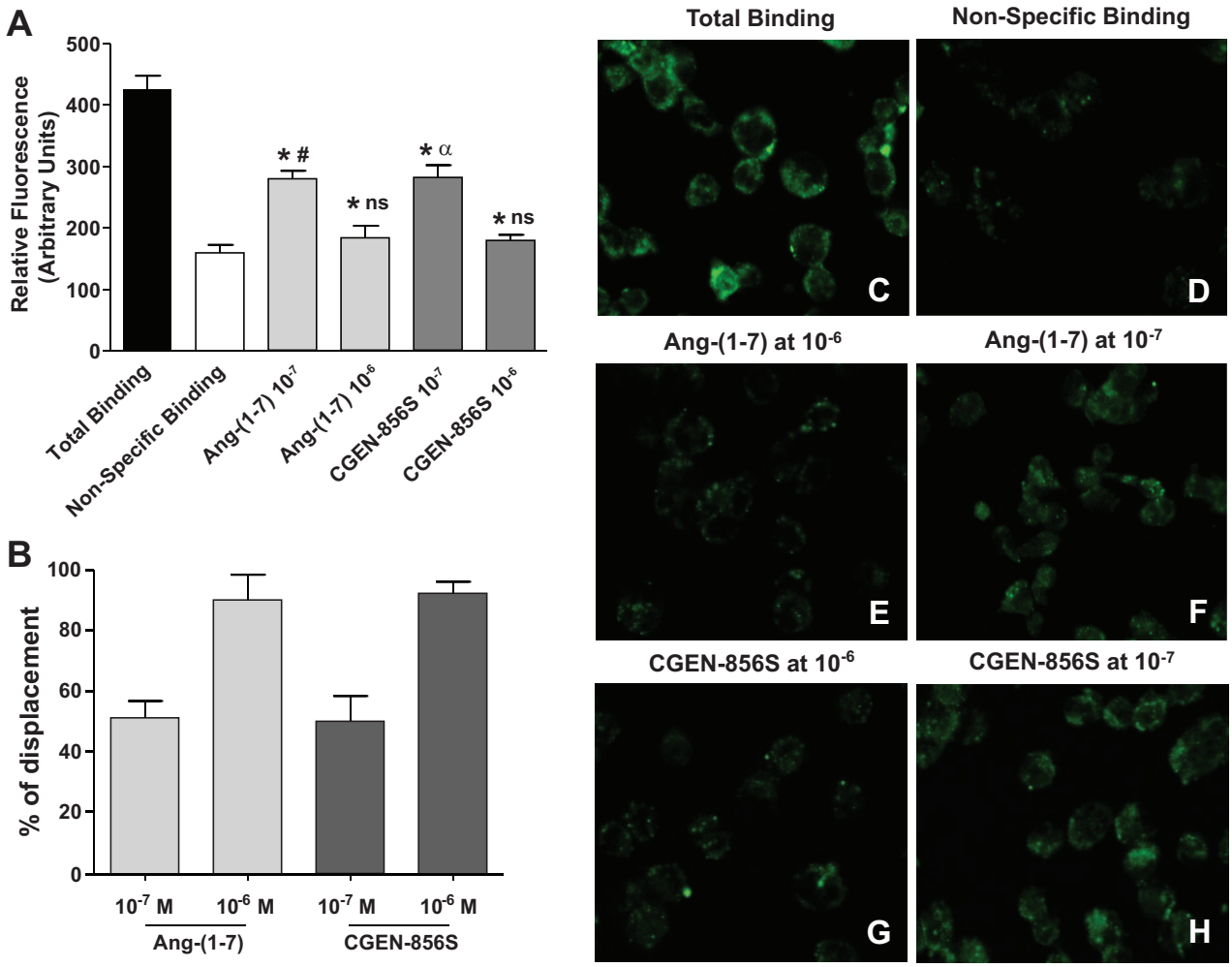


**Figure 6.** Binding assay on the Mas receptor. CGEN-856S at $10^{-7}$ and $10^{-6}$ mol/L and Ang-(1-7) at $10^{-7}$ and $10^{-6}$ mol/L displaced binding of FAM-Ang-(1-7) at $10^{-8}$ mol/L on *Mas*-transfected CHO cells (A and B). Graphs show the relative fluorescence captured on confocal microscopy (A) and the percentage of FAM-Ang-(1-7) displacement by CGEN-856S and Ang-(1-7) (B). Confocal microphotographs of the FAM-Ang-(1-7) binding on *Mas*-transfected CHO cells (C through H); C, total binding of FAM-Ang-(1-7) at $10^{-8}$ mol/L; D, nonspecific binding; E and G, Ang-(1-7) displacement of the FAM-Ang-(1-7) as a positive control; F and H, CGEN-856S displacement of the FAM-Ang-(1-7). Each column represents the mean±SEM; *$P<0.001$, significantly different from total binding; #$P<0.05$, significantly different comparing Ang-(1-7) at $10^{-7}$ mol/L with Ang-(1-7) at $10^{-6}$ mol/L; $\alpha P<0.001$, significantly different comparing CGEN-856S at $10^{-7}$ mol/L with CGEN-856S at $10^{-6}$ mol/L (1-way ANOVA followed by Bonferroni multiple comparison test).

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nmol/L, Ang-(1-7) presents an antiarrhythmogenic effect. Ang-(1-7) thus displays a clear tendency for a bell-shaped dose-response curve or biphasic effect.[32,33] Taken together, these findings suggest that the effect of Mas agonists on reperfusion arrhythmias in vitro is biphasic and concentration dependent. On the other hand, no evidence for a proarrhytmogenic effect of CGEN-856S (this study) or Ang-(1-7) was obtained in vivo.[3,34]

We have also observed an antihypertensive effect of CGEN-856S in SHRs. This observation is in accordance with ongoing studies in our laboratory using Ang-(1-7)[29] and with previous reports by others, suggesting that Mas stimulation could be a strategy to treat hypertension.[35] Indeed, we have shown recently that genetic deletion of Mas in FVB/N mice produced a significant increase in blood pressure associated with endothelium dysfunction.