Impairment of In Vitro and In Vivo Heart Function in Angiotensin-(1-7) Receptor Mas Knockout Mice

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Abstract—In this study we investigated the effects of the genetic deletion of the angiotensin (Ang)-(1-7) receptor Mas on heart function. Localization of Mas in the mouse heart was evaluated by binding of rhodamine-labeled Ang-(1-7). Cardiac function was examined using isolated heart preparations. Echocardiography was used to confirm the results obtained with isolated heart studies. To elucidate the possible mechanisms involved in the cardiac phenotype observed in Mas−/− mice, whole-cell calcium currents in cardiomyocytes and the expression of collagen types I, III, and VI and fibronectin were analyzed. Ang-(1-7) binding showed that Mas is localized in cardiomyocytes of the mouse heart. Isolated heart techniques revealed that Mas-deficient mice present a lower systolic tension (average: 1.4 ± 0.09 versus 2.1 ± 0.03 g in Mas+/+ mice), ±dT/dt, and heart rate. A significantly higher coronary vessel resistance was also observed in Mas-deficient mice. Echocardiography revealed that hearts of Mas-deficient mice showed a significantly decreased fractional shortening, posterior wall thickness in systole and left ventricle end-diastolic dimension, and a higher left ventricle end-systolic dimension. A markedly lower global ventricular function, as defined by a higher myocardial performance index, was observed. A higher delayed time to the peak of calcium current was also observed. The changes in cardiac function could be partially explained by a marked change in collagen expression to a profibrotic profile in Mas-deficient mice. These results indicate that Ang-(1-7)-Mas axis plays a key role in the maintenance of the structure and function of the heart. (Hypertension. 2006;47:996-1002.)

Key Words: receptors, angiotensin • extracellular matrix • echocardiography • heart failure • cardiac function

The renin–angiotensin system (RAS), a potent regulator of blood pressure (BP), plays a major role in the pathogenesis of cardiovascular diseases.1–3 Although angiotensin (Ang) II is the major effector of this system, several other Angs are now recognized as being biologically active. The heptapeptide Ang-(1-7) is particularly interesting, because it appears to counterbalance most of the Ang II effects. Thus, Ang-(1-7) may have a pivotal role in the cardiovascular system.4–7 Accordingly, Ang-(1-7) has been reported to present important effects in coronary vessels and heart function.8–14 This peptide produced a significant increase in cardiac output and stroke volume in anesthetized Wistar rats,15 decreased the incidence and duration of ischemia-reperfusion arrhythmias,11 apparently by activating the sodium pump,14 and improved the postischemic contractile function12 in isolated perfused rat hearts. In keeping with these data, chronic infusion of Ang-(1-7) improved endothelial aortic function and coronary perfusion and preserved cardiac function in a rat model of heart failure.10 More recently, using a transgenic rat that expresses an Ang-(1-7)-producing fusion protein, an improvement in cardiac function was observed.16 Ang-(1-7) also reduces the overall growth of cardiomyocytes.16–18 Concerning the effects in the coronary vasculature, it has been shown that Ang-(1-7) elicits vasorelaxation in isolated canine and porcine coronary artery rings. In isolated perfused mouse hearts, Ang-(1-7) produced complex vascular effects involving interaction of its receptor with angiotension II type 1 (AT1) and AT2 receptor–related mechanisms, leading to vasodilation.20

We have reported recently that Ang-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas.21 The genetic deletion of Mas abolished the binding of Ang-(1-7) to mouse kidneys and abrogated the antidiuretic effect of Ang-(1-7) in mice after acute water load. In addition, aortic rings from Mas-deficient mice lack the endothelium-dependent Ang-(1-7)-induced relaxation. Ang-(1-7) also induced an increase of 3H-arachidonic acid release and NO release from Mas-transfected cells that were blocked by the specific Ang-(1-7) receptor Mas antagonist, A-779, but not by AT1 or AT2 antagonists.21,22

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In rodents, Mas mRNA has been detected in the heart in addition to other tissues, such as testis, kidney, and brain. Recent in vitro studies have suggested that Ang-(1-7) induces antifibrotic and antitrophic effects in cardiac cells when it binds to cardiac fibroblasts and reduces the growth of cardiomyocytes through activation of the Mas receptor. However, there are little data available concerning the potential role of Mas in the heart. Thus, the aim of this study was to investigate the effects of genetic deletion of Mas on cardiac function and coronary vascular resistance. In addition, the effect of genetic deletion of Mas on the expression of extracellular matrix protein in the heart was also investigated.

