Angiotensin-(1–9) regulates cardiac hypertrophy *in vivo* and *in vitro*

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Background Angiotensin-(1–9) is present in human and rat plasma and its circulating levels increased early after myocardial infarction or in animals treated with angiotensin-converting enzyme inhibitor. However, the cardiovascular effects of this peptide are unknown.

Objective To determine whether angiotensin-(1–9) is a novel anti-cardiac hypertrophy factor *in vitro* and *in vivo* and whether this peptide is involved in the pharmacological effects of cardiovascular drugs acting on the renin–angiotensin system.

Methods and results The administration of angiotensin-(1–9) to myocardial infarcted rats by osmotic minipumps (450 ng/kg per min, $n = 6$) vs. vehicle ($n = 8$) for 2 weeks decreased plasma angiotensin II levels, inhibited angiotensin-converting enzyme activity and also prevented cardiac myocyte hypertrophy. However, cardiac myocyte hypertrophy attenuation triggered by angiotensin-(1–9) was not modified with the simultaneous administration of the angiotensin-(1–7) receptor antagonist A779 (100 ng/kg per min, $n = 6$). In experiments *in vitro* with cultured cardiac myocytes incubated with norepinephrine (10 μmol/l) or with insulin-like growth factor-1 (10 nmol/l), angiotensin-(1–9) also prevented hypertrophy. In other experimental setting, myocardial infarcted rats ($n = 37$) were randomized to receive either vehicle ($n = 12$), enalapril (10 mg/kg per day, $n = 12$) or angiotensin II receptor blocker candesartan (10 mg/kg per day, $n = 13$) for 8 weeks. Both drugs prevented left ventricle hypertrophy and increased plasma angiotensin-(1–9) levels by several folds. Angiotensin-(1–9) levels correlated negatively with different left ventricular hypertrophy markers even after adjustment for blood pressure reduction.

Conclusion Angiotensin-(1–9) is an effective and a novel anti-cardiac hypertrophy agent not acting via the Mas receptor. *J Hypertens* 28:1054–1064 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Journal of Hypertension 2010, **28**:1054–1064

Keywords: angiotensin-converting enzyme 2, angiotensin-(1–9), cardiac dysfunction, cardiac hypertrophy, cardiac myocyte, myocardial infarction

Abbreviations: ACE, angiotensin-converting enzyme; ACEI, ACE inhibitor; ANF, atrial natriuretic factor; Ang, angiotensin; BW, body weight; C, candesartan; E, enalapril; LCA, left coronary artery; LV, left ventricle; LVAWT, left ventricular anterior wall thickness; LVEDD, left ventricular end-diastolic diameter; LVEDV, LV end-diastolic volume; LVEF, LV ejection fraction; LVESD, left ventricular end-systolic diameter; LVESV, LV end-systolic volume; LVFS, left ventricular fractional shortening; LVH, left ventricular hypertrophy; LVP, left ventricular protein; LVPWT, left ventricular posterior wall thickness; LVW, left ventricular weight; LVWT, left ventricular wall thickness; MI, myocardial infarction; RAS, renin–angiotensin system; S, sham; SBP, systolic blood pressure; TL, tibial length; β-MHC, β-myosin heavy chain

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Received 24 July 2009 **Revised** 16 October 2009
Accepted 26 November 2009

Introduction

Accumulating evidence indicates that the renin–angiotensin system (RAS) and its primary effector angiotensin (Ang) II are linked to the pathophysiology of cardiac hypertrophy and heart failure [1,2]. In the classic concept of the RAS, renin released from kidneys catalyzes the formation of Ang I in circulating blood from liver-derived angiotensinogen. From Ang I, Ang II is generated through the action of the angiotensin-converting enzyme (ACE) mainly in the pulmonary circulation [1,2]. The resulting octapeptide Ang II is the major mediator of the diverse biological functions of the RAS, attaining effects through its binding to specific receptors in target cells. The traditional concept of the RAS in the circulation has been expanded to its local existence in many organs, including the heart and arteries [3–5]. The effects of Ang II result from its binding to specific receptors AT1 and AT2 [6]. Both cell surface receptors belong to the large family of G protein-coupled receptors, though the signaling pathways are completely different and signal in apparent opposition [6]. Most of the classically recognized actions of Ang II, including vasoconstriction, stimulation of aldosterone release and the antinatriuretic effects in the kidney are mediated by AT1 receptors [6].

0263-6352 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI:10.1097/HJH.0b013e328335d291

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However, the RAS is a more complex system than originally thought. ACE2 has been identified as a homologue of ACE [7]. ACE2 is also a metalloprotease with a considerable degree of homology to ACE [8,9]. Both ACE and ACE2 are bound to the plasma membrane and must be cleaved to release the soluble enzyme [8]. Their cellular and tissue distributions are also different in that ACE is expressed in the endothelium throughout the vasculature, whereas ACE2 is distributed to most tissues with the most abundant expression in heart, kidney and testis [9]. Their substrate specificities also differ; ACE2 cleaves Ang I to Ang-(1–9). This reaction is negligibly slow because it is several hundred times slower than Ang II hydrolysis by ACE2 to form Ang-(1–7), a vasodepressor peptide counterbalancing the vasopressor effect of Ang II [10–12]. Ang-(1–9) is a relatively more stable intermediate and then is cleaved by ACE to Ang-(1–7) [13]. However, ACE2 does not act on bradykinins and its activity is not inhibited by classic ACE inhibitors (ACEIs) [8].

After myocardial infarction (MI), there are changes in the enzymatic activities of both ACE and ACE2 as well as in the circulating levels of various Angs [14,15]. We have previously observed that circulating and left ventricular activities of ACE2 were downregulated in the long-term phase of left ventricular dysfunction in myocardial infarcted rats; these effects were prevented by the conventional ACEI enalapril; and plasma Ang-(1–9) levels were significantly increased when myocardial infarcted rats or sham-operated rats were treated with enalapril for 8 weeks but circulating Ang-(1–7) levels did not change at that time [16]. Based on these findings, we proposed that Ang-(1–9) rather than Ang-(1–7) acts as a counter-regulator of Ang II [15]. The aims of the present study were to determine whether Ang-(1–9) is a novel anti-cardiac hypertrophy factor *in vitro* and *in vivo* and whether this peptide is involved in the pharmacological effects of cardiovascular drugs that act on the RAS.

Methods

Experimental protocols

The present investigation complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the Research Commission from the School of Medicine, Pontifical Catholic University of Chile. Male Sprague–Dawley rats, aged 6–7 weeks (200 ± 10 g), were housed in cages with *ad libitum* access to rat chow and water. Rats were subjected to either sham operation (S) or left coronary artery (LCA) ligation (MI) as described [15]. MI was confirmed by electrocardiography 24 h after surgery. The size of the infarct in survivors (mortality $\approx 25\%$) was determined by planimetry of the endocardial circumference of the left ventricle (LV) on histological sections [15].

