Autocrine and Paracrine Function of Angiotensin 1–7 in Tissue Repair During Hypertension

Weixin Meng,1 Wenyuan Zhao,2 Tieqiang Zhao,2 Chang Liu,1 Yuanjian Chen,2 Hongyu Liu,1 and Yao Sun2

BACKGROUND
Angiotensin-converting enzyme 2 (ACE2) cleaves angiotensin (Ang) II to generate Ang1–7, which mediates cellular actions through Mas receptors (MasR). Hypertension is accompanied by high or low circulating AngII levels and cardiac/renal injury. The purpose of this study is to explore (i) whether circulating AngII affects ACE2/MasR expressions in the hypertensive heart and kidney; and (ii) whether Ang1–7 regulates cardiac repair/remodeling responses through MasR during hypertension.

METHODS
In the first portion of the study, rats received either an AngII infusion (400 ng/kg/min) for 4 weeks, leading to hypertension with high circulating AngII, or an aldosterone (ALDO, 0.75 μg/h) infusion for 4 weeks, leading to hypertension with low/normal circulating AngII. Cardiac and renal ACE2/MasR expressions were examined. We found that cardiac ACE2 was increased and MasR attenuated in both AngII and ALDO groups. However, renal ACE2 and MasR remained unchanged in both AngII- and ALDO-treated animals.

The renin-angiotensin system (RAS) plays an integral role in maintaining vascular tone, salt and water homeostasis, and cardiac function in humans. Chronic activation of RAS causes hypertension, accompanied by cardiac, renal, and vascular injury/remodeling that eventually leads to end-organ disease, such as renal failure, heart failure, and coronary disease.1,2

Recently, the classical view of the RAS has been challenged by the discovery of angiotensin-converting enzyme 2 (ACE2). This membrane-bound enzyme is identified in rodents and humans and is found mainly in the heart and kidney.3,4 The major role for ACE2 is to cleave angiotensin (Ang) II to generate Ang1–7. The latter is an active peptide whose cellular actions are mediated by Mas receptors (MasR).

In effect, Ang1–7 counterbalances the role of AngII. It has been shown that administration of Ang1–7 and genetic manipulations of ACE2 or MasR expression (by either targeted disruption or overexpression) have significant effects on cardiac and renal protection.5–7 It remains to be elucidated whether cardiac and renal ACE2 and MasR expressions during hypertension are differentially expressed.

Hypertension is often accompanied by elevated circulating AngII.8 Conversely, hyperaldosteronism-induced hypertension is accompanied by low/normal levels of circulating AngII.9 The morphology of cardiac and renal remodeling, however, is similar during hypertension irrespective of circulating AngII levels.10–12 It remains uncertain whether circulating AngII level is associated with ACE2 and MasR in the hypertensive heart and kidney. Studies from our and other laboratories have shown that chronic infusion of AngII or aldosterone (ALDO) leads to comparable hypertension and cardiac repair/remodeling in rats. However, circulating AngII is elevated in AngII-infused rats, whereas circulating AngII remains normal or low in ALDO-infused rats.

In addition to its endocrine function, RAS is well recognized to have autocrine, paracrine, and intracrine functions. Studies have revealed that tissue RAS activation is independent of systemic RAS (i.e., cardiac ACE is significantly increased in the

RESULTS
In the second portion, rats received AngII infusion with/without MasR antagonist (A779, 1 mg/kg/day) for 4 weeks. The roles of MasR blockade in cardiac inflammation, fibrosis, apoptosis, and ventricular function were examined. Chronic AngII infusion caused scattered cardiac injuries, and A779 cotreatment exacerbated cardiac injury, resulting in aggravated inflammatory, fibrogenic, and apoptotic responses compared with the AngII group. Cardiac function, however, was unaltered in the AngII and A779 groups.

CONCLUSIONS
ACE2 and MasR expressions in the hypertensive heart and kidney are not regulated by circulating AngII levels. Ang1–7 is involved in multiple repair responses, suggesting that therapeutic strategies aimed at administering Ang1–7 hold potential for the management of cardiac remodeling.

Keywords: angiotensin 1–7; blood pressure; heart; hypertension; kidney; Mas receptor blockade; repair.

