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Antagonism of angiotensin 1–7 prevents the therapeutic effects of recombinant human ACE2

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Abstract

Activation of the angiotensin 1–7/Mas receptor (MasR) axis counteracts angiotensin II (Ang II)-mediated cardiovascular disease. Recombinant human angiotensin-converting enzyme 2 (rhACE2) generates Ang 1–7 from Ang II. We hypothesized that the therapeutic effects of rhACE2 are dependent on Ang 1–7 action. Wild type male C57BL/6 mice (10–12 weeks old) were infused with Ang II (1.5 mg/kg/d) and treated with rhACE2 (2 mg/kg/d). The Ang 1–7 antagonist, A779 (200 ng/kg/min), was administered to a parallel group of mice. rhACE2 prevented Ang II-induced hypertrophy and diastolic dysfunction while A779 prevented these beneficial effects and precipitated systolic dysfunction. rhACE2 effectively antagonized Ang II-mediated myocardial fibrosis which was dependent on the action of Ang 1–7. Myocardial oxidative stress and matrix metalloproteinase 2 activity was further increased by Ang 1–7 inhibition even in the presence of rhACE2. Activation of Akt and endothelial nitric oxide synthase (eNOS) by rhACE2 were suppressed by the antagonism of Ang 1–7 while the activation of pathological signaling pathways was maintained. Blocking Ang 1–7 action prevents the therapeutic effects of rhACE2 in the setting of elevated Ang II culminating in systolic dysfunction. These results highlight a key cardioprotective role of Ang 1–7, and increased Ang 1–7 action represents a potential therapeutic strategy for cardiovascular diseases.

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Authors contribution VBP, AT, TR, SKD, RB, MBG, DAH, and ZK performed experiments, analyzed, and interpreted the data. GYO designed the research and supervised the project. All authors read and approved the final version of the paper.

Keywords

Renin–angiotensin system; Angiotensin-converting enzyme 2; Angiotensin 1–7; PI3K/Akt signaling

Introduction

The renin-angiotensin system (RAS) is a crucial regulator of cardiovascular homeostasis and consists of a series of enzymatic reactions culminating in the generation of angiotensin II (Ang II) in plasma as well as in various tissues including the heart, kidneys, and lungs. Ang II is a well-known agonist whose action leads to concentric hypertrophy and diastolic dysfunction [1, 2]. Angiotensin-converting enzyme 2 (ACE2) is a carboxypeptidase that degrades Ang II to generate the beneficial heptapeptide, angiotensin 1–7 (Ang 1–7) [3,4]. Ang 1–7 is a biologically active metabolite of the RAS and is an endogenous ligand for the G protein-coupled receptor Mas receptor (MasR), whose actions are often opposite to those attributed to Ang II [5, 6]. Ang II-mediated oxidative stress, cardiac hypertrophy, contractile dysfunction, and fibrosis are exacerbated in ACE2-deficient mice, whereas recombinant human ACE2 (rhACE2) attenuates these responses and improves cardiac function, with a marked reversal of Ang II-mediated signaling [1, 7]. Thus, the ACE2/Ang 1–7/MasR axis provides a vasoprotective/anti-proliferative mechanism resulting in counter-regulation of the RAS [8, 9].

Activation of key signaling pathways is a central feature of Ang 1–7/MasR action. Endothelial nitric oxide synthase (eNOS) produces nitric oxide (NO), and its activity is regulated by various kinases. Akt, a serine/threonine kinase, also appears to have a central role in Ang 1–7 signaling, and phosphorylation of eNOS at Ser1177 by Akt results in a twofold increase in eNOS catalytic activity [10]. The phosphorylation of Akt at the regulatory sites Ser473 and Thr308 is stimulated by Ang 1–7 in human endothelial cells in vitro, as well as in the rat heart, liver, skeletal muscle, and adipose tissue in vivo [11]. Ang 1–7 exerts its beneficial effects through a NO/ cGMP pathway [12, 13]. Based on the well-known interactions between NO and Ang 1–7/MasR axis, we hypothesized that enhanced Ang 1–7 action plays a key protective role in mediating the therapeutic effects of recombinant ACE2. In this study, we demonstrate that inhibition of Ang 1–7 action abolishes the beneficial effects of rhACE2 and potentiates Ang II-mediated adverse myocardial remodeling.

Materials and methods

Experimental model

Wild type C57BL/6 mice (10–12 weeks old) were used. The mice were implanted with mini osmotic pumps to deliver Ang II (1.5 mg/kg/d) or A779 (200 ng/kg/ min) over a 14-day period, and rhACE2 (2 mg/kg/d) was given by daily IP injection. rhACE2 was kindly provided by GlaxoSmithKline, Stevenage, UK. A779 was used as a potent and selective antagonist of Ang 1–7 [14, 15]. All experiments were performed in accordance with the GSK policy on the care, welfare, and treatment of laboratory animals and institutional

guidelines, Canadian Council on Animal Care, and the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (revised 2011). All studies were approved by the Animal Care and Use Committee at the University of Alberta.

Echocardiography and tissue Doppler imaging

Transthoracic echocardiography and tissue Doppler imaging were performed non-invasively in anesthetized mice (1 % isoflurane) and analyzed in a blinded manner, as described previously [16, 17] using a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B, VisualSonics, Toronto, Canada).

Plasma ACE2 activity assay

Plasma ACE2 activity was assessed using fluorescent assay protocol involving 20 μM 7-methoxycoumarin-YVADAPK-(2,4-dinitrophenyl)-OH (R&D Systems) as a fluorogenic substrate, as previously described [18]. DX-600 (Phoenix Pharmaceuticals, Burlingame, CA) was used as a specific ACE2 inhibitor. Activities were normalized using a standard curve created by using the calibration standard, 7-methoxycoumarin-PL-OH (Bachem Torrance, CA).

Plasma angiotensin peptide levels

Murine plasma Ang II and Ang 1–7 levels were measured at the Hypertension Core Laboratory, Wake Forest University, Winston-Salem, NC, as previously described [1, 7].

Histological analysis

LV fibrosis and cardiomyocyte hypertrophy and renal and pulmonary fibrosis were measured by Masson trichrome and picrosirius red (PSR) staining, as described previously [1], and visualized and imaged using light microscopy (DM4000B, Leica) and fluorescence microscopy (Olympus 1×81), respectively.