To study the effects of Ang-(1–9) chronic administration after MI on hypertrophy, circulating Ang II levels as well as plasma and left ventricular ACE activities, rats were randomized into two groups, sham operation (S, $n = 8$) or coronary artery ligation (MI, $n = 20$). Myocardial infarcted rats received randomly vehicle ($n = 8$) or Ang-(1–9) (450 ng/kg per min, $n = 6$) with or without the Ang-(1–7) blocker A779 (100 ng/kg per min, $n = 6$) [16] for 14 days using osmotic minipumps Alzet implanted in the jugular vein under ketamine HCl/xylazine [35 and 7 mg/kg intraperitoneally (i.p.), respectively]. Systolic blood pressure (SBP) was determined using tail-cuff method by investigators blinded to treatment. Left ventricular function was assessed by bidimensional transthoracic echocardiography [15] using a Sonos 5000 equipped with 5–12 MHz sectorial electronic ultraband S12 Philips transducer under ketamine HCl/xylazine (35 and 7 mg/kg i.p., respectively). The following echocardiographic indices of left ventricular size (dimensions and volume) and function (fractional shortening and ejection fraction) were measured: left ventricular end-systolic diameter (LVESD), left ventricular end-diastolic diameter (LVEDD), infarcted and noninfarcted wall thickness (LVAWT and LVPWT, respectively) [15]. Left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV) were calculated by a modified Simpson's method in M-mode and stroke volume values were derived as follows: LVEDV−LVESV. Left ventricular fractional shortening (LVFS %) in the short axis view made from M-mode was calculated as (LVEDD−LVESD)/LVEDD $\times$ 100 and LV ejection fraction (LVEF %) was derived as LVEF = (LVEDV−LVESV)/LVEDV $\times$ 100 [17].

To evaluate the long-term effects of ACE inhibition and AT1 receptor blocking after MI on plasma RAS components, myocardial function and cardiac hypertrophy, rats were randomized to sham operation (S, $n = 34$) or LCA ligation (MI, $n = 37$) as described [15]. Sham and myocardial infarcted rats received either vehicle ($n = 11$ and 12, respectively), enalapril (10 mg/kg per day, $n = 10$ and 12, respectively) [15] or candesartan (10 mg/kg per day, $n = 13$) [18] by gavage for 8 weeks, starting 48 h after MI.

Evaluation of left ventricular hypertrophy

Left ventricular hypertrophy (LVH) was quantified by left ventricular weight (LVW, mg), the ratios between LVW, and body weight (g) or tibial length (mm) and by the total left ventricular protein (LVP) contents. Furthermore, other molecular LVH markers such as relative levels of cardiac atrial natriuretic factor (ANF) mRNA and β-myosin heavy chain (β-MHC) protein levels were also determined [15]. Morphological and morphometric analysis were done in transverse mid-ventricular slices (10 μm) of the hearts embedded in paraffin, stained with hematoxylin and eosin and examined by light microscopy

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(20×). Cardiac myocyte size was determined as described by Nakamura *et al.* [19]. Briefly, cell images viewed with a video camera fixed to a microscope were projected onto a monitor and traced. Image J analysis software directed the computation of the cardiac myocyte area and perimeter by a blinded observer (at least 70 cells per animal).

Plasma angiotensins and bradykinin levels

Plasma Angs and bradykinins were determined as described [15,20]. Briefly, rats were anesthetized with a combination of ketamine and xylazine. Blood was collected from the inferior vena cava directly into a syringe containing 5 ml of 4 mol/l guanidine thiocyanate. The blood was centrifuged and the plasma (approximately 3 ml) was immediately extracted with Sep-Pak C18 cartridges (Waters Chromatography Division, Milford, Massachusetts, USA) and the peptides were acetylated before by high-performance liquid chromatography (HPLC) [20]. After reconstitution in water, each HPLC fraction was assayed in duplicate with the amino terminal-directed antibody A41, which measures acetyl-Ang II, acetyl-Ang I, acetyl-Ang-(1–9) with 100% and acetyl-Ang-(1–7) with 79% cross-reactivity or with the amino terminal-directed antibody B-24, which measures acetyl-bradykinin-(1–7) with 79% and acetyl-bradykinin-(1–9) with 100% cross-reactivity. Both antibodies were kindly donated by Dr D.J. Campbell, St Vincent's Institute of Medical Research, University of Melbourne, Fitzroy. Acetylation, HPLC and radioimmunoassay were performed as described by Campbell *et al.* [20]. The coefficients of variation for interassay and intraassay variances were 8.5 and 7.1% for Ang I, 7.0 and 6.9% for Ang II, 5.0 and 3.4% for Ang-(1–9) and 4.0 and 2.8% for Ang-(1–7), 3.7 and 4.5% for bradykinin-(1–9) and 4.0 and 8.0% for bradykinin-(1–7), respectively. The recoveries from plasma were 73% for Ang I, 65% for Ang II, 70% for Ang-(1–9), 58% for Ang-(1–7), 62% for bradykinin-(1–7) and 82% for bradykinin-(1–9) ($n = 8$). The lower limit of detection was 0.3 fmol/ml for Angs and bradykinin assays.

Plasma and tissue angiotensin-converting enzyme activity

Plasma and tissue ACE activities were measured fluorometrically by following the hydrolysis of *Z*-phenyl-L-histidyl-L-leucine (Bachem Bioscience Inc., King of Prussia, Pennsylvania, USA) as described previously [15]. Plasma and left ventricular ACE activities were expressed in U/ml (1 U = 1 nmol L-histidyl-L-leucine/min) and U/mg prot, respectively.

Culture of cardiac myocyte and evaluation of hypertrophy

Cardiac myocytes were isolated from neonatal Sprague–Dawley rat ventricles as described [21]. Cell cultures were at least 95% pure. Cardiac myocytes were plated at 70% final density in gelatin-coated wells (12-well plates) or in 60-mm Petri dishes and maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air for 24 h in Dulbecco's Modified Eagle's medium [(DMEM)/M199] (4 : 1) containing 10% fetal bovine serum and 5% fetal calf serum. Serum was withdrawn 24 h before preincubation with 1, 10 or 100 μmol/l Ang-(1–9) for 1 h; then, 10 μmol/l norepinephrine (Sigma, St Louis, Missouri, USA) or 10 nmol/l human insulin-like growth factor-1 (IGF-1, kindly donated by Dr C. George-Nascimento; Austral Biologicals, San Ramon, California, USA) were added and the cultures were incubated for 24 h. The effect of A779 (1 μmol/l) was evaluated preincubating the cells with A779 (1 μmol/l) for 30 min. Then the cells were incubated with 1 μmol/l Ang-(1–9) for 1 h and then with 10 μmol/l norepinephrine. The sarcomerization and cell size were analyzed as follows. Cells were grown on coverslips, stimulated and fixed, incubating with cytoskeleton stability buffer [10 mmol/l 2-(*N*-morphiline)-ethane sulfonic acid (MES), pH 6.0, 150 mmol/l NaCl, 5 mmol/l ethylenediaminetetraacetic acid (EDTA), 3% sucrose, 5 mmol/l $MgCl_2$] for 5 min. Cell size was determined in cells fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X100 for 6 min. Nonspecific sites were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h. Cells were washed with PBS and incubated with phalloidin-rhodamine 1 : 400 (to stain F-actin) at room temperature for 45 min. Coverslips were mounted in DakoCytomation Fluorescent Mounting Medium. Cell staining was evaluated by epifluorescence microscopy. At least 100 cells from randomly selected fields were analyzed using Image J software (NIH).

Statistical analysis

Results are shown as mean ± SEM and analyzed by *t*-test or analysis of variance (ANOVA). Pearson and Spearman correlation analysis and multivariate linear regression analysis were also used. All *P* values less than 0.05 were considered statistically significant.

Results

Effects of chronic administration of angiotensin-(1–9) in myocardial infarcted rats on hemodynamic, echocardiographic indices of left ventricular size (dimensions or volume) and function (fractional shortening and ejection fraction), plasma angiotensins and angiotensin-converting enzyme activity

In order to evaluate the effect of Ang-(1–9) on in-vivo cardiac remodeling, rats were subjected to MI by LCA ligation and immediately Ang-(1–9) was administered by continuous perfusion with Alzet minipumps during 14 days. Table 1 summarizes body weight, SBP, LVW/body weight ratio and LVP contents at death in the S, MI, MI + Ang-(1–9) and MI + A779 + Ang-(1–9) groups.