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Correspondence: Yao Sun (yasun@uthsc.edu).

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1Division of Cardiac Surgery, Department of Surgery, First Affiliate Hospital of Harbin Medical University, Harbin, China; 2Division of Cardiovascular Diseases, Department of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee, USA.

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infarcted hearts of rats with normal circulating AngII and the hypertensive hearts with high circulating AngII induced by AngII infusion and low circulating AngII induced by ALDO infusion.\textsuperscript{11,13,14} Cells expressing ACE in the injured heart are primarily repairing cells, including macrophages, myofibroblasts, and endothelial cells. The relationship of tissue ACE2/MasR expressions and circulating AngII levels, however, remains unknown. The first purpose of the study is to determine whether ACE2 and MasR expressions in the hypertensive heart and kidney are dependent on the circulating AngII levels.

Hypertension results in myocyte hypertrophy. The protective role of Ang1–7 on cardiac hypertrophy has been well established. Studies have shown that Ang1–7 attenuates hypertension-induced myocyte hypertrophy.\textsuperscript{5} In addition to hypertrophy, cardiac repair/remodeling is a major morphologic feature of the hypertensive heart, characterized by inflammation, fibrosis, angiogenesis, and apoptosis. Extensive cardiac repair/remodeling is a risk factor for heart failure.\textsuperscript{15,16} The potential regulation of Ang1–7 on various cardiac repair responses and ventricular function during hypertension is not fully understood. The second purpose of the study is to explore the role of MasR blockade on inflammation, fibrosis, and apoptosis in the hypertensive heart and the potential underlying mechanisms.

METHODS

Animal model

Eight-week-old male Sprague-Dawley rats were used to generate hypertension models. This study was approved by the University of Tennessee Health Science Center Animal Care and Use Committee.

Experiment 1. Three animal groups (n = 6/group) were studied: (i) untreated rats, which served as controls; (ii) rats receiving AngII infusion (400 ng/min, given subcutaneously by implanted minipump), leading to hypertension with high circulating AngII; and (iii) uninephrectomized rats receiving ALDO (0.75 μg/h) given subcutaneously by implanted minipump together with 1% sodium chloride in drinking water,\textsuperscript{17} causing hypertension with low circulating AngII. At week 4, the heart and kidney were removed and frozen in liquid nitrogen to determine ACE2 and MasR expressions.

Experiment 2. Three animal groups (n = 6/group) were included: (i) untreated control rats; (ii) rats receiving AngII infusion (400 ng/min); and (iii) rats receiving an AngII infusion together with the MasR antagonist A779 (1 mg/day, given subcutaneously by minipump).\textsuperscript{17,18} At week 4, cardiac function was assessed by echocardiography. The hearts were then removed, weighted, and frozen to determine the effect of Ang1–7 on cardiac inflammatory, fibrogenic, and apoptotic responses.

Morphology

The heart and kidney morphologies were examined with hematoxylin and eosin–stained cryostat sections (6 μm). Cardiac and renal fibrosis was determined by collagen-specific picrosirius red–stained cryostat sections. Cardiac collagen volume fraction was determined as previously reported.\textsuperscript{19}

Reverse-transcription polymerase chain reaction

The total RNA was extracted from the heart and kidney. Gene expressions of cardiac and renal ACE2 and MasR, cardiac monocyte chemotactic protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, gp91\textsuperscript{phox} (a subunit of NADPH oxidase), tumor necrosis factor (TNF) α, transforming growth factor (TGF) β, tissue inhibitors of metalloproteinases (TIMP) 1, and TIMP-2 were deduced by reverse-transcription polymerase chain reaction (RT-PCR) as previously reported.\textsuperscript{20} Fold change was used to compare the difference among groups.

Western blotting

The expression of cardiac ACE2 and MasR, as well as cardiac apoptotic proteins (caspase3, Bax, and Bcl2), were measured using Western blot as previously reported.\textsuperscript{20} The amount of protein detected by each antibody was measured by a computer image analysis system.