[36] An increase of vascular resistance in many organs[37] and a marked endothelium dysfunction[38] was also observed in Mas$^{-/-}$ C57BL/6 mice. The absence of an increase of blood pressure in this strain may be related to an impairment of heart function.[39] Infusion of CGEN-856S did not change HR in normotensive rats, whereas in SHRs only transient and dose-independent slight changes in HR were observed. The mechanism(s) of this selective effect in SHRs remain to be clarified.

One may argue that the antihypertensive effect of CGEN-856S could be because of interference with ACE activity or could be mediated by an Ang II receptor, acting as an $AT_1$ receptor antagonist or $AT_2$ receptor agonist. However, our results show that CGEN-856S did not inhibit ACE activity and did not significantly bind the $AT_1$ receptor. The very low affinity of this peptide for the $AT_2$ receptor (1000-fold lower than Ang II), together with the lack of effect of pure $AT_2$ agonists on the blood pressure of awake SHRs,[40] strongly suggest the absence of $AT_2$ receptor involvement in the cardiovascular action of CGEN-856S.

One important point that should be considered is related to the similarities and differences among CGEN-856S, Ang-(1-

7), and AVE 0991.[16,22] These 3 Mas agonists display different properties and applications. The peptide CGEN-856S (amino acid sequence: FLGY*S*IYLNRKRRGDPAFKRRLRD) is apparently more stable than Ang-(1-7) and has no ACE inhibitory activity. Because of its hydrophobicity, AVE 0991 could cross the blood-brain barrier producing central effects. Because some central effects of Ang-(1-7) are opposite to those evoked peripherally, the possibility that some peripheral effects of AVE 0991 could be masked by its central actions should be evaluated. On the other hand, Ang-(1-7) is endogenous and has quite well-characterized Mas-dependent actions.

In summary, in the current study we have identified a novel Mas agonist peptide, CGEN-856S, and further validated our bioinformatic platform for identifying novel GPCR ligands. Importantly, we present considerable evidence for antihypertensive and cardioprotective effects for CGEN-856S.

