Journal of the American Heart Association

ORIGINAL RESEARCH

Prolylcarboxypeptidase Alleviates Hypertensive Cardiac Remodeling by Regulating Myocardial Tissue Angiotensin II

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BACKGROUND: Prolonged activation of angiotensin II is the main mediator that contributes to the development of heart diseases, so converting angiotensin II into angiotensin 1-7 has emerged as a new strategy to attenuate detrimental effects of angiotensin II. Prolylcarboxypeptidase is a lysosomal pro-X carboxypeptidase that is able to cleave angiotensin II at a preferential acidic pH optimum. However, insufficient attention has been given to the cardioprotective functions of prolylcarboxylpeptidase.

METHODS AND RESULTS: We established a CRISPR/CRISPR-associated protein 9–mediated global prolylcarboxylpeptidase-knockout and adeno-associated virus serotype 9–mediated cardiac prolylcarboxylpeptidase overexpression mouse models, which were challenged with the angiotensin II infusion (2 mg/kg per day) for 4 weeks, aiming to investigate the cardioprotective effect of prolylcarboxylpeptidase against hypertensive cardiac hypertrophy. Prolylcarboxylpeptidase expression was upregulated after 2 weeks of angiotensin II infusion and then became downregulated afterward in wild-type mouse myocardium, suggesting its compensatory function against angiotensin II stress. Moreover, angiotensin II–treated prolylcarboxylpeptidase-knockout mice showed aggravated cardiac remodeling and dampened cardiac contractility independent of hypertension. We also found that prolylcarboxylpeptidase localizes in cardiomyocyte lysosomes, and loss of prolylcarboxylpeptidase led to excessive angiotensin II levels in myocardial tissue. Further screening demonstrated that hypertrophic prolylcarboxylpeptidase-knockout hearts showed upregulated extracellular signal-regulated kinases 1/2 and downregulated protein kinase B activities. Importantly, adeno-associated virus serotype 9–mediated restoration of prolylcarboxylpeptidase expression in prolylcarboxylpeptidase-knockout hearts alleviated angiotensin II–induced hypertrophy, fibrosis, and cell death. Interestingly, the combination of adeno-associated virus serotype 9–mediated prolylcarboxylpeptidase overexpression and an antihypertensive drug, losartan, likely conferred more effective protection than a single treatment protocol to mitigate angiotensin II–induced cardiac dysfunction.

CONCLUSIONS: Our data demonstrate that prolylcarboxylpeptidase protects the heart from angiotensin II–induced hypertrophic remodeling by controlling myocardial angiotensin II levels.

Key Words: hypertension ■ hypertrophy ■ lysosome ■ prolylcarboxylpeptidase

The renin-angiotensin system (RAS) is a coordinated hormonal signaling pathway regulating cardiovascular, renal, and adrenal function. Angiotensin II is the key effector peptide with pleiotropic actions via the angiotensin type I receptor (AT1R), including stimulation of oxidative stress, hypertrophic growth, fibrogenesis,

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This manuscript was sent to Daniel T. Eitzman, MD, Senior Guest Editor, for review by expert referees, editorial decision, and final disposition.

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.122.028298

For Sources of Funding and Disclosures, see page 16.

JAHA is available at: www.ahajournals.org/journal/jaha

RESEARCH PERSPECTIVE

What Is New?

- The causation of prolylcarboxylpeptidase loss-induced hypertensive remodeling and cardiac dysfunction is independent of angiotensin II–induced hypertension, indicating that prolylcarboxylpeptidase might not be responsible for the regulation of angiotensin II at the systemic level like angiotensin-converting enzyme 2.
- Prolylcarboxylpeptidase localizes in lysosomes, and loss of prolylcarboxylpeptidase leads to excessive production of myocardial angiotensin II, indicating prolylcarboxylpeptidase's roles in the regulation of the renin-angiotensin system system at the intracellular level.

What Question Should Be Addressed Next?

- In this study, we showed that the combined therapy of prolylcarboxylpeptidase and losartan demonstrated a better efficiency compared with losartan only to slow down the progression of hypertrophic remodeling toward heart failure.
- In the future, we will investigate whether prolylcarboxylpeptidase and a broader choice of renin-angiotensin system–based drugs could be used to treat ischemic cardiac disease and diabetic cardiomyopathy.

Nonstandard Abbreviations and Acronyms

AAV9	adeno-associated virus serotype 9
AT1R	angiotensin type 1 receptor
AT2R	angiotensin type 2 receptor
ERK1/2	extracellular signal-regulated kinases 1/2
GFP	green fluorescent protein
MAPK	mitogen-activated protein kinase
PKB	protein kinase B
RAS	renin-angiotensin system

inflammation, and cell death.[1] Pharmacological angiotensin II antagonists, including angiotensin-converting enzyme inhibitors (ACEis) and angiotensin type 1 receptor blockers, are some of the most successful clinical interventions in the treatment of hypertension and heart failure.[2] Over the past decades, the existence of a counterregulatory arm of the RAS, including angiotensin 1-7, angiotensin 1-9, and angiotensin III, has been discovered. Of those, evidence over the past decade has shown that angiotensin 1-7 via the Mas1 receptor cascade is able to counteract and prevent the detrimental effects caused by angiotensin II overactivity.[3]

There are 3 enzymes that negatively regulate angiotensin II, including angiotensin-converting enzyme 2 (ACE2), prolylcarboxypeptidase, and prolyloligopeptidase.[4] There is increasing evidence in rodent models that human recombinant ACE2 could prevent many cardiac diseases such as angiotensin II–induced hypertension, myocardial hypertrophy, diastolic and systolic dysfunction, and myocardial fibrosis by lowering angiotensin II and increasing angiotensin 1-7 levels.[3] Moreover, a pilot study involving 5 patients with pulmonary arterial hypertension demonstrated beneficial effects of angiotensin 1-7 on cardiac performance and pulmonary vascular resistance in company with increased superoxide dismutase 2 and reduced inflammatory markers levels in plasma.[5] In another study, 44 patients with acute respiratory distress syndrome receiving intravenous administration of human recombinant ACE2 were found to be well tolerated, but the improvement in oxygenation in these patients was not detected.[6] Thus, further clinical trials of ACE2 need a larger sample size to powerfully evaluate clinical outcomes. In addition, ACE2 has recently been identified as the SARS-CoV-2 receptor to allow viral cell entry. This raises the awareness of the urgent need to examine the regulatory functions of the other 2 enzymes in heart diseases in detail for safer treatment options.[7] Interestingly, a recent study showed that angiotensin II conversion to angiotensin 1-7 in plasma in the circulation is prolyloligopeptidase dependent and ACE2 independent.[8] Thus, these discrepancies suggest a lack of knowledge of the physiological and pathophysiological mechanisms involved in this noncanonical cascade.

Prolylcarboxylpeptidase is a lysosomal Pro-X carboxypeptidase that is capable of cleaving angiotensin II to produce angiotensin 1-7 at a preferential acidic pH optimum. Prolylcarboxylpeptidase$^{gt/gt}$ mice experienced significantly elevated blood pressure and increased reactive oxygen species.[9] Furthermore, spontaneous hypertensive rats showed decreased prolylcarboxylpeptidase mRNA and protein levels in left ventricles, along with the development of ventricular hypertrophy.[10] Similarly, alcohol-mediated left ventricular systolic dysfunction in spontaneous hypertensive rats also was associated with the downregulation of prolylcarboxylpeptidase.[11] However, a study by Maier et al[12] indicated that cardiac dysfunction in global prolylcarboxylpeptidase deficiency mice was mainly due to the lack of prolylcarboxylpeptidase activity in the kidney collecting tubules, where the prevailing pH is low. These conflicting findings have inspired us to explore the mechanistic action of prolylcarboxylpeptidase in metabolizing angiotensin II in the heart and its therapeutic potential for the treatment of heart diseases.

Increasing evidence supports the notion that some organs, such as hearts and kidneys, are able to express or uptake different components of the RAS, leading to the local angiotensin II synthesis.[13] In addition, increasing evidence showing the intracellular expression of several RAS components such as renin and intracellular angiotensin II and its receptors indicates the presence of functional intracrine RAS.[14] However, the regulatory mechanism of the intracrine angiotensin II cascade and its roles in the progression of heart failure are not fully understood. In contrast with ACE2 and prolyloligopeptidase, prolylcarboxylpeptidase is expressed predominantly in lysosomes, where its activity is essentially optimal for angiotensin II degradation under the acidic range.[12,15,16] Therefore, we proposed that prolylcarboxylpeptidase is important for protecting hearts from angiotensin II–mediated hypertrophy through the lysosomal control of intracellular angiotensin II.

In the present study, we aimed to investigate the role and the underlying mechanism of prolylcarboxylpeptidase in pathological cardiac hypertrophy subjected to 4 weeks of angiotensin II infusion. Moreover, we also characterize the therapeutic values of prolylcarboxylpeptidase in alleviating the progression of pathological hypertrophy.

METHODS

All laboratory mice and rats used in this study were maintained in a pathogen-free facility at the University of Manchester. Animal studies were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and were approved by the University of Manchester Ethics Committee. Data, methods, and study materials are available from the corresponding author upon reasonable request. Additional details of experimental procedures are included in Data S1 and Table S1.

Statistical Analysis

The power calculation was performed on the basis of pilot data of systolic blood pressure from wild-type mice after 2 weeks of angiotensin II infusion, where that in the vehicle group was 120.25±4.92 mm Hg versus that in angiotensin II–infused mice was 131±5.47 mm Hg (pooled SD=5.19). Based on the calculation at the power of 0.9 and α=0.05, the minimum number of 5 mice was required per group for functional study. For all in vivo and in vitro analyses, normal distribution (Gaussian distribution) was first determined by the Shapiro–Wilk test. Data were then analyzed using Student's *t*-test for comparisons between 2 experimental groups, and 1-way or 2-way ANOVA with Bonferroni post hoc tests for comparisons among multiple experimental groups. For nonnormal distribution data, the nonparametric Mann–Whitney *U* test and the Kruskal–Wallis test were used as alternatives for the *t*-test and ANOVA, respectively. Data were analyzed using GraphPad software (La Jolla, CA) and expressed as mean±SEM, and a $P<0.05$ was considered statistically significant.

RESULTS

Alteration of Prolylcarboxylpeptidase Expression During Angiotensin II–Mediated Hypertrophic Development

To determine how prolylcarboxylpeptidase expression responds during the development of hypertrophic growth of the heart, adult wild-type mice were subjected to 4 weeks of angiotensin II infusion (2 mg/kg per day). As a result, wild-type hearts under angiotensin II–induced hypertrophic stress displayed an increase (≈1.5-fold) in prolylcarboxylpeptidase protein expression level after a 2-week angiotensin II infusion, which was then dramatically reduced in the fourth week of angiotensin II infusion. In contrast, ACE2 expression progressively declined during the time course of angiotensin II treatment (Figure 1A). Moreover, we also tested whether these changes in prolylcarboxylpeptidase expression could be mirrored in human induced pluripotent stem cell–derived cardiomyocytes under angiotensin II treatment (100 nmol/L). As expected, prolylcarboxylpeptidase expression was significantly increased after 24 hours of angiotensin II treatment and then decreased at 48 hours as compared with control groups (Figure 1B). Next, we examined the change of prolylcarboxylpeptidase expression in the hearts of patients with end-stage dilated cardiomyopathy. Similarly, prolylcarboxylpeptidase expression was decreased by 50% in dilated cardiomyopathy hearts (Figure 1C). These results suggest that the upregulation of prolylcarboxylpeptidase expression might be essential to protect the heart from the angiotensin II stress, and loss of prolylcarboxylpeptidase might contribute to the failure stage of hypertrophic myocardium.