Methods
An expanded Methods section detailing the techniques and procedures mentioned here is available in an online supplement at http://www.hypertensionaha.org.

Mas-Knockout Mice
Wild-type (Mas+/+) and Mas knockout (Mas−/−) C57BL/6 mice were obtained from the transgenic animal facilities of the Laboratory of Hypertension, Federal University of Minas Gerais. All of the experimental protocols were performed in accordance with the guidelines for the humane use of laboratory animals established at our institution.

In Vitro Fluorescent–Labeled Ang-(1-7) Binding
Hearts from 3-month-old Mas+/+ (n=3) and Mas−/− (n=2) mice were snap-frozen in cooled isopentane. Cryostat sections (6 µm) were serially cut, mounted on gelatin-coated slides, and dried at 4°C before the assay. The slices were preincubated in the assay buffer (10−5 M Na-phosphate buffer, pH 7.4; 1.2×10−4 M NaCl; 5 mmol/L MgCl2; 0.2% BSA; and 0.005% bacitracin) for 30 minutes. The experiments were performed using assay buffer containing 2.5×10−3 M phenylmethylsulfonyl fluoride; 6×10−5 M 1,10-phenanthroline; 2×10−5 M rhodamine-labeled Ang-(1-7) in the presence (non-specific binding) or absence (specific binding) of Ang-(1-7) (10−8 mol/L) for 1 hour at 22°C to 24°C. Relative fluorescence measurements were performed on a Zeiss 510 Meta confocal microscope using a ×63 oil-immersion objective lens.

Isolated Mouse Heart Technique
Seven male Mas+/+ and 4 Mas−/− mice (3 months old) were used for isolated hearts experiments as described previously.

Echocardiography Studies
Echocardiographic features were obtained using the recommendations of American Society of Echocardiography. All of the transthoracic echocardiograms were performed in male Mas+/+ and Mas−/− mice (6 months old; n=10) by a single, blinded observer with the use of a Sequoia 512 (ACUSON Corp), which offers a 10- to 14-MHz multifrequency linear transducer.

Electrophysiological Analysis
Individual mouse ventricular myocytes (n=8 cardiomyocytes from 3 different animals) were isolated from Mas+/+ and Mas−/− animals from the same litters at 3 months of age. All of the experiments were performed using conventional whole-cell patch-clamp techniques at room temperature.

Immunohistochemical Analysis
Immunofluorescence labeling and quantitative confocal microscopy were used to investigate the distribution and quantity of collagen types I, III, and VI and fibronectin present in Mas+/+ and Mas−/− mice hearts.

BP Measurement
A polyethylene catheter was inserted into right carotid artery of Mas+/+ and Mas−/− mice (6 months old, n=3) for BP measurements. All of the surgical procedures, basal mean arterial pressure, and heart rate (HR) measurements were performed under halothane anesthesia (0.75 to 1.25%, 1 L/min). An additional measurement was made in conscious mice after recovery of anesthesia. BP and HR (derived from the BP measurement) were recorded on a data acquisition system (MP 100, Biopac Systems).

Statistical Analysis
Data are reported as mean±SEM. Statistical analyses for the binding assay, echocardiography, electrophysiological studies, and BP measurements were performed using unpaired Student t test. Immunohistochemistry statistical analysis was performed using unpaired Student t test followed by the Mann Whitney test. Statistical significance for isolated perfused heart experiments was estimated using 2-way ANOVA followed by Bonferroni test. P<0.05 was considered significant.

Results
Fluorescent-labeled Ang-(1-7) binding was found in the cardiomyocytes of Mas−/− mice as displayed by confocal analysis (Figure 1). Figure 1A shows total binding in Mas−/− cardiomyocytes, which presented a punctuated staining pattern as would be expected for a G protein-coupled receptor. Conversely, specific binding of rhodamine-labeled Ang-(1-7) to heart slices from Mas−/− evaluated by quantitative confocal microscopy was essentially abolished (Figure 1E).