NADPH oxidase activity, dihydroethidium and nitroryrosine staining

The lucigenin chemiluminescence assay was used to measure the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity using a single tube luminometer (Berthold FB12, Berthold Technologies, Germany) modified to maintain the sample temperature at 37 °C as described previously [16]. Lucigenin (5mM) and NADPH (1mM) were added to the samples, and light emission was recorded every 2 s over an 8 min period. The NADPH oxidase specific inhibitor apocynin (1 mM) was used to confirm superoxide generation from the NADPH oxidase. All measurements were performed in triplicates, and results were normalized per 1 mg of protein. Myocardial superoxide level was assayed by lucigenin-enhanced chemiluminescence in LV myocardium frozen and fixed in optical coherence tomography (OCT). Dihydroethidium (DHE) fluorescence was performed on 5-μm OCT myocardial sections, which were washed with Hank's balanced salt solution (HBSS), incubated at 37 °C for 30min with DHE (5 μM) in HBSS, and then imaged using fluorescence microscopy (Olympus 1×81). Nitrotyrosine immunofluorescence staining was performed in 5-μM thick cryosections using a rabbit anti-nitrotyrosine primary antibody

(Millipore) and Alexa Flour 488 conjugated anti-rabbit secondary antibody (Life Technologies).

Taqman real-time PCR, western blot analysis

Messenger RNA expression levels of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), α-skeletal muscle actin (α-SKA), β-myosin heavy chain (β-MHC), procollagen type I α, procollagen type III α, and TGF-β1 were determined using 18S rRNA as an internal standard in an Roche Lightcycler RT-PCR machine (primers and probes are listed in Supplemental Table 1). Western blot analysis was carried out for eNOS, p-eNOS (S1177), Akt, p-Akt(T308), p-Akt(S473), p38, p-p38, Janus-activated kinase (JNK), p-JNK, extracellular-activated kinase 1/2 (ERK1/2), and pERK 1/2 (Cell Signaling, ON, Canada) with standard procedures.

In situ gelatin zymography and in vitro zymography

In situ zymography was performed to assess the myocardial gelatinase activity as previously described [19]. In brief, OCT-embedded frozen LV tissues were cut into 14-μM thick sections and incubated for an hour in reaction buffer (Tris-HCl 50 mM, NaCl 150 mM, $CaCl_2$ 5 mM, andNaN_3 2 mM). Separately, a substrate was prepared by dissolving 10 μg of dye-quenched gelatin (DQG, Molecular Probes®) and then imaged using a fluorescence microscope (Olympus 1×81) using GFP filter. Negative controls were obtained by imaging myocardial sections not incubated with DQ-gelatin as well as quenching of the fluorescence signal by incubation with EDTA (200 μM). Gelatin zymography was carried out as previously described [16, 20]. Following electrophoresis using 8 % SDS-polyacrylamide gels copolymerized with gelatin (2 mg/mL, Sigma), the gels were incubated for 48 h at 37 °C in an incubation buffer, stained with 0.05 % Coomassie Brilliant Blue (G-250; Sigma), and then destained in a mixture of methanol to acetic acid to water ratio (2.5:1:6.5*v/v*).

Statistical analysis

All of the statistical analyses were performed using an SPSS software (Chicago, IL; version 10.1). Hypothesis testing methods included a one-way analysis of variance (ANOVA) followed by the Student–Neuman–Keuls test for multiple comparison testing. For all comparisons, differences were considered significant at a value of $p<0.05$. All the results were expressed as mean±SEM.

Results

Inhibition of Ang 1–7 prevents the therapeutic effects of rhACE2 and leads to systolic dysfunction

Treatment with rhACE2 resulted in a marked increase in plasma ACE2 activity (Fig. 1a). Ang II infusion resulted in a marked increase in plasma Ang II levels which was markedly reduced by rhACE2 with an accompanying increase in plasma Ang 1–7 levels (Fig. 1b, c). A non-invasive functional assessment by echo-cardiography showed a diastolic dysfunction with preserved systolic function in Ang II-treated mice which was prevented by rhACE2 supplementation (Table 1; Fig. 2a – e). Blocking Ang 1–7 action using A779 prevented the anti-hypertrophic effects of rhACE2, worsened the diastolic dysfunction, increased the LV

end-systolic dimension, and precipitated the systolic dysfunction as indicated by reduced ejection fraction and fractional shortening (Table 1; Fig. 2a–e). The expression of hypertrophic disease markers ANF, BNP, α-SKA, and β-MHC was markedly reduced by rhACE2 supplementation in the Ang II-treated mice (Fig. 2f–i). Notably, Ang II-induced pathological effects in the heart were not suppressed by rhACE2 when the Ang 1–7 action was inhibited by A779 in the Ang II + rhACE2 + A779 group (Fig. 2f–i). These results clearly demonstrate that the therapeutic effects of rhACE2 in the setting of the elevated Ang II is clearly dependent on the Ang 1–7 action.

Blocking Ang 1–7 action prevents the anti-fibrotic effects of rhACE2

Ang II is a well-known profibrotic agonist of the RAS [1, 7, 21] while Ang 1–7 is anti-fibrotic [7, 22]. Ang II infusion resulted in a predictable increase in myocardial interstitial and perivascular fibrosis as assessed by Masson trichrome and picrosirius red (PSR) staining (Fig. 3a–c). The increased myocardial fibrosis was effectively suppressed by rhACE2 which was abrogated and further exacerbated by the use of A779 (Fig. 3a–c). We next examined the myocardial mRNA expression profile of procollagen type I α, procollagen type III α, and TGF-β1. Consistent with our histological findings, rhACE2 was effective at suppressing the increased expression of the pro-fibrotic genes, and antagonism of Ang 1–7 further exacerbated the Ang II-mediated pro-fibrotic increases in gene expression (Fig. 3c–f). Ang II-mediated fibrotic effects are widespread including increased pro-fibrotic effects in the kidneys and lungs [23, 24]. Given the systemic delivery of Ang II, we also showed that blocking Ang 1–7 action using A779 exacerbated Ang II-mediated renal and pulmonary fibrosis (Fig. 4). Clearly, the ability of rhACE2 to antagonize the Ang II-mediated tissue fibrosis is clearly dependent on the action of Ang 1–7.