Characterization of Blood Pressure and Cardiac Function of Prolylcarboxylpeptidase-Knockout Mice at 2 and 6 Months Old

First, we recruited CRISPR/CRISPR-associated protein 9 technology to produce the global prolylcarboxylpeptidase-knockout mice. The deletion of prolylcarboxylpeptidase expression in different organs was confirmed by Western blot, which showed a >90% removal of prolylcarboxylpeptidase in different organs (Figure S1A through S1E). A trace of prolylcarboxylpeptidase protein residual was

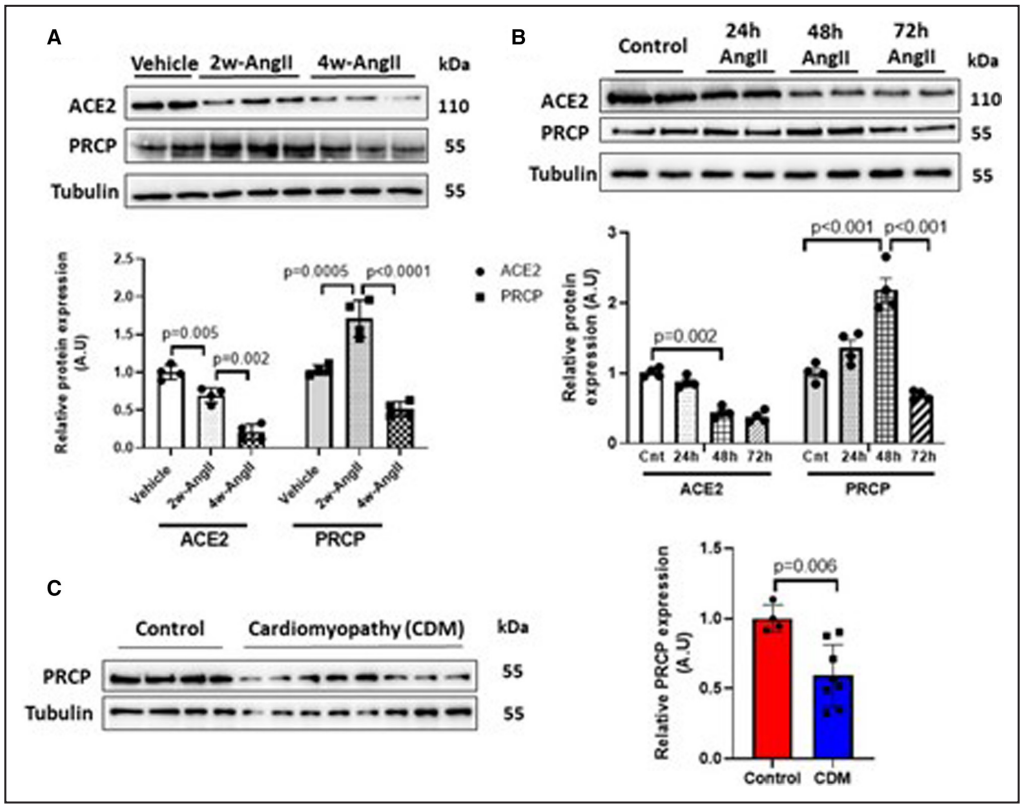

Figure 1. Alteration of cardiac prolylcarboxylpeptidase expression in response to angiotensin II stress.

A, While cardiac ACE2 expression was continuously downregulated, cardiac prolylcarboxylpeptidase expression was upregulated after 2 weeks of angiotensin II infusion and then became downregulated (n=4 per group). One-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. Data are expressed as means±SEM. **B**, ACE2 expression was decreased after angiotensin II (100 nmol/L) treatments, whereas prolylcarboxylpeptidase was increased after 24 hours of angiotensin II treatment and then decreased in human induced pluripotent stem cell–derived cardiomyocytes. One-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. Data are expressed as means±SEM (n=4 per group). **C**, Prolylcarboxylpeptidase expression was downregulated in heart tissue samples of patients with CDM, compared with controls. Unpaired 2-tailed Student's *t*-test was used for analysis. Data are expressed as means±SEM (n=4 for control, n=8 for CDM). ACE2 indicates angiotensin-converting enzyme 2; AngII, angiotensin II; A.U, Arbitrary unit; CDM, cardiomyopathy; and PRCP, prolylcarboxylpeptidase.

observed in prolylcarboxylpeptidase-knockout mice, which was likely due to a small degree of mosaicism that occurred during pronuclear coinjection of guided RNA and CRISPR-associated protein 9 transcript rather than CRISPR-associated protein 9.

Because angiotensin II plays an important role in the regulation of blood pressure and cardiac function, we first examined whether knockout of prolylcarboxylpeptidase alters blood pressure and cardiac functions in the 2- and 6-month-old prolylcarboxylpeptidase-knockout mice at a basal level. Blood pressure measured by tail-cuff method revealed that the 2-month prolylcarboxylpeptidase-knockout mice exhibited no alteration in diastolic and systolic blood pressures compared with the wild-type littermates (Figure S2A). To assess any mild alterations of blood pressure caused by prolylcarboxylpeptidase loss, arterial blood pressure was quantified by invasive hemodynamic assessment at the 6-month-old time point. However, we still could not detect any hypertensive phenotypes developed in 6-month-old prolylcarboxylpeptidase-knockout mice (Figure S2D). Consistently, no significant changes were detected in echocardiographic assessments (Figure S2B, S2C, S2E, and S2F).

Prolylcarboxylpeptidase Deficiency Accelerates Hypertrophic Remodeling in Response to Long-Term Angiotensin II Infusion

Prolylcarboxylpeptidase was previously proposed to possess a negative regulating function of angiotensin

II.[16] Thus, to determine the functional significance of prolylcarboxylpeptidase to the angiotensin II signaling cascades, wild-type and prolylcarboxylpeptidase-knockout littermates were subjected to 4 weeks of angiotensin II infusion (2 mg/kg per day^{-1}). Both the tail-cuff method and invasive hemodynamic assessment showed that although angiotensin II infusion for 28 days resulted in dramatic increases in diastolic and systolic blood pressures in both wild-type and prolylcarboxylpeptidase-knockout mice compared with vehicle groups, angiotensin II–treated groups did not elicit significant differences in their blood pressure (Figure 2A and Figure S3A). Despite this, after 4 weeks of angiotensin II infusion, we found significantly enhanced left ventricular hypertrophy in angiotensin II–treated prolylcarboxylpeptidase-knockout mice, indicated by a significant increase in heart weight over tibial length ratio and the cardiac hypertrophic index (Figure S3B). In addition, the quantification of the cross-sectional area of cardiomyocytes by wheat germ agglutinin staining showed that greater cell size was present in angiotensin II–treated prolylcarboxylpeptidase-knockout mice compared with angiotensin II–treated wild-type (Figure 2B). Moreover, the expressions of the hypertrophic gene markers atrial natriuretic peptide and brain natriuretic peptide were significantly upregulated in prolylcarboxylpeptidase-knockout mice in comparison with the wild-type group (Figure S3C and S3D). Additionally, echocardiographic M-mode assessments revealed that the left ventricular end-diastolic posterior wall was also significantly increased in angiotensin II–treated prolylcarboxylpeptidase-knockout mice compared with angiotensin II–treated wild-type mice, indicating the greater concentric remodeling in the prolylcarboxylpeptidase-knockout mice (Figure 2C). Notably, within 4 weeks of angiotensin II infusion, prolylcarboxylpeptidase-knockout mice showed greater diastolic dysfunction, assessed by transmitral Doppler filling, than wild-type mice since the second week, indicated by significant increases in the E/A ratio and left ventricle isovolumetric relaxation time (Figure 2D, Figure S3E). However, contractile function, determined by fractional shortening, remained unchanged among groups within the first 2 weeks, and that started to progressively decrease from the third week. As a result, the prolylcarboxylpeptidase-knockout mice had dramatically lower fractional shortening compared with wild-type littermates after 4 weeks of angiotensin II infusion (Figure 2E). In accordance with the exacerbation of angiotensin II–mediated pathological effects in prolylcarboxylpeptidase-knockout mice, Masson's trichrome staining demonstrated that interstitial fibrosis area in the angiotensin II–treated prolylcarboxylpeptidase-knockout mice was almost doubled compared with the angiotensin II–treated wild-type littermates (Figure 2F). Consistently, upregulation of the transcripts of the fibrosis marker genes procollagen type 1α, procollagen type 3α, and transforming growth factor-beta 1 were significantly upregulated in prolylcarboxylpeptidase-knockout ventricles (Figure S3F and S3H).

To delineate whether prolylcarboxylpeptidase is required for angiotensin II–induced cardiomyocyte hypertrophy, prolylcarboxylpeptidase expression in adult rat cardiomyocytes was knocked down by small interfering RNA for 48 hours (Figure S4A). Then, the cells were treated with angiotensin II (100 nmol/L) for 48 hours. The result demonstrated that the knockdown of prolylcarboxylpeptidase led to an increase in the cell size in adult rat cardiomyocytes in response to angiotensin II, showing a significantly increased cell surface area concomitant with an ≈1.5-fold upregulation of atrial natriuretic peptide expression relative to controls (Figure S4B through S4D). These findings together suggest that prolylcarboxylpeptidase is involved in cardioprotection against angiotensin II–induced hypertrophy.