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Table 1 Effects of angiotensin-(1–9) administered for 2 weeks with or without A779 on blood pressure, echocardiographic indexes of left ventricular size (dimensions and volume) and function (fractional shortening and ejection fraction) following coronary artery ligation

Parameter	S	MI	MI + Ang-(1–9)	MI + A779 + Ang-(1–9)
N	8	8	6	6
BW (g)	255 ± 6	264 ± 6	274 ± 24	277 ± 4
SBP (mmHg)	115 ± 4	110 ± 3	112 ± 2	117 ± 9
LVW (mg)	830 ± 20	915 ± 18*	810 ± 56†	828 ± 35†
LVW/TL (mg/cm)	235 ± 7	279 ± 9*	247 ± 4†	241 ± 6†
LVW/BW (mg/g)	3.2 ± 0.1	3.6 ± 0.1*	3.1 ± 0.2†	3.0 ± 0.2†
LVP content (mg)	8.2 ± 0.4	9.8 ± 0.3*	8.0 ± 0.3†	8.4 ± 0.2†
Heart rate (beat/min)	276 ± 14	271 ± 7	259 ± 14	260 ± 10
LVESD (mm)	4.7 ± 0.1	6.7 ± 0.2*	5.7 ± 0.3†	5.5 ± 0.3†
LVEDD (mm)	7.3 ± 0.1	8.7 ± 0.2*	8.0 ± 0.1†	8.2 ± 0.1†
LVAWT (mm)	1.3 ± 0.0	0.8 ± 0.0*	0.9 ± 0.03*	0.9 ± 0.0*
LVPWT (mm)	1.3 ± 0.0	1.7 ± 0.0*	1.2 ± 0.0†	1.1 ± 0.0†
LVEDV (ml)	2.4 ± 0.1	3.4 ± 0.2*	2.7 ± 0.1*,†	2.8 ± 0.1*,†
LVESV (ml)	0.8 ± 0.0	1.7 ± 0.1*	1.2 ± 0.1*,†	1.4 ± 0.1*,†
Stroke volume (ml)	1.6 ± 0.1	1.8 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
LVEF (%)	66 ± 0.8	51 ± 2.0*	53 ± 1.3*	53 ± 0.6*
LVFS (%)	36 ± 0.8	23 ± 1.6*	27 ± 1.9*	27 ± 1.5*
LV infarct size (%)	NA	29 ± 6	26 ± 2	27 ± 3

Values as mean ± SEM. A779, Ang-(1–7) blocker receptor; BW, body weight; LV, left ventricle; LVAWT, left ventricular anterior wall thickness; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; LVESV, left ventricular end-systolic volume; LVFS, left ventricular fractional shortening; LVP, left ventricular protein; LVPWT, left ventricular posterior wall thickness; LVW, left ventricle weight; LVWT, left ventricular wall thickness; MI, myocardial infarction; S, sham; SBP, systolic blood pressure; TL, tibial length. * $P < 0.05$ vs. S. † $P < 0.05$ vs. MI (after ANOVA).

As expected, in the MI group, LVW/body weight, LVW/tibial length ratio and LVP contents increased significantly at week 2. Echocardiographic studies of left ventricular size performed in hearts showed significant increases in the LVESD, LVEDD, LVPWT, LVEDV and LVESV indexes in the MI groups as compared with the S groups, with concomitant decreases in the LVFS, LVEF and LVAWT values at both time points. Ang-(1–9) levels did not change the body weight, SBP, heart rate and stroke volume as compared with the MI and sham groups (Table 1). Continuous administration of Ang-(1–9) to myocardial infarcted rats significantly decreased LVW, LVW/body weight, LVW/tibial length ratios and LVP in comparison to the MI group. Ang-(1–9) prevented the effect of LCA ligation on LVESD, LVEDD, LVPWT, LVEDV and LVESV but not on LVEF, LVFS and LVAWT parameters. The average infarct size was similar in the MI and MI + Ang-(1–9) groups.

Because Ang-(1–9) can be cleaved by ACE to form Ang-(1–7), we evaluated the coadministration of A779, an Ang-(1–7) receptor antagonist, with Ang-(1–9) to myocardial infarcted rats for 2 weeks. A779 administration had no effect on the Ang-(1–9)-dependent suppression of LVH (Table 1). Thus, the in-vivo antihypertrophic action of Ang-(1–9) in cardiac tissue is not mediated by Ang-(1–7).

Table 2 Effects of angiotensin-(1–9) administered for 2 weeks on circulating angiotensins and angiotensin-converting enzymes after coronary artery ligation

Parameter	S	MI	MI-Ang-(1–9)
N	11	12	6
Ang I (fmol/ml)	26.9 ± 2.8	19.2 ± 0.9*	21.6 ± 4.5
Ang-(1–7) (fmol/ml)	6.0 ± 0.9	5.2 ± 0.7	6.7 ± 0.9
Ang-(1–9) (fmol/ml)	5.6 ± 0.5	6.5 ± 0 .5	34.0 ± 3.2†
Ang II (fmol/ml)	19.2 ± 0.4	31.5 ± 1.8*	23.5 ± 3.3†
Plasma ACE activity (U/ml)	96 ± 1	153 ± 6*	119 ± 2†
LV ACE activity (U/mg prot)	1.6 ± 0.4	3.5 ± 0.5*	1.5 ± 0.3†

Values as mean ± SEM. ACE, angiotensin-converting enzyme; Ang, angiotensin; LV, left ventricle; MI, myocardial infarction; *N*, number of animals; S, sham. * $P < 0.05$ vs. S. † $P < 0.05$ vs. MI (after ANOVA).

To assess whether Ang-(1–9) could be an endogenous inhibitor of ACE [22], we measured the effect of Ang-(1–9) administration on circulating and left ventricular ACE activities as well as on plasma Ang II levels. Table 2 shows that Ang-(1–9) administration prevented the effect of LCA ligation on plasma Ang II levels and plasma and left ventricular ACE activities.

Cardiac myocyte hypertrophy after chronic administration of angiotensin-(1–9) in myocardial infarcted rats

Cardiac myocyte area increased by 69% relative to sham rats ($P < 0.01$), whereas Ang-(1–9) administration prevented this increase relative to myocardial infarcted rats ($P < 0.01$; Fig. 1a–b). The cardiac myocyte perimeter followed the same trend, increasing by 28% in myocardial infarcted rats as compared with sham rats ($P < 0.01$) and decreasing by 31% in Ang-(1–9)-treated myocardial infarcted rats as compared with myocardial infarcted rats ($P < 0.01$; Fig. 1c). Left ventricular ANF mRNA levels were 215% higher in myocardial infarcted rats than in sham rats ($P < 0.01$; Fig. 2a–b) and were reduced by 80% in Ang-(1–9)-treated myocardial infarcted relative to myocardial infarcted rats ($P < 0.01$). Left ventricular β-MHC protein levels (Fig. 2c–d) increased by 90% in myocardial infarcted rats relative to sham rats ($P < 0.01$) and decreased by 51% in Ang-(1–9)-treated myocardial infarcted rats relative to myocardial infarcted rats ($P < 0.01$). Taken together, these results indicate that

