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Circ Res. 2013;112:1104-1111; originally published online February 27, 2013;
doi: 10.1161/CIRCRESAHA.113.301077

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Discovery and Characterization of Alamandine A Novel Component of the Renin–Angiotensin System

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Rationale: The renin–angiotensin system (RAS) is a key regulator of the cardiovascular system, electrolyte, and water balance. Here, we report identification and characterization of alamandine, a new heptapeptide generated by catalytic action of angiotensin-converting enzyme-2 angiotensin A or directly from angiotensin-(1–7).

Objective: To characterize a novel component of the RAS, alamandine.

Methods and Results: Using mass spectrometry we observed that alamandine circulates in human blood and can be formed from angiotensin-(1–7) in the heart. Alamandine produces several physiological actions that resemble those produced by angiotensin-(1–7), including vasodilation, antifibrosis, antihypertensive, and central effects. Interestingly, our data reveal that its actions are independent of the known vasodilator receptors of the RAS, Mas, and angiotensin II type 2 receptor. Rather, we demonstrate that alamandine acts through the Mas-related G-protein–coupled receptor, member D. Binding of alamandine to Mas-related G-protein–coupled receptor, member D is blocked by D-Pro⁷-angiotensin-(1–7), the Mas-related G-protein–coupled receptor, member D ligand β -alanine and PD123319, but not by the Mas antagonist A-779. In addition, oral administration of an inclusion compound of alamandine/ β -hydroxypropyl cyclodextrin produced a long-term antihypertensive effect in spontaneously hypertensive rats and antifibrotic effects in isoproterenol-treated rats. Alamandine had no noticeable proliferative or antiproliferative effect in human tumoral cell lines.

Conclusions: The identification of these 2 novel components of the RAS, alamandine and its receptor, provides new insights for the understanding of the physiological and pathophysiological role of the RAS and may help to develop new therapeutic strategies for treating human cardiovascular diseases and other related disorders. (*Circ Res.* 2013;112:1104-1111.)

Key Words: angiotensin II ■ antihypertensive treatment ■ cardiovascular system ■ hypertension ■ renin–angiotensin system ■ vasoactive peptides ■ vascular reactivity

In the past few years, novel components of the renin–angiotensin system (RAS) have been described, including the prorenin/renin receptor,¹ angiotensin-converting enzyme-2 (ACE2),^{2,3} and Mas.⁴ ACE2 and Mas are now considered to be part of a novel

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axis of the RAS, the ACE2/angiotensin 1 to 7 [Ang-(1–7)]/Mas axis,⁴⁻¹¹ which counteracts most of the action of the classical

Original received February 7, 2013; revision received February 22, 2013; accepted February 27, 2013. In January 2013, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 12.2 days.

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From the Department of Physiology and Biophysics, National Institute of Science and Technology in Nanobiopharmaceutics (R.Q.L., D.C.V., R.A.F.-S., N.S., T.V.-B., F.C.-F., A.A., E.S., C.B., A.O., J.B., S.S., G.M., A.B.P., D.P.-S., A.F., F.A., A.M., R.P., D.M.-S., M.J.C.-S., R.S.), Department of Morphology (A.F.), Department of Biochemistry and Immunology (A.P.), and Department of Chemistry, National Institute of Science and Technology in Nanobiopharmaceutics (F.S., R.S.), Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil; Charite University Berlin, Campus Benjamin Franklin, Berlin, Germany (J.J., V.J.); Department of Biological Sciences, Institute of Exact and Biological Sciences, Federal University of Ouro Preto, Ouro Preto, Minas Gerais, Brazil (A.A., E.S., C.B.); Department of Physiology and Functional Genomics, College of Medicine, University of Florida, Gainesville, FL (M.R.); Max-Delbrück Center for Molecular Medicine, Berlin, Germany (F. Kemplin, N.A., M.B.); and Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark (T.V.-B., F. Kjeldsen).

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The online-only Data Supplement is available with this article at <http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.113.301077/-/DC1>.

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DOI: 10.1161/CIRCRESAHA.113.301077

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Nonstandard Abbreviations and Acronyms

ACE2	angiotensin-converting enzyme-2
Ang-(1–7)	angiotensin-(1-7)
AT₂R	angiotensin II type 2 receptor
MrgD	Mas-related G-protein-coupled receptor, member D
RAS	renin-angiotensin system

ACE/angiotensin II/angiotensin II type 1 receptor axis.^{2,12} Here, we describe the identification and characterization of a novel peptide of the renin-angiotensin system, the heptapeptide, Ala-Arg-Val-Tyr-Ile-His-Pro, alamandine, in rats, mice, and humans.

Methods

Forming of Alamandine by ACE2

Angiotensin A (10 μ mol/L) was incubated with hACE2 (10 nmol/L). After the reaction and evaporation, angiotensin peptides were analyzed by mass spectrometry (matrix-assisted laser desorption ionization-time-of-flight/time-of-flight).

Alamandine Detection in Blood

Plasma preparation, size-exclusion chromatography, isolation in mass spectrometry, synthesis, and detection of peptide were described previously.¹³

Aortic Rings Assay

The vessels were precontracted with submaximal concentrations of phenylephrine (0.1 μ mol/L). Agonists were added in increasing cumulative concentrations once the response to phenylephrine had stabilized. To study the mechanism of alamandine vasorelaxation, the following compounds were used: L-NAME, A-779, D-Pro⁷-Ang-(1–7), PD123319, and β -alanine.

Caudal Ventrolateral Medulla and Rostral Ventrolateral Medulla Microinjections

Unilateral microinjections of Ang-(1–7), alamandine, A-779, D-Pro⁷-Ang-(1–7), or vehicle (100 nL) were made over a 20- to 30-s period into the caudal ventrolateral medulla or rostral ventrolateral medulla of urethane-anesthetized Fisher rats.

Cell Culture

Transfected and nontransfected Chinese hamster ovary (CHO) cells and A549 human lung adenocarcinoma cells and DU 145 (prostate cancer cell line) were grown in DMEM-F12 medium (Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum, 100 μ g/mL penicillin, and 100 U/mL streptomycin in a humidified incubator with 5% CO₂ and 95% room air.

Fluorescent Binding in Mas-Related G-protein-Coupled Receptor, Member D-Transfected Cells

Binding of fluorescent-labeled alamandine was tested in Mas-related G-protein-coupled receptor, member D-stably transfected CHO cells (CHO-MrgD). Fluorescent-labeled alamandine (0.01 μ mol/L) was incubated for 30 minutes at room temperature alone or 5 minutes after preincubation with nonfluorescent alamandine (1 μ mol/L, nonspecific binding), A-779 (1 μ mol/L), D-Pro⁷-Ang-(1–7) (1 μ mol/L), β -alanine (1 μ mol/L), and the angiotensin II type 2 receptor (AT₂R) antagonist PD123319 (1 μ mol/L). Relative fluorescence measurements were performed by laser scanning confocal microscopy.

Evaluation of Nitric Oxide Release From CHO Cells

Nitric oxide (NO) release from transfected and nontransfected CHO cells was evaluated using the NO

indicator 4-amino-5-methylamino-2,7-difluoro fluorescein diacetate (DAF-FM).¹⁴

Inclusion Complex Preparation

The inclusion compound HP β CD/alamandine was prepared as previously described¹⁵ (see also Online Figure IIIA–IIIC).

Effects of Oral Administration of HP β CD-Alamandine in Isoproterenol-Treated Rats

The protocol used for treatment with isoproterenol and the inclusion compound was the same as the one described previously.¹⁶

Effects of Oral Administration of HP β CD-Alamandine in Conscious Hypertensive Rats

Spontaneously hypertensive rats were instrumented for acute blood pressure recording as described previously.¹⁴ After oral administration of the inclusion compound (132 μ g/kg equivalent to 50 μ g/kg of alamandine) or vehicle (HP β CD, 82 μ g/kg), mean arterial pressure and heart rate were continuously recorded for 5 hours.

Results

Using mass spectrometry (Figure 1A) we have identified alamandine as a product of the catalytic hydrolysis of the octapeptide Ala¹-AngII (angiotensin A)¹³ by human ACE2. We also demonstrate that alamandine can be formed in the rat heart after Ang-(1–7) perfusion (Figure 1B–1J) with the use of selected reaction monitoring-mass spectrometry.¹⁷ This indicates that the cardiac tissue contains all necessary components to promote the decarboxylation of the Ang-(1–7) N-terminal aspartate amino acid residue. Moreover, we show that alamandine is an endogenous peptide of this tissue (Figure 1I). Next, we determined if this peptide is also present in human blood. As shown in Online Figure 1A through 1D, alamandine circulates in human blood; and consistent with previous observations for angiotensin A,¹³ our data revealed that nephropathic patients have increased plasmatic concentration of alamandine (Online Figure 1E).