Blocking Ang 1–7 increased myocardial oxidative stress, activated matrix metalloproteinases, and altered myocardial signaling pathways

Ang II-induced heart disease is linked to increased oxidative stress [25, 26], and ACE2 is known to reduce Ang II-induced oxidative stress [1, 7]. Ang II increased myocardial oxidative stress as measured by the DHE fluorescence, nitrotyrosine levels, and NADPH oxidase activity which was prevented by rhACE2 (Fig. 5a–e). However, the beneficial action of rhACE2 was mitigated when A779 was co-administered confirming that blocking Ang 1– 7 completely prevented the anti-oxidant effects of rhACE2 (Fig. 5a–e). Ang II-mediated generation of ROS activates matrix metalloproteinases in a $p47^{phox}$-dependent manner [16]. Ang II treatment significantly increased the myocardial gelatinase activity as confirmed by in situ zymography which was further attenuated and exacerbated by rhACE2 and A779, respectively (Fig. 5f, g, and Supplemental Figure 1). Gelatin zymography showed the anticipated Ang II-mediated increase in MMP9 and pro and active MMP2, which were suppressed by rhACE2; blocking Ang 1–7 action prevented the rhACE2 effects and further increased the levels of active MMP2 (Fig. 5h).

The ACE2/Ang-(1–7)/MasR axis directly activates eNOS in the heart [12, 27], and impairment of NO production and eNOS uncoupling can precipitate systolic dysfunction [28, 29]. In response to Ang II, phosphorylation of myocardial Akt at serine-473 and threonine-308 was enhanced in response to rhACE2 which was completely prevented by

A779 treatment (Fig. 6a, b). Importantly, increased Akt phosphorylation correlated with increased phosphorylation of eNOS at serine-1177 which markedly reduced by treatment with A779 (Fig. 6c). The mitogen-activated protein kinase (MAPK) family such as ERK1/2, JNK1/2, and p38 kinase mediates adverse myocardial remodeling and heart failure [30]. Phosphorylation of myocardial ERK1/2, JNK1/2, and p38 were markedly increased by Ang II, prevented by rhACE2, and completely reversed by blocking Ang 1–7 action (Fig. 6d–f). These results suggest that antagonism of Ang 1–7 exacerbates Ang II-mediated myocardial oxidative stress, activates MMP2, and prevents the activation of eNOS and suppression of MAPK signaling.

Discussion

Activation of the RAS plays a key pathogenic role in cardiovascular, kidney, and pulmonary diseases. The ACE2/Ang 1– 7/MasR axis of the RAS has emerged as the physiological antagonist of the ACE/Ang II/AT1R axis [5, 31, 32]. As such, enhancing ACE2 action such as the use of rhACE2 has become a key therapeutic strategy [33, 34]. Our current results and previous studies [1, 35, 36] have clearly demonstrated a reduction in plasma Ang II levels and an increase in plasma Ang 1–7 levels in response to rhACE2 and are consistent with in vitro effects of rhACE2 which degrades plasma Ang II into Ang 1–7 [37] and in vivo effects in healthy human volunteers whereby 100–200 μg/kg of rhACE2 lowered Ang II levels and increased Ang 1–7 levels [38]. While blocking Ang II and enhancing Ang 1–7 effects can both contribute to the beneficial action of rhACE2 [1, 7], the direct in vivo role of Ang 1–7 remains unresolved. These effects are consistent with the ability of rhACE2 to reduce Ang II-pressor effects [1, 36] with A779 having a neutral effect on Ang II-induced pressor response [39]. Our data demonstrate that in a murine model of Ang II-induced heart disease, Ang 1–7 action plays a dominant role in the therapeutic effects mediated by rhACE2.

Ang II infusion is a well-accepted model of heart disease characterized by concentric hypertrophy and diastolic dysfunction with preserved systolic function [1, 2]. Ang II-mediated hypertrophy, oxidative stress, myocardial fibrosis, and pathological signaling were reduced by rhACE2. These results are in agreement with previous studies showing a detrimental effect of ACE2 deficiency [1, 16, 20, 40] and the ability of rhACE2 to metabolize Ang II into Ang 1–7 thereby conferring protective effects [1, 33, 37, 41]. Blocking Ang 1– 7 action prevented the therapeutic effects of rhACE2 and resulted in adverse myocardial remodeling and systolic dysfunction which correlated with increased oxidative stress and myocardial fibrosis and altered key signaling pathways. These findings are consistent with the beneficial effects of the Ang 1–7/MasR axis both in vitro and in vivo [5, 22, 31, 42]. Impaired systolic function correlates with increased myocardial oxidative stress and inhibition of oxidative stress prevents heart failure [43, 44]. While rhACE2 counter-regulated the action of Ang II and reduced the oxidative stress and nitrotyrosine level conforming that ACE2 serves as a negative regulator of the RAS.

While the systemic effects of our experimental interventions cannot be ruled out, Ang 1–7 mediates direct protective effects of Ang 1–7 on cardiofibroblasts and cardiomyocytes [1, 5, 7, 12, 22, 27]. Loss of Ang 1–7 action likely results in unopposed negative inotropic effects of Ang II thereby further worsening the systolic dysfunction. Importantly, these results show

a critical role in Ang 1–7 pathways in preventing the transition of heart disease associated with preserved ejection fraction to reduced ejection fraction. Ang II is known to induce the production of collagen and TGFβ1 [22], both of which were largely inhibited by rhACE2. Ang II-upregulated transcription of procollagen-I, III, and TGF-β1 was markedly suppressed by rhACE2 and exacerbated by inhibition of Ang 1–7. Ang 1–7, acting via the Mas receptor, stimulates eNOS activation and NO production via Akt-dependent pathways [12]. Moreover, eNOS and PI3K/Akt are downstream effectors of Ang 1–7 in ventricular cardiomyocytes [45] and endothelial cells [12, 27]. In aorta of Mas-deficient mice, endothelium-dependent vasodilator effects of Ang 1–7 are abolished, while Ang 1–7 causes endothelium-dependent vasodilation mediated by NO in intact blood vessels [15, 46]. Recombinant human ACE2 stimulated an increased phosphorylation of Akt and eNOS which was markedly blunted by antagonism of Ang 1–7 action. Blocking Ang 1–7 action also resulted in a greater increase in active MMP2 levels which has been linked to systolic dysfunction and progression to heart failure [47, 48]. In the myocardium, the MAPK transduction cascade regulates the hypertrophic response and plays a key role in cardiomyopathy and heart failure [30]. Similarly, in response to A779 treatment, reduced activation of myocardial ERK1/2, and p38 MAPK signaling pathways were restored to pathological levels with a greater phosphorylation of the JNK1/2 pathway.