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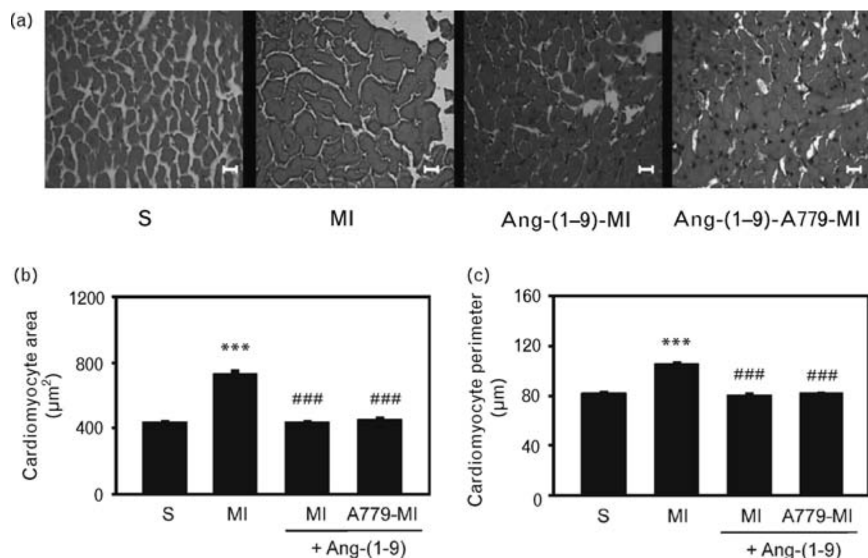
Fig. 1



Ang-(1–9) attenuates increases in cardiac myocyte area and perimeter secondary to myocardial infarction. Myocardial infarcted (MI) rats received either vehicle or Ang-(1–9) for 14 days. (a) Microphotographs of cross-sectional noninfarcted left ventricular slices stained with hematoxylin and eosin (400×). Scale bar, 25 μm. (b) Cardiomyocyte area and (c) perimeter in noninfarcted left ventricles, respectively. Results are presented as mean ± SEM (6–8 rats, $n = 70$ cells). $^{***}P < 0.001$ vs. S and, $^{###}P < 0.001$ vs. MI.

Ang-(1–9) administration prevented cardiac myocyte hypertrophy after MI. The Ang-(1–7) blocker A779 administration had no effect on the Ang-(1–9)-dependent suppression of cardiac myocyte hypertrophy induced by MI (Fig. 1a–c). Thus, the in-vivo anti-hypertrophic action of Ang-(1–9) in cardiac tissue after MI is not mediated by Ang-(1–7).

The in-vivo anti-hypertrophic effects of Ang-(1–9) were corroborated *in vitro* by experiments using cultured neonatal cardiac myocytes and 10 μmol/l norepinephrine or 10 nmol/l IGF-1 as hypertrophic stimuli. As shown in Fig. 3a–b, norepinephrine and IGF-1 induced increases in cardiac myocyte area. This effect was significantly prevented by Ang-(1–9). A779 incubation had no effect on the Ang-(1–9)-dependent suppression of cardiomyocyte hypertrophy (Fig. 3c–d). Similar effects on cardiac myocyte perimeter and sarcomerization were observed with Ang-(1–9) (data not shown). These results suggest that the in-vivo antihypertrophic actions produced by chronic administration of Ang-(1–9) in myocardial infarcted rats could be explained, at least in part, by a direct action of Ang-(1–9) on cardiomyocytes.

Hemodynamic and morphometric parameters and left ventricular function in myocardial infarcted rats after treatment with enalapril or candesartan

To analyze whether standard RAS-associated antihypertensive/antiremodeling drugs actions could be associated with the modification of Ang-(1–9) plasma levels, rats were subjected to MI by LCA and then treated by enalapril or candersartan during 8 weeks. Markers of LVH and Angs and bradykinin plasma levels were determined at basal and 8 weeks of treatment.

SBP, body weight, LVW/body weight ratio, relative ANF mRNA levels, LVP contents and β-MHC protein levels at 8 weeks after surgery in the six experimental groups are shown in Table 3. Body weight and SBP were similar in sham and in the myocardial infarcted rats and were decreased in the rats treated with candesartan or enalapril for 8 weeks. In rats treated with candesartan, the SBP was significantly lower than that in rats treated with enalapril. The average infarct size was similar in all treated and untreated myocardial infarcted rats. As expected, the four hypertrophy markers (LVW/body weight ratio, LVP content, ANF mRNA and β-MHC protein levels) were increased significantly at 8 weeks after LCA ligation and were reduced by administration of candesartan or enalapril.

Table 3 also shows that LVESD, LVEDD, LVEDV and LVESV indexes increased significantly in the MI group (by 33, 19, 30 and 50%, respectively) compared with the sham rats, with concomitant reduction in the LVFS, LVEF and LVAWT values after 8 weeks post-MI. Both candesartan and enalapril significantly prevented the left ventricular dilation induced by the MI, but they did not modify the changes in LVFS, LVEF and LVAWT. However, with candesartan, the LVPWT and LVEDV were significantly lower than in rats treated with enalapril.

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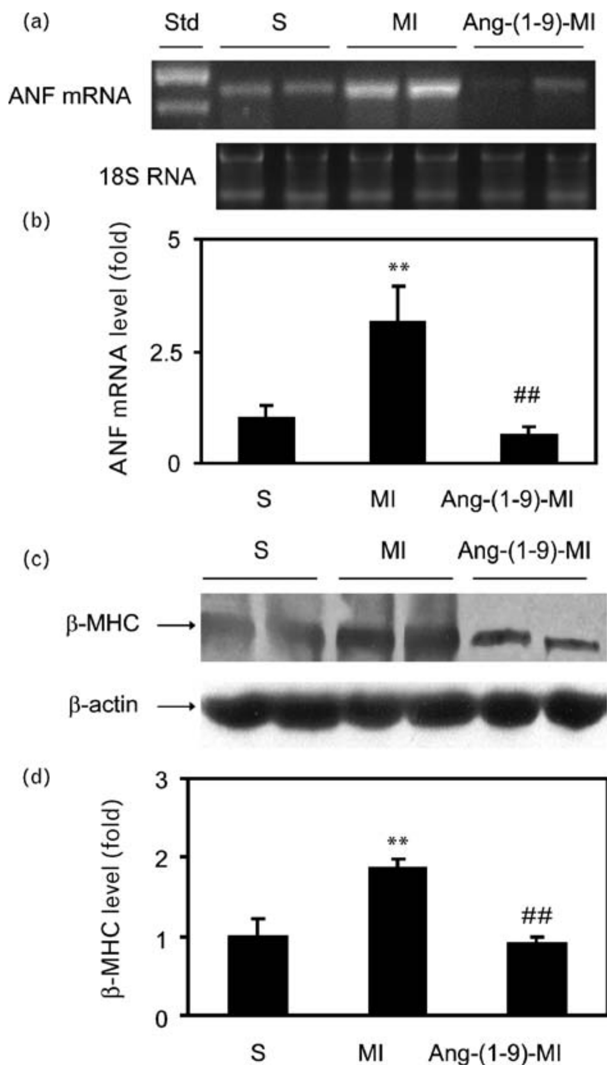
Fig. 2



Ang-(1–9) prevents increases in left ventricular atrial natriuretic factor mRNA expression and β-myosin heavy chain protein levels induced by myocardial infarction. Myocardial infarcted (MI) rats received either vehicle or Ang-(1–9) for 14 days. (a) DNAse-treated total RNA was isolated with Trizol and the integrity of 18S RNA was assessed by agarose gel electrophoresis. Band intensities were quantified by computerized densitometry and normalized to 18S RNA. (b) Quantification of atrial natriuretic factor (ANF) mRNA in noninfarcted left ventricles. Data are presented as mean ± SEM ($n = 6-8$). $^{**}P < 0.01$ vs. S and $^{\#\#}P < 0.01$ vs. MI. (c) β-Myosin heavy chain (β-MHC) protein levels in noninfarcted left ventricles were determined by western blot analysis. (d) Results are reported as mean ± SEM ($n = 6-8$). For quantification, the films were scanned and digitized images were analyzed with UN-SCAN-IT software; the values are expressed as fold over S. $^{**}P < 0.01$ vs. S and $^{\#\#}P < 0.01$ vs. MI.