Subcellular Localization of Prolylcarboxylpeptidase and an Excess of Myocardial Angiotensin II Level in Prolylcarboxylpeptidase-Knockout Hearts

Prolylcarboxylpeptidase was previously reported to localize mainly in lysosomes, where it has the capability of degrading angiotensin II in an acidic condition.[12,15,16] Therefore, to discern whether loss of prolylcarboxylpeptidase will lead to alterations in angiotensin II and angiotensin 1-7 levels in response to angiotensin II stress, we first confirmed the localization of prolylcarboxylpeptidase in cardiac lysosomes where lysosomal-associated membrane protein 1 protein was used as a marker. As expected, coimmunofluorescent staining showed prolylcarboxylpeptidase is colocalized with lysosomal-associated membrane protein 1 in lysosomes (Figure 3A). This was further confirmed by examining prolylcarboxylpeptidase expression in the isolated cardiac lysosomes. Interestingly, prolylcarboxylpeptidase does not solely reside in lysosomes but also in the cytosol (Figure 3B). Next, we employed ELISA assays to measure alterations of angiotensin II and angiotensin 1-7 in plasma and myocardial tissues. After 4 weeks of exogenous angiotensin II infusion, angiotensin II and angiotensin 1-7 significantly increased in both plasma and cardiac tissues in both wild-type and prolylcarboxylpeptidase-knockout mice (Figure 3C through 3F). However, prolylcarboxylpeptidase deficiency did not cause any significant change in angiotensin II and angiotensin 1-7 concentrations in plasma in prolylcarboxylpeptidase-knockout mice compared with wild-type mice (Figure 3C and 3D). In contrast, prolylcarboxylpeptidase-knockout myocardial tissues displayed a significant increase in angiotensin II level compared with wild-type hearts (Figure 3E). To

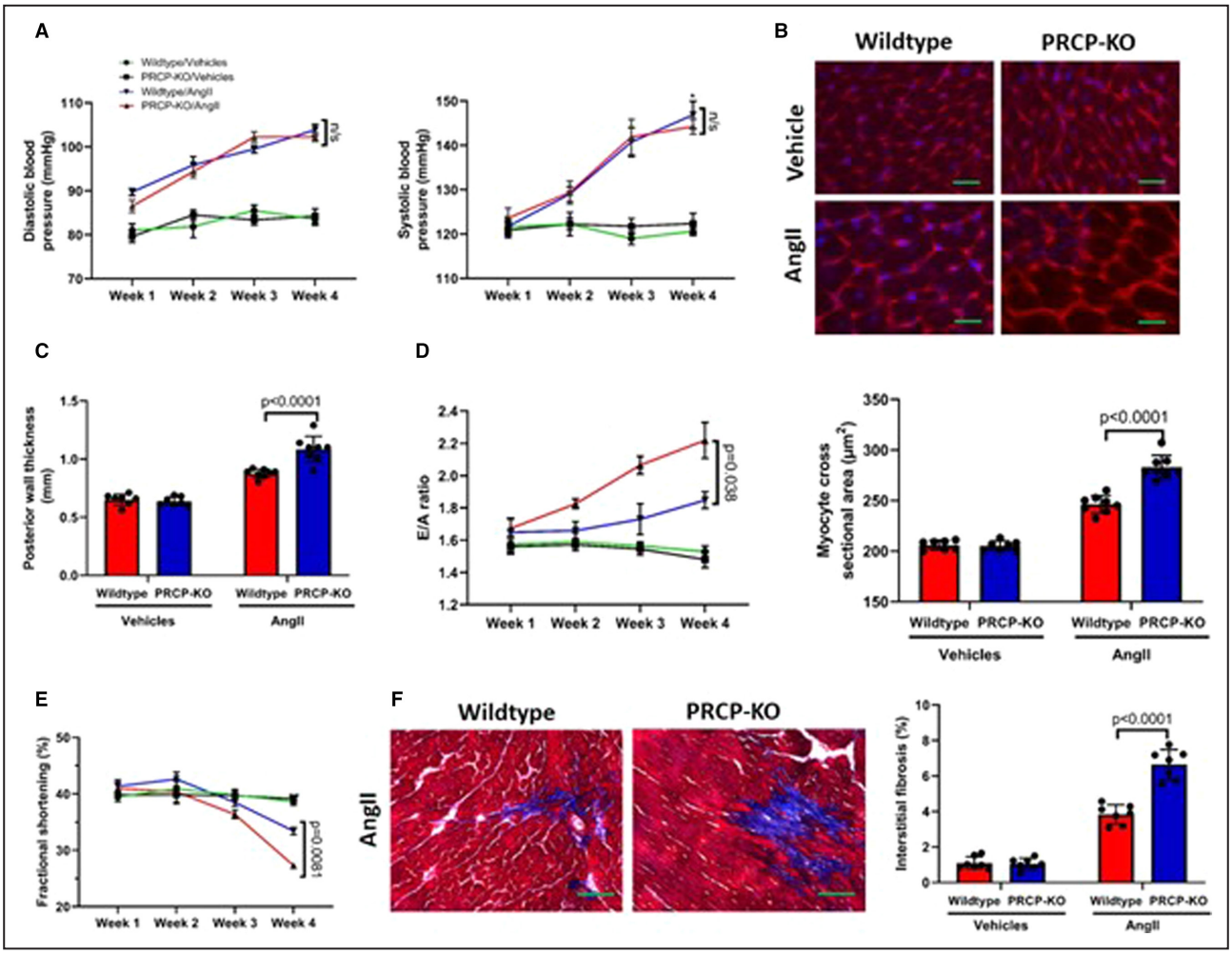

Figure 2. Prolylcarboxylpeptidase deficiency led to reduced cardiac function with augmented hypertrophic remodeling upon 4 weeks of angiotensin II infusion.

A, Diastolic and systolic blood pressure after 4 weeks of angiotensin II infusion. **B**, WGA staining of heart cross-sections (scale bar: 50 μm). **C**, Left ventricle end-diastolic posterior wall. **D**, Mitral Doppler showed the ratio of peak velocity blood flow from left ventricular relaxation in early diastole (the E wave) to peak velocity flow in late diastole caused by atrial contraction (the A wave) indicated worsen diastolic function in prolylcarboxylpeptidase-knockout mice. **E**, Fractional shortening (%) was significantly decreased in prolylcarboxylpeptidase-knockout mice. **F**, Masson's trichrome-staining of cross-sections showed significantly increased interstitial fibrosis in prolylcarboxylpeptidase-knockout mice compared with the same cohort of control mice (scale bar: 30 μm), followed by quantification of fibrosis area against total tissue area (n=8 mice per group). Data are expressed as means±SEM. Two-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. AngII indicates angiotensin II; PRCP-KO, prolylcarboxylpeptidase knockout; and WGA, wheat germ agglutinin.

explore the prolylcarboxylpeptidase-mediated angiotensin II conversion in vitro, small interfering RNA–mediated knockdown of prolylcarboxylpeptidase was performed in human induced pluripotent stem cells–derived cardiomyocytes, followed by 48 hours of angiotensin II treatment (100 nmol/L). Angiotensin II and angiotensin 1-7 levels from cell culture media and cell lysis were quantified by ELISA. Consistent with the above results, only the angiotensin II level from the prolylcarboxylpeptidase knockdown group was significantly higher in the control group (Figure S5A through S5D). Moreover, we assessed lysosomal acidic condition–dependent prolylcarboxylpeptidase function by treating induced pluripotent stem cell–derived cardiomyocytes with chloroquine (10 μmol/L), which is a lysosome inhibitor, by altering the acidic environment of lysosomes, for 24 hours, followed by 48 hours of angiotensin II treatment. Quantification of angiotensin II and angiotensin 1-7 levels from human induced pluripotent stem cell–derived cardiomyocytes showed that angiotensin II but not angiotensin 1-7 level was significantly increased in the group of chloroquine and angiotensin II treatment (Figure S5E through S5F). These observations suggest that lysosomal prolylcarboxylpeptidase might play an important role in the prevention of excessive accumulation of angiotensin II in the cardiomyocytes.

Increased Extracellular Signal-Regulated Kinases 1/2 Activity and Reduced Protein Kinase B/Endothelial Nitric Oxide Synthase Signaling Lead to Profound Cell Death in Prolylcarboxylpeptidase-Knockout Hearts

The angiotensin II pathway plays important role in the regulation of oxidative stress and cell death.[3] After 4 weeks of angiotensin II infusion, dihydroethidium staining showed that angiotensin II–treated prolylcarboxylpeptidase-knockout mice had a greater level of superoxide production than angiotensin II–treated wild-type mice (Figure 4A). Moreover, the accumulation of reactive oxygen species is known as a critical trigger for cardiomyocyte loss. As expected, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling staining demonstrated that prolylcarboxylpeptidase-knockout hearts displayed a pronounced increase in cell death, nearly twice more than that of wild-type hearts (Figure 4B and 4C). To confirm a higher level of cardiomyocyte loss, molecular pathways responsible for apoptosis were examined. Dramatic upregulation of proapoptotic proteins including p53 and Bax was observed, whereas the expression of antiapoptotic protein, B-cell lymphoma 2, was downregulated in the prolylcarboxylpeptidase-knockout hearts. In line with that, the enhanced activity of caspase 3 was detected in association with prolylcarboxylpeptidase deletion (Figure S6A).

To further dissect the mechanism by which loss of prolylcarboxylpeptidase enhances the hypertrophic response, we examined the expression and phosphorylation of several prohypertrophic MAPKs (mitogen-activated protein kinases) that are also downstream of the angiotensin II signaling pathway, including ERK1/2, MAPK kinase 7, MAPK kinase 4, and c-Jun N-terminal kinase 1/2. While we found the other MAPK proteins expression and phosphorylation levels, including MAPK kinase 4, MAPK kinase 7, c-Jun N-terminal kinase 1/2, remained unchanged between wild-type littermates and prolylcarboxylpeptidase-knockout mice, prolylcarboxylpeptidase-knockout hearts demonstrated a significant increase in phosphorylation of ERK1/2 at p44/p42 sites (Figure 4D and Figure S6B). In addition, a previous study has shown that prolylcarboxylpeptidase is critical for PI3K/PKB (phosphoinositide-3-kinase/protein kinase B) signaling pathway by stabilizing insulin receptor substrate 1 through which it can maintain survival in breast cancer cells.[17] Using Western blotting, we also discerned that phosphorylation levels of PKB at Ser437 and its downstream target, endothelial nitric oxide synthase, dramatically downregulated in prolylcarboxylpeptidase-knockout hearts compared with wild-type hearts (Figure 4D and Figure S6C and S6D). Taken together, our screening results suggest that while the high level of cell death detected in prolylcarboxylpeptidase-knockout hearts might be attributable to reduced PKB activities, the greater hypertrophic phenotypes might be due to increased ERK1/2 activities.

Adeno-Associated Virus Serotype 9–Mediated Overexpression of Prolylcarboxylpeptidase Attenuated Hypertrophic Remodeling in Prolylcarboxylpeptidase-Knockout Hearts

To investigate whether restoration of cardiac prolylcarboxylpeptidase expression would protect the heart from angiotensin II–induced hypertrophic stress, prolylcarboxylpeptidase-knockout mice were first injected with adeno-associated virus serotype 9 (AAV9)-prolylcarboxylpeptidase with a dose of 1×10^{11} viral genome per mouse via tail-vein route 3 days in advance, which was followed by 4 weeks of angiotensin II infusion (2 mg/kg per day), and AAV9-GFP (green fluorescent protein) was used as a control. The cardiac overexpression of exogenous prolylcarboxylpeptidase was regulated under the control of cardiac troponin T promoter to ensure cardiac specificity. The amount of AAV9-mediated prolylcarboxylpeptidase overexpression in the heart was validated by Western blot (Figure S7A through S7C). After 4 weeks of angiotensin II infusion, the blood pressures were not notably different between angiotensin II–treated groups (Figure 5A). Hence, overexpression of prolylcarboxylpeptidase did not detain raised blood pressures in response to angiotensin II.