Immunohistochemistry

Cardiac expression of ED-1 (a marker of macrophages) was detected by immunohistochemistry. Cardiac sections (6 μm) were incubated with a primary antibody against ED1 (Sigma, St. Louis, MO) for 1 hour at room temperature. The sections were then incubated with the immunoglobulin G peroxidase–conjugated secondary antibody (Sigma) for 1 hour at room temperature and incubated with 0.5 mg/ml dianinobenzidine tetrahydrochloride 2-hydrate + 0.05% hydrogen peroxide for 5 minutes. Negative control sections were incubated with the secondary antibody alone.\textsuperscript{20}

Echocardiographic assessment of left ventricular function

Rats were lightly anesthetized with 1.5% isoflurane at a constant volume and anesthetic time. Transthoracic echocardiography was performed in the supine position using an echocardiographic system (Sonos 4500 with a 7- to 11-MHz transducer). Heart rate, respiration rate, and body temperature were monitored, and additional warmer was applied upon necessity. The parasternal short-axis view at the level of the papillary muscle was recorded under 2-dimensional guided M-mode. Left ventricle (LV) end-systolic and end-diastolic dimensions, as well as systolic and diastolic wall thickness, were measured from the M-mode. For each measurement, at least 3 consecutive cardiac cycles were sampled. LV ejection fraction was calculated from the short-axis wall thickness and chamber dimension measurements by assuming a spherical LV geometry. Mitral valve inflow velocities were measured in the apical 4-chamber view at the level of the chordal attachments to the papillary muscles, and the E/A ratio was calculated. All measurements and calculation were made using the software installed in the echocardiographic system.\textsuperscript{21}
Statistical analysis

Statistical analyses of RT-PCR, Western blot, heart weight, and echocardiography data were performed using analysis of variance. The values were expressed as mean ± SEM, with P < 0.05 considered significant. Multiple group comparisons among the controls and each group were made by the Scheffe F test.

RESULTS

Cardiac and renal morphology in AngII- or ALDO-induced hypertension

Our data showed that compared with the control hearts (Figure 1a), hypertension induced by chronic AngII or ALDO treatments leads to similar morphological changes of the heart, represented as hypertrophy and scattered microscopic damages (Figure 1b,c). Compared to the normal kidney (Figure 1d), chronic infusion of AngII or ALDO also leads to morphological changes in the kidney, characterized as glomerular and tubular damage accompanied by inflammatory response and interstitial fibrosis (Figure 1f).

Cardiac and renal ACE2 in AngII- or ALDO-induced hypertension

Accessed by RT-PCR and Western blot, ACE2 mRNA and protein were observed in the normal rat heart and kidney. Cardiac ACE2 mRNA and protein levels were increased in both AngII- and ALDO-infused rats compared with the control rats (Table 1; Figure 1g). Renal ACE2 gene and protein expressions, however, remained unchanged in both AngII and ALDO groups compared with controls (Table 1; Figure 1i).

Cardiac and renal MasR in AngII or ALDO-induced hypertension

Compared with controls, cardiac and renal MasR mRNA levels were not significantly changed in AngII and ALDO groups (Table 1). Cardiac Mas protein levels were reduced in the ALDO group (Figure 1h). Cardiac MasR protein levels in the AngII group were also decreased but did not reach statistical significance (Figure 1h). Renal MasR levels remained unchanged in both AngII and ALDO groups compared with controls (Figure 1i).

Heart weight and blood pressure

Chronic AngII infusion significantly increased the heart weight and blood pressure in treated rats compared with controls (Table 2). MasR blockade did not alter the heart weight and blood pressure in AngII-treated rats (Table 2).

Effect of MasR blockade on inflammatory response of the hypertensive heart

Chronic AngII infusion resulted in microscopic injuries in the heart. Macrophages are the major inflammatory cells. Immunohistochemical ED-1 staining showed that macrophages were rarely present in the normal heart (Figure 2a), while they are accumulated at sites of cardiac injury in the AngII-treated animals (Figure 2b). A779 treatment further increased macrophage population in the hypertensive heart (Figure 2c).

Macrophages migrate to the damaged area drawn by MCP-1 and ICAM-1. Macrophage-derived NADPH oxidase and TNF-α are the key inflammatory mediators. Our data showed that, compared with control hearts, the expressions of MCP-1, ICAM-1, TNF-α, and gp91phox (a subunit of NADPH oxidase) were elevated in the left ventricle of the AngII-treated rats. A779 treatment significantly increased the cardiac expression of MCP-1, ICAM-1, and gp91phox compared with the AngII group (Figure 3a).

Effect of MasR blockade on fibrogenic response of the hypertensive heart

A small amount of collagen detected by collagen-specific picrosirius red staining was seen in the interstitial space of the heart (Figure 2d). Chronic AngII infusion led to accumulated collagen in the injured regions of the left ventricle, represented by microscopic scars (Figure 2e). Compared with the AngII group, cardiac interstitial fibrosis became more extensive in the A779 cotreatment group (Figure 2f). The quantitative cardiac collagen volume is shown in Figure 3b.

Fibrogenesis is stimulated by TGF-β and TIMPs. AngII infusion resulted in elevated TGF-β1, TIMP-1, and TIMP-2 in the left ventricle of the AngII-treated rats. A779 cotreatment further increased cardiac TGF-β and TIMP-2 levels compared with the AngII-infused rats (Figure 3c).

Effect of MasR blockade on apoptosis of the hypertensive heart

Caspase 3 plays a central role in the transduction of apoptotic signals, and Bax/Bcl2 ratio determines the occurrence of apoptosis. Detected by Western blot, cardiac caspase 3 level was slightly increased in the AngII group compared with the controls, whereas A779 cotreatment significantly increased cardiac caspase 3 level compared with the AngII group (Figure 4a).

A779 cotreatment also significantly elevated cardiac Bax level (Figure 4b), but not Bcl2 (Figure 4c), thus increasing the Bax/Bcl2 ratio in the hypertensive heart.

Cardiac function

Occurrence of ventricular dysfunction in the hypertension model depends on the dosage and length of AngII infusion. The dose and time of AngII treatment in this study did not alter ventricular function compared with normal rats. In addition, A779 cotreatment resulted in unchanged ventricular function compared with control and AngII groups (Table 2).
Figure 1. Morphology, angiotensin-converting enzyme 2 (ACE2) and Mas receptor (MasR) expression of the hypertensive heart and kidney. (a) Normal morphology of the heart. Cardiac damage occurs in rats receiving (b) angiotensin II (AngII) and (c) aldosterone (ALDO). (d) normal morphology of the kidney. (e) Glomerular and tubular damages and (f) interstitial fibrosis were evident in ALDO-infused rats and AngII-infused rats (not shown for AngII-infused rats). (g–j) Cardiac and renal ACE2 and MasR expressions. *P < 0.05 vs. controls (CTL). Magnification ×200.
Table 1. Angiotensin-converting enzyme 2 (ACE2) and MasR gene expressions in the heart and kidney

<table>
<thead>
<tr>
<th></th>
<th>Fold vs. control</th>
<th>Control</th>
<th>AngII</th>
<th>AngII+A779</th>
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<tr>
<td>Cardiac ACE2 mRNA</td>
<td></td>
<td>1.00 ± 0.09</td>
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<td>Cardiac MasR mRNA</td>
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<td>1.21 ± 0.09</td>
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<tr>
<td>Renal MasR mRNA</td>
<td></td>
<td>1.00 ± 0.06</td>
<td>1.23 ± 0.09</td>
<td>1.31 ± 0.13</td>
</tr>
</tbody>
</table>

Abbreviations: AngII, angiotensin II; MasR, Mas receptor. *P < 0.05 vs. control.

Table 2. Heart weight, blood pressure and ventricular function

<table>
<thead>
<tr>
<th></th>
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<th>AngII+A779</th>
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<tr>
<td>Heart/body weight, mg/g</td>
<td>2.93 ± 0.03</td>
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<td>Blood pressure, mm Hg</td>
<td>115 ± 5.5</td>
<td>182 ± 9.5*</td>
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<tr>
<td>Ejection fraction, %</td>
<td>66.8 ± 3.4</td>
<td>60.6 ± 3.5</td>
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<td>Fraction shortening, %</td>
<td>36.5 ± 2.6</td>
<td>37.8 ± 3.2</td>
<td>34.7 ± 2.8</td>
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<tr>
<td>Average wall thickness, mm</td>
<td>2.0 ± 0.1</td>
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<tr>
<td>Mitral valve E:A</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.1</td>
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</table>

Abbreviation: AngII, angiotensin II. *P < 0.05 vs. control.

DISCUSSION

RAS has recently been extended by the addition of a novel axis consisting of ACE2, Ang1–7, and the G protein-coupled MasR. The ACE2/Ang1–7/MasR axis has been shown to exert protective actions in hypertension, diabetes, and other cardiovascular disorders and represents a promising therapeutic target for cardiovascular and metabolic diseases.

The first purpose of the study was to determine the expression of ACE2 and MasR in the hypertensive heart and kidney and its relationship to circulating AngII. Our data have revealed that ACE2 expression is increased in the hypertensive heart, suggesting that cardiac Ang1–7 production is enhanced during hypertension. Other studies have shown that the ACE2 level is upregulated in the infarcted heart. Moreover, ACE2 expression is elevated in the failing human heart in idiopathic dilated cardiomyopathy and ischemic cardiomyopathy. These observations indicate that ACE2 expression is enhanced in the diseased heart irrespective of its etiologic origins. Elevated ACE2 in the hypertensive heart can reduce cardiac AngII and increase Ang1–7 production, which should be beneficial to cardiac repair/remodeling.

MasR expression is, however, reduced in the hypertensive heart. It has been reported that Ang1–7 infusion to normal rats downregulates cardiac MasR expression. These studies have demonstrated the negative feedback loop of Ang1–7 on MasR in the heart.

Chronic AngII and ALDO infusions result in hypertensive renal disease, characterized as glomerular and tubular damage and interstitial fibrosis. Our study shows that, unlike the heart, renal ACE2 and MasR expressions remain unchanged in hypertensive rats. ACE2 protein is reported to increase in kidneys from diabetic mice. By contrast, other studies show that ACE2 expression in the kidney is significantly decreased in hypertensive, diabetic, and pregnant rats. The difference in ACE2 expressions in kidney diseases is unidentified and is likely related to the stages of renal damage and repair. It is unclear why ACE2 and MasR are differentially expressed in the hypertensive heart and kidney, and further study is needed.

This study shows that cardiac and renal ACE2 and MasR expressions are comparable in rats with high or low circulating AngII. This finding suggests that ACE2 and MasR expressions in the hypertensive heart and kidney are not related to circulating AngII levels. This is supported by the elevated ACE2 level in the infarcted myocardium in rats with normal levels of circulating AngII level. Numerous studies have shown that ACE is significantly increased at sites of cardiac damage in rats with AngII or ALDO infusion and myocardial infarction. These observations indicate that AngII and Ang1–7 productions in tissue are irrespective of circulating AngII level. It remains unknown why ACE2 is differently expressed in the hypertensive heart and kidney. It is likely because of the different cell types in the heart and kidney as well as stage of organ damage.

The second purpose of this study was to explore the potential regulation of Ang1–7 on different repairing responses. RAS plays a relevant role in the pathogenesis of inflammatory diseases. AngII promotes inflammatory response by free radical generation; activation of protein kinases and nuclear transcription factors; recruitment of inflammatory cells; upregulation of adhesion molecules; and stimulation of expression, synthesis, and release of cytokines and chemokines. More recently, several studies have shown that the counter-regulatory role of Ang1–7 may influence inflammatory responses. Studies by Sukumaran and colleagues have shown that Ang1–7 is relevant to the anti-inflammatory effects of losartan in a rat model of autoimmune myocarditis by increasing ACE2 and MasR expression in line with reduction of proinflammatory cytokines.

This study shows that MasR blockade upregulates cardiac MCP-1 and ICAM-1, leading to increased macrophage population. A779 cotreatment also elevates macrophage-derived TNF-α and NADPH oxidase expressions, resulting in an aggravated inflammatory response. It has been reported that recombinant human ACE2 significantly blunts AngII infusion–induced NADPH oxidase and reactive oxygen species (ROS) production in the heart. Thus, Ang1–7 plays an anti-inflammatory role in the heart through multiple mechanisms.

Cardiac inflammatory response is accompanied by fibrogenesis, characterized as microscopic scars and interstitial fibrosis in the hypertensive heart. Cells responsible for fibrous tissue formation consist principally of phentoypically transformed fibroblast-like cells termed myofibroblasts. These cells contain α-smooth muscle actin microfilaments and are responsible for collagen synthesis and scar formation.
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contraction. TGF-β is the key mediator of myofibroblast differentiation and collagen synthesis. AngII promotes fibrogenesis by upregulation of TGF-β synthesis.

This study shows that MasR blockade upregulates TGF-β1 expressions in the hypertensive heart, resulting in more extensive cardiac fibrosis. Recently, other studies have evaluated the role of Ang1–7 in modifying the expression of TGF-β and key components of the TGF-β pathway. Ang1–7 decreases TGF-β1 mRNA levels in cultured cardiac fibroblasts and improves vascular remodeling through down-regulation of TGF-β and inhibition of the Smad2 pathway.

Collagen synthesis and degradation coexist in the repairing tissue, and its balance determines tissue collagen volume. Metalloproteinases are responsible for collagen degradation, with metalloproteinate activity inhibited by TIMPs. The current study shows that MasR blockade upregulates TIMP expression in the hypertensive heart, which controls metalloproteinase activity and promotes collagen accumulation. Altogether, these studies suggest that the antifibrogenic effect of Ang1–7 is through its regulation of TGF-β and TIMP synthesis.

Apoptosis of cardiac muscle cells has been identified as an essential process in the progression to heart failure. Unlike necrosis, apoptosis is an orderly biological process that regulates the balance between prodeath and prosurvival cell signals, the outcome of which is the key to cell fate. The Bcl-2 family of proteins has emerged as a key regulatory component of the cell death process, including suppressors of apoptosis, such as Bcl2, and promoters of apoptosis, such as Bax. Another logical target to modulate myocyte apoptosis more directly in the heart would be caspases.

AngII participates in the stimulation of cardiac apoptosis in essential hypertension. This study shows that MasR blockade with A779 significantly increases Bax and caspase 3 expressions in the hypertensive heart, indicating that Ang1–7 counteracts Ang II-induced apoptosis in the hypertensive heart.

According to Loot et al., chronic infusion of Ang1–7 improves coronary perfusion and preserves cardiac function in the infarcted heart. HPβCD/Ang1–7 attenuates heart function impairment and cardiac remodeling induced by isoproterenol. Cardiac overexpression of Ang1–7 exerts a protective influence on heart function after cardiac injury and attenuates the cardiac remodeling process. The dose and length of AngII treatment in this study result in hypertension without ventricular dysfunction. MasR blockade with A779 does not alter cardiac function. Further study is required to study the role of Ang1–7 in the late stage of hypertension with heart failure.

In addition to endocrine function, AngII has well-established autocrine and paracrine functions in regulating molecular and cellular actions in hypertensive heart disease and myocardial infarction. It has been shown that Ang1–7 infusion into hypertensive rats attenuates myocyte hypertrophy without alteration of blood pressure. This study shows that MasR blockade is involved in various repairing responses in the hypertensive heart without change of blood pressure and heart weight. This observation demonstrates that Ang1–7 regulates cardiac repair/remodeling in an autocrine/paracrine manner.

This study has shown that ACE2 and MasR are differently expressed in the hypertensive heart and kidney, which are
Ang1-7 and Cardiac remodeling

not related to circulating AngII level. Ang1–7 is involved in multiple repair responses and protects the hypertensive heart from remodeling in an autocrine/paracrine manner. The beneficial effect of ACE2/Ang1–7/MasR axis is a novel therapeutic target in cardiac repair/recovery during hypertension.

Figure 3. Influence of Mas receptor (MasR) blockade on (a) gene expressions of proinflammatory factors, (b) cardiac collagen volume fraction (CVF), and (c) profibrotic factors in hypertensive rats. *P < 0.05 vs. controls (CTL); #P < 0.05 vs. angiotensin II (AngII) group.

Figure 4. Effect of Mas receptor (MasR) blockade on apoptotic-related proteins. A779 treatment significantly increased cardiac caspase 3 (a) and Bax (b) protein levels, but not Bcl2 (c) compared with controls (CTL). *P<0.05 vs. CTL; #P < 0.05 vs. angiotensin II (AngII) group.
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DISCLOSURE

The authors declared no conflict of interest.

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