The sequence of alamandine is very similar to Ang-(1–7), differing only by the presence of an alanine residue in place of an aspartate residue in the amino terminus. This structural modification (Online Figure 1F) is because of a decarboxylation of the aspartate radical group, which has already been reported for angiotensin II.¹³ Because of structural similarity between alamandine and Ang-(1–7), we decided to test whether the biological actions of these 2 peptides were similar, too. As shown in Figure 2A, and previously reported for Ang-(1–7),^{4,10} alamandine produces endothelial-dependent vasorelaxation in aortic rings from FVB/N mice. Similar data were obtained in aortic rings from Wistar rats (data not shown). Accordingly, the alamandine-induced vasorelaxation was attenuated by pretreatment with the nitric oxide synthase inhibitor, L-NAME (Figure 2B). Strikingly, treatment with the Mas antagonist, A-779, did not block the alamandine-induced vasorelaxation (Figure 2C) as previously described for Ang-(1–7).^{4,14} In addition, the alamandine-induced vasorelaxation was preserved in aortic rings of Mas-deficient mice (Figure 2D). These observations suggest that Mas is not a putative alamandine receptor. Therefore, we conclude that alamandine is not a Mas agonist, although the vascular effects of this novel RAS peptide resemble those elicited by Ang-(1–7).

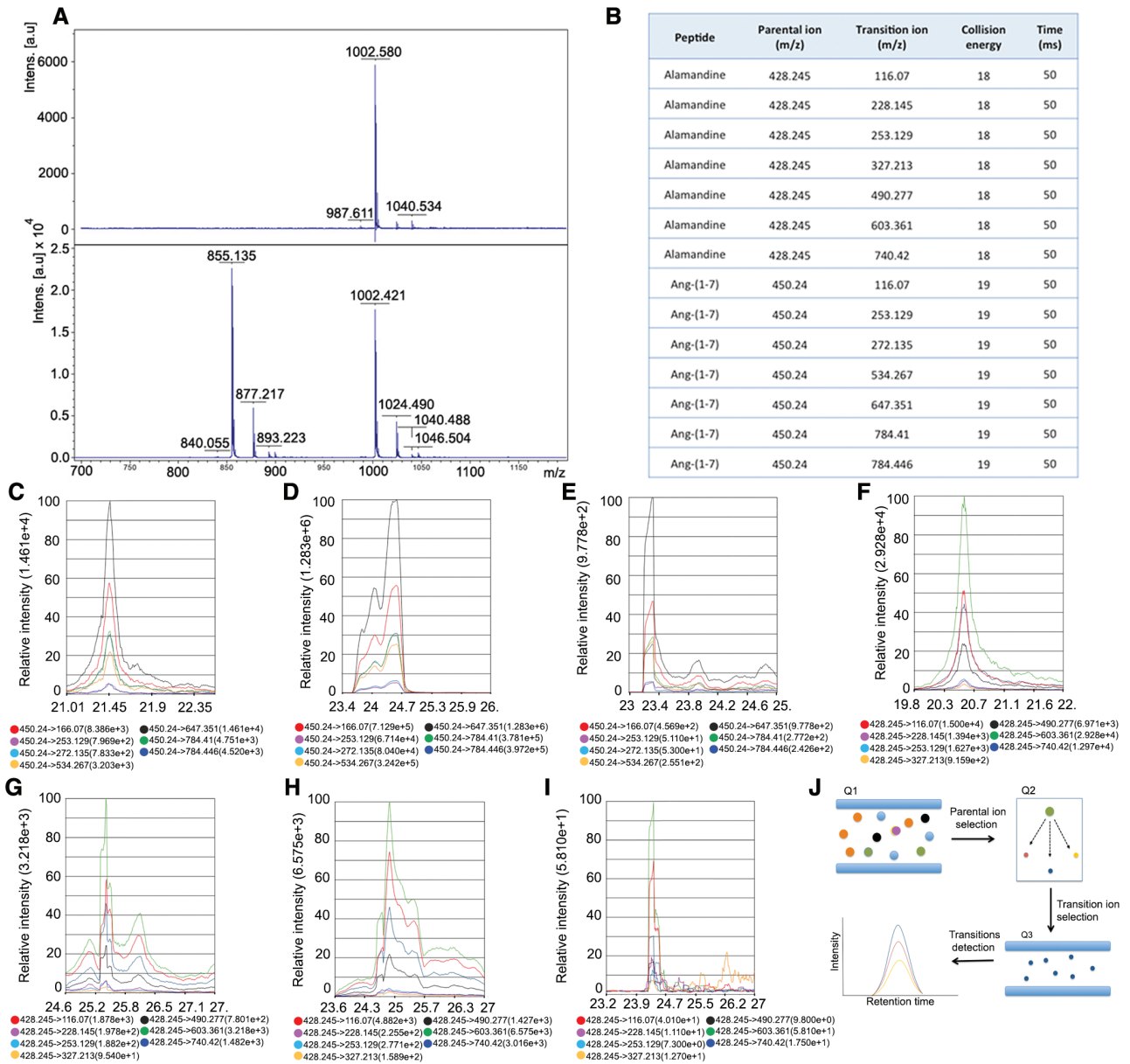


Figure 1. Formation of alamandine by recombinant human angiotensin-converting enzyme-2 (ACE2) and in the isolated rat heart. **A**, ACE2 forms alamandine from angiotensin A (Ang A). Ang A was incubated with rhACE2 and the assay product was analyzed by matrix-assisted laser desorption ionization-time-of-flight/time-of-flight mass spectrometry. **Top**, Negative control (Ang A without rhACE2). **Bottom**, Mass spectrometry (MS) spectrum observed after 15 minutes of incubation. **B**, Table containing the transitions monitored to detect alamandine or angiotensin 1 to 7 [Ang-(1-7)]. **C** to **E**, The chromatogram profiles of the selected transition ions from Ang-(1-7). **F** to **I**, The profiles for the alamandine transitions. **C**, 3×10^{-15} mol of synthetic Ang-(1-7) in 0.1% formic acid. **D**, Isolated heart was perfused with Krebs solution (NaCl 118; KCl 4.7; KH_2PO_4 0.9; MgSO_4 1.1; CaCl_2 2.5; NaHCO_3 25, D-glucose 11 [mmol/L]) containing 10^{-6} mol/L Ang-(1-7). The collected sample was desalted and resuspended with 0.1% formic acid. It is possible to note that because of matrix interactions, the Ang-(1-7) transitions elute slightly later. **E**, Isolated heart was perfused with Krebs solution without Ang-(1-7). It was possible to detect all Ang-(1-7) transitions in the expected retention time, indicating the presence of endogenous Ang-(1-7) in the heart. **F**, 3×10^{-15} mol of synthetic alamandine in 0.1% formic acid. **G**, Isolated heart was perfused with Krebs solution containing 10^{-6} mol/L Ang-(1-7). It was possible to detect all the transitions of the alamandine, indicating that this peptide can be formed in the heart after Ang-(1-7) perfusion. **H**, 3×10^{-15} mol of synthetic alamandine was spiked in the perfused heart solution to confirm the later retention time of alamandine transition because of matrix interference observed in (**G**). **I**, Isolated heart was perfused with Krebs solution without Ang-(1-7). It was possible to detect the transitions of the alamandine being eluted in the expected retention time, suggesting that alamandine is heart endogenous peptide. All experiments were performed with 2 biological replicates. **J**, Detection of alamandine in heart using selected reaction monitoring mass spectrometry (SRM-MS). Typical SRM method performed in a triple quadrupole MS. In the Q1, the parental ions are selected based on their mass-to-charge (m/z) ratio. The selected parental ions are fragmented in the collision cell Q2 and the fragment ions (transitions) are selected and mass-resolved in the Q3.

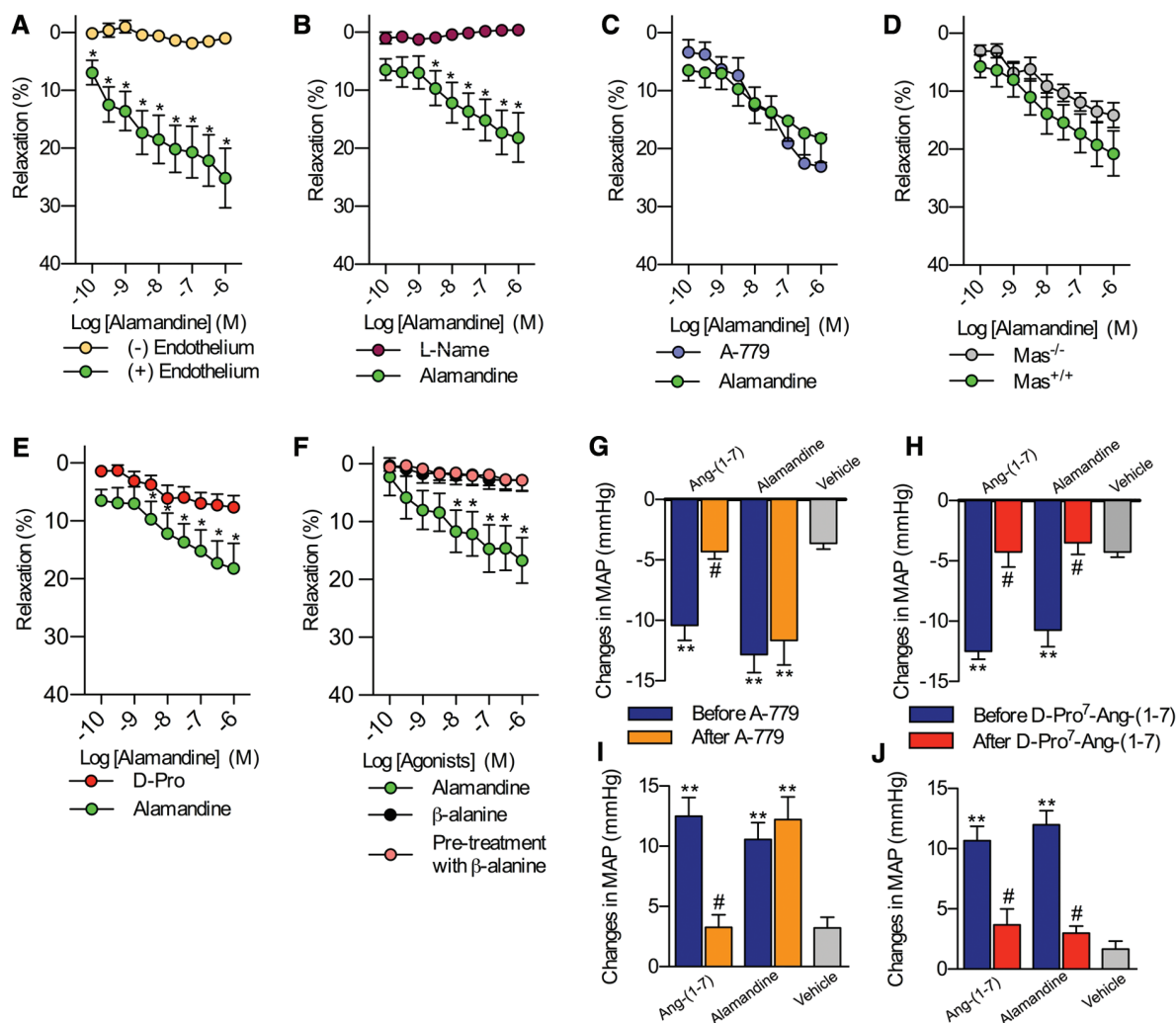


Figure 2. Functional effects of alamandine in aortic rings from FVB/N mice and after central microinjection into the caudal ventrolateral medulla (CVLM) or rostral ventrolateral medulla of Fisher rats. **A**, Vessels containing or lacking functional endothelium (n=5–6), **(B)** in the absence or presence of L-NAME (100 μ mol/L; n=6–8), **(C)** in the absence or presence of Mas antagonist A-779 (1 μ mol/L; n=6–8), **(D)** in wild-type or Mas knockout mice (Mas^{-/-}; n=7–4), **(E)** in the absence or presence of the Mas antagonist D-Pro⁷-angiotensin 1 to 7 [Ang-(1–7); 1 μ mol/L; n=8 for each condition], **F**, Vessels pretreated or not with β -alanine (0.1 μ mol/L; n=4–5). **G–H**, Effects of Ang-(1–7) (40 pmol) or alamandine (40 pmol) before and 5 minutes after microinjection of A-779 (50 pmol; **G**) or D-Pro⁷-Ang-(1–7) (50 pmol) (**H**) into the CVLM (n=4). **I–J**, Ang-(1–7) (40 pmol) or alamandine (40 pmol) before and 5 minutes after microinjection of A-779 (50 pmol; **I**) or D-Pro⁷-Ang-(1–7) (**J**; 50 pmol) into the rostral ventrolateral medulla (n=6). Each point or column represents the mean \pm SEM; * P <0.05 (2-way ANOVA followed by Bonferroni post hoc test), ** P <0.01 vs vehicle, # P <0.05 versus to respective before (1-way ANOVA). MAP indicates mean arterial pressure.

Studies have shown that in some circumstances Ang-(1–7) effects are not blocked by A-779, but can be blocked by another Ang-(1–7) antagonist, D-Pro⁷-Ang-(1–7).^{18–20} We next tested whether this antagonist could also block alamandine effects. Intriguingly, D-Pro⁷-Ang-(1–7) completely blocked the vasorelaxation produced by alamandine in mice aortic rings (Figure 2E). To ascertain whether this blockade was not limited to vessels, we performed experiments using microinjection of alamandine in central areas critically involved in blood pressure control, the caudal and rostral ventrolateral medulla. As shown in Figure 2G through 2J, both Ang-(1–7) and alamandine produced a pressor effect when microinjected in the rostral ventrolateral medulla and a depressor effect when microinjected into the caudal ventrolateral medulla of anesthetized Fisher rats. However, although the effect of Ang-(1–7)

was altered by co-microinjection of A-779 and D-Pro⁷-Ang-(1–7), as observed in blood vessels, the effect of alamandine was only blocked by D-Pro⁷-Ang-(1–7).

Besides being a Mas agonist, Ang-(1–7) has been reported to be a weak agonist of the Mas-related receptor, MrgD.²¹ Therefore, we hypothesized that alamandine could be a possible endogenous ligand for MrgD. The amino acid β -alanine has been reported to be an agonist of MrgD; however, the effective concentration of β -alanine in MrgD-transfected cells is considerably high (10⁻³ to 10⁻⁶ mol/L).^{22,23} We first tested whether β -alanine and alamandine could interact in blood vessels. As shown in Figure 2F, β -alanine did not induce a direct relaxing effect in aortic rings. However, preincubation of aortic rings with β -alanine (10⁻⁷ mol/L) abolished the vasorelaxation induced by alamandine in aortic rings of FVB/N mice.