## Perspectives

The RAS is a key target for treating cardiovascular and renal diseases. The current therapy to block the activity of the RAS includes ACE inhibitors, $AT_1$ receptors antagonists, and direct renin inhibitors. The accumulating evidence that stimulation of the ACE2/Ang-(1-7)/Mas axis produces cardioprotection and antihypertensive effects[8,19,35,39] raises the possibility of development of a next generation of RAS-related drugs aimed to increase the activity of this axis.[34,41] The results presented here, using CGEN-856S, in addition to reinforcing the concept that Mas stimulation produces beneficial cardiovascular effects, illustrates the potential of this novel therapeutic strategy for treating cardiovascular diseases.

## Acknowledgments

We are grateful to José Roberto da Silva and Marilene Luzia de Oliveira for their helpful technical assistance.

## Sources of Funding

S.Q.S. was supported by a fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico, and R.Q.L. was supported by a fellowship from Fundação de Amparo à Pesquisa de Minas Gerais. This study was supported by a Compugen Grant and by Ministério de Ciência e Tecnologia/Fundação de Amparo à Pesquisa de Minas Gerais/Instituto Nacional de Ciencia e Tecnologia-INCT-NanoBiofar, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, and Conselho Nacional de Desenvolvimento Científico e Tecnológico/Fundação de Amparo à Pesquisa de Minas Gerais/Programa de Apoio a Núcleos de Excelência.

## Disclosures

R.A.S.S. is a consultant of Compugen Ltd. M.B., G.C., Y.C., and G.R. are employees of Compugen Ltd.

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# Vascular Relaxation, Antihypertensive Effect, and Cardioprotection of a Novel Peptide Agonist of the Mas Receptor

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*Hypertension.* 2010;56:112-120; originally published online May 17, 2010;
doi: 10.1161/HYPERTENSIONAHA.110.152942
*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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**ONLINE SUPPLEMENT**

# VASCULAR RELAXATION, ANTI-HYPERTENSIVE EFFECT AND CARDIO-PROTECTION OF A NOVEL PEPTIDE AGONIST OF THE MAS RECEPTOR

Silvia Quintão Savergnini;[1] Merav Beiman;[2] Roberto Queiroga Lautner;[1] Vanice de Paula-Carvalho;[1] Kyan Allahdadi;[1] Dalton Caires Pessoa;[1] Fabiana Pereira Costa-Fraga;[1] Rodrigo Araújo Fraga-Silva;[1] Gady Cojocaru;[2] Yossi Cohen;[2] Michael Bader;[3] Alvair Pinto de Almeida;[1] Galit Rotman;[2] Robson Augusto Souza dos Santos.[1]

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**Running title:** Anti-hypertensive effect of a novel Mas agonist.

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## METHODS

### *Rat Aortic Rings Preparation and Mounting*

Rings (3-4 mm) from the descending thoracic aorta, free of adipose and connective tissue, were set up in gassed (5% $CO_2$-95% $O_2$) Krebs-Henseleit solution (mmol/L): NaCl 110.8, KCl 5.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.07, $CaCl_2$ 2.49, $NaH_2PO_4$ 2.33 and glucose 11.51, at 37°C, under a tension of 1.0 g, for 1 hour to equilibrate. The presence of functional endothelium was assessed by the ability of Acetylcholine (1 µmol/L) to induce more than 70% relaxation of vessels pre-contracted with phenylephrine (0.1 µmol/L). When necessary, the endothelium was removed by rubbing the intimal surface with a wooden stick. Mechanical activity, recorded isometrically by a force transducer, was fed to an amplifier-recorder (Powerlab 4/20, ADInstruments, Inc.) and to a personal computer equipped with an analogue-to-digital converter board (AD16JR; World Precision Instruments, Inc.), using CVMS data acquisition/recording software (World Precision Instruments, Inc.).

The vasorelaxant activity of the peptide was measured in rat vessels with or without functional endothelium pre-contracted to the same tension level (approximately 1.5 g of tension) induced by submaximal concentration of phenylephrine ($10^{-7}$ mol/L). CGEN-856S was added in increasing cumulative concentrations (0.0001 to 1 µmol/L) once the response to phenylephrine had stabilized. The effect of the Ang-(1-7)/Mas antagonist, A-779, was tested by adding this compound at $10^{-6}$ mol/L, 3 minutes prior to the addition of cumulative concentration of CGEN-856S. In order to verify the participation of endothelium-derived NO in the relaxant effect of peptides, experiments were performed in the presence of 100 µmol/L L-NAME (*N*ω-nitro-L-arginine methyl ester), a nitric oxide synthase (NOS) inhibitor. In these experiments, vessels were pre-contracted with 0.03 µmol/L of phenylephrine, to achieve the same tension level as the others. L-NAME was added to the bath 20 min prior to the addition of phenylephrine. As a control for all the above-mentioned protocols, another vessel segment from each rat was simultaneously monitored for effects of the peptide alone.

The vasorelaxant activity of the peptides CGEN-856S and Ang-(1-7) were compared in isolated aortic rings with functional endothelium, pre-contracted to the same tension level (approximately 1.5 g of tension) induced by submaximal concentration of phenylephrine ($10^{-7}$ mol/L). CGEN-856S or Ang-(1-7) was added in increasing cumulative concentrations (0.0001 to 1 µmol/L) once the response to phenylephrine stabilized.

### *Mouse Aortic Rings Preparation and Mounting*

Rings (2-3 mm) from the descending thoracic aorta of $Mas^{+/+}$ and $Mas^{-/-}$ mice, free of adipose and connective tissue, were set up in gassed (5% $CO_2$-95% $O_2$) Krebs-Henseleit solution, at 37°C, under a tension of 0.5 g, for 1 hour to equilibrate. The presence of functional endothelium was assessed by the ability of acetylcholine (10 µmol/L) to induce more than 70% relaxation of vessels pre-contracted with phenylephrine (0.1 µmol/L). CGEN-856S was added in increasing

cumulative concentrations (0.0001 to 1 μmol/L) once the response to 0.1 μmol/L phenylephrine had stabilized. Mechanical activity, recorded isometrically by a force transducer, was fed to an amplifier-recorder (Powerlab 4/20, ADInstruments, Inc.) and to a personal computer equipped with an analogue-to-digital converter board (AD16JR; World Precision Instruments, Inc.), using CVMS data acquisition/recording software (World Precision Instruments, Inc.).

**Angiotensin-converting Enzyme (ACE) activity assay**

Plasma ACE activity was measured by a fluorometric method utilizing Hip-His-Leu as substrate as described previously.[1] Duplicate aliquots of a pool of rat plasma (10 µL) were incubated with 440 µL of 1 mmol/L Hip-His-Leu in 0.4 mol/L sodium borate buffer, pH 8.3, containing 0.9 mol/L NaCl for 15 min at 37°C. The reaction was stopped by the addition of 1.2 mL 0.34 mol/L NaOH, 100 µL of orthophthaldehyde (20 mg/mL in methanol) was added and after 10 min at room temperature, 200 µL of 3 N HCl was added. After centrifugation at 800 × g for 5 min, fluorescence of the supernatant solution (365 nm excitation and 495 nm emission) was measured against water. Blanks were prepared by inverting the order of addition of rat plasma and NaOH. A standard curve of 0.5 to 20 nmol His-Leu/ tube was prepared for each assay. Enzyme activity was calculated as nmol His-Leu/ min/ mL. The specificity of the assay was demonstrated by 98% inhibition with 5 µmol/L enalaprilat. To test for ACE inhibitory activity of Ang-(1-7) and CGEN-856S, final concentrations ranging from $10^{-9}$ to $10^{-5}$ mol/L were used for each peptide. Captopril was used as a positive control ($10^{-9}$ to $10^{-5}$ mol/L). In all assays the peptide solutions were mixed with the substrate solution immediately before addition of the rat plasma.

**Radioligand Binding Competition Assay at Human AT1 and AT2 receptors.**

CGEN-856S was tested in competitive radioligand binding assays at the human AT1 and AT2 Ang II receptors, at eight concentrations: 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μmol/L, in duplicate. The assays were performed at Euroscreen (Belgium).

Recombinant chinese hamster ovary (CHO)-K1-AT1 membranes were thawed on ice and diluted at 0.5 µg/well in assay buffer (50 mmol/L Tris-HCl pH 7.4, 5 mmol/L $MgCl_2$, Saponine 10µg/mL, 0.1% Bacitracin). The ligand ($Sar^1$, $Ile^8$) Angiotensin II, and the radioligand [$^{125}$I]-($Sar^1$, $Ile^8$) Angiotensin II were diluted in assay buffer. The following reagents were successively added in the wells of a 96 well plate: 50 µL of test compound at increasing concentrations, 25µL of radioligand (at a final concentration of 0.03 nmol/L) and 25µL of membrane extracts. The samples were incubated 60 min at 25°C and filtered over filter plate (presoaked in washing buffer 50 mmol/L Tris-HCl pH 7.4, 5 mmol/L $MgCl_2$, 0.05% BSA for 2h at RT) with a Filtermate Harvester (Perkin Elmer). After wash of the filters 6 times with 0.5 mL of ice-cold washing buffer, 50 µL of Microscint 20 (Packard) were added and the samples were incubated 15 min on an orbital shaker and then counted with a TopCount™ for 1 min/well.

Recombinant CHO-K1-AT2 membranes thawed on ice and diluted at 2.5 µg/well in assay buffer (25 mmol/L Hepes pH 7.4, 5 mmol/L $MgCl_2$, Saponine 10µg/mL, 0.5% BSA protease). The ligand, Angiotensin II and the radioligand: [$^{125}$I]-(Sar$^1$, Ile$^8$) Angiotensin II were diluted in assay buffer. The following reagents were successively added in the wells of a 96 well plate: 50µL of test compound at increasing concentrations, 25µL of radioligand (at a final concentration of 0.1 nmol/L) and 25µL of membrane extracts. The samples were incubated 60 min at 25°C and filtered over filter plate (presoaked in washing buffer 25 mmol/L Hepes pH 7.4, 5 mmol/L $MgCl_2$, 0.5% PEI for 2h at RT) with a Filtermate Harvester (Perkin Elmer). After wash of the filters 6 times with 0.5 mL of ice-cold washing buffer, 50µL of Microscint 20 (Packard) were added and the samples were incubated 15 min on an orbital shaker and then counted with a TopCount$^{TM}$ for 1 min/well.

On each day of experimentation and prior to the testing of compounds, reference agonist was tested at several concentrations in duplicate (n=2) to obtain a dose-response curve and an estimated EC50. Reference values thus obtained for the test were compared to historical values obtained from the same receptor and used to validate the experimental session.


## ACKNOWLEDGEMENTS

We are grateful to José Roberto da Silva and Marilene Luzia de Oliveira for their helpful technical assistance.

## SOURCES OF FOUNDING

Silvia Savergnini was supported by a fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Roberto Lautner was supported by a fellowship from Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG). This study was supported by a Compugen Grant and by Ministério de Ciência e Tecnologia (MCT)/FAPEMIG/CNPq-INCT-NanoBiofar, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and CNPq/FAPEMIG/PRONEX (Programa de Apoio a Núcleos de Excelência).


## DISCLOSURES

Robson A. S. Santos is a consultant of Compugen Ltd.

Merav Beiman, Gady Cojocaru, Yossi Cohen and Galit Rotman are employees of Compugen Ltd.

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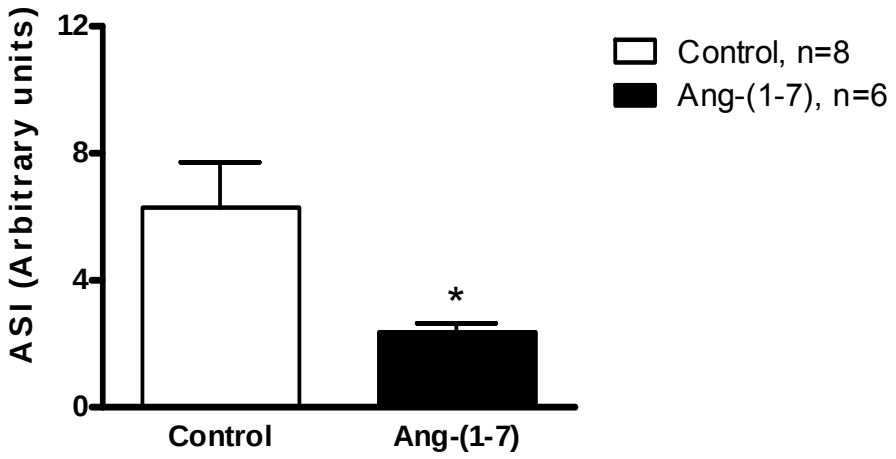
**SUPPLEMENTAL FIGURE 1**