Figure 1. In vitro localization of rhodamine–Ang-(1-7) binding in mice left ventricle slices. Figure shows total binding in Mas−/− cardiomyocytes (A), nonspecific binding in Mas−/− cardiomyocytes (B), total binding in Mas−/− cardiomyocytes (C), and non-specific binding in Mas−/− cardiomyocytes (D). Concentration of rhodamine-Ang-(1-7) was 2×10−8 mol/L in the presence (non-specific binding) or absence (specific binding) of Ang-(1-7) (10−6 mol/L). Quantification of relative fluorescence in Mas−/− and Mas−/− cardiomyocytes (E). Data are shown as the SD. *P<0.01 (unpaired Student t test).
Mas−/− mice presented an impairment of in vitro heart function. As shown in Figure 2A, systolic tension was significantly lower in hearts from Mas−/− mice (average: 1.4±0.09 versus 2.1±0.03 g in age-matched Mas+/+ mice; P<0.0001). Diastolic tension was similar in both groups (Figure 2B). Mas-deficient mice presented a lower +dT/dt and −dT/dt (average: 49.5±1.3 versus 65.2±0.3 and 49.5±1.5 versus 64.2±1.0 g/s in age-matched Mas+/+ mice, P<0.0001, respectively; Figure 2C and 2D) and a lower HR (average: 250.8±9.5 versus 333.3±2.6 bpm in age-matched Mas+/+ mice; P<0.0001; Figure 2E). In addition, isolated hearts from Mas−/− mice presented a significantly higher coronary perfusion pressure (average of the 30-minute recording: 172.7±3.0 versus 126.9±2.8 mm Hg in age-matched Mas+/+ mice; P<0.0001; Figure 2F).

The Table summarizes the echocardiographic parameters obtained in Mas+/+ and Mas−/− mice. In agreement with the results obtained with isolated hearts, intact hearts in Mas-deficient mice showed a significantly lower fractional shortening (19.01±0.71 versus 22.90±0.25% in Mas+/+ mice; P<0.001). Morphologically, Mas−/− mice displayed a significantly lower posterior wall thickness in systole (0.085±0.001 versus 0.090±0.001 mm in Mas+/+ mice; P<0.05) and left ventricle end-diastolic dimension (0.387±0.001 versus 0.395±0.003 cm in Mas+/+ mice; P<0.05) and a higher left ventricle end-systolic dimension (0.313±0.002 versus 0.305±0.002 cm in Mas+/+ mice; P<0.01). A substantially lower global ventricular function, expressed by a higher myocardial performance index, was also observed (0.73±0.05 versus 0.54±0.03 in Mas+/+ mice; P<0.01; Figure 3).

To elucidate the possible mechanisms underlying the cardiac function impairment, we analyzed the whole-cell L-type Ca2+ current in individual cardiomyocytes and cardiac expression of collagen types I, III, and VI and fibronectin. Although it is now generally accepted that L-type Ca2+ current is the major trigger for sarcoplasmic reticulum Ca2+ release, no significant differences were observed between L-type Ca2+ current density from age-matched Mas+/+ and Mas−/− mice (Figure 4A through 4D). Interestingly, the time to the peak of Ca2+ current was statistically different when
Echocardiographic Parameters Obtained in Mas+/+ and Mas−/− Mice

<table>
<thead>
<tr>
<th>Echocardiographic Parameters</th>
<th>Mas+/+</th>
<th>Mas−/−</th>
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<tbody>
<tr>
<td>Fractional shortening, %</td>
<td>22.90±0.25</td>
<td>19.01±0.71*</td>
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<tr>
<td>Posterior wall thickness in diastole, mm</td>
<td>0.068±0.001</td>
<td>0.066±0.001</td>
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<tr>
<td>Posterior wall thickness in systole, mm</td>
<td>0.090±0.001</td>
<td>0.085±0.001†</td>
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<tr>
<td>Interventricular septal thickness in diastole, mm</td>
<td>0.067±0.001</td>
<td>0.066±0.001</td>
</tr>
<tr>
<td>Interventricular septal thickness in systole, mm</td>
<td>0.089±0.001</td>
<td>0.084±0.001</td>
</tr>
<tr>
<td>Left ventricle end-diastolic dimension, cm</td>
<td>0.395±0.003</td>
<td>0.387±0.001†</td>
</tr>
<tr>
<td>Left ventricle end-systolic dimension, cm</td>
<td>0.305±0.002</td>
<td>0.313±0.002‡</td>
</tr>
<tr>
<td>Myocardial performance index</td>
<td>0.54±0.03</td>
<td>0.73±0.05‡</td>
</tr>
<tr>
<td>Isovolumic relaxation time, ms</td>
<td>19.50±1.18</td>
<td>20.60±0.88</td>
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<tr>
<td>Isovolumic relaxation time/HR</td>
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<td>1.88±0.07</td>
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<tr>
<td>Heart rate, bpm</td>
<td>512.9±9.4</td>
<td>501.8±10.5</td>
</tr>
<tr>
<td>Left ventricular mass, g</td>
<td>0.085±0.002</td>
<td>0.082±0.003</td>
</tr>
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</table>

Values are mean±SEM. Transthoracic echocardiogram was performed in male Mas+/+ and Mas−/− mice (n=10 for each group). Data were analyzed by unpaired Student t test.

*P<0.0001; †P<0.05; ‡P<0.01.

comparing the 2 groups (8.5±0.4 ms for the Mas+/+ versus 10.8±0.6 ms for the Mas−/− at −10 mV; P<0.01; and 7.3±0.6 ms for the Mas+/+ versus 9.4±0.4 ms for the Mas−/− at 0 mV; P<0.01). This change could contribute to the cardiac dysfunction described.

Strikingly, we observed that the expression of several matrix proteins were significantly and markedly higher in the ventricles of adult Mas−/− mice hearts compared with Mas+/+ control mice (Figure 5A and 5B): that is, type I collagen 94.22±4.90 versus 59.00±2.35 in Mas+/+ mice (P<0.0001); type collagen III 111.20±8.09 versus 45.67±2.21 in Mas+/+ mice (P<0.0001); and fibronectin 78.89±2.90 versus 45.67±2.21 in Mas+/+ mice (P<0.0001). Conversely, the expression of type VI collagen in Mas−/− mice was decreased 20.78±2.29 versus 43.22±3.29 in Mas+/+ mice (P<0.0001).

No major differences were detected in the atria (data not shown, E. Gava and G.T. Kitten, unpublished data, 2005). A similar change was observed in the left ventricle of neonate mice (Figures I and II, available online).

To further discard the possibility that the changes in collagen expression in cardiac function in vivo were secondary to increased BP, an additional group of animals at the same age of those used for echocardiograph measurements (6 months) were instrumented for acute BP recordings. As expected,24,25 no differences in BP between Mas−/− and Mas+/+ mice were observed (BP: 79.3±4.9 versus 90.3±6.4 mm Hg, P=0.2438; HR: 467.0±56.1 versus 511.3±24.2 bpm, P=0.5086 in age-matched Mas+/+ mice under halothane anesthesia and BP: 100.0±3.0 versus 104.3±2.9 mm Hg, P=0.3622; HR: 559.7±21.2 versus 613.3±27.2 bpm, P=0.1957 in Mas+/+ mice after recovery of anesthesia). Actually, halothane-anesthetized Mas−/− animals presented slight lower BP in comparison to Mas+/+, probably resulting from the lower cardiac output.

**Discussion**

In this study, we have unmasked a key functional role of the Ang-(1-7) receptor Mas in the heart. We observed that systolic tension, +dT/dt, and −dT/dt were significantly lower in isolated hearts of Mas-deficient mice. Accordingly, an impaired heart function was observed in vivo by means of echocardiography. Echocardiographic measurements revealed a decreased fractional shortening, posterior wall thickness in systole and left ventricle end-diastolic dimension, and a higher left ventricle end-systolic dimension. A markedly lower global ventricular function, as defined by a higher myocardial performance index, was observed. In addition, Mas−/− mice presented a higher coronary perfusion pressure compared with Mas+/+ mice. These findings are in keeping with several studies suggesting that Ang-(1-7) has beneficial effects in myocardial function.8 –13,16,28 We have shown that Ang-(1-7), at subnanomolar concentration, has an antiarhythmic effect on reperfusion11 and that it preserves the cardiac function in the postischemic period12 in isolated perfused rat hearts. These effects were completely blocked by the selective Ang-(1-7) receptor Mas antagonist A-779, suggesting that these Ang-(1-7) actions in the heart are mediated by its specific receptor. One possible mechanism that may contribute to the beneficial effects of Ang-(1-7) in the heart is the modulation of cardiac Ang II levels, as described recently by Mendes et al.29 The present results indicate that the Ang-(1-7) receptor Mas mediates the effects of Ang-(1-7) on the cardiac function.