Given the increasing importance of the ACE2/Ang 1–7/ Mas receptor axis, our study provides strong evidence of the important protective role of the Ang 1–7/Mas receptor axis against the development of cardiac pathology. The protective role of Ang 1–7 has been documented in several models of cardiovascular [8, 31, 49, 50] and metabolic diseases [31, 32, 51]. These in vivo studies coupled with extensive in vitro studies on the protective role of Ang 1–7 in various cell types including cardiomyocytes [1, 7], cardiofibroblasts [1, 52], and endothelial cells [27], are clearly supportive of a protective action of Ang 1–7 in heart disease. Enhancing Ang 1–7 action represents an important targeted therapeutic application in diseases processes characterized by an activated RAS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key messages

- Activation of the renin-angiotensin system (RAS) plays a key pathogenic role in cardiovascular disease.
- ACE2, a monocarboxypeptidase, negatively regulates pathological effects of Ang II.
- Antagonizing Ang 1–7 prevents the therapeutic effects of recombinant human ACE2.
- Our results highlight a key protective role of Ang 1–7 in cardiovascular disease.

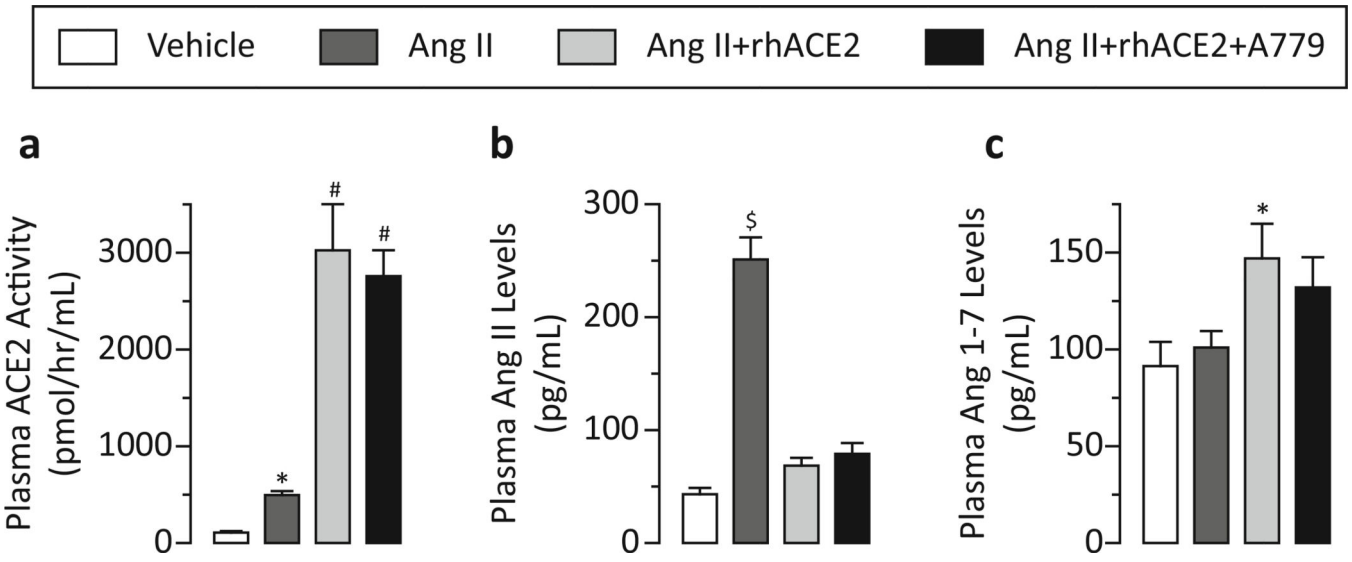

Fig. 1.

Plasma ACE2 activity and angiotensin peptide levels in response to Ang II and treatment with rhACE2 and A779. Plasma ACE2 activity was markedly elevated in response to rhACE2 (**a**). Ang II infusion increased plasma Ang II levels which was prevented by treatment with rhACE2 (**b**) which also resulted in elevated plasma Ang 1–7 levels (**c**). $n = 8$ for the vehicle group and $n=10$ for all other groups. $^{*}p<0.05$ compared to the vehicle group; $^{\#}p<0.05$ compared to the Ang II group; $^{\$}p<0.05$ compared with all other groups

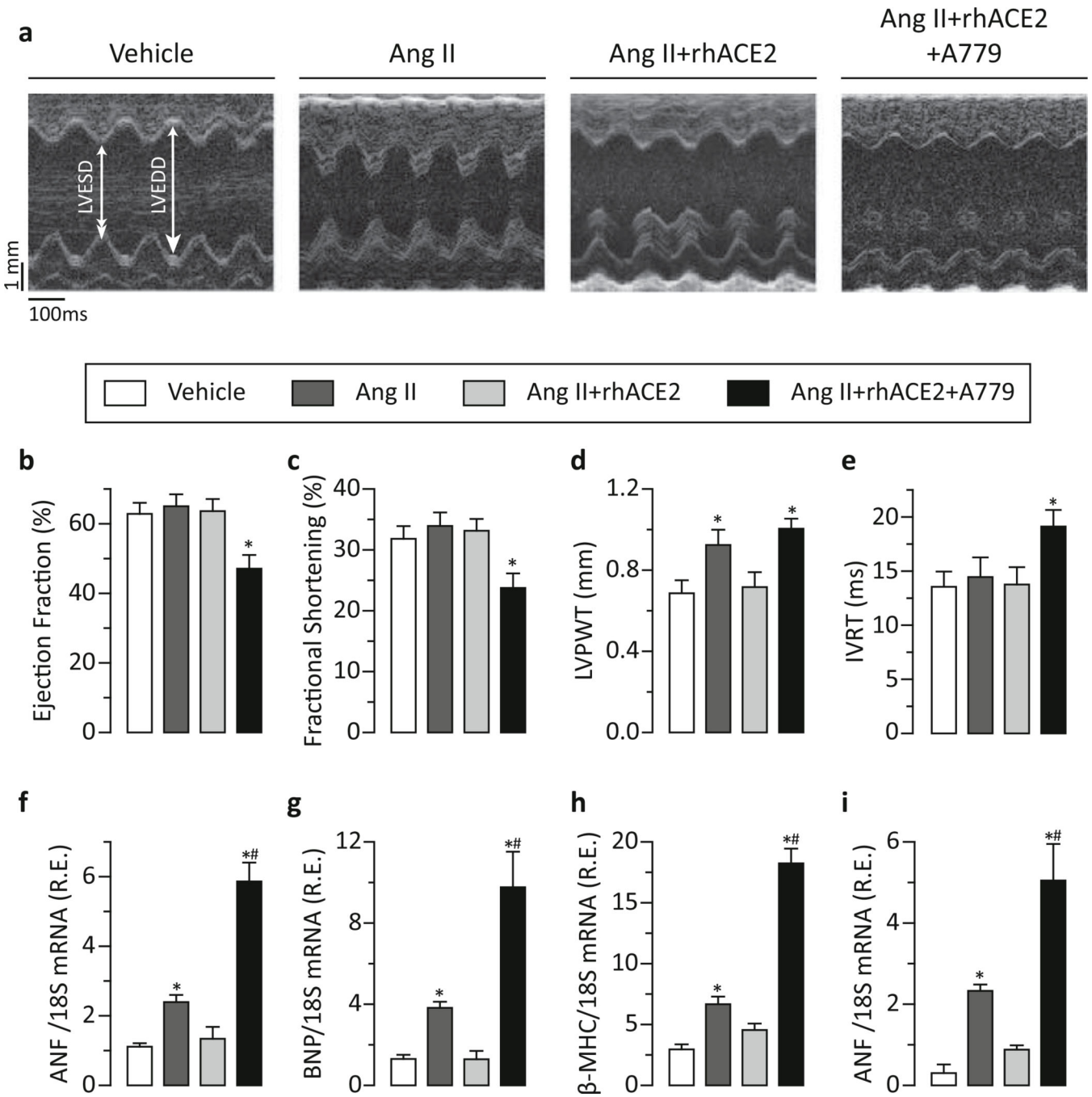

Fig. 2.

Antagonism of Ang 1–7 inhibits cardioprotective effects of rhACE2 and leads to systolic dysfunction in response to Ang II. Echocardiographic assessment of heart function as illustrated by M-mode images (**a**) and quantitative evaluation of heart function showing reduced LV ejection fraction (**b**) and LV fractional shortening (**c**), increased LV posterior wall thickness (LVPWT) (**d**) and isovolumic relaxation time (IVRT) (**e**) in response to Ang 1–7 blockade in the setting of elevated Ang II and treatment with rhACE2. *n* = 12 for each group. Taqman PCR analysis showing increased mRNA expression of atrial natriuretic

factor (ANF, **f**), brain natriuretic peptide (BNP, **g**), β-myosin heavy chain (β-MHC, **h**), and α-skeletal actin (α-SKA, **i**) in response to Ang 1–7 blockade in the setting of elevated Ang II and treatment with rhACE2. n=6 for each group. *p<0.05 compared to the vehicle group; #p<0.05 compared to the Ang II group