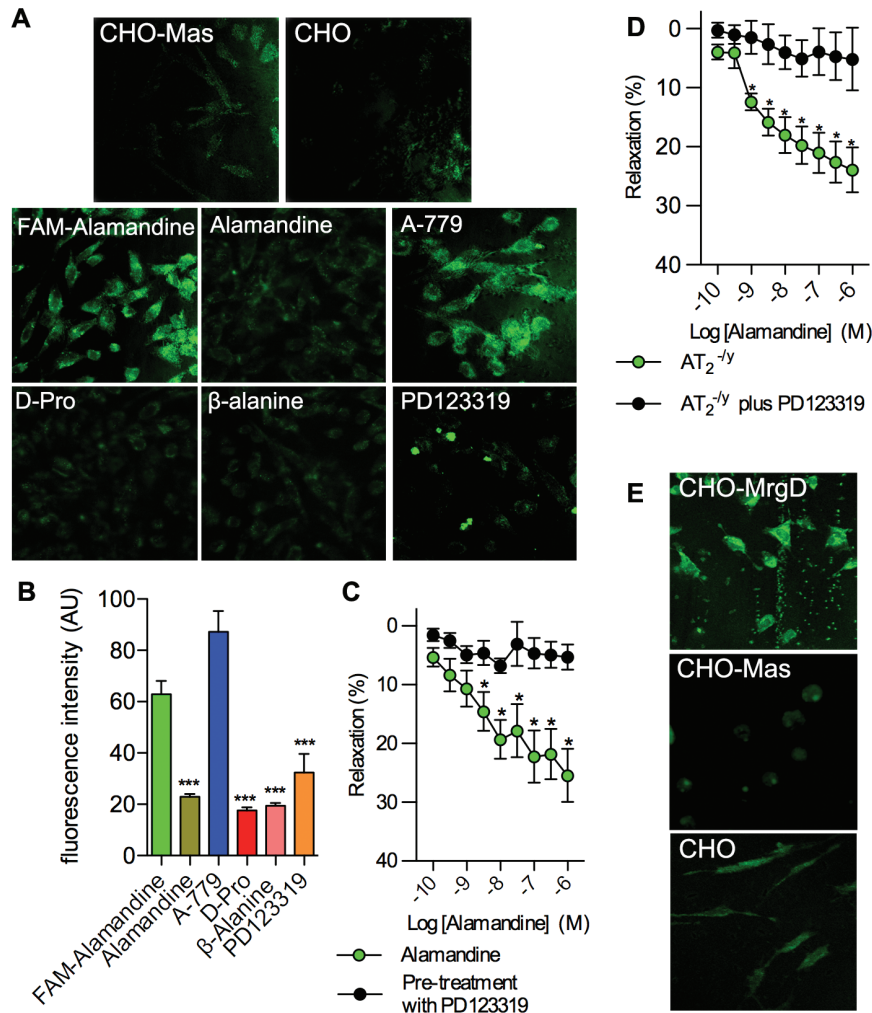


Figure 3. Pharmacodynamics and functional effects of alamandine. **A**, Representative confocal photomicrograph images of fluorescently labeled alamandine peptide (FAM-alamandine; 0.01 μ mol/L) binding to stably expressed Mas receptors in Chinese hamster ovary (CHO) cells, nontransfected CHO cells, and to MrgD receptors stably expressed in CHO cells alone or in the presence of nonfluorescent alamandine (alamandine), the Mas receptors antagonist A-779 (1 μ mol/L) or D-Pro⁷-angiotensin 1 to 7 [Ang-(1–7); 1 μ mol/L], β -alanine (1 μ mol/L), and the angiotensin II type 2 receptor antagonist PD123319 (1 μ mol/L). **B**, Summary of data of displacement of FAM-alamandine bound to MrgD receptors (170 cells analyzed). **C**, Vascular effects of increasing concentrations of alamandine (0.1 pmol/L to 1 μ mol/L) on aortic rings of wild-type mice in the presence or absence of PD123319 (1 μ mol/L). **D**, Vascular effects of increasing concentrations of alamandine (0.1 pmol/L to 1 μ mol/L) on aortic rings of wild-type or angiotensin II type 2 (AT_2) receptor knockout mice. **E**, Representative confocal photomicrograph image of NO production induced after 15 minutes of treatment with alamandine (0.01 μ mol/L) in CHO cells expressing the MrgD receptor (MrgD-CHO), in CHO cells expressing the Mas receptor (Mas-CHO) or in nontransfected CHO cells. Columns represent mean \pm SEM relative fluorescence. * P <0.05 and *** P <0.001 (2-way ANOVA or 1-way ANOVA followed by Bonferroni post hoc test).

To further address the role of MrgD in the alamandine effects, we performed binding and functional studies in human MrgD-transfected cells (see online Data Supplement). For the binding experiments we used mono-5-(and-6)-carboxy-fluorescein, FAM- labeled alamandine. As shown in Figure 3A through 3B, fluorescent-labeled alamandine specifically binds to MrgD-stably transfected CHO cells. No significant binding was observed in nontransfected CHO or Mas-stably transfected CHO cells (Figure 3A). Similar with the data obtained in aortic rings and central microinjection experiments, the binding of alamandine to MrgD-transfected cells was competed by D-Pro⁷-Ang-(1–7) and β -alanine but not by A-779 (Figure 3A–3B). To verify whether the binding of alamandine to MrgD-transfected cells would produce a functional response, we evaluated NO release using DAF.¹⁴

Incubation with alamandine led to NO release in MrgD-transfected cells but not in Mas-transfected cells or in non-transfected CHO cells (Figure 3E).

PD123319 is a putative AT_2 R antagonist^{24–26} and has been reported to block some of the Ang-(1–7) effects.^{27,28} In wild-type animals, PD123319 blocked the alamandine effects in aortic rings (Figure 3C). However, alamandine continues to induce vasorelaxation in aortic rings isolated from AT_2 R KO mice (Figure 3D), excluding a role for AT_2 R in the alamandine vascular effects. When alamandine was tested in the presence of PD123319 (10⁻⁷ mol/L), its vasorelaxation-induced effect in AT_2 R KO mice was abolished (Figure 3D). These findings indicate that PD123319 binds to another receptor sensitive to alamandine. Indeed, PD123319 efficiently competed the binding of alamandine to MrgD-transfected CHO cells (Figure

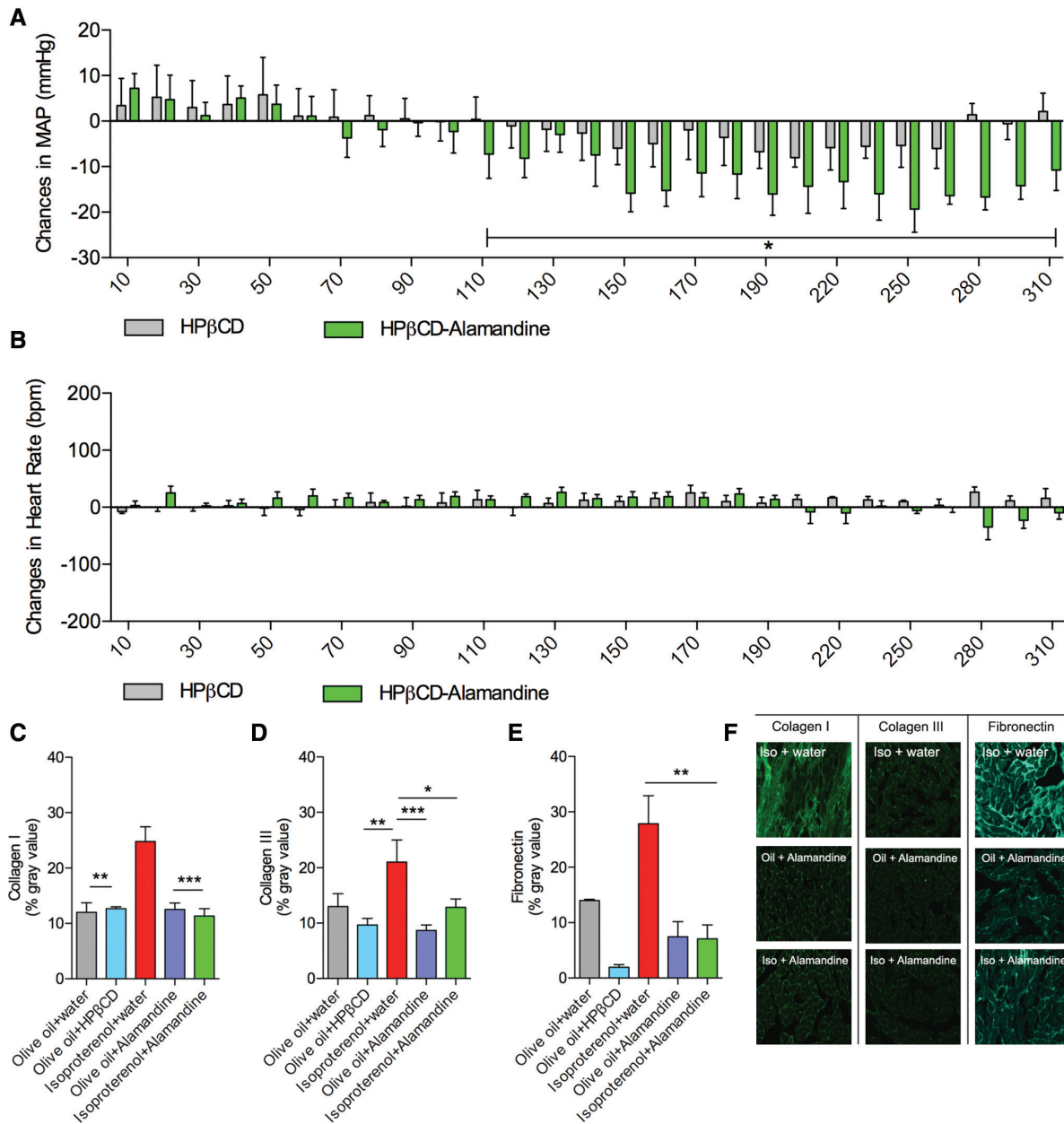


Figure 4. Effect of the inclusion compound alamandine/HPβCD in spontaneously hypertensive rats (SHR) and on cardiac fibrosis. **A, B, SHR.** The baseline blood pressure (**A**) and heart rate (**B**) were recorded for at least 90 minutes, and the baseline values were calculated as the average of 5 points during this time. After that, the SHR received a single oral dose of vehicle (HPβCD; 82 μg/kg) or HPβCD/alamandine (132 μg/kg, equivalent to 50 μg/kg of alamandine) by gavage. Bars represent the difference between values before and after treatment, at every 10 min after gavage. **P*<0.05 alamandine vs vehicle (2-way ANOVA followed by Bonferroni post hoc test). **C to E,** The effect of alamandine on the isoproterenol-induced increase in collagen I, collagen III, and fibronectin, respectively, in heart tissue of Wistar rats was evaluated by confocal microscopy on a Zeiss LSM 510 META laser scanning confocal microscope excited at 488 nm with an argon-ion laser (oil-immersion objective lens, ×63). **F,** First column, alamandine reduces collagen I; second column, alamandine reduces collagen III; and third column, alamandine reduces fibronectin. **P*<0.05 vs isoproterenol+water; ***P*<0.01 vs isoproterenol+water; and ****P*<0.001 vs isoproterenol+water (1-way ANOVA followed by the Bonferroni post hoc test). MAP indicates mean arterial pressure.

3A–3B). Taken together, these findings are strong evidence that in addition to be an AT₂R antagonist, PD123319 is also a MrgD antagonist/ligand.

We next tested whether alamandine could influence the growth of human tumoral cells. As shown in the Online Figure IIA through IIB, alamandine had no noticeable proliferative or antiproliferative effect in the human tumoral cell lines A549 and DU145, which express MrgD (Online Figure

IID). Similar results were obtained with β-alanine (Online Figure IIA–IIB).

Finally, we evaluated the potential therapeutic application of alamandine by including it in HP-β cyclodextrin^{15,16} (Online Figure IIIA–IIIC). A single oral administration of the inclusion compound alamandine/HP-β cyclodextrin produced a long-term antihypertensive effect in spontaneously hypertensive rats (Figure 4A). In addition, a remarkable decrease

in collagen I, III, and fibronectin accumulation was observed in isoproterenol-treated rats receiving 50 µg/kg of alamandine once a day (Figure 4B–4E).

In summary, we here report the identification of alamandine, a novel RAS hormone. Alamandine circulates in human blood and presents unique properties. Its biological activities resemble those of Ang-(1–7), including vasodilatation, antihypertensive effect in spontaneously hypertensive rats, and central cardiovascular effects. However, unlike Ang-(1–7), these effects are not mediated by binding to Mas. We identified a novel RAS-related receptor, MrgD, which is the binding site for alamandine. This receptor can be blocked by D-Pro⁷-Ang-(1–7), and its identification solves some earlier discrepant findings with the Mas antagonist A-779.^{18–20} This observation is in line with our finding that Ang-(1–7) can be processed to alamandine in the isolated rat heart. Furthermore, we demonstrated that human ACE2 forms alamandine from angiotensin A, reinforcing the central role of this enzyme in the processing of angiotensin peptides. A simplified updated RAS cascade based on our present findings is presented in Online Figure IV.

It is pertinent to point out that previous observations suggesting a physiological or pathophysiological role for AT₂R or its participation in the effects of Ang-(1–7), based on the use of PD123319, must be reevaluated in light of our current data.

Furthermore, recent data suggested a proliferative role for MrgD, which in contrast to what has been proposed in the past,²⁹ is not expressed exclusively in nociceptive sensory neurons^{21,29,30} (Online Figure V). However, we could not find any evidence for proliferative or antiproliferative activity of alamandine and of β-alanine in 2 different human tumoral cell lines, which also express MrgD (Online Figure IIA–IIB).

The fact that the new RAS hormone alamandine can be easily administered orally as a HPβCD inclusion compound and produced antihypertensive effects in spontaneously hypertensive rats and pronounced cardioprotective effects in isoproterenol-treated rats, opens new perspectives for exploring the therapeutic potential of angiotensin-(1–7)-related peptides. The identification of 2 novel components of the RAS, alamandine and its receptor, will be important for improving our understanding of the physiological and pathophysiological role of the this key regulatory system.

Acknowledgments

We thank José Roberto Silva and Marilene Oliveira for their skilled technical assistance.

Sources of Funding

This work was partially supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundação de Amparo à Pesquisa do Estado de Minas Gerais, Instituto Nacional de Ciência e Tecnologia em Nanobiofarmacêutica, a grant FP7-HEALTH-2009-2.4.5-2 to SysKid (grant agreement 241544) and Mascara (grant agreement 278249) from the European Union.

Disclosures

None.

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Novelty and Significance

What Is Known?

- The renin angiotensin system (RAS) is a key regulator of the cardiovascular system. The main end products of the RAS are angiotensin II and angiotensin-(1–7).
- Angiotensin-(1–7) acts on the G-protein-coupled receptor Mas to produce vasodilation and it antagonizes most angiotensin II actions.

What New Information Does This Article Contribute?

- The heptapeptide Ala-Arg-Val-Tyr-Ile-His-Pro, alamandine is a new RAS component.
- Alamandine produces vasodilation by a mechanism not involving the known vasodilation receptors of the RAS (Mas and angiotensin II type 2).

This article describes the discovery and characterization of the heptapeptide alamandine (Ala-Arg-Val-Tyr-Ile-His-Pro). Alamandine can be formed from angiotensin A by angiotensin-converting enzyme-2 or directly from angiotensin-(1–7) by decarboxylation of its aspartate residue. It produces effects resembling those of Ang-(1–7). However, it acts independently of the 2 known vasodilators receptors of the RAS (Mas and angiotensin II type 2). To produce its effects, alamandine binds to the Mas-related receptor, MrgD. A novel orally active formulation of alamandine produced a long-term antihypertensive effect in spontaneously hypertensive rats and cardioprotective effects. These novel findings will be helpful for developing a new understanding of the RAS, a key regulator of blood pressure and fluid balance.

Supplemental Material

Full Methods

Isolation of Plasma

We obtained peripheral blood (10 mL) by catheterisation of the cubital vein and collected the blood in tubes containing K₂-EDTA (7.2 mg). The blood samples were centrifuged at 300 g for 30 min at 4°C for isolation of plasma.

Size-exclusion chromatography

Human plasma was fractionated by size-exclusion-chromatography. For this, the size-exclusion chromatography gel (Sephacryl S-100 High Resolution; 1000 x 16 mm, S100 HR, Pharmacia BioTech, Uppsala, Sweden) was equilibrated with 0.9 % NaCl in water. Human plasma was loaded onto the column to elute it with 0.9 % NaCl in water at a flow rate of 1 mL min⁻¹. The elution with a UV-detector was monitored at 280 nm.

Analytical reversed-phase chromatography

The eluate was loaded onto a monolithic reversed phase chromatography column (ChromolithTM; 100 x 4.6 mm I.D., Merck, Darmstadt, Germany). 0.1 % trifluoroacetic acid (TFA) in water was used as an equilibration buffer (flow rate: 1 mL min⁻¹). The retained substances were eluted using 0.1 % trifluoroacetic acid (TFA) in water-acetonitrile (20:80, v/v-%) and the following gradient: 0-2 min: 0 % eluent B, 2-32 min: 0-75 % B, 32-32.5 min: 75- 100 % eluent B; 32.5-33.5 min: 100 % B; flow rate: 2.0 mL min⁻¹. The elution with a UV-detector was monitored at 280 nm.

Identification of the isolated peptide by Mass spectrometry

Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) measurements were performed using a Ultraflex-III (Bruker-Daltronic, Bremen, Germany). One mL of the peptide solution was mixed with 1 mL of α -cyano-4-hydroxycinnamic acid matrix solution consisting of 10 mg of matrix dissolved in 1 mL of 0.3% TFA in acetonitrile/water (50:50, v/v-%). From the resulting mixture, 1 mL was applied to the sample plate. Samples were air-dried at ambient temperature (20°C). Measurements were performed in the reflectron mode at an acceleration voltage of 20 kV, 70% grid voltage, and a delay of 150 ns. Each spectrum represented the accumulation of 150 laser shots. Five spectra of one spot measured at different positions were averaged. MS/MS fragment spectra were recorded using a matrix-assisted laser desorption/ionisation time-of-flight / time-of-flight (MALDI-TOF/TOF) instrument (Ultraflex-III, Bruker-Daltronic, Bremen, Germany) equipped with a smart-beam laser operating at a frequency of 200 Hz. Air was used as the collision gas. Spectra were obtained by accumulation of up to 20,000 consecutive laser shots. Fragment data were analysed using the Bio-Tools software (version V 3.1; Bruker-Daltronic, Bremen, Germany).

Synthesis of Alamandine

Alamandine was purchased from Biosynthan (Berlin, Germany) or synthesized automatically by the solid-phase method using standard Fmoc chemistry in a continuous flow mode.

Quantification of Alamandine

Alamandine and Angiotensin II were quantified by a MALDI-MS based method as recently described¹. Briefly, the adrenocorticotrophic hormone (ACTH) fragment 18-39 (Arg-Pro-Val-Lys-Val-

Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe; 10 µg per sample) was added as internal standard. Hereby local differences in the peptide concentration on the MALDI spot were neutralized. The lyophilised fractions of the reversed-phase chromatography of the plasma were resuspended in 10 µL 0.1% TFA before quantification. 1 µL of each fraction was prepared on a prestructured MALDI sample support (MTP AnchorChip™ 400/384, Bruker-Daltonics, Bremen, Germany) using the α -4-hydroxycinnamic acid affinity sample preparation method [Gobom, 2001]. The mass-spectrometric measurements were performed on a Bruker Reflex III instrument (Bruker-Daltonics, Bremen, Germany). The instrument was equipped with a nitrogen laser (VSL-337 ND, Laser Science, USA), emitting at 337 nm. The laser beam was focused to a typical diameter of 50 µm at an angle of 45° to the surface of a target. Samples were checked microscopically. On average, 200 single-shot spectra were accumulated to improve the signal-to-noise ratio.

Controls and Patients

A total of 5 healthy control subjects (3 males, 2 females; mean age 52.6 ± 5.0 years) and 5 patients with stage 5 chronic kidney disease (4 males, 1 females; mean age 60.2 ± 5.2 years) who were on regular hemodialysis were investigated. Written informed consent was obtained from each patient and ethical approval by the local ethics committee was obtained for the study. None of the patients was a smoker. Mean systolic blood pressure of CRF patients vs. healthy controls was 130.0 ± 11.3 vs. 121.0 ± 3.3 mmHg and mean diastolic blood pressure was 81.2 ± 5.3 vs. 82.0 ± 2.0 mmHg. All data are given as mean \pm SEM.

Detection of Alamandine in isolated heart by selected reaction Monitoring-Mass Specometry (SRM-MS)

The samples obtained from the perfusion of isolated hearts were lyophilized, resuspended with 0.1 % formic acid and separated by reverse-phase liquid chromatography on an in-house packed Reprosil-Pur C18-AQ column (3 µm; Dr. Maisch GmbH - Ammerbuch, Germany) (length: 15 cm; inner diameter: 75 µm) using an Easy-LC nanoHPLC (Thermo Fisher, San Jose CA) coupled to a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose CA). The peptides were eluted using a gradient of 0-34 % phase B (0.1% formic acid, 90% MeCN) over 39 min at $250 \text{ nL} \times \text{min}^{-1}$. The TSQ Vantage was operated in the selected reaction monitoring (SRM) mode. The selectivity for the Q1 and Q3 was set to 0.7 FWHM. The collision gas pressure in the Q2 was set to 1.5 mTorr. The collision energy was calculated by the software Pinpoint 1.0 (Thermo Fisher, San Jose CA). In total, 16 transitions were monitored (8 transitions from the alamandine and 8 transitions from the Ang-(1-7)). Each transition was monitored for 50 ms, leading to 800 ms cycle time.

Aortic Ring Preparation and Mounting

Mouse aortic rings (2mm) from the descending thoracic aorta, free of adipose and connective tissue, were set up in gassed (95% O₂ and 5%CO₂) Krebs-Henseleit solution (mmol/L): NaCl 110.8, KCl 5.9, NaHCO₃ 25.0, MgSO₄ 1.07, CaCl₂ 2.49, NaH₂PO₄ 2.33 and glucose 11.51, at 37°C, under a tension of 0.5 g, for 1 h to equilibrate. The presence of a functional endothelium was assessed by the ability of acetylcholine [ACh] (10 µM) to induce more than 70% relaxation of vessels pre-contracted with phenylephrine (0.1 µM). Rat rings (4mm) were stabilized under 1.0 g of tension. For endothelium-denuded assay, the endothelium was removed by rubbing the intimal surface with a wooden stick. Mechanical activity, recorded isometrically by a force transducer (World Precision Instruments, Inc., Sarasota, FL, USA), was fed to an amplifier-recorder (Model TMB-4; World Precision Instruments, 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 vessels were pre-contracted with submaximal concentrations of phenylephrine (0.1 µM). Alamandine was added in increasing cumulative concentrations once the response to phenylephrine

had stabilized. To study the mechanism of alamandine follow compounds were used: N^G-nitro-L-arginine methyl ester (L-NAME, at 10⁻⁶M) a nitric oxide synthase inhibitor; indometacin (10⁻⁶M) a prostaglandins inhibitor; A-779 (10⁻⁶M) and D-Pro⁷ (10⁻⁶M) both Mas antagonists and β-alanine (10⁻⁶M). In addition, Mas and AT₂ gene-deleted mice was used.

CVLM and RVLM Microinjections

The rats were anesthetized with urethane (1.2 g/ Kg, *i.p.*) and underwent a tracheostomy. Next, a polyethylene catheter was inserted into the abdominal aorta, through the femoral artery for arterial pressure measurement, and another catheter was inserted into the inferior cava vein, through the femoral vein for drugs injection. The animals were placed in a stereotaxic frame (David Kopf instruments, CA) as previously described by Alzamora et al².

Unilateral microinjections of Ang-(1-7) (40 pmol), alamandine (40 pmol), A-779 (50 pmol), D-Pro⁷-Ang-(1-7) (50 pmol) or sterile saline (vehicle - NaCl 0,9%, 100nL) were made over a 20-30 s period into the CVLM (0.7 mm anterior, 1.8 mm lateral to the obex, and just above pia mater in the ventral surface) or into the RVLM (2.1 mm anterior, 1.8 mm lateral to the obex, and just above pia mater in the ventral surface) as previously described by Alzamora et al. ¹. The dose of the Ang-(1-7), A-779 and D-Pro⁷-Ang-(1-7) were based in previous studies ¹⁻³.

Cell Culture

Transfected and non-transfected CHO, A549 human lung adenocarcinoma cells and DU 145 (prostate cancer cell line) (Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil) were grown in DMEM-F12 medium (Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 units/mL streptomycin in a humidified incubator with 5% CO₂ and 95% room air. Cells were allowed to grow in poly-lysine treated glass slides for 2 days. The cells were incubated with DMEM-F12 medium without fetal bovine serum for 1 h before the experiment.

Fluorescent Binding on MrgD-Transfected Cells

The plasmid containing the Human MrgD receptor gene (Genecopoeia clone) and neomycin (G428) resistance was stably transfected in CHO cells using the polyethyleimine (PEI) reagent according to the manufacturer's instructions (Polysciences). Clonal cell lines that permanently expressed MrgD receptor were selected by 800 μg/ml G418. Binding of fluorescent-labeled [FAM-Alamandine];Bioshyntan, Berlin, Germany] was tested in MrgD-stably transfected Chinese hamster ovary cells (CHO-MrgD), Mas-stably transfected Chinese hamster ovary cells (CHO-Mas) and non-transfected CHO cells. FAM-Alamandine (0.01 μmol/L) was incubated for 30 minutes at room temperature. In CHO-Mrgd cells FAM-Alamandine was incubated alone or 5 minutes after the incubation of non-fluorescent alamandine (1 μmol/L), the Mas receptors antagonists A-779 (1 μmol/L) or D-Pro⁷-Ang-(1-7) (1 μmol/L), β-alanine (1 μmol/L) and the AT₂ receptor antagonist PD123319 (1 μmol/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 (1 μmol/L), of alamandine. 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: x63) and the total fluorescence was quantified using ImageJ software. Four images were captured in each slide.

Evaluation of Release of NO from CHO Cells

For fluorescence images, CHO cells stably expressing or not the MrgD or MAS receptors were plated in 6-wellPlates. Confluent cells were preincubated in serum free medium containing the NO indicator 4-amino-5 methylamino-2, 7-difluorofluorescein diacetate (DAF-FM) at 5 μM for 30

min. Subsequently, cells were stimulated with alamandine (0,01nM; 30 min) at 37°C in the humidified incubator under an atmosphere with 5% CO₂. Fluorescent images were obtained using a Zeiss LSM 510 Meta laser-scanning confocal microscope excited at 488 nm with an argon-ion laser (63 oil-immersion objective lens; Carl Zeiss, Oberkochen, Germany).

Inclusion Complex Preparation

Alamandine and Hydroxypropyl- β -Cyclodextrin were purchased from Bachem Bioscience Inc., Pennsylvania (USA) and from Xiamen Mchem, Xiamen (China), respectively. Inclusion complex between these molecules was prepared by the freeze drying process using the 1:1 molar ratio. An aqueous solution, using Milli-Q[®] water, of host and guest molecules was stirred for 3 h to ensure that equilibrium had been reached. Then, this solution was frozen in liquid nitrogen and lyophilized (Savant ModulyoD - Freeze-Dryer, Thermo Electron Corp., Waltham, MA, USA) for 48 h to obtain the solid inclusion complex.

Nuclear Magnetic Resonance

Alamandine: Hydroxypropyl- β -Cyclodextrin inclusion complex prepared by the freeze drying process was dissolved in D₂O (Cambridge Isotope Laboratories, Inc. - 99.9% of isotopic purity) and was used in order to investigate the guest inclusion by nuclear magnetic resonance spectroscopy (NMR). NMR spectra were recorded at 27.0 °C on a Bruker DRX 400 – AVANCE spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 400 MHz, equipped with a 5 mm inverse probe with a z-gradient coil. ¹H NMR spectrum was acquired using the WATERGATE technique for suppression of the residual water signal. Rotating frame Overhauser effect spectroscopy (2D ROESY) experiment (mixing time 600 ms) was applied to identify the cross peak correlation between host and guest hydrogens and to propose the supramolecular structure.

Induction of Cardiac Fibrosis

Twenty three male Sprague Dawley rats (SD) (approximately 3 months of age) were used. The animals were housed in a temperature- and humidity controlled room maintained on a 12 hours of schedule light and dark with free access to water and food.

The SD rats were divided in the five following groups: olive oil plus water (n=3), olive oil plus Hydroxypropyl β -cyclodextrin (HP β CD) (n=3), isoproterenol (ISO) plus water (n=5), olive oil plus alamandine included in HP β CD (n=6) and ISO plus alamandine included in HP β CD (n=6).

The olive oil (0.1ml/100g) or ISO (2 mg/Kg) dissolved in olive oil were injected subcutaneously in the dorsal region followed, by oral gavage of distilled water (0.1ml/100g), HP β CD (82 ug/Kg) or alamandine included in HP β CD (132 ug/Kg, equivalent to 50 ug/Kg of alamandine) dissolved in distilled water, for seven days.

After the treatment the SD rats were sacrificed by decapitation, the heart were removed and placed in a Langendorff preparation to wash out the blood cells with Krebs solution. The left ventricles were cryofixed in a solution with 20% dimethyl sulfoxide (DMSO) plus 80% methanol for at least 1 week in a -80°C freezer.

The left ventricles were rehydrated with serial wash of ethanol (70%, 50% and 30% in PBS), PBS and sucrose in a shaker at 4°C for one hour each and submerged in Tissue Teck (OCT compound, Miles, USA), frozen at 20°C and stored at -80°C.

Immunofluorescence staining

The left ventricles were cut in sections of 10 μ m thick in cryostat at -25°C and placed in slides. The section were washed in water and PBS for 10 minutes and incubated in blocking solution

(1% BSA and 0.1% Tween 20 in PBS) for 1 hour at room temperature. Then the sections were incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-human Collagen type I (1:200, Rockland), rabbit anti-human Collagen type III (1:200, Rockland) or rabbit anti-human fibronectin (1:200, Rockland). All antibodies were diluted in blocking solution 10%. The control sections were incubated without primary antibodies.

In the following day the sections were washed 4-5 times in PBS and incubated with the secondary antibody, donkey anti-rabbit IgG conjugated with Alexa fluor 488 (1:200, Molecular Probes) diluted in blocking solution 1%, at room temperature for 1 hour in the dark. After the sections were washed 3 times for 5 minutes in PBS and mounted in 25% glycerol plus 75% PBS and then coverslipped.

The images of the sections were captured in a immunofluorescence confocal microscope (Zeiss). The optimal settings (white balance, black reference) were determined at the beginning of each imaging session and then held constant during the analysis of all the sections.

For quantitative analysis of the fluorescence for each primary antibody tested, the Image J program was used. Images were captured in RGB and analyzed in the gray scale. The fluorescence intensity was measured as an average of the area (sum of gray values of all pixels divided by the number of pixels in the area) and values noted as arbitrary units (% gray value).

Data were expressed as mean \pm SEM. Statistical analysis was performed by One-Way ANOVA followed by Bonferroni's multiple comparison test.

Cytotoxicity of alamandine and β -alanine

Alamar blue metabolism was used for measurement of cytotoxicity. The cells A549 lung carcinoma cell line and DU 145 prostate cancer cell line were grown in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) supplemented with 10% FBS and standard antibiotics concentrations at 37°C and 5% CO₂. These cells were diluted to 2×10^4 cells.ml⁻¹ in DMEM-F12 medium containing Fetal Bovine Serum and 250 μ l were distributed in each well of a 96 well plate, except the last line. After overnight attachment, the cells were treated with different doses of alamandine (10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} M), β -Alanine (10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} M) or LY294002 (500, 250, 125, 62,5 and 31,25 μ M) for 48 hours in a 37°C incubator. Following, these cells were incubated for 3 hours with resazurine 0,125% in PBS (Sigma), the plates were read in spectrophotometer in 570 nm and 600 nm wavelength (Wallac Envision, 2102 Multi-label) and data were analyzed in MS Excel and GraphPrism. Percentage cytotoxicity was calculated by comparison with control wells.

RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to manufacturer's recommendations and were treated with RNase free DNase I (Invitrogen). Total RNA (1 μ g) was reverse transcribed using M-Mlv (Promega), following manufacturer's recommendations. The RT products (cDNA) were amplified by GoTaq® Green Master Mix (Promega), using oligonucleotide primers specific for MgrD (Forward 5'-GGGGTCTTAACCCAGTGAT-3' and Reverse 5'-CTGCTTACGGACGAGGAGAG-3'). The PCR cycling conditions were 95°C for 5 min followed by 35 cycles of a three-step amplification program (95°C for 30s, 60°C for 30s and 72°C for 40s) and a final step of 72°C for 5 min. The PCR products were visualized by agarose gel. For negative controls, no RT products were used as templates and verified by the absence of gel-detected bands. For positive control, a plasmid containing the MgrD cDNA cloned was used.

Online Figure I

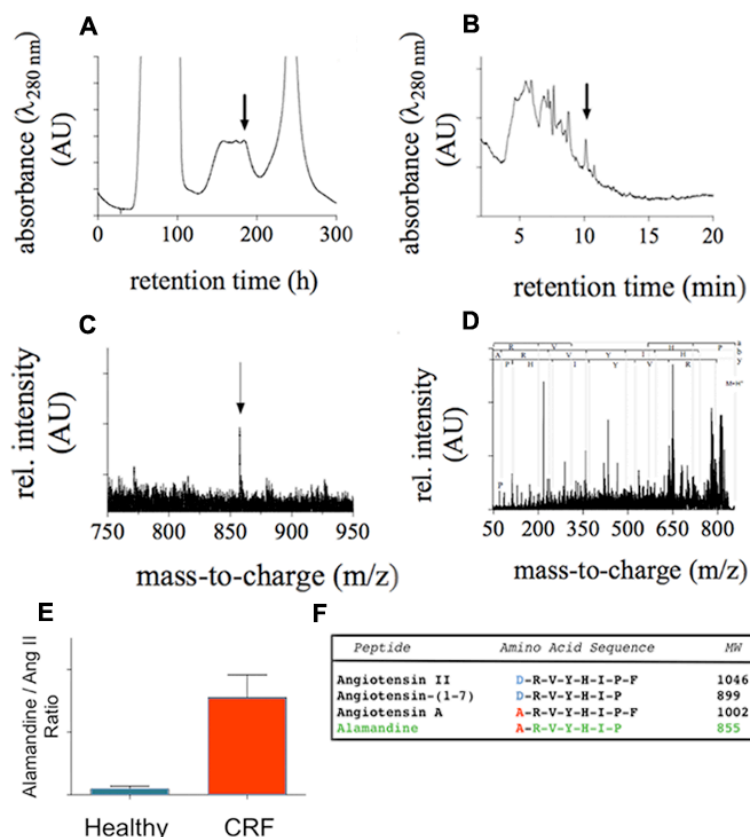


Figure I. Formation of Alamandine by ACE2 and its detection in human blood. **A**, Size-exclusion chromatogram of human plasma (conditions: Sephacryl S-100 high-resolution, Pharmacia BioTech, Sweden; column dimension: 1000 x 16 mm; eluent: 0.9 % NaCl in water; flow rate: 1.0 mL min⁻¹; abscissa: retention time (h); ordinate: UV-absorption at 280 nm (arbitrary units)). The arrow indicates a fraction containing Angiotensin-A 1-7. **B**, Reversed phase chromatography of the size-exclusion fraction labelled by an arrow in **Online Figure 1A**, using an analytical reversed-phase high performance liquid chromatographic column (conditions: ChromolithTM (100 x 4.6 mm I.D., Merck, Darmstadt, Germany; eluent A: 0.1 % trifluoroacetic acid (TFA) in water; eluent B: 0.1 % trifluoroacetic acid (TFA) in water-acetonitrile (20:80, v/v-%); gradient: 0-2 min: 0 % eluent B, 2-32 min: 0-75 % B, 32-32.5 min: 75-100 % eluent B; 32.5-33.5 min: 100 % B; flow rate: 2.0 mL min⁻¹; abscissa: retention time (min); ordinate: UV-absorption at 280 nm (arbitrary units)). The arrow indicates a fraction containing Angiotensin-A 1-7. **C**, MALDI-TOF mass spectrum of the fraction labelled by arrow in **Online Figure 1B**. The peak with a m/z ratio of 856.62 [M+H⁺] (calculated m/z 856.43 [M+H⁺]) corresponds to peptide sequence Ala-Arg-Val-Tyr-Ile-His-Pro. **D**, MALDI-TOF/TOF-MS/MS spectrum of the peak with an m/z ratio of 856.62 [M+H⁺] shown in **Online Figure 1C**. Both the b ions and the y ions confirm the amino acid sequence of Ala-Arg-Val-Tyr-Ile-His-Pro. **E**, Angiotensin-A 1-7 / Angiotensin-II ratio in plasma from chronic renal failure patients and healthy controls. **F**, amino acid sequence of Angiotensinergic peptides.

Online Figure II

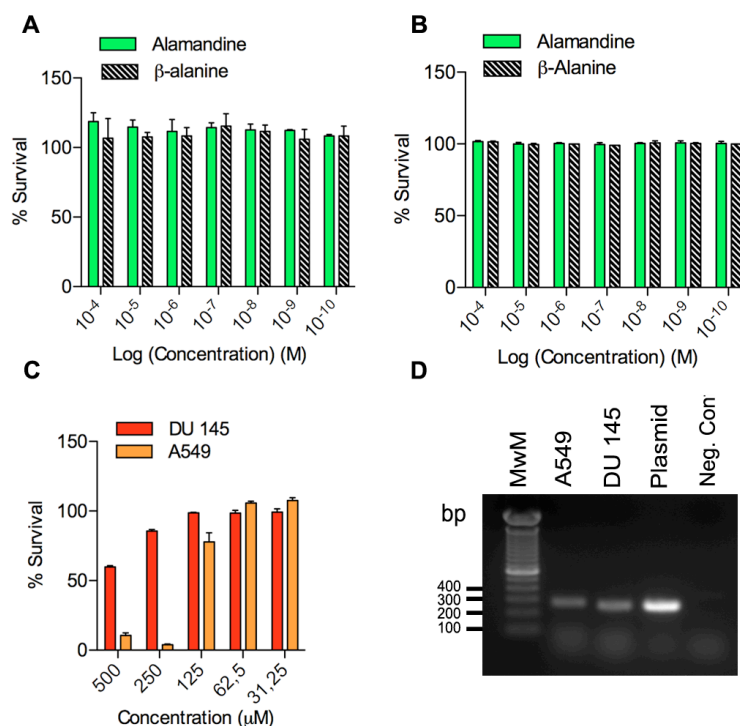


Figure II. Evaluation of proliferative or cytotoxicity of Alamandine in human tumoral cell lines. **A**, DU 145 prostate cancer cell line. **B**, A549 lung carcinoma cell line were treated with different concentrations of Alamandine (green) or β -Alanine (dashed). **C**, positive control, red (DU 145) and orange (A549) treated with LY294002. Percentage cytotoxicity was calculated by comparison with control wells. **D**, PCR detection of MrgD receptor expression in tumoral cell lines DU 145 and A549. Agarose-gel electrophoresis of PCR products from cDNA of A549 and DU 145 cells, positive control plasmid (plasmid) and PCR negative control without DNA (Neg. Control). The PCR products presented the same size, related to the amplification of a region of MrgD cDNA. bp: Base pairs. MwM: 100bp DNA molecular weight marker (Invitrogen).

Online Figure III

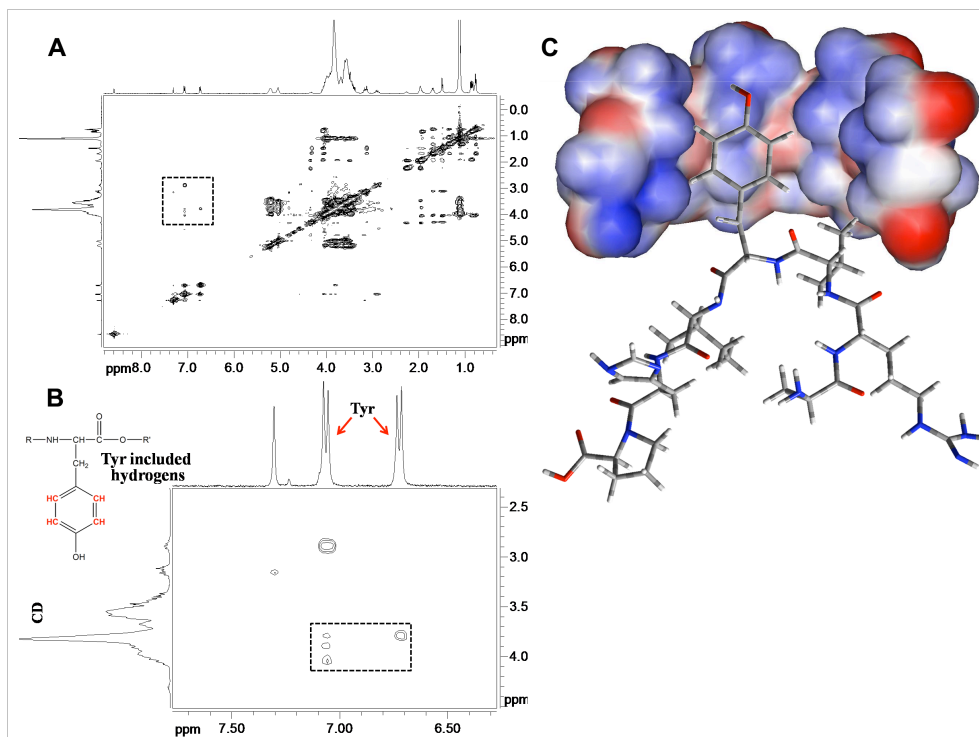


Figure III. Nuclear magnetic resonance spectra for the Alamandine Hydroxypropyl- β -Cyclodextrin inclusion compound. **A**, Hydroxypropyl- β -Cyclodextrin inclusion complex acquired at 400 MHz in a Bruker DRX 400 – AVANCE spectrometer using D₂O as solvent, at 27 °C (mixing time 600 ms): 2D ROESY contour map δ from 0 to 9 (F1 and F2) and **B**, expansion for the 2D ROESY contour map in the Alamandine aromatic region (δ 6.3 – 7.7) and Hydroxypropyl- β -Cyclodextrin region (δ 2.4 – 4.5). **C**, schematic representation for the Angiotensin-(1-7) inclusion into the Hydroxypropyl- β -Cyclodextrin cavity through the Tyr residue.

Online Figure IV

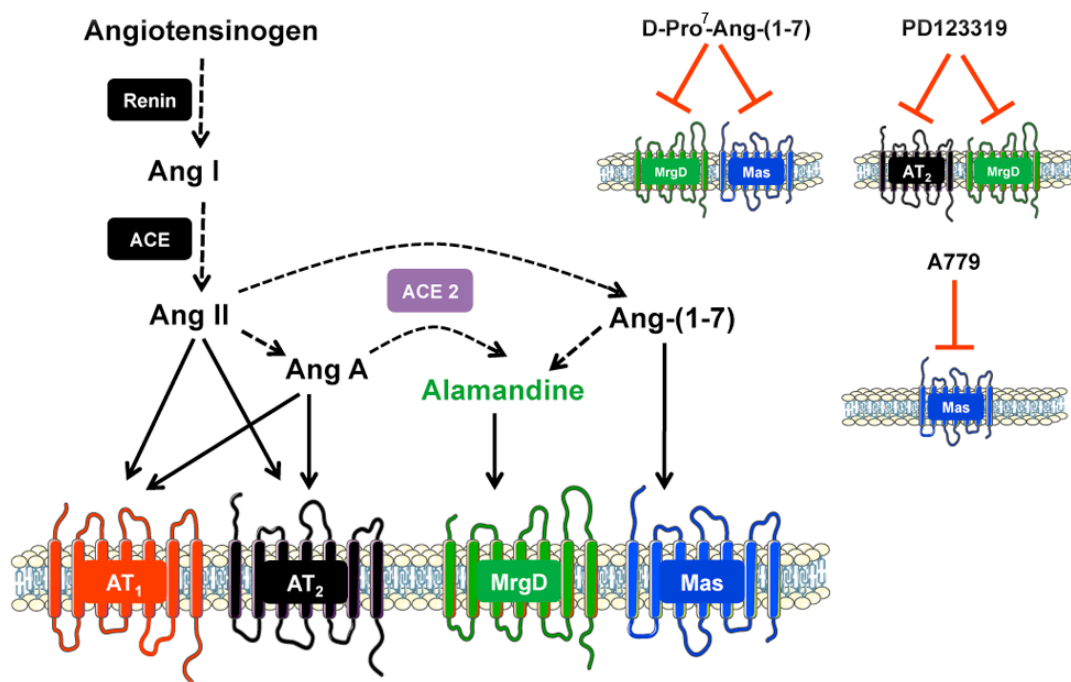


Figure IV. Simplified updated view of the renin–angiotensin system cascade.

Abbreviations: ACE, angiotensin-converting enzyme; ACE 2, angiotensin-converting enzyme type 2, Ang, angiotensin; AT₁, Ang II type 1 receptor; AT₂, Ang II type 2 receptor; Mas, Ang(1–7) receptor Mas; MrgD, MAS-related G-protein coupled receptor D; A779, selective Ang-(1-7) antagonist; D-Pro⁷-Ang-(1-7), Ang-(1-7) antagonist; PD123319, AT₂ receptor antagonist.

Online figure V

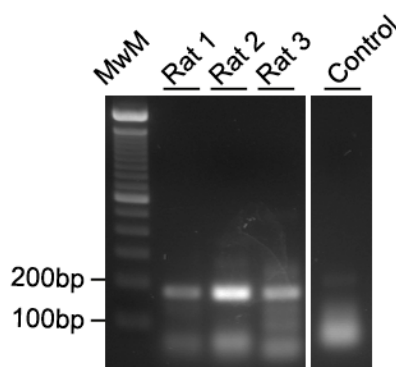


Figure V. Evaluation of the expression of MrgD in the aorta of sprague dawley (SD) rats. Agarose-gel electrophoresis of PCR products from cDNA from the aorta of three SD rats and PCR negative control without DNA (Control). The PCR products presented the same size, related to the amplification of a region of MrgD cDNA. bp: Base pairs. MwM: 100bp DNA molecular weight marker (Invitrogen).

Supplementary References

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