**A**




**B**

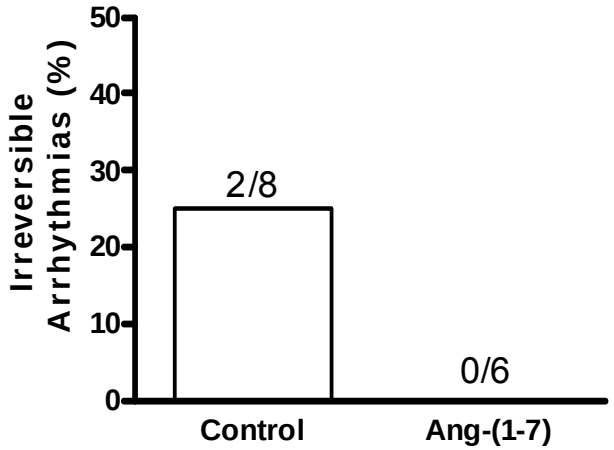



**Figure S1 -** Effect of Ang-(1-7) on ischemia/ reperfusion arrhythmias in isolated hearts. Arrhythmias were produced by 15 minutes occlusion of the LAD in isolated rat hearts, followed by reperfusion. The hearts were perfused with KRS in the absence or presence of Ang-(1-7) (0.2 nmol/L). (A) Averaged ASI (arrhythmia severity index). (B) Percentage of irreversible reperfusion arrhythmias. Numbers above the bars indicate the incidence of irreversible arrhythmias (>30 min) during the reperfusion period. *$P$<0.05 vs control group (Student's $t$ test).