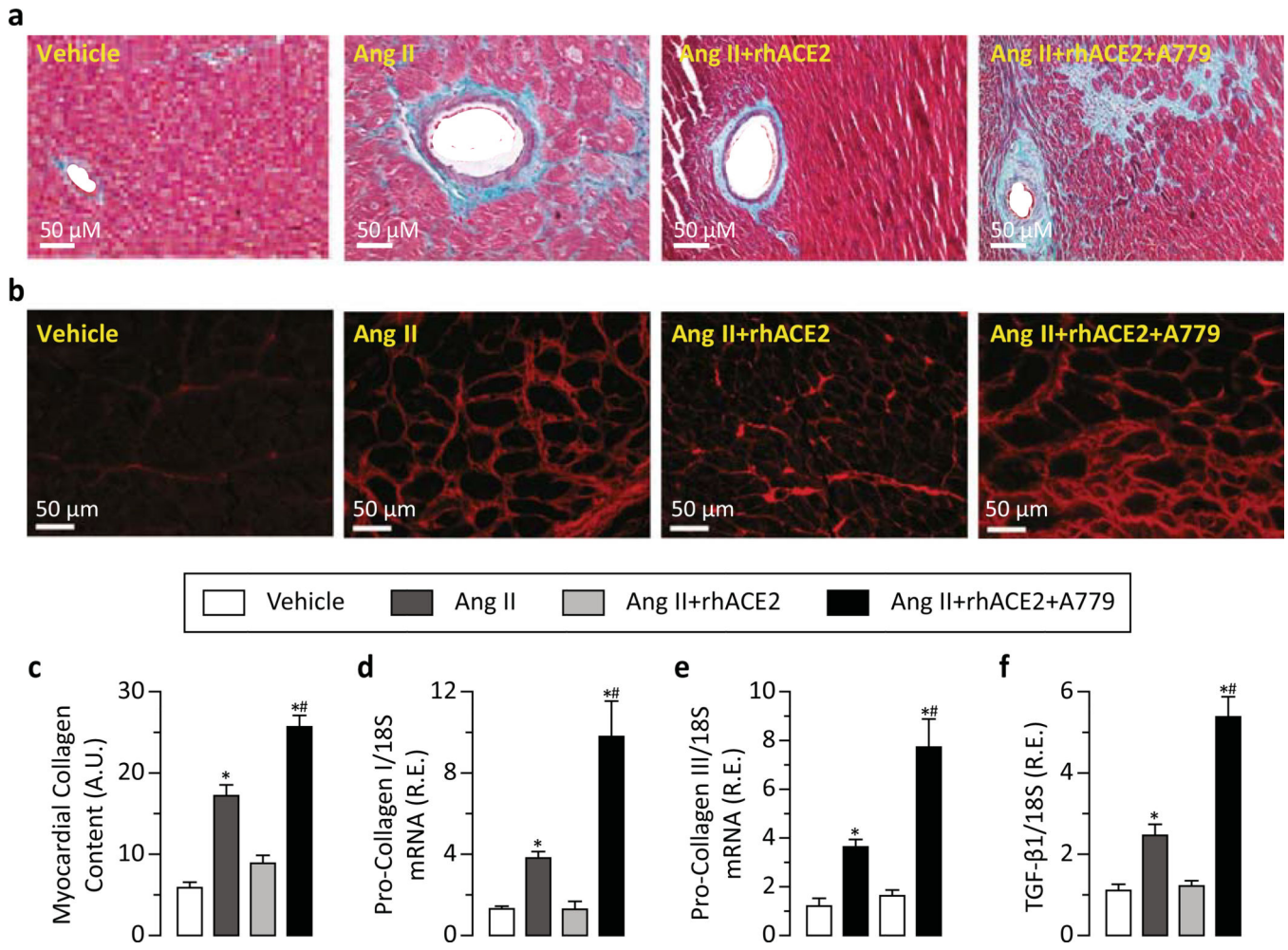

Fig. 3.

Antagonism of Ang 1–7 inhibits the anti-fibrotic effects of rhACE2 and exacerbates Ang II-induced cardiac fibrosis. Masson trichrome (**a**) and picrosirius red (**b**) staining showing Ang II-mediated increased perivascular and interstitial myocardial fibrosis which was inhibited by rhACE2 and further exacerbated by blocking Ang 1–7 action using A779. Quantitative evaluation of myocardial collagen levels derived from PSR staining images showing A779 co-treatment with rhACE2 ameliorated Ang II-mediated increase in myocardial collagen levels (**c**). *n* = 4 for each group. Similarly, higher mRNA expression of procollagen Iα (**d**), procollagen IIIα (**e**) and transforming growth factor betal (TGF-β1, **f**) normalized with 18S in response to Ang II was blocked and exacerbated by rhACE2 and A779, respectively, *n*=8 for each group. **p*<0.05 compared to the vehicle group; $^{\#}p$<0.05 compared to the Ang II-treated group

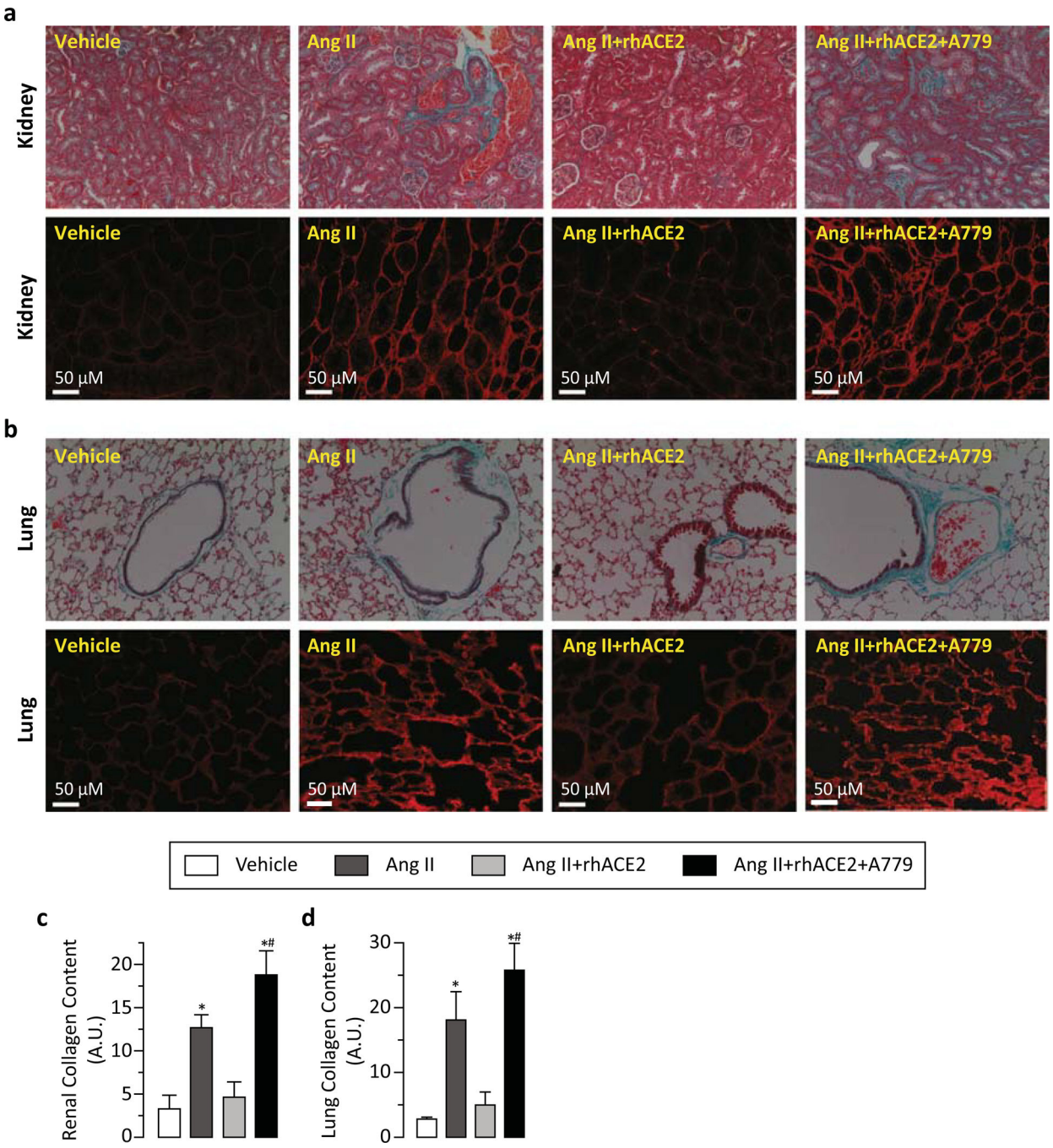

Fig. 4.

Inhibition of Ang 1–7 action inhibits the anti-fibrotic effects of rhACE2 and exacerbates Ang II-induced renal and lung fibrosis. Masson trichrome staining images (**a, c**) and picrosirius red staining images (**b, d**) showing enhanced renal (**a, b**) and lung fibrosis (**c, d**) in Ang II treated mice which was suppressed by rhACE2 treatment and exacerbated by blocking Ang 1–7 with A779 (**a-d**). Quantitative evaluation of renal (**e**) and lung (**f**) collagen levels derived from PSR staining images confirming that rhACE2 and A779 co-treatment

suppressed and potentiated Ang II-mediated increase in tissue fibrosis, respectively. n=4 for each group. *p<0.05 compared to the vehicle group; $^{\#}p$<0.05 compared to the Ang II group

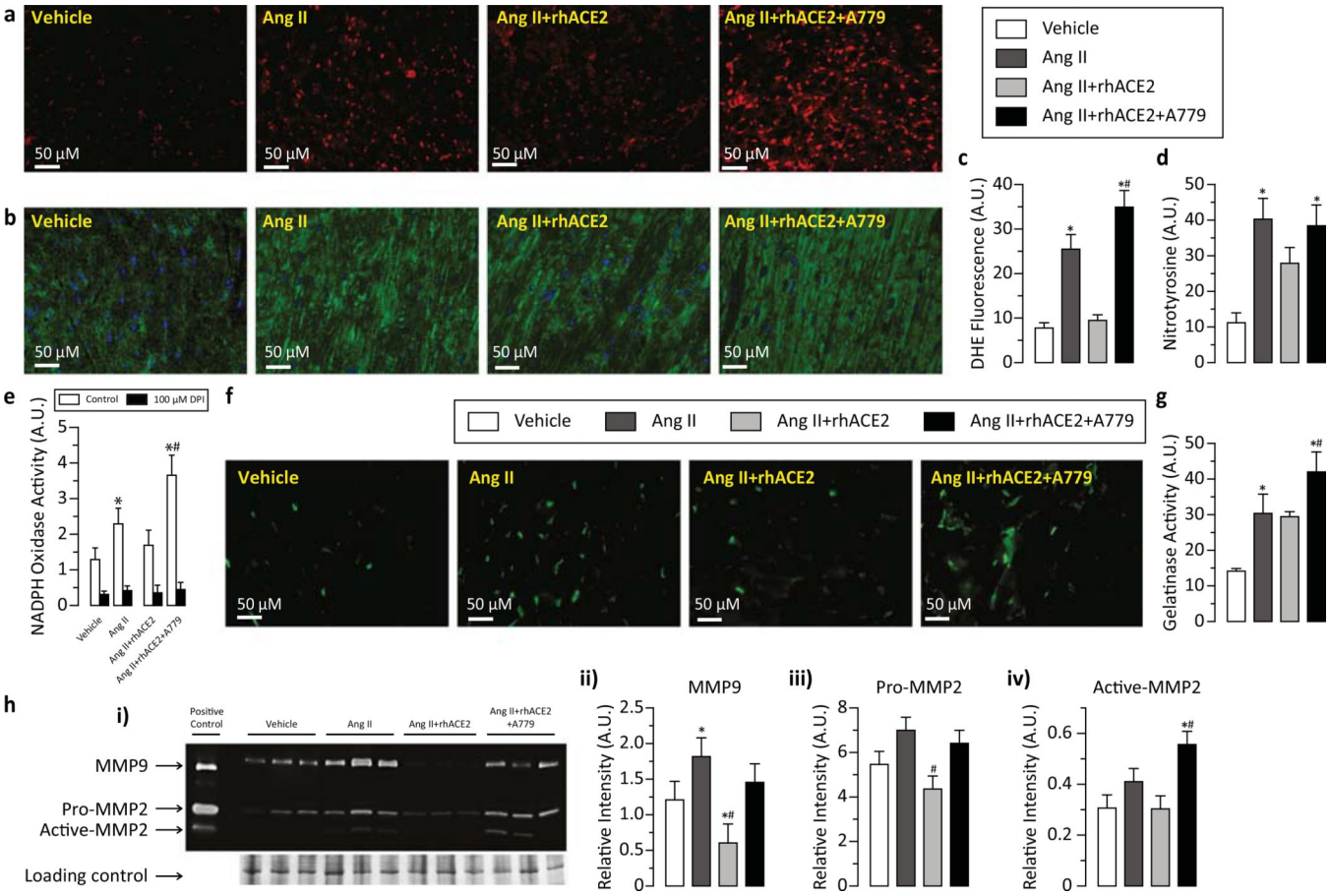

Fig. 5.

Myocardial oxidative stress induced by Ang II is suppressed and exacerbated by rhACE2 and A779, respectively. Ang II treatment increased myocardial oxidative stress as shown by dihydroethidium (DHE) fluorescence imaging and quantification of superoxide levels (**a, c**), increased nitrotyrosine level (**b, d**) and increased NADPH oxidase activity (**e**). Recombinant human ACE2 prevented Ang II-induced oxidative stress while A779 restored nitrotyrosine levels and further exacerbates DHE fluorescence and NADPH oxidase activity (**a–e**). Apocynin (1 mM) were used to suppress NADPH oxidase activity (*gray bars*). In situ zymography (**f**) and quantification (**g**) showing increased myocardial gelatinase activity in response to Ang II, suppression by rhACE2 and a further increase in response to blocking Ang 1–7 action. Gelatin zymography (i) showing increased MMP9 (ii), pro and active MMP2 (iii and iv) in response to Ang II which was suppressed by rhACE2; blocking Ang 1–7 action prevented these effects and further increased the levels of active MMP2 (**h**). *n*=6 for each group. *RE* relative expression, *AU* arbitrary unit. **p*<0.05 compared to the vehicle group; $^{\#}p$<0.05 compared to the Ang II group