ELISA assays on myocardial tissues showed that angiotensin II concentration was dramatically decreased by ≈30% in prolylcarboxylpeptidase-knockout/AAV9-prolylcarboxylpeptidase compared with that in prolylcarboxylpeptidase-knockout/AAV9-GFP mice (Figure 5B). However, overexpression of prolylcarboxylpeptidase had no effects on myocardial angiotensin 1-7, plasma angiotensin II, and angiotensin 1-7 concentrations in response to chronic angiotensin II infusion (Figure S8A through S8C).

Regarding cardiac morphology and function, prolylcarboxylpeptidase-knockout/AAV9-GFP mice showed a significant increase in heart weight/tibia length compared with prolylcarboxylpeptidase-knockout/AAV9-prolylcarboxylpeptidase mice (Figure S8D). Furthermore, a smaller cross-sectional area of cardiomyocytes was observed in prolylcarboxylpeptidase-knockout/AAV9-prolylcarboxylpeptidase mice, compared with prolylcarboxylpeptidase-knockout/AAV9-GFP mice (Figure 5C). Moreover, echocardiography demonstrated

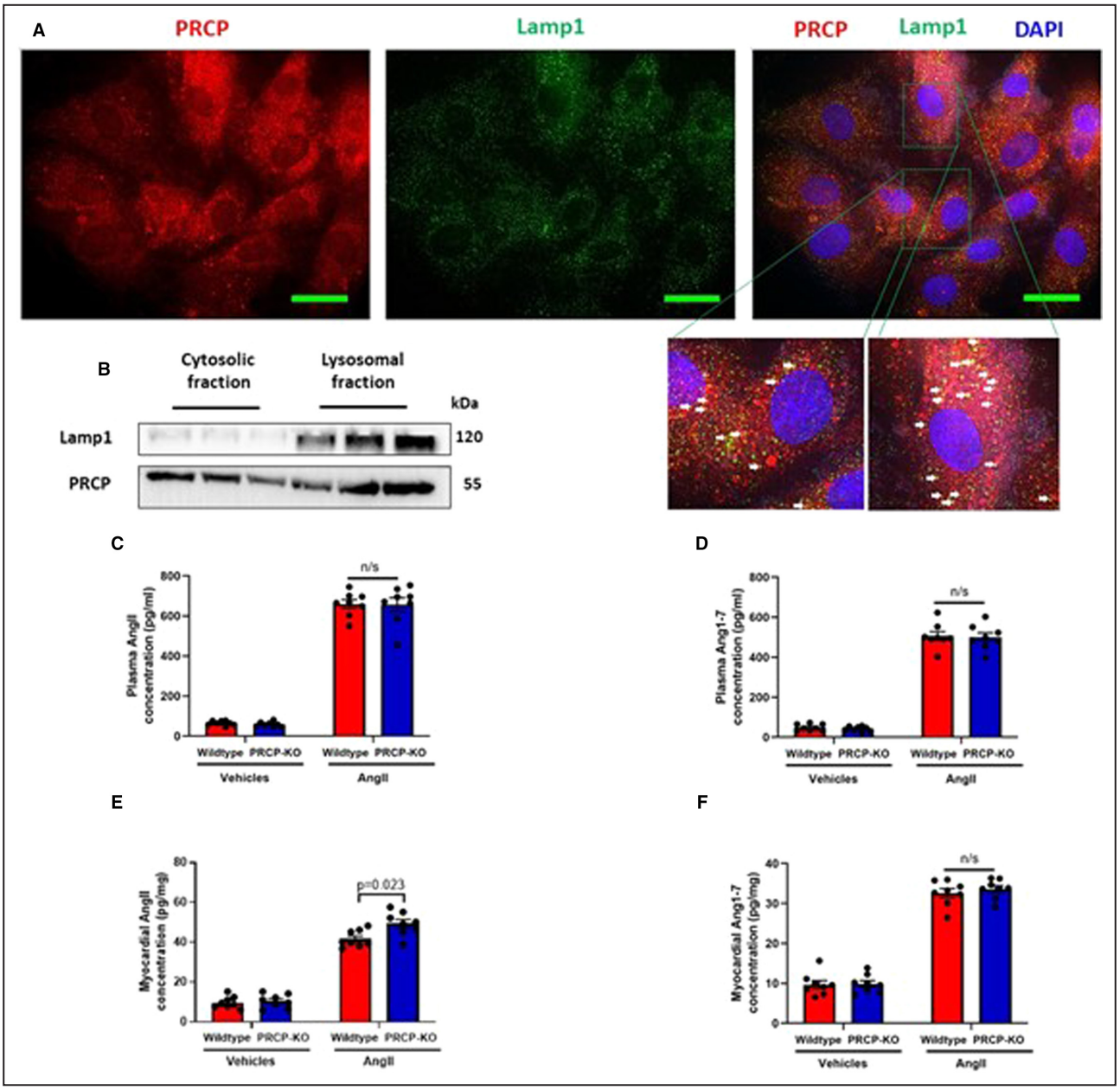

Figure 3. Prolylcarboxylpeptidase localizes in lysosomes and mediates intracardiac angiotensin II degradation through the lysosomal pathway.

A, Immunostaining of prolylcarboxylpeptidase (red) and Lamp1 (lysosome marker) in H9C2 cells shows prolylcarboxylpeptidase partially resides in lysosomes. White arrows indicate colocalization of prolylcarboxylpeptidase and Lamp1 (yellow dots) (scale bar: 15 μm). **B**, Cytosolic and lysosomal fractions of wild-type hearts show the localization of prolylcarboxylpeptidase in both cytosol and lysosome. Lamp1 is used as a marker for lysosomes. **C**, Angiotensin II levels in plasma. **D**, Angiotensin 1-7 levels in plasma. **E**, Angiotensin II levels in myocardial tissues. **F**, Angiotensin 1-7 levels in myocardial tissues (n=8 mice per group). Data are expressed as means±SEM. Two-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. AngII indicates angiotensin II; DAPI, 4′, 6-diamidino-2-phenylindole; Lamp1, lysosomal-associated membrane protein 1; PRCP, prolylcarboxylpeptidase; and PRCP-KO, prolylcarboxylpeptidase knockout.

that prolylcarboxylpeptidase-knockout/AAV9-prolylcarboxylpeptidase mice exhibited significant decreases in posterior wall thickness compared with the angiotensin II–treated prolylcarboxylpeptidase-knockout/AAV9-GFP mice (Figure S8E). Consistent with these results, the mRNA levels of the hypertrophic gene markers including atrial natriuretic peptide and brain natriuretic peptide were significantly decreased in prolylcarboxylpeptidase-knockout/AAV9-prolylcarboxylpeptidase mice in comparison

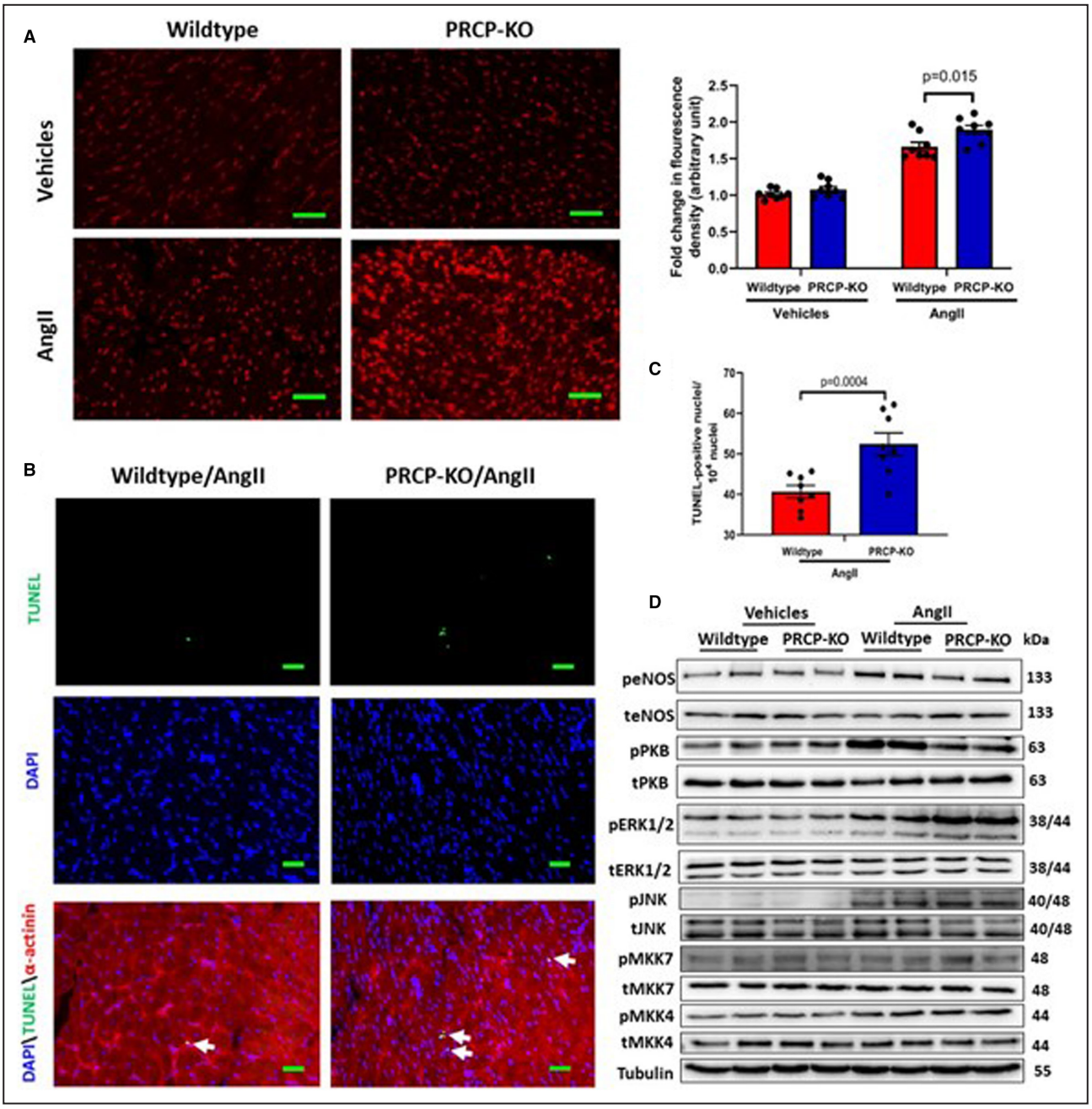

Figure 4. Prolylcarboxylpeptidase-knockout hearts revealed more reactive oxygen species production and apoptosis than wild-type hearts in response to 2 weeks of angiotensin II infusion.