The higher perfusion pressure observed in the isolated heart preparation is in agreement with the previous reports of a vasorelaxant activity of Ang-(1-7) in the coronary bed.8,19 Brosnihan et al10 found that this heptapeptide elicits a vasodilator response in canine coronary arteries. This effect was completely blocked by the nonselective Ang antagonist, [Sar1Thr8]-Ang II, but not by either an AT1 receptor antagonist or an AT2 receptor antagonist, suggesting that the Ang-(1-7)-induced vasodilation in coronary arteries is mediated by a non-AT1/AT2 receptor. Our data indicate that the Ang-(1-7) receptor Mas may account for Ang-(1-7)-induced vasodilation in the coronary bed. Accordingly, the Mas

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Representative echocardiographic measurements of contracting hearts from Mas+/+ and Mas−/−. LVESD indicates left ventricle end-systolic dimension; LVEDD, left ventricle end-diastolic dimension.
receptor antagonist A-779 abolished the potentiating effect of Ang-(1-7) on bradykinin-induced vasodilation in an isolated perfused rat heart preparation and the vasodilation induced by Ang-(1-7) in losartan-treated isolated mouse heart. This later effect was also abolished in isolated hearts of Mas-deficient mice.

Recent reports have suggested that Ang-(1-7) could be a modulator of ion channel functioning similar to Ang II. Both peptides augment the release of [3H] norepinephrine at the neuromuscular junction, Ang-(1-7) increased acetylcholine release in a dose-dependent manner. Thus, the lower contractility observed in Mas–/– mice provides additional evidence that interactions between Ang-(1-7) and Mas receptor play an important modulatory role in calcium homeostasis in the heart tissue. Our data also indicate that the time to the peak of Ca2+ current measured at −10 and 0 mV for the L-type Ca2+ current was slower for the Mas–/– cells, which could impair the sarcoplasmic reticulum refilling process and, thus, lead to less Ca2+ being released during excitation.

We have suggested that Ang-(1-7) could produce changes in pacemaker ionic currents. Isolated hearts from transgenic rats [TGR(A1-7)3292] that chronically overproduce Ang-(1-7) resulting in 2.5-fold increase in circulating Ang-(1-7) concentration showed a significantly higher basal intrinsic HR observed in vivo and in ex vivo conditions. On the other hand, in Mas–/– mice, the basal intrinsic HR was significantly lower compared with Mas+/+ mice. In line with these data, De Mello et al. reported that Ang-(1-7) activates the sodium pump, hyperpolarizes the heart cell, and re-establishes the impulse conduction during ischemia/reperfusion, indicating that the antiarrhythmicogenic effect of Ang-(1-7) could be because of changes in ionic currents. These observations suggest that Ang-(1-7) could actually modulate some component of the pacemaker ionic currents through Ang-(1-7) Mas receptor stimulation. However, the exact mechanism involved in the change in HR in Mas-deficient mice remains to be established.

The changes in time to the peak of Ca2+ current could be related to the actual amount of Ca2+ that is mobilized during an action potential, but because we did not measure the global Ca2+ transients elicited by electrical stimulation, we cannot address this point. However, a major mechanism for the impairment in heart function in Mas–/– appears to be the strikingly changes in extracellular matrix proteins. Our results provide strong evidence for a previously unexpected key role for the Ang-(1-7)-Mas axis in the control of expression of extracellular matrix proteins. The fact that collagen VI undergoes an opposite change as that observed for collagen I, III, and fibronectin suggests that this influence is selective. These observations are in accordance with a recent report by Iwata et al., showing that Ang-(1-7) produces antifibrotic effects. As expected, a contribution of BP to the changes in collagen expression or in cardiac function could be excluded by the direct BP measurements in halothane-anaesthetized and in nonanaesthetized mice. Actually, under halothane anesthesia, the mean arterial pressure of Mas–/– mice was slightly lower than that of Mas+/+ mice, probably because of the lower cardiac output. Because the expression of extracellular matrix proteins presented a similar pattern in neonate mice, we can further exclude a contribution of BP or systemic vascular resistance in Mas–/– as a primary cause for the cardiac phenotype observed. Furthermore, Walther et al. showed that Mas–/– are healthy, grow normally, and show no obvious developmental abnormalities. They also reported no difference in drinking behavior. It is well known that fibrosis leads to alterations in diastolic function. Accordingly, associated with the important deficit of systolic function, we have also found a significantly lower dimension of the left ventricle at the end of diastole and a decreased −dT/dt in isolated
hearts of Mas−/−. Walther et al27 have reported an increased BP variability and indirect evidence for an increased sympathetic tonus in male Mas−/− mice as compared with Mas+/+ mice, whereas the expression of type VI collagen was decreased. The images in the control column show the level of immunostaining obtained when the primary antibody is omitted from the incubation procedure (A). Quantification of extracellular matrix proteins in the left ventricles of Mas+/+ and Mas−/− mice (B). Data are shown as the SEM. *P<0.001; **P<0.0001. A.U. indicates arbitrary unit.

Figure 5. Immunofluorescent localization of matrix proteins in the left ventricles of adult Mas+/+ and Mas−/− mice hearts. Expression of type I collagen, type III collagen, and fibronectin were increased in the left ventricles of Mas−/− compared with Mas+/+ mice, whereas the expression of type VI collagen was decreased. The images in the control column show the level of immunostaining obtained when the primary antibody is omitted from the incubation procedure (A). Quantification of extracellular matrix proteins in the left ventricles of Mas+/+ and Mas−/− mice (B). Data are shown as the SEM. *P<0.001; **P<0.0001. A.U. indicates arbitrary unit.

One of the limitations of our study is related to the echocardiogram measurements. Reporting of E and A peaks and E/A relation, as well as E deceleration time would add an important information. However, because the halothane anesthetic regimen keeps HR near physiological levels and the HR of mice is very high, choosing an anesthetic regimen that keeps it near to physiological levels should be an important concern. In this study, halothane has been used to perform echocardiogram, because it is more convenient and reliable with respect to rate of induction, reversibility, and control of anesthetic depth.34 In addition, HR is less affected with inhaled anesthetics than with intraperitoneal ones. Although further studies with other anesthetics, such as isoflurane, would be interesting to confirm our findings with halothane, the similarity between our in vivo and in vitro data provides a very strong evidence for a key role of the Ang-(1-7)-Mas axis in heart function.

Perspectives
The changes in extracellular matrix proteins to a profibrotic profile in Mas−/− mice provide additional evidence that the Ang-(1-7)-Mas axis plays a key role in the beneficial effects of angiotensin-converting enzyme (ACE) inhibitors, AT1 receptor blockers (ARBs), and aldosterone antagonists in the heart. It has been shown that chronic treatment with ACE inhibitors or ARBs increases the activity of Ang-(1-7)-forming enzymes,35–37 in particular, heart ACE2.38 More recently, it has been demonstrated in humans that treatment with aldosterone antagonists substantially increased ACE2 activity.38 The prominent decrease in cardiac function in Mas−/− mice, which can be correlated with the marked changes in extracellular matrix proteins and the antihypertrophic effects of Ang-(1-7),16,17 apparently mediated by its interaction with Mas,18 suggest that activation of the Ang-(1-7)-forming pathways by RAS blockade produces beneficial cardiac effects through activation of Mas. One could argue that ACE2 knockout mice50 showed more pronounced cardiac function impairment than Mas knockout mice. However, the ACE2 knockout mice presented a higher Ang II level, which may contribute to the cardiac phenotype. Of note is the fact that, in ACE2 knockout mice, the cardiac dysfunctions appeared only after 6 months of age, whereas the cardiac fibrotic phenotype in Mas−/− mice was already observed in the neonatal period.

In conclusion, our findings suggest that Ang-(1-7)-Mas axis plays a key role in the heart. Furthermore, our data also suggest that this RAS axis may be a potential target for the development of new cardiovascular drugs.7

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