Plasma angiotensins and bradykinins in myocardial infarcted rats after treatment with enalapril or candesartan

As shown in Table 4, in the MI group, plasma Ang I levels were lower and Ang II levels were higher than those in sham animals. However, Ang-(1–7) and Ang-(1–9) levels were similar in sham and in myocardial infarcted rats. As expected, with enalapril, plasma Ang I levels increased by 147% in myocardial infarcted rats compared with untreated myocardial infarcted rats. With candesartan, plasma Ang II levels increased significantly by 29% in myocardial infarcted rats compared with untreated myocardial infarcted rats. Both enalapril and candesartan increased circulating Ang-(1–9) levels as well as Ang-(1–7)/Ang II and Ang-(1–9)/Ang I ratios. Plasma Ang-(1–7) levels were increased only by candesartan. In the experimental groups, only Ang-(1–9) (Fig. 4a), but not Ang II or Ang-(1–7), plasma levels (Fig. 5a–b), were significantly correlated with LVW ($r = -0.553$, $P < 0.01$, $n = 62$). Considering that the observed effect of Ang-(1–9) on LVW might depend on SBP reduction, we tested for a significant interaction between both variables in a multiple linear regression model. The effect of Ang (1–9) on LVW remained significant after adjustment for SBP reduction ($r = -0.532$, $P < 0.01$, Fig. 4a). The relationship between LVP content ($r = -0.324$; $P = 0.01$, Fig. 4b) and LVW/body weight ($r = -0.401$; $P < 0.01$) with plasma Ang-(1–9) levels also remained significant after SBP reduction adjustment ($r = -0.310$, $P = 0.02$, Fig. 4b for LVP content; $r = -0.423$, $P < 0.01$ for LVW/body weight). SBP reduction became nonsignificant when included in the multivariate analysis. Collectively, these results indicate that both ACE inhibition and AT1 receptor blockade increased plasma Ang-(1–9) levels and that increased circulating Ang-(1–9) levels are associated with attenuation of LVH and left ventricular remodeling.

Table 4 also depicts that in the MI group, circulating bradykinin-(1–7), bradykinin-(1–9) levels and bradykinin-(1–7)/bradykinin-(1–9) ratio were not different from those from sham rats. Candesartan did not modify the plasma bradykinin levels, whereas enalapril increased the bradykinin-(1–9) plasma levels and decreased bradykinin-(1–7) plasma levels and bradykinin-(1–7)I/bradykinin-(1–9) ratio in sham and myocardial infarcted rats.

These results indicate that both candesartan and enalapril increase plasma levels of Ang-(1–9) in myocardial infarcted rats. Moreover, in these animals, cardiac hypertrophy inversely correlates with Ang-(1–9) plasma levels, suggesting an association between left ventricular anti-hypertrophic actions of enalapril and candesartan and their ability to increase Ang-(1–9) plasma levels.

Discussion

Our main findings were as follows. First, administration of Ang-(1–9) for 2 weeks after MI prevented the increases in plasma Ang II levels, plasma and left ventricular ACE activities and cardiac myocyte hypertrophy development. These effects were not mediated by Ang-(1–7) or the Mas receptor. Second, the in-vivo anti-hypertrophic effects of Ang-(1–9) were confirmed by in-vitro experiments using cultured cardiac myocytes. Third, after experimental MI in the rat, both ACE inhibition and AT1 receptor blockade for 8 weeks increased plasma

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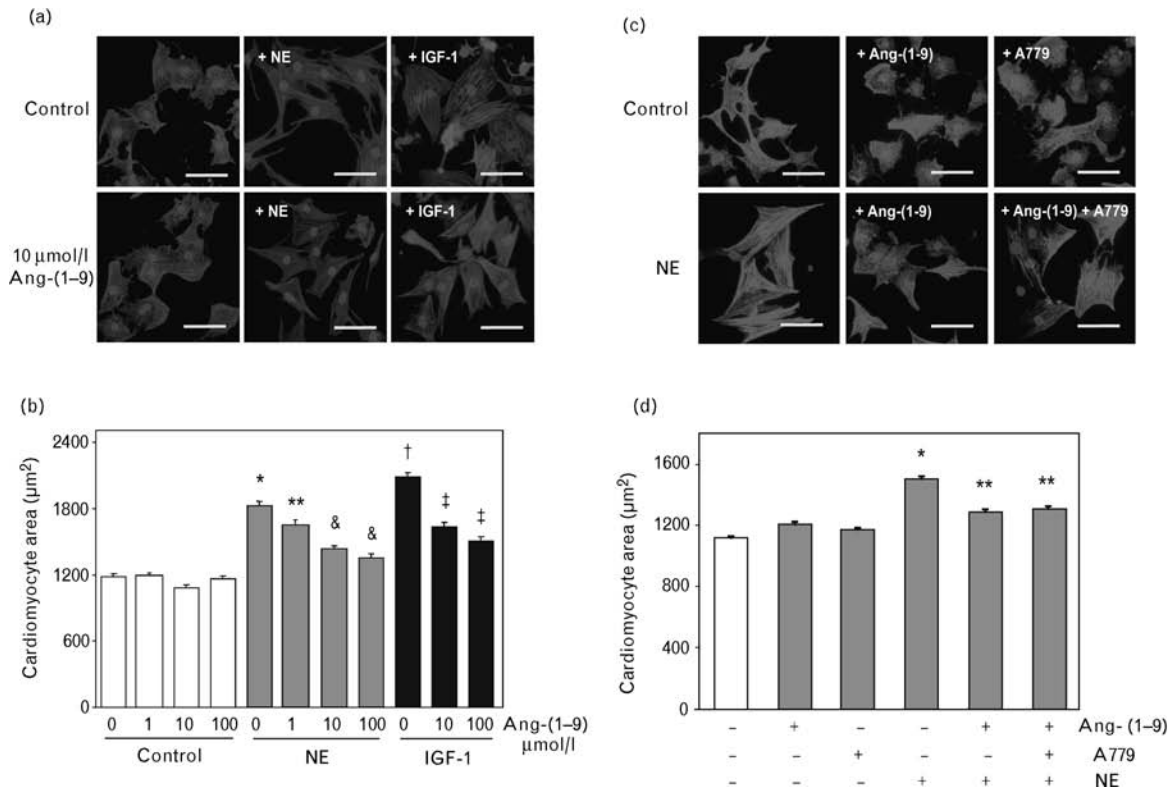
Fig. 3



Ang-(1–9) prevents norepinephrine and insulin-like growth factor-1-induced cardiomyocyte hypertrophy *in vitro*. (a, c) Representative microphotographs of cultured rat neonatal cardiomyocytes preincubated with or without 1, 10 or 100 μmol/l Ang-(1–9) or 1 μmol/l Ang-(1–9) + A779 for 1 h, and then incubated with or without 10 μmol/l norepinephrine or 10 nmol/l insulin-like growth factor-1 (IGF-1) for 24 h. Cardiomyocyte size was determined in cells fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 6 min. Cells were then incubated with phalloidine and rhodamine for 1 h at room temperature. (b, d) Analysis and quantification of cardiac myocyte area of at least 100 cells per independent experiment from randomly selected fields using Image J-software (NIH). Values are presented as mean ± SEM ($n = 3$). $^{*}P < 0.001$ vs. control, $^{**}P < 0.001$ vs. norepinephrine, $^{\&}P < 0.001$ vs. norepinephrine + 1 μmol/l Ang-(1–9), $^{\dagger}P < 0.001$ vs. control and norepinephrine, $^{\ddagger}P < 0.001$ vs. IGF-1.

Table 3 Effects of the angiotensin AT1 receptor antagonist candesartan and the angiotensin-converting enzyme inhibitor enalapril on blood pressure, echocardiographic indexes of left ventricular size (dimensions and volumes) and function (fractional shortening and ejection fraction) following coronary artery ligation (8 weeks)

Parameter	S	MI	C-S	C-MI	E-S	E-MI
N	11	12	13	13	10	12
BW (g)	380 ± 8	372 ± 10	$343 \pm 11^{*,\dagger}$	$357 \pm 10^{*,\dagger}$	$350 \pm 7^{*,\dagger}$	$345 \pm 4^{*,\dagger}$
SBP (mmHg)	123 ± 2	117 ± 2	$84 \pm 4^{*,\dagger,\dagger\dagger}$	$88 \pm 4^{*,\dagger,\dagger\dagger}$	$113 \pm 2^{*,\dagger}$	$110 \pm 3^{*,\dagger}$
LV infarct size (%)	NA	29 ± 1	NA	30 ± 1	NA	31 ± 2
LVW/BW (mg/g)	3.0 ± 0.1	$3.6 \pm 0.1^{*}$	$2.7 \pm 0.1^{*,\dagger}$	$2.8 \pm 0.1^{*,\dagger}$	$2.7 \pm 0.2^{*,\dagger}$	$2.9 \pm 0.3^{*,\dagger}$
Relative ANF mRNA	1.0 ± 0.1	$1.7 \pm 0.2^{*}$	0.7 ± 0.2	$1.1 \pm 0.2^{\dagger,\dagger\dagger}$	0.9 ± 0.1	1.6 ± 0.1
LVP content (mg)	11.1 ± 0.3	$30.1 \pm 3.3^{*}$	11.6 ± 1.1	$12.3 \pm 1.4^{\dagger}$	12.4 ± 0.8	$12.8 \pm 0.5^{\dagger}$
Relative β-MHC	1.0 ± 0.2	$2.2 \pm 0.2^{*}$	1.1 ± 0.2	$1.0 \pm 0.1^{\dagger}$	1.3 ± 0.2	$1.2 \pm 0.1^{\dagger}$
LVESD (mm)	5.2 ± 0.1	$6.9 \pm 0.3^{*}$	5.1 ± 0.1	$5.9 \pm 0.4^{\dagger}$	5.2 ± 0.1	$5.4 \pm 0.3^{\dagger}$
LVEDD (mm)	7.4 ± 0.2	$8.8 \pm 0.2^{*}$	7.7 ± 0.2	$8.0 \pm 0.3^{\dagger}$	7.5 ± 0.3	$7.1 \pm 0.2^{\dagger}$
LVAWT (mm)	1.5 ± 0.1	$1.0 \pm 0.1^{*}$	1.1 ± 0.4	$0.9 \pm 0.4^{*}$	1.3 ± 0.2	$0.9 \pm 0.1^{*}$
LVPWT (mm)	1.4 ± 0.1	$1.6 \pm 0.1^{*}$	1.1 ± 0.0	$1.2 \pm 0.0^{*,\dagger,\dagger\dagger}$	1.4 ± 0.0	1.5 ± 0.1
LVEDV (ml)	2.6 ± 0.1	$3.4 \pm 0.1^{*}$	2.5 ± 0.1	$2.6 \pm 0.0^{\dagger}$	2.2 ± 0.1	$2.9 \pm 0.2^{*}$
LVESV (ml)	1.0 ± 0.0	$1.5 \pm 0.2^{*}$	1.0 ± 0.0	$1.4 \pm 0.0^{*}$	0.9 ± 0.0	$1.4 \pm 0.1^{*}$
Stroke volume (ml)	1.6 ± 0.1	1.7 ± 0.0	1.5 ± 0.1	1.5 ± 0.2	1.3 ± 0.1	1.5 ± 0.1
LVEF (%)	61 ± 2.5	$52 \pm 2.8^{*}$	59 ± 2.0	$52 \pm 2.0^{*}$	60 ± 1.7	$49 \pm 3.0^{*}$
LVFS (%)	29 ± 2	$22 \pm 3^{*}$	30 ± 2	$22 \pm 2^{*}$	30 ± 1	$21 \pm 1^{*}$

Values as mean ± SEM. BW, body weight; C, candesartan; E, enalapril; LV, left ventricle; LVAWT, left ventricular anterior wall thickness; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; LVESV, left ventricular end-systolic volume; LVFS, left ventricular fractional shortening; LVP, left ventricular protein; LVPWT, left ventricular posterior wall thickness; LVW, left ventricular weight; LVWT, left ventricular wall thickness; MI, myocardial infarction; *N*, number of animals; NA, not applicable; S, sham; SBP, systolic blood pressure. $^{*}P < 0.05$ vs. S. $^{\dagger}P < 0.05$ vs. MI. $^{\dagger\dagger}P < 0.05$ vs. E-MI (after ANOVA).

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Table 4 Effects of the angiotensin AT1 receptor antagonist candesartan and the angiotensin-converting enzyme inhibitor enalapril on circulating angiotensins and bradykinin levels 8 weeks after coronary artery ligation

Parameter	S	MI	C-S	C-MI	E-S	E-MI
N	10	9	12	11	10	10
Ang I (fmol/ml)	29.8 ± 4.0	$13.1 \pm 1.6^{*}$	22.6 ± 5.3	$30.7 \pm 1.9^{\dagger}$	30.7 ± 1.0	$32.4 \pm 0.8^{\dagger}$
Ang-(1–7) (fmol/ml)	4.3 ± 1.1	2.9 ± 0.5	$10.9 \pm 1.6^{*,\dagger,\dagger\dagger}$	$14.4 \pm 2.7^{*,\dagger,\dagger\dagger}$	4.1 ± 0.5	3.8 ± 0.6
Ang-(1–9) (fmol/ml)	5.3 ± 0.8	5.7 ± 0.9	$36.2 \pm 4.1^{*,\dagger}$	$43.8 \pm 2.9^{*,\dagger,\dagger\dagger}$	$25 \pm 5^{*}$	$29 \pm 3^{*,\dagger}$
Ang II (fmol/ml)	24.3 ± 3.0	$33.0 \pm 2.5^{*}$	$38.6 \pm 7.2^{*}$	$42.6 \pm 2.2^{*,\dagger,\dagger\dagger}$	$15.4 \pm 3.8^{*}$	$23.3 \pm 5.4^{\dagger}$
Ang II/Ang I	0.86 ± 0.1	$2.83 \pm 0.1^{*}$	$1.7 \pm 0.0^{*}$	$1.5 \pm 0.1^{*,\dagger,\dagger\dagger}$	$0.45 \pm 0.0^{*}$	$0.72 \pm 0.1^{*,\dagger}$
Ang-(1–9)/Ang I	0.18 ± 0.0	$0.42 \pm 0.1^{*}$	$1.6 \pm 0.3^{*}$	$1.8 \pm 0.4^{*,\dagger,\dagger\dagger}$	$0.75 \pm 0.0^{*}$	$0.89 \pm 0.0^{*,\dagger}$
Ang-(1–7)/Ang II	0.16 ± 0.0	$0.10 \pm 0.0^{*}$	$0.29 \pm 0.0^{*}$	$0.32 \pm 0.0^{*,\dagger,\dagger\dagger}$	0.25 ± 0.1	$0.26 \pm 0.0^{*,\dagger}$
Bk-(1–7) (fmol/ml)	3.2 ± 0.8	2.8 ± 0.5	4.4 ± 1.5	$5.4 \pm 1.6^{\dagger\dagger}$	$1.5 \pm 0.5^{*,\dagger}$	$1.4 \pm 0.2^{*,\dagger}$
Bk-(1–9) (fmol/ml)	3.9 ± 1.1	4.2 ± 1.0	4.3 ± 1.4	$4.1 \pm 1.4^{\dagger\dagger}$	$9.5 \pm 3.0^{*}$	$7.9 \pm 1.2^{*,\dagger}$
Bk-(1–7)/Bk-(1–9)	1.1 ± 0.8	0.8 ± 0.4	1.7 ± 0.5	$1.2 \pm 0.2^{\dagger\dagger}$	$0.2 \pm 0.1^{*,\dagger}$	$0.2 \pm 0.1^{*,\dagger}$

Values as mean ± SEM. Ang, angiotensin; Bk, bradykinin; C, candesartan; E, enalapril; MI, myocardial infarction; *N*, number of animals; S, sham. $^{*}P < 0.05$ vs. S. $^{\dagger}P < 0.05$ vs. MI $^{\dagger\dagger}P < 0.05$ vs. E-MI (after ANOVA).

Ang-(1–9) levels. In these experiments, increased Ang-(1–9) levels were associated with attenuation of LVH even after adjustment for blood pressure reduction. These in-vivo and in-vitro data show that Ang-(1–9) is an effective and novel anti-cardiac hypertrophy agent not acting via the Mas receptor.

LVH has traditionally been considered a compensatory mechanism of the heart to maintain cardiac output under stresses that compromise cardiac function. However, both clinical and basic studies have shown that sustained cardiac hypertrophy is rather a maladaptive process, ultimately leading to heart failure [23]. Mechanical stretch and neurohumoral factors induce changes in intracellular signaling pathways resulting in cardiac growth, remodeling and dysfunction [24]. In our model, as a consequence of the RAS overactivation [25], myocardial infarcted rats had increased plasma Ang II levels and developed cardiac hypertrophy, reflected by a higher LVW/tibial length ratio and LVP content as well as increased levels of cardiac ANF mRNA expression and relative levels of β-MHC protein. LVH secondary to MI was prevented by enalapril and by candesartan, confirming that overstimulation of the RAS is indeed responsible for these changes.

We hypothetized that Ang-(1–9) itself might be a negative regulator of cardiac hypertrophy *in vivo*. Thus, administration of 450 ng/kg per min Ang-(1–9) by minipumps increased 5.5-fold the circulating levels of Ang-(1–9) – similar to levels found in myocardial infarcted rats treated with enalapril or candesartan – and completely reproduced the effects of ACEI or AT_1 receptor antagonist on cardiac hypertrophy secondary to MI, which was independent of blood pressure modification.

Because there is in-vitro evidence that the incubation of Ang-(1–9) with ACE generates Ang-(1–7) [8] and Ang-(1–7) negatively regulates hypertrophy [16,26], we used the Ang-(1–7) receptor blocker A779 to investigate whether Ang-(1–7) could mediate the effects of Ang-(1–9). Even though A779 was bioactive, with significant increase in circulating Ang-(1–7) levels by 2.7-fold, this compound did not modify the Ang-(1–9)-dependent suppression of cardiac myocyte hypertrophy secondary to MI. However, Ang-(1–7) has also been shown to act at the Ang type 2 receptor (AT2R) [27]. Therefore, we have not excluded the possibility that the Ang-(1–9) is acting via Ang-(1–7) through AT2R. Thus, these findings lead to suggest that antihypertrophic action of Ang-(1–9) is not mediated by the Mas receptor and provide the first evidence for a role of Ang-(1–9) in the prevention of cardiac hypertrophy.

Our in-vitro results also demonstrated that Ang-(1–9), at μmol/l levels, directly prevents cardiomyocyte hypertrophy induced by two different stimuli (norepinephrine and IGF-1). Studies in genetically modified mouse models have confirmed that norepinephrine-dependent Gαq/α11 coupling is a necessary event in the induction of pathological cardiac hypertrophy, whereas IGF-I signaling regulates physiological cardiac hypertrophy, as well as the growth of the heart during normal development [28]. Interestingly, Ang-(1–9) antagonized both types of cardiac hypertrophy. This effect was not affected by the Ang-(1–7) receptor blocker A779. All these data suggest that Ang-(1–9) *per se* might selectively modulate similar downstream signaling effectors of cardiac remodeling. Our in-vivo data show that Ang-(1–9) at pmol/l levels prevented LVH development. The discrepancy between in-vivo and in-vitro experiments regarding Ang-(1–9) concentrations is not clear. However, the activity of local RAS could explain that Ang-(1–9) levels may be higher in tissues than in circulation.

Although Ang-(1–9) is present in human and rat plasma in the range of 2–6 fmol/ml [15,29] and its circulating level was increased by pathological condition (i.e. early after MI) [15] or in animals treated with ACEIs [15,25,30], this peptide has almost not been investigated because it was initially thought to be active only after conversion to Ang-(1–7). Ang-(1–9) can be generated by several carboxypeptidase-type enzymes, including ACE2 or by cathepsin A [31,32]. Ang-(1–9) was hydrolyzed 18 times slower than Ang I and 30% slower than Ang-(1–7) in monolayers of Chinese hamster ovary cells transfected

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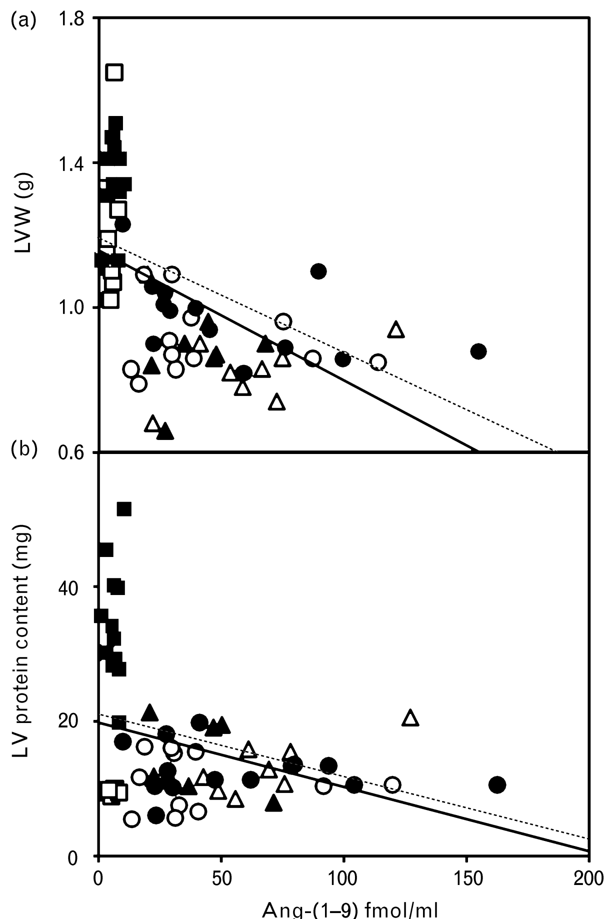
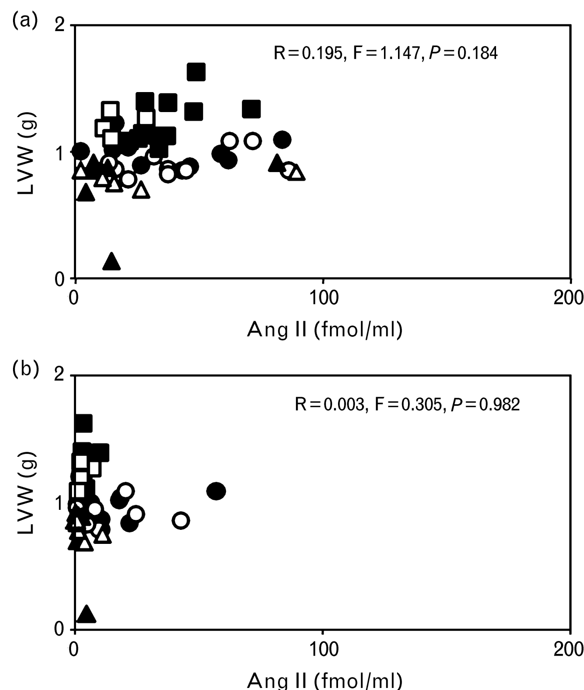
Fig. 4



Correlations between left ventricular weight (a) or left ventricular protein content (b) with circulating Ang-(1–9) levels. The associations between both variables remained significant after adjustment for systolic blood pressure (SBP) reduction. Continuous lines show unadjusted correlations (a and b, $r = -0.553$, $P < 0.01$; $r = -0.324$, $P = 0.01$, respectively); dashed lines show correlations after adjustment for SBP reduction (a and b, $r = -0.532$, $P < 0.01$; $r = -0.310$, $P = 0.02$, respectively). All these parameters were determined in sham and myocardial infarcted rats treated or untreated with the angiotensin AT1 receptor antagonist candesartan or the angiotensin-converting enzyme inhibitor enalapril as described in 'Methods'. C, candesartan; LVW, left ventricular weight; MI, myocardial infarction. Symbols: □ = S ($n = 10$), ■ = MI ($n = 9$), ○ = C-S ($n = 12$), ● = C-MI ($n = 11$), Δ = E-S ($n = 10$), ▲ = E-MI ($n = 10$).

Fig. 5



Correlations between left ventricular weight and circulating Ang II (a) or Ang-(1–7) (b) levels. All these parameters were determined in myocardial infarcted rats treated or untreated with the angiotensin AT1 receptor antagonist candesartan or the angiotensin-converting enzyme inhibitor enalapril as described in 'Methods'. C, candesartan; E, enalapril; LVW, left ventricular weight; MI, myocardial infarction; S, sham-operated rats. Symbols: □ = S ($n = 10$), ■ = MI ($n = 9$), ○ = C-S ($n = 12$), ● = C-MI ($n = 11$), Δ = E-S ($n = 10$), ▲ = E-MI ($n = 10$).

to express human ACE [13]. Incubation of Ang-(1–9) with primary rat neonatal cardiac myocytes, which produces ACE, also yielded Ang-(1–7), Ang-(1–5), as well as Ang-(1–4) [8]. Ang-(1–9) biological function is unknown [8] and might be an endogenous inhibitor of ACE [22]. In this regard, Donoghue *et al.* [8] reported that Ang-(1–9) is a competitive inhibitor of ACE because it is an ACE substrate itself [8]. Our results support this last hypothesis because Ang-(1–9) at pmol/l levels prevented LVH development and also the increases in plasma Ang II levels and plasma and left ventricular ACE activities induced by LCA ligation.

Cardiac hypertrophy is controlled simultaneously by stimulatory (prohypertrophic) and counter-regulatory (antihypertrophic) pathways [33]. Given the potent prohypertrophic effects of the Ca^{2+}-calcineurin-nuclear factor of activated T-cell (NFAT) pathway in cardiac myocytes, the activity of this pathway is tightly controlled at multiple levels. Several inhibitory mechanisms upstream (including nitric oxide/cyclic GMP) and downstream from calcineurin (glycogen synthase kinase-3, mitogen-activated protein kinases) have been described [33]. At the same concentration, Ang-(1–9) was significantly more active than Ang-(1–7) to potentiate the release of nitric oxide and arachidonic acid by bradykinin kininase-resistant analogue on the B2 receptor, in human pulmonary arterial endothelial cells [34]. It remains to be determined whether Ang-(1–9) regulates cardiac myocyte hypertrophy through arachidonic acid/nitric oxide release or through other mechanisms.

Because ACE2 was initially described as a carboxypeptidase converting Ang I to Ang-(1–9) [8], it can be suggested that the cardiac effects of ACE2 and Ang-(1–9) should be similar. Overexpression of ACE2 protects the heart from

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Ang II-induced hypertrophy and fibrosis [35], whereas the disruption of its regulatory function accelerates the development of cardiac hypertrophy mediated by Ang II and shortens the transition period from compensated hypertrophy to cardiac failure [36]. ACE2 also converts Ang II into Ang-(1–7) and chronic overproduction of Ang-(1–7) in the heart from transgenic mice reduces hypertension-induced cardiac remodeling through a direct effect on the heart [37]. These observations shed new light on the beneficial effects of the ACE2/Ang-(1–7) axis within the heart. Our work suggests that Ang-(1–9) itself also regulates cardiac hypertrophy. Further research should clarify whether other effectors, different from Ang-(1–7), mediate the antihypertrophic action of Ang-(1–9).

The discovery of new RAS components suggests that cardiovascular drugs like enalapril and candesartan could also affect the levels of other Angs [i.e. Ang-(1–7); Ang-(1–9)] and/or bradykinins [bradykinin-(1–9); bradykinin-(1–7)] [14,38,39]. In the MI group, plasma Ang I levels were lower and Ang II levels were higher than those in sham animals. However, Ang-(1–7) and Ang-(1–9) levels were similar in sham and in myocardial infarcted rats. Enalapril increased plasma Ang I levels by 147% in myocardial infarcted rats compared with untreated myocardial infarcted rats and decreased Ang II levels by 30%, indicating effective inhibition of Ang I conversion. Campbell *et al.* [20] reported that perindopril increased Ang I levels, reaching a maximal increase of approximately 18-fold after perindopril administration (0.467 mg/kg per day). By contrast, plasma Ang II levels fell only to 20–30% of control [20]. Our data with candesartan showed that plasma Ang II, Ang I and Ang-(1–7) levels increased significantly by 29, 134 and 220%, respectively in myocardial infarcted rats compared with untreated myocardial infarcted rats. Ishiyama *et al.* [14] have reported that the blockade of Ang II receptors with olmesartan during the 28-day post-MI was associated with higher plasma levels of Ang I (169%) when compared with sham-operated or vehicle-treated coronary artery ligation rats. In summary, our results with enalapril and candesartan agreed with previous reports in the literature.

Ferrario *et al.* [40] proposed that ACE/ACE2 is involved in the metabolism of Ang-(1–7) and that this peptide may, in part, mediate the ACEI cardioprotective effect [41]. Our previous findings suggest that Ang-(1–9) might be also involved in the cardioprotective effect of enalapril in the late phase of cardiac remodeling post-MI [15]. Here we extended this observation and demonstrated that the treatment of myocardial infarcted rats with the AT_1 receptor antagonist candesartan also resulted in significant increases in Ang-(1–9) levels, consistent with other observations [38,42]. Donoghue *et al.* [8] proposed that this increase in Ang-(1–9) steady-state levels could be due to decreased catabolism of Ang-(1–9) by ACE or due to increased production by ACE2 as a result of increased availability of Ang I substrate [8].

In the current study, an inverse relationship between cardiac hypertrophy and circulating Ang-(1–9) levels was observed, which was independent of blood pressure modification. However, a limitation of our study is that the tail-cuff method may not detect small changes in blood pressure. This effect was specific as neither Ang-(1–7), Ang II nor bradykinins were correlated with LVH.

In conclusion, Ang-(1–9), acting as a counter-regulator of Ang II, is an effective, possibly direct and a novel anti-cardiac hypertrophy agent. We propose that the noncanonical RAS arm has two biological effectors [(Ang-(1–9) and Ang-(1–7)] to counterregulate the classical RAS.

Acknowledgements

We thank Soledad Veliz, Fidel Albornoz and Ruth Marquez for the excellent technical assistance. The study was supported by Comisión Nacional de Ciencia y Tecnología (CONICYT), Chile (FONDECYT 1070662 to M.P.O and FONDAP 15010006 to S.L).

There are no conflicts of interest.

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