A, Dihydroethidium staining was used to detect levels of reactive oxygen species production (scale bar: 30 μm), followed by quantification (n=8 per group). **B**, TUNEL staining assay was applied to detect levels of apoptotic cells in heart cross sections (scale bar: 30 μm). TUNEL: green; nuclei: blue/DAPI; a-actinin: red. Arrows indicate TUNEL-positive nuclei. **C**, The quantification of TUNEL positive nuclei is shown in bar graphs (n=7–8 per group). **D**, Immunoblot analysis shows expression levels of several mitogen-activated protein kinase proteins, including eNOS, pPKB, tPKB, pERK1/2, tERK1/2, pJNK, tJNK, pMKK7, tMKK7, pMKK4, tMKK4. Student's *t*-test or 2-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. AngII indicates angiotensin II; DAPI, 4′, 6-diamidino-2-phenylindole; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinases1/2; JNK, c-Jun N-terminal kinase; MKK4, mitogen-activated protein kinase kinase 4; MKK7, mitogen-activated protein kinase kinase 7; p, phhosphorylation; PKB, protein kinase B; PRCP-KO, Prolylcarboxylpeptidase knockout; t, total protein; and TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

with the controls (Figure S8F and S8G). Regarding cardiac function, cardiac overexpression of prolylcarboxylpeptidase alleviated the progression of diastolic dysfunction, which was indicated by the lower E/A ratio and isovolumetric relaxation time compared with prolylcarboxylpeptidase-knockout/AAV9-GFP mice

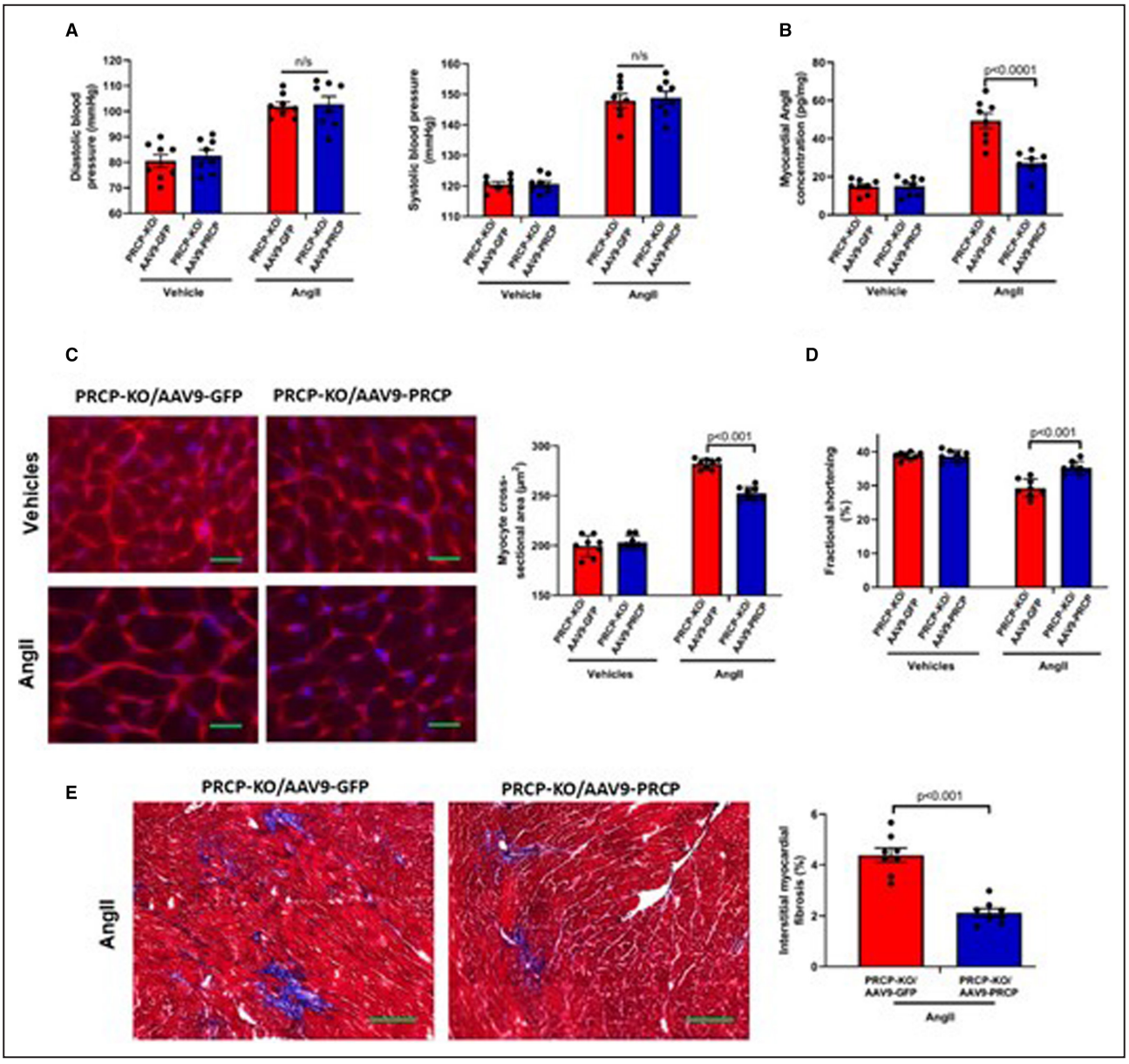

Figure 5. AAV9-mediated restoration of cardiac prolylcarboxylpeptidase expression alleviated the progression of angiotensin II–induced hypertrophic remodeling in the prolylcarboxylpeptidase-knockout heart.
A, Diastolic and systolic blood pressure. **B**, Myocardial angiotensin II concentration. **C**, Wheat germ agglutinin staining of heart cross-sections (scale bar: 50 μm). **D**, Echocardiographic assessment of fractional shortening %. **E**, Masson's trichome staining of representative myocardial sections (scale bar: 45 μm) and quantification of interstitial fibrosis. Data are presented as means±SEM. Student's *t*-test or 2-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. AngII indicates angiotensin II; AAV9, adeno-associated virus serotype 9; GFP, green fluorescent protein; PRCP, prolylcarboxylpeptidase; and PRCP-KO, prolylcarboxylpeptidase-knockout.

after 4 weeks of angiotensin II infusion (Figure S8H). Also, prolylcarboxylpeptidase-knockout/AAV9-prolylcarboxylpeptidase displayed improved contractility compared with prolylcarboxylpeptidase-knockout/AAV9-GFP mice (Figure 5D). Masson's trichrome staining illustrated less fibrosis in prolylcarboxylpeptidase-knockout/AAV9-prolylcarboxylpeptidase hearts (Figure 5E). Besides, prolylcarboxylpeptidase-knockout/AAV9-prolylcarboxylpeptidase hearts showed the downregulation of the fibrosis marker genes including procollagen type 1α and transforming growth factor-beta (Figure S8I).

In line with this, overexpression of prolylcarboxylpeptidase also attenuated the increases in oxidative stress and cardiomyocyte loss in response to chronic angiotensin II stress. After 4 weeks of

angiotensin II infusion, the number of terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling–positive nuclei in the heart of prolylcarboxylpeptidase-knockout/AAV9-prolylcarboxylpeptidase mice was almost half that in controls, indicating less cardiomyocyte apoptosis (Figure S8J). Our study further showed that the restoration of prolylcarboxylpeptidase in prolylcarboxylpeptidase-knockout hearts also blocked downstream effects of angiotensin II overactivation in which increased phosphorylated ERK1/2 and decreased phosphorylated PKB levels were reversed (Figure S8K). Together, prolylcarboxylpeptidase restoration was able to slow down the progress of cardiac hypertrophy and remodeling in response to angiotensin II stress by the control of myocardial angiotensin II level.

Combination Therapy of AAV9-Mediated Prolylcarboxylpeptidase Overexpression and Losartan Conferred More Cardioprotection Against Angiotensin II–Induced Cardiac Remodeling

Although the restoration of prolylcarboxylpeptidase expression could alleviate the progression of cardiac hypertrophic remodeling and improve cell survival in prolylcarboxylpeptidase-knockout hearts, it could not correct a high level of plasma angiotensin II and resultant hypertension in response to angiotensin II stress. In addition, angiotensin II can be produced and works at both intracellular and systemic levels, and the notion of the intracardiac angiotensin II regulation by prolylcarboxylpeptidase suggests that lone prolylcarboxylpeptidase use as the monotherapy might not provide a complete treatment regimen by blocking excess angiotensin II activities at different levels. Thus, we further hypothesized that the combination of the gene therapy-mediated prolylcarboxylpeptidase overexpression with other RAS-based antihypertensive drugs might reduce the susceptibility to angiotensin II–induced cardiac hypertrophy more effectively. To test this hypothesis, wild-type C57BL/6J mice were subjected to 4 weeks of angiotensin II (2 mg/kg per day) and of the treatment course, followed by either AAV9-GFP or AAV9-prolylcarboxylpeptidase plus losartan (50 mg/kg per day) at the fourth week. The AAV9 was injected 3 days in advance to allow sufficient overexpression of prolylcarboxylpeptidase, and losartan was delivered daily by intraperitoneal injections for 7 days (Figure S9A).

As expected, the wild-type mice that received either the losartan or the combination treatment groups exhibited a significant reduction in systolic blood pressure compared with other treatment groups (Figure 6A). This indicated that the losartan treatment attenuated the progressive development of hypertension. In addition, echocardiographic assessments showed that AAV9-mediated prolylcarboxylpeptidase overexpression or losartan treatment could reduce the hypertrophic growth of the posterior wall and mitigate the angiotensin II–induced diastolic and systolic dysfunction compared with angiotensin II–treated wild-type mice, and these beneficial effects tended to be further enhanced in the combination treatment despite no statistical significance indicated (Figure 6B and 6C, Figure S9B and S9C). In line with this, wheat germ agglutinin staining showed that the combination therapy tends to have a smaller cross-sectional area compared with the angiotensin II–treated monotherapy groups (Figure 6D). Masson's trichrome and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling staining showed that angiotensin II–treated wild-type hearts, which received the combination therapy, tended to display less fibrosis and apoptotic cells compared with those receiving a single AAV9-prolylcarboxylpeptidase gene therapy (Figure 6E).

Interestingly, while the AAV9-prolylcarboxylpeptidase-treated group showed comparable levels of plasma angiotensin II to the AAV9-GFP-treated group, the combination therapy could reduce the angiotensin II level in plasma. Moreover, prolylcarboxylpeptidase overexpression could alleviate the high level of angiotensin II in myocardial tissues, which was further relieved as combined with losartan (Figure 6F, Figure S9D). Collectively, the combination treatment of AAV9-mediated prolylcarboxylpeptidase overexpression and losartan might reduce the susceptibility to cardiac hypertrophy induced by angiotensin II more effectively than a single therapy via blood pressure control and myocardial angiotensin II level control. However, longer treatment is required to further validate the efficiency of the combination for the treatment of heart diseases and any potential side effects.

DISCUSSION

Prolylcarboxylpeptidase is a serine protease that can metabolize angiotensin II to generate angiotensin 1-7 in an acidic condition. With the support of novel technologies, we were able to create various in vivo models including the prolylcarboxylpeptidase-knockout mice and AAV9-mediated cardiac prolylcarboxylpeptidase overexpression mice to investigate the cardioprotective roles of prolylcarboxylpeptidase in response to angiotensin II–induced cardiac hypertrophy.