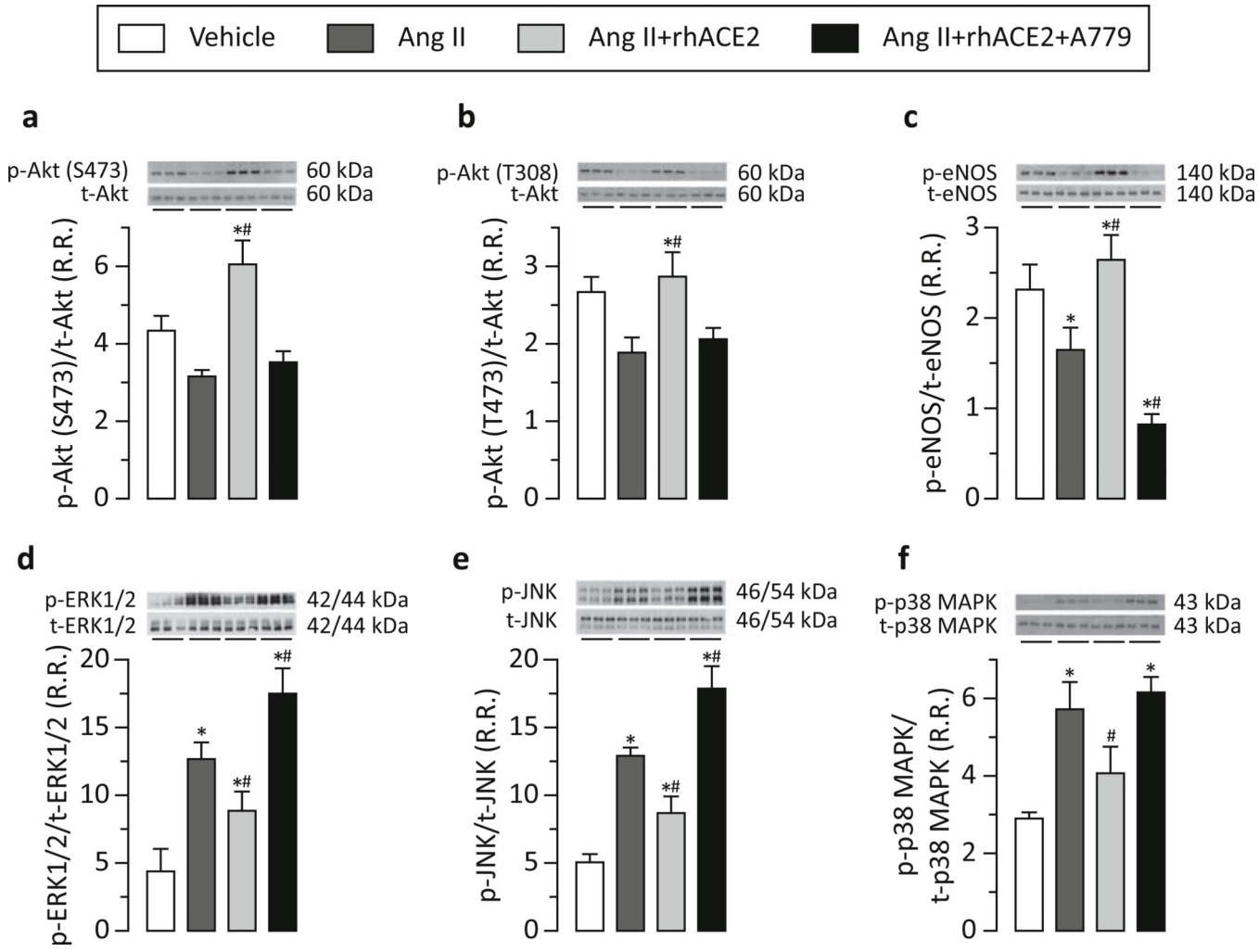

Fig. 6.

Blocking Ang 1–7 action results in loss of phosphorylation of Akt and eNOS and maintained activation of the MAPK signaling pathways. Phosphorylation of myocardial Akt (serine-473) (**a**) and Akt (threonrne-308) (**b**) and eNOS (serinel 177) (**c**) were reduced by Ang II which was restored in response to rhACE2 and further suppressed by A779 treatment. Phosphorylation of myocardial ERK1/2 (**d**), JNK1/2 (**e**) and p38 (**f**) signaling pathways were increased by Ang II, suppressed by rhACE2 which was loss when Ang 1–7 action was blocked with A779 treatment. *RE* relative expression. $n = 6$ for each group. $^{*}p<0.05$ compared to the vehicle group; $^{\#}p<0.05$ compared to the Ang II group

Table 1

Echocardiographic assessment of heart function

Parameter N	Vehicle 10	Ang II 12	Ang II + rhACE2 12	Ang II + rhACE2 + A779 12
HR (bpm)	498±12	491±14	501±16	507±15
E-wave (mm/s)	742±18	715±23	736±31	707 ±27
A-wave (mm/s)	419±16	536±19	432±15	484±21
E/A Ratio	1.77±0.09	1.33±0.11*	1.70±0.08	1.46±0.12*
IVRT (ms)	13.6±1.3	14.5±1.7	13.8±1.5	19.2±1.4*
E′ (mm/s)	26.1±1.5	18.1±1.2	27.2±1.4	20.1±1.3
E/E′ Ratio	28.4±1.9	39.5±2.3*	27.1±2.1	35.2±2.2*
A′ (mm/s)	17.9±1.2	25.1±1.4	20.1±1.7	25.9±2.9
E′/A′	1.46±0.06	0.72±0.07	1.35±0.08	1.07±0.05
LA size (mm)	1.53±0.05	1.85±0.07*	1.64±0.06	1.96±0.08*
LVEDD (mm)	3.75±0.07	3.44±0.06	3.71±0.08	3.72±0.09
LVESD (mm)	2.51±0.06	2.27±0.07	2.47±0.06	2.83±0.08
LVFS(%)	33.1±1.8	34.1±2.1	33.3±1.8	23.9±2.1#
LVEF (%)	63.2±2.8	65.4±3.1	63.6±3.2	48.01±3.73#
LVPWT (mm)	0.69±0.06	0.93±0.07*	0.72±0.07	1.01±0.02*

HR heart rate, *IVRT* isovolumic relaxation time, *LVEDD-LV* end diastolic diameter, *LVESD-LV* end-systolic diameter, *LVFS-LV* fractional shortening, *LVEF-LV* ejection fraction, *LVPWT-LV* posterior wall thickness, *LA* left atrium

* $p<0.05$ compared to the vehicle group;

# $p<0.05$ compared to all other groups