## SUPPLEMENTAL FIGURE 2

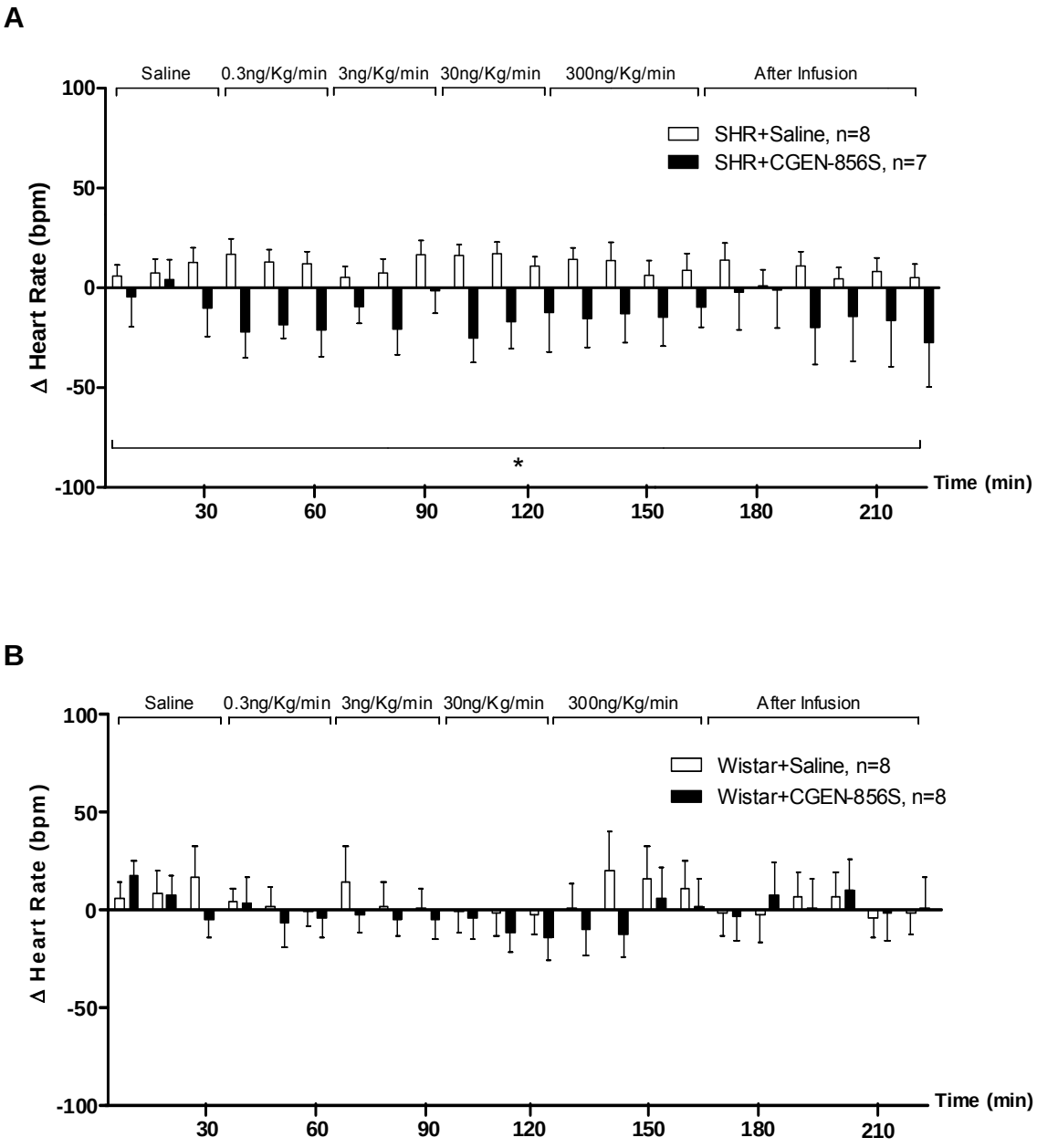



**Figure S2 -** Changes in heart rate (HR) produced by acute infusion of graded doses of CGEN-856S (0.3, 3, 30 and 300 ng/Kg/min) in conscious SHR (A) and in concious Wistar rats (B). Bars represent values obtained every 10 min of infusion. **P*<0.05 SHR+CGEN-856S vs SHR+Saline group (Two-Way ANOVA followed by Bonferroni test).

**SUPPLEMENTAL FIGURE 3**

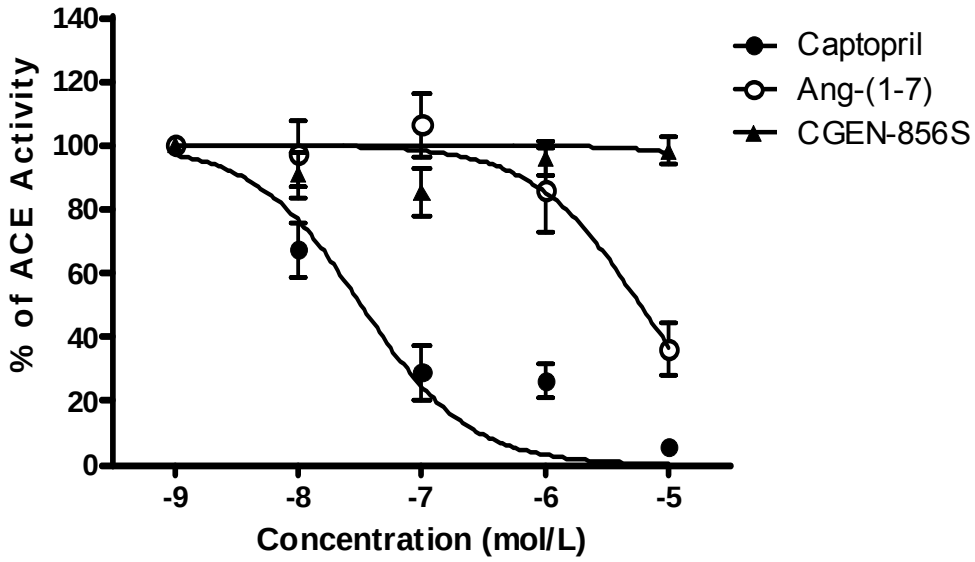
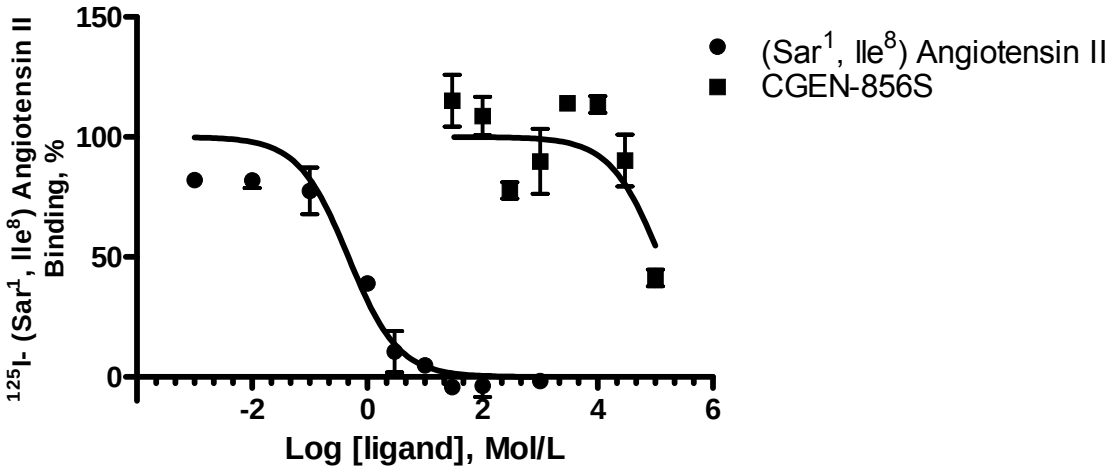



**Figure S3 -** Effect of CGEN-856S and Ang-(1-7) on plasma ACE activity. Final concentrations ranging from $10^{-9}$ to $10^{-5}$ mol/L were used for each peptide. Captopril was used as a positive control ($10^{-9}$ to $10^{-5}$ mol/L). Each point represents the mean ± SEM generated from 3 separate experiments.

**SUPPLEMENTAL FIGURE 4**

**A**




**B**

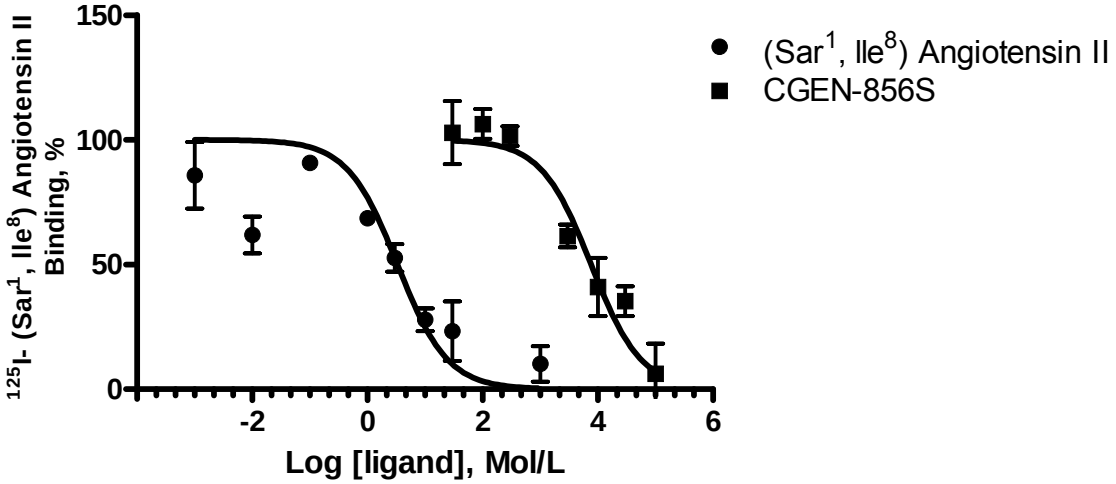



**Figure S4 -** Binding assays on the Ang II receptors AT1 and AT2. Competitive inhibition of AT1 binding of the radioligand $^{125}$I [Sar-$^{1}$,Ile-$^{8}$] Ang II by Ang II or CGEN-856S (A). Competitive inhibition of AT2 binding of the radioligand $^{125}$I [Sar-$^{1}$,Ile-$^{8}$] Ang II by Ang II or CGEN-856S (B).