Although our prolylcarboxylpeptidase-knockout model is not a complete knockout likely due to mosaicism caused by a pronuclear injection of transcript form of CRISPR-associated protein 9, the pathological phenotypes observed in the heart are evident

compared with wild-type littermates. This highlights the physiological importance of prolylcarboxylpeptidase in the heart. Similar to the global ACE2 knockout model,[18] 2-month-old prolylcarboxylpeptidase-knockout mice did not exhibit hypertension and ventricular remodeling at a basal condition, so either the presence of

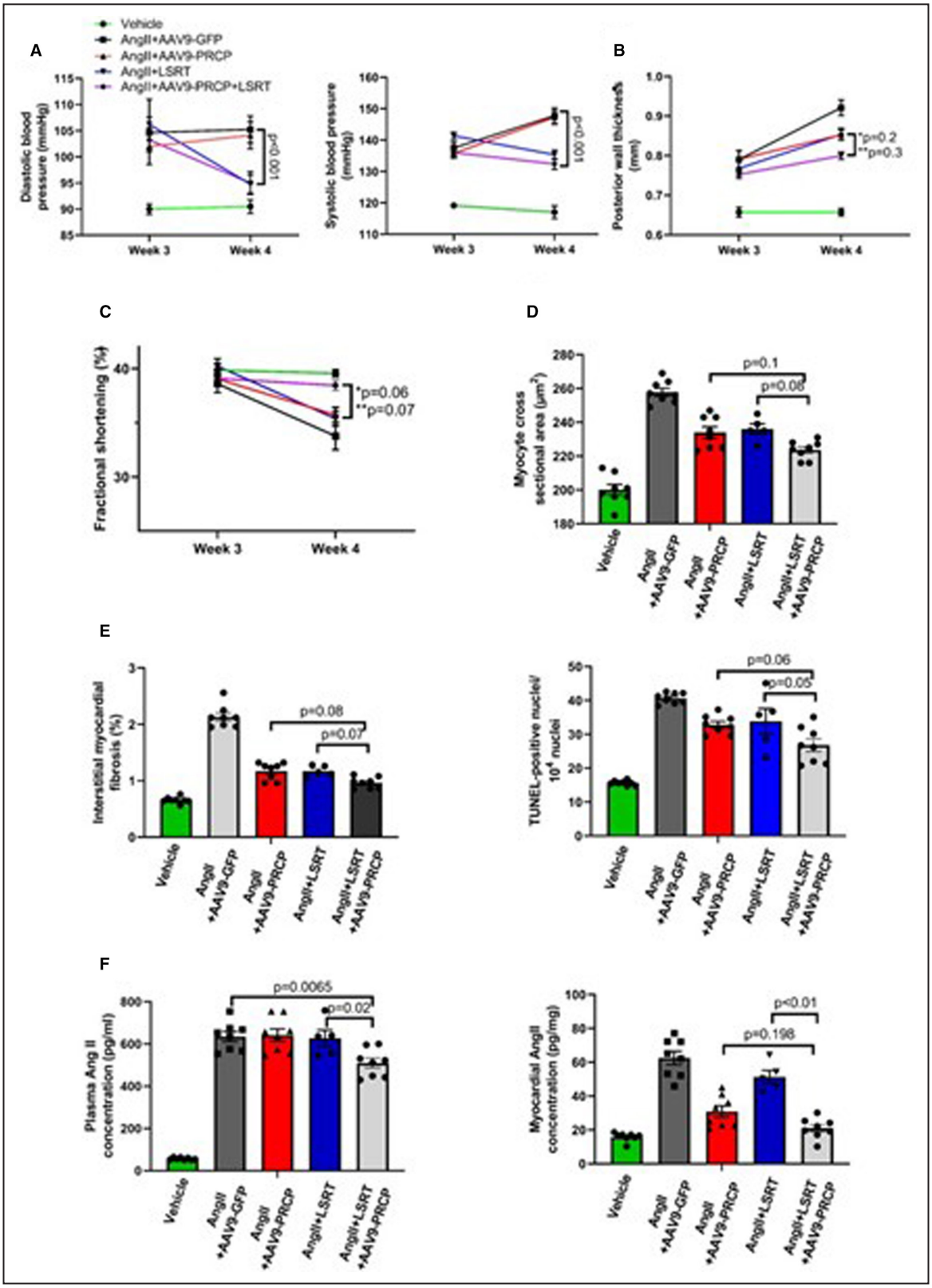

Figure 6. The combination therapy of AAV9-mediated prolylcarboxylpeptidase overexpression and LSRT gave better efficacy than the monotherapies to treat angiotensin II–induced cardiac malfunction.
A, Diastolic and systolic blood pressure. **B**, Left ventricle end-diastolic posterior wall. **C**, Echocardiographic assessment of fractional shortening percentage. **D**, Wheat germ agglutinin staining of heart cross sections. **E**, Masson's trichome staining for the quantification of interstitial fibrosis and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay to detect levels of apoptotic cells. **F**, Angiotensin II levels in plasma and myocardial tissues. n=5 for angiotensin II + LSRT group and n=8 for other groups. Data are presented as means±SEM. One- or 2-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. **P* value: angiotensin II + AAV9-prolylcarboxylpeptidase vs angiotensin II + AAV9-prolylcarboxylpeptidase + LSRT; ***P* value: angiotensin II + LSRT vs angiotensin II + AAV9-prolylcarboxylpeptidase + LSRT. AAV9 indicates adeno-associated virus serotype 9; angiotensin II, angiotensin II; GFP, green fluorescent protein; LSRT, losartan; and PRCP, prolylcarboxylpeptidase.

prolylcarboxylpeptidase or ACE2 expression is sufficient to maintain the physiology of the heart. In this study, noninvasive tail-cuff and invasive hemodynamic assessments showed that prolylcarboxylpeptidase-knockout mice did not display a significant difference in blood pressure compared with the wild-type littermates. This phenotype seems to be contradictory to a previous finding by Adams et al,[9] where blood pressure was detected by telemetry and showed notably higher blood pressure in the 6-month-old prolylcarboxylpeptidase$^{gt/gt}$ mice compared with the wild-type mice. However, they did not describe the phenotypes of younger mice. Interestingly, we observed that 4 weeks of angiotensin II infusion did not induce a significant difference in blood pressure between prolylcarboxylpeptidase-knockout and wild-type mice despite the hypertension phenotypes observed, which is consistent with a previous result by Maier et al.[12] The difference in basal blood pressure between our results and previous findings could be explained by the genetic background of the mouse strain used to produce the lines. While prolylcarboxylpeptidase$^{gt/gt}$ mice were generated with 129svj background, our prolylcarboxylpeptidase-knockout mice were produced from the DBA/2J strain. It has been previously reported that 129svj mice develop higher blood pressure compared with C57 mice, whereas blood pressure and cardiac function are within the normal range in the DBA/2J mice.[19,20]

In addition, our data showed that prolylcarboxylpeptidase expression was significantly increased after 2 weeks of angiotensin II induction despite a decrease in ACE2 expression, which was consistent with previous results.[18] Some studies have reported that either overexpression of angiotensin type 2 receptor (AT2R) or stimulating AT2R with the agonist CGP42112A increased prolylcarboxylpeptidase expression, so AT2R is required for prolylcarboxylpeptidase upregulation, which might be necessarily important during a compensatory stage of cardiac hypertrophy.[21,22] Moreover, the downregulation of both ACE2 and prolylcarboxylpeptidase expression after 4 weeks of angiotensin II infusion led to deleterious function in both diastole and systole. Thus, the presence of both ACE2 and prolylcarboxylpeptidase is essential for maintaining cardiac functions under stress conditions.

Prolylcarboxylpeptidase Degrades Angiotensin II at the Cellular Level

The synergetic actions of both ACE2 and prolylcarboxylpeptidase for the metabolism of angiotensin II are essential for preserving the heart function upon angiotensin II stress. However, the exact mechanism remains elusive. ACE2 is classified as an ectoenzyme that is found to be mainly membrane bound and works optimally at a neutral pH.[12] Membranous ACE2 has a catalytic site exposed outward to the extracellular surface, where angiotensin II conversion happens.[23] Loss of ACE2 leads to increased angiotensin II concentration in both plasma and myocardial tissues.[18] In contrast, prolylcarboxylpeptidase is not only found in the membrane and plasma but is also found to locate predominantly in lysosomes, where an acidic pH condition is optimum for its angiotensin II–cleaving activity.[15,24,25] This might suggest an alternate mechanism of metabolizing angiotensin II in the heart, in which prolylcarboxylpeptidase is likely to degrade angiotensin II intracellularly through a lysosomal pathway rather than extracellularly like ACE2. Consistently, our biochemical analysis demonstrated that loss of prolylcarboxylpeptidase results in increased angiotensin II levels but not angiotensin 1-7 in myocardial tissues. No change in angiotensin 1-7 levels suggests that lysosomal angiotensin II degradation may also lead to angiotensin 1-7 degradation. However, the mechanism of how lysosomal prolylcarboxylpeptidase regulates myocardial angiotensin II level has not been fully elucidated. Several studies showed that the angiotensin II could be degraded through the β-arrestin1/2–dependent desensitization and endocytosis of the binding complex of angiotensin II and AT1R.[26,27] Furthermore, other studies showed that the AT1R–angiotensin II complex was dissociated after endocytosis, in which AT1R is recycled and angiotensin II is transferred to lysosomes via microautophagy.[28,29] Thus, lysosomal prolylcarboxylpeptidase might be important in the regulation of angiotensin II circulating in the cardiac microenvironment through AT1R internalization. Additionally, lysosomal

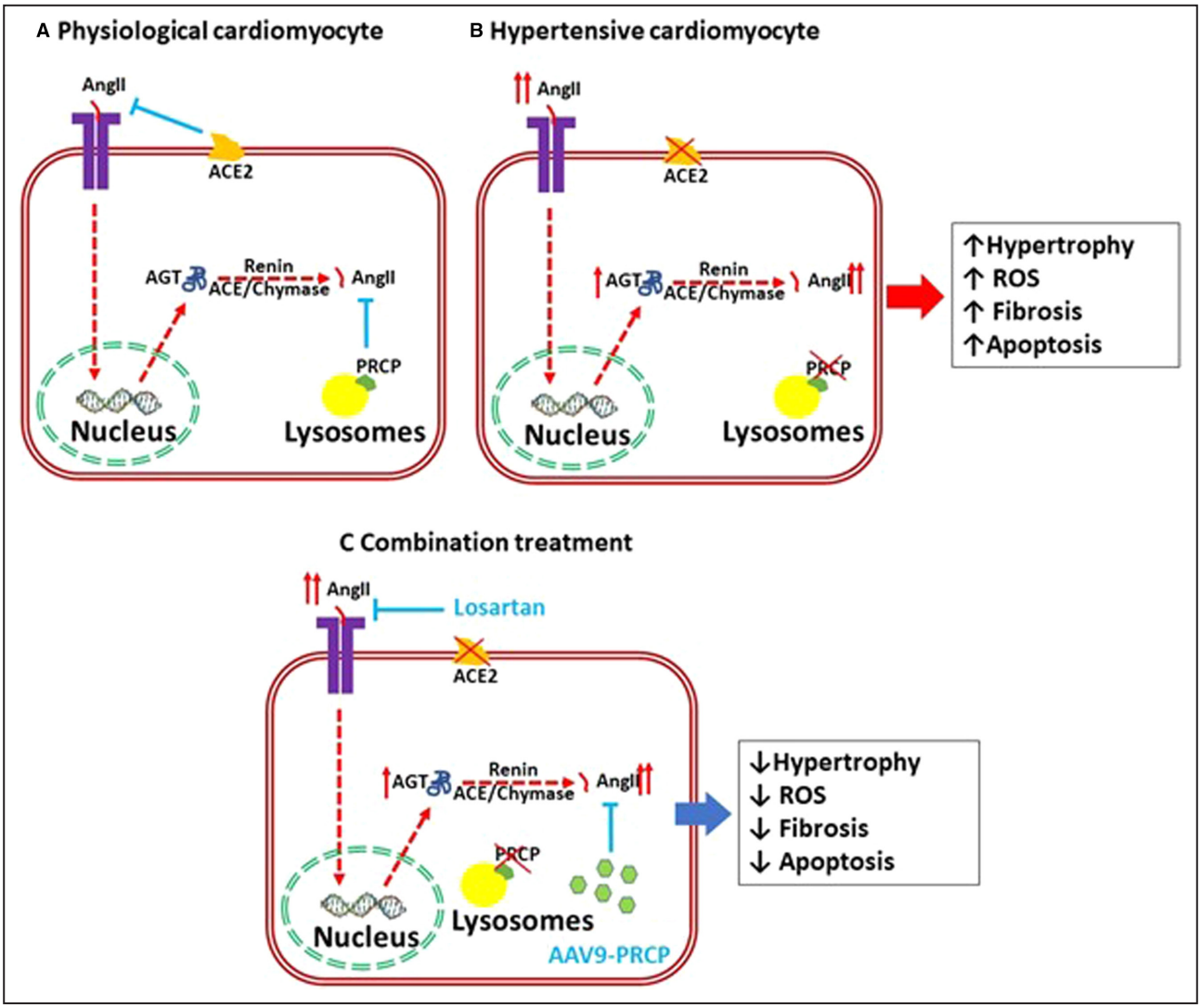

Figure 7. Schematic figure proposing prolylcarboxylpeptidase cardioprotection against hypertensive cardiac remodeling. **A**, At a normal state, the cardiac function is regulated by angiotensin II, which is produced in the circulatory system and in cardiomyocytes. ACE2 and prolylcarboxylpeptidase work synergically to protect the hearts from the overaction of angiotensin II at both systemic and intracellular levels. **B**, In hypertensive diseases, the angiotensin II levels, however, are significantly upregulated due to loss of cardiac ACE2 and prolylcarboxylpeptidase expressions, leading to the development of hypertrophic remodeling and heart failure. **C**, In this study, we proposed that a combination treatment of AAV9-mediated overexpression of prolylcarboxylpeptidase and losartan could ameliorate cardiac functions by improving the regulation of angiotensin II at both systemic and intracellular levels. AAV9 indicates adeno-associated virus serotype 9; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AGT, angiotensinogen; AngII, angiotensin II; and ROS, reactive oxygen species.

prolylcarboxylpeptidase might be critical in regulating intracardiac angiotensin II. Increasing evidence supports the notion that some organs, such as hearts and kidneys, express different components of the RAS, leading to the local angiotensin II synthesis.[13,30] Moreover, angiotensin II acts not only as an endocrine/paracrine factor but also as an intracrine peptide, with the ability to trigger signaling cascades in the cell, without stimulating plasma membrane receptors.[31,32] Interestingly, Abadir et al[14] described a functional mitochondrial angiotensin system in a variety of cells of human and mouse origin, such as skeletal muscle cells, mouse cardiac myocytes, renal tubular cells, neuronal cells, and hepatocytes, where the colocalization of endogenous angiotensin II and AT2R on the inner mitochondrial membrane was reported to regulate mitochondrial NO production. Moreover, another study confirmed the presence of both AT1R and AT2R in mitochondria from dopaminergic neurons by immunofluorescence and confocal microscopy.[33] Interestingly, a recent study has shown that prolylcarboxylpeptidase plays an important role in mitophagy.[32] These indicate that prolylcarboxylpeptidase might negatively regulate the angiotensin II level inside cardiomyocytes

through the mitophagy pathway, where mitochondrial membrane-bound angiotensin II could be delivered to the lysosome and degraded there.

Our findings demonstrated that loss of prolylcarboxylpeptidase leads to increased cell death, which is an important indicator of cardiac remodeling. An increase in apoptosis is directly derived from elevated reactive oxygen species production. Interestingly, prolylcarboxylpeptidase-knockout hearts showed less PKB activity upon the angiotensin II stress. Because the phosphoinositide-3-kinase/PKB signaling pathway is responsible for cardiomyocyte growth and survival at physiological conditions, impairment of PKB activity can reduce cell survival.[34,35] Moreover, another study demonstrated that prolylcarboxylpeptidase inhibition caused destabilization of insulin receptor substrate 1 in response to rapamycin-induced cytotoxicity, resulting in a disruption in the PKB pathway in pancreatic cancer cells.[17] In addition, several studies indicated that PKB is responsible for physiological cardiomyocyte growth and survival, so the reduced PKB activity might result in maladaptive responses.[36,37] In addition, our screening in prolylcarboxylpeptidase-knockout mice revealed greater levels of phosphorylated ERK1/2. The enhanced ERK1/2 phosphorylation in cardiomyocytes can promote the growth pathway in hearts, resulting in hypertrophic development.[38,39] It is worth mentioning that increased ERK1/2 could associate with increased p53 transcriptional activity, enhanced proapoptotic pathways, and inhibited antiapoptotic proteins.[40,41] Our data so far suggest that prolylcarboxylpeptidase might protect the heart against angiotensin II–mediated cardiac hypertrophy via its interaction with the RAS at the intracellular level. However, prolylcarboxylpeptidase is a multifunctional peptidase that targets other substrates, which might relate to the involvement of prolylcarboxylpeptidase in the regulation of Des-Arg^{9} -bradykinin, (Pyr)-apelin-13, and α-melanocyte-stimulating hormone 1–13.[42] Thus, it could not be excluded that our prolylcarboxylpeptidase-knockout mice might have alterations of these peptides, which could contribute to the development of observed phenotypes.

Therapeutic Potential of Prolylcarboxylpeptidase

To investigate the therapeutic potential of cardiac prolylcarboxylpeptidase, AAV9-mediated restoration of cardiac prolylcarboxylpeptidase expression in prolylcarboxylpeptidase-knockout hearts by AVV9 was first employed. The study revealed that restoration of cardiac prolylcarboxylpeptidase leads to mitigation of the development of hypertrophic remodeling of prolylcarboxylpeptidase-knockout hearts in response to angiotensin II infusion. This evidence reinforces our concept that maintenance of cardiac prolylcarboxylpeptidase expression holds a promising therapy for cardiovascular diseases.

Although the single use of gene therapy–mediated prolylcarboxylpeptidase overexpression could ameliorate cardiac performance and hypertrophic remodeling to a certain extent, it could not halt stressed hearts from progressing to failing hearts due to sustained systemic high blood pressure. To reinforce the therapeutic purpose of prolylcarboxylpeptidase in the treatment of cardiovascular diseases, we further designed the experiment as a proof-of-concept strategy that blocks angiotensin II action at both systemic and intracellular levels to improve the efficacy of the single use of classic anti-RAS drugs. We combined the cardiac-specific overexpression of prolylcarboxylpeptidase and losartan, a classic angiotensin type 1 receptor blocker, and investigated their efficacy compared with the lone treatment. In our study, losartan was chosen because it is more advantageous than an ACEi by not affecting the bradykinin pathway and having a better tolerability profile.[43–45] In contrast, ACEi has been shown to block the degradation of bradykinin, leading to an increase in bradykinin levels and troublesome side effects in patients such as dry cough and angioedema. Therefore, the combined use of prolylcarboxylpeptidase and ACEi might cause an overload of bradykinin levels and induce undesirable side effects. Additionally, angiotensin II breakthrough and aldosterone breakthrough are described as factors that potentially happen during long-term use of ACEis.[46] In addition, the generation of plasma and tissue angiotensin II by non–ACE-related enzymes such as chymase suggests that alternative options of complement therapy may be required in the future of the RAS-based new drug discovery. Our observations in the combinatory use of prolylcarboxylpeptidase overexpression and losartan against angiotensin II–induced cardiac hypertrophy provide the first line of evidence supporting a novel notion for the treatment of heart disease via limiting the pathological effects of an activated systemic and local RAS. However, our combination treatment was conducted for only 7 days, so this outcome might not reflect the complete picture of the long-term treatment. For example, a long-term losartan treatment might lead to an unprecedented surge of plasma angiotensin II, which was not detected in our experimental setting.[47] Hence, this work, hopefully, will inspire further studies to investigate the synergistic functions of each component of the RAS signaling pathway that can provide a refined therapeutic guideline.

CONCLUSIONS

The present study demonstrates that prolylcarboxylpeptidase exerts a cardioprotective effect against

angiotensin II stress-induced hypertrophic remodeling through the negative regulation of angiotensin II at the tissue level. This finding thus adds another level of complexity to the RAS signaling pathway in the heart. With chronic heart diseases, excessive angiotensin II levels can occur in both tissues and blood, and we believe that the treatment of a single agent, either an ACEi or angiotensin type 1 receptor blocker, might only delay the development of heart failure through the management of hypertension caused by systemic angiotensin II. Thus, to provide a more comprehensive therapy, we combined the gene therapy–mediated cardiac-specific prolylcarboxylpeptidase overexpression with losartan, an angiotensin type 1 receptor blocker, to evaluate whether this combination therapy could protect angiotensin II–induced stressed hearts more effectively. Hence, the synergetic actions of exogenous cardiac prolylcarboxylpeptidase and losartan have limited adverse myocardial remodeling through the controls of tissue angiotensin II level and hypertensive effects of plasma angiotensin II (Figure 7). We conclude that prolylcarboxylpeptidase plays an important role in the alleviation of myocardial hypertrophy, and the use of prolylcarboxylpeptidase represents a novel therapeutic strategy for cardiovascular diseases.

ARTICLE INFORMATION

Received October 4, 2022; accepted May 25, 2023.

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Sources of Funding

This study was supported by the British Heart Foundation (PG/17/31/32988 and PG/19/53/34499 to X. Wang).

Disclosures

None.

Supplemental Material

Data S1
Table S1
Figures S1–S9
References[48–50]

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SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

We used the ARRIVE reporting guidelines (Percie du Sert N, et al. The ARRIVE Guidelines 2.0: updated guidelines) for reporting animal research.

Generation of PRCP-knockout mice

For our project, we applied CRISPR/Cas9 technology to knockout *prcp* gene in mice. The gene knockout was mediated by either homology-directed repair (HDR) or non-homologous end joining (NHEJ) after a double-stranded break introduced by the CRISPR/Cas9 complex. Firstly, guideRNA (gRNA) with a target sequence (5'-ATTACTCGGGGCCGCCACAA-3') was designed to target exon 1 of the *PRCP* gene. Then, a two-vector system encoding a Cas9 endonuclease (Plasmid #44758) and the gRNA (Plasmid #51132) underwent *in vitro* transcription (MEGAshortscript™ T7 Transcription Kit for gRNA vector and mMESSAGE mMACHINE® T7 ULTRA Transcription Kit for Cas9 vector) (Shen et al., 2013). RNAs of both were co-injected with the exon 1- targeted single-stranded DNA template, which contains homology sequences immediately upstream and downstream of the exon 1 to mediate HDR, into a pronuclear stage to generate a global PRCP-KO model. All the works were carried out at Transgenic Center in FBMH Research and Innovation, University of Manchester.

Animal works

All *in vivo* studies were blinded for both genotype and surgical procedure/treatment during the measurement and analysis stages. Only male mice aged between 9-10 weeks old were included in all in vivo experiments in this study. The animal studies were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and institution guidelines for laboratory animal research. Cervical dislocation followed by removal of the heart prior to permanent cessation of the circulation was used for euthanasia in mice. Rats were sacrificed by intraperitoneal injection of Pentobarbitone Sodium 20% w/v and 250µl Heparin Sodium (1000 U/ml). Cardiac hypertrophy was induced by subcutaneous administration of Angiotensin II (Sigma-Aldrich, A9525-10MG) at 2mg/g/day for 4 weeks via osmotic mini-pumps (Alzet, model 1004). The pumps were subcutaneously implanted in PRCP-KO mice and wildtype littermates. After mini-pump installation, buprenorphine (0.1mg/kg, subcutaneously) was administrated daily in the following 3 days to reduce pain and suffering.

Amounts of required Angiotensin-II were calculated based on body weight of individual animals. For the study of the combination therapy, wildtype mice C57BL/6J were purchased from a specific-pathogen free colony (Envigo) to be used in this study. A well-known blocker for AT1R, losartan (Sigma-Aldrich, PHR1602-1G) (50mg/kg/day) was delivered through intraperitoneal injection.

Human Heart Tissues

The study complies with the Declaration of Helsinki and was reviewed and approved by the ethics committee of the Technische Universität Dresden (Az.: EK 422092019 and EK EK 446122011). Left ventricle tissues were obtained from dilated heart failure patients (n=8), and tissue from non-failing donor hearts (n=4) served as reference. Details of human heart tissues are provided in the Table S1.

Echocardiography

Mice were anesthetized with 1.5% isoflurane and echocardiographic assessment of cardiac function was carried out. Transthoracic M-mode echocardiographic recordings were performed using an Acuson Sequoia C256 system (Siemens) ultrasound machine.

Non-invasive blood pressure measurement

The CODA 2-Channel Non-Invasive Blood Pressure system was used to measure blood pressure of conscious mice. The mice were acclimated to restraint and to tail-cuff inflation a day before the actual experiment. The restraint platform was maintained at approximately 33-34°C. The procedure used was followed via the manufacture's instruction manual.

Invasive Blood Pressure measurement by hemodynamic Analysis

Mice were anesthetized with Avertin (Sigma, 200 mg/kg) via intraperitoneal injection. We used a 1.4F pressure-volume catheter (SPR-839) following a protocol described previously[48].

Wheat germ agglutinin (WGA) staining

Hearts were fixed in paraformaldehyde (PFA), embedded in paraffin and sectioned at 5µm thick. Paraffin sections were deparaffinized and rehydrated. Then, the sections were incubated with WGA (Texas Red™-X Conjugate, W21405) (5µg/ml) for 1 hour at room temperature in a dark humid chamber. The sections were finally washed with PBS for 10 minutes and mounted with vectashield. The images were taken at 60x magnification, and for each heart, around sizes of 400 cells were quantified by ImageJ software.

TUNEL Assay

Hearts were fixed in PFA, embedded in paraffin and sectioned at 5µm thick. Paraffin sections were deparaffinized and rehydrated. TUNEL assay was performed to detect apoptotic cardiomyocytes using the in-situ Cell Death Detection kit (Roche, Cat: 11684817910). A total of 10,000 cardiomyocytes from random fields per heart were analyzed. The images were obtained by Zeiss Axioplan2 microscope and then analyzed by Image J software.

Masson's Trichrome Staining

Hearts were fixed in PFA, embedded in paraffin and sectioned at 5µm thick. Paraffin sections were deparaffinized and rehydrated. Next, the heart sections were fixed in Bouin's solution for two hours at room temperature following staining in Harris' haematoxylin for 5 minutes. Sections were washed in water and stained in red solution (90ml 1%(w/v) Biebrich Scarlet, 10ml 1%(w/v) Acid Fuchsin, 1ml Glacial Acetic acid) and then treated for 15 minutes in 2.5%(w/v) phosphomolybdic acid before being stained with aniline blue (Sigma) for 3 minutes. Slides were rinsed briefly before being treated in 1% acetic acid for 1 minute. Slides were subsequently dehydrated and cleared. Coverslips were mounted using Eukitt quick-hardening mounting medium (Sigma-Aldrich). The images were obtained by Zeiss Axioplan2 microscope and then analyzed by Image J software. Percentage of tissue area stained blue, was calculated and expressed as percentage of interstitial fibrosis.

Dihydroethidium Fluorescence (DHE) staining

DHE, an oxidative fluorescence dye, was used to detect levels of superoxide (O^{2-}) in heart tissues. Hearts were fixed in PFA, embedded in paraffin and sectioned at 5µm thick. Paraffin sections were deparaffinized and rehydrated. Then, the sections were incubated with DHE (Invitrogen™, D1168) diluted in PBS (20µM) at 37^0C with coverslips on for 30 minutes in a dark humid chamber. Fluorescence images were immediately taken with an Olympus microscope selected with Terex (red) channel. The red fluorescent intensity was measured using ImageJ software.

Construction and administration of adeno-associated virus.

The pSSV9-TnT-eGFP plasmid was modified by replacing GFP with Flag-tagged constitutively active PRCP cDNA. To obtain the recombinant AAV9 virus, HEK293T cells were co-transfected with an adenoviral helper plasmid (pDGΔVP), a pAAV2-9 Rep-Cap plasmid (p5E18-VD2/9) and an adeno-associated virus genome plasmids pSSV9-TnT-PRCP and pSSV9-TnT-eGFP. AAV9 viruses were purified and characterized as previously described[49]. Mice were anesthetized with 2% isoflurane and 1×10^{11} genomic particles were administered by tail vein injection.

Human induced pluripotent stem cell (iPSC)-derived cardiomyocytes

Human iPSCs were derived from peripheral blood cells and maintained with E8 medium (Thermo Fisher Scientific) on Geltrex-coated plates. Cardiomyocyte differentiation of iPSCs was performed as previously described[50]. iPSC-derived cardiomyocytes (iPSC-CMs) were cultured in cardiac culture medium (RPMI1640 HEPES Glutamax, B27) up to 120 days for maturation.

Adult rat cardiomyocytes (ARCMs) isolation and culture

Six weeks old, male, Wistar rats (150g) were sacrificed by intraperitoneal injection of 750µl Pentobarbitone Sodium 20% w/v (Pentoject) and 250µl Heparin Sodium (1000 U/ml, Wockhardt UK). The heart was excised and placed in an ice-cold perfusion buffer containing heparin (Hank's balanced salt solution, HBSS, Sigma), 10mM Butandione monoxime, 11mM glucose, 1mM $MgSO_4$; 10% heparin). Next, the heart was cannulated and mounted on a Langendorff perfusion apparatus and perfused with calcium-free digestion buffer containing Collagenase type II (65 U/ml, Worthington Biochemical) and protease from Streptomyces Griseus (0.28 U/ml, Sigma) for 20 minutes. After perfusion, the heart was carefully transferred into trypsin solution (4.4 U/ml Trypsin, Worthington; 70 U/ml DNase I, Worthington) and sliced into small pieces. Digestion buffer (115U/ml Collagenase type II, Worthington; 0.14 U/ml Protease from Streptomyces Griseus (Sigma), 4.4 U/ml Trypsin, Worthington and 70 U/ml DNase I, Worthington in perfusion buffer) was added and incubated in a 37^{o}C water bath for 20 minutes. Cell suspensions were passed through a 100µm filter (Corning). The filtered cell suspensions were centrifuged at 1200rpm for 3 minutes and the cell pellet was washed twice with wash buffer (1:1 perfusion buffer and ACCT media consisting in low glucose, GlutaMAX™, pyruvate, no HEPES DMEM, 1g BSA, 5mM L-carnitine, 2mM Creatine, 5mM Taurine and 100U/ml penicillin/1mg/ml streptomycin). Afterwards, the cell suspension was slowly pipetted on top of a BSA solution (1mM BSA in ACCT media) and left to pass through the gradient for 40 minutes to remove dead cells and non-cardiomyocytes. The resultant cells were resuspended in ACCT media containing blebbistatin (20µM, Abcam) and plated on plates coated with Geltrex (Gibco, LDEV-Free, hESC-qualified Reduced Growth Factor Basement Membrane).

Real-time and quantitative PCR

Total RNA was isolated from ventricular tissues or cells using TRIzol (Invitrogen; Carlsbad, CA, USA). Total RNA (1µg) was reverse-transcribed to cDNA using LunaScript® RT SuperMix Kit (NEB #E3025). qPCR reactions were performed in triplicate with SYBR Select PCR Master Mix according to the manufacturer's instruction in the Step One Plus PCR System (Applied Biosystems). The fold change was estimated by the $2^{-\Delta\Delta CT}$ relative quantification method, using Gapdh for normalization.

Lysate preparation and immunoblotting

Total protein from tissues or cells was obtained with Triton lysis buffer (Tris 20 mM, NaCl 137 mM, EDTA 2 mM, 1% Triton X-100, β-glycerophosphate 25 mM, Na_3VO_4 1 mM, phenylmethanesulfonylfluoride 1 mM, aprotinin 1.54 μM, leupeptin 21.6 μM, 10% glycerol; pH 7.4). Protein concentration was determined by Bio-Rad protein assay. Protein extracts (20 μg) were subject to immunoblot analyses with under reducing conditions on SDS–polyacrylamide gels, transferred to nitrocellulose membranes, and detected with PRCP (biorbyt, orb538025), ACE2 (Santa Cruiz, AC18Z), MKK7(Cell signalling, 4172), pMKK7(Cell signalling, 4171), JNK(Cell signalling, 9252), pJNK(Cell signalling, 4668), MMK4 (Cell signalling, 9152), MKK6 (Cell signalling, 8550), peNOS (Cell signalling, 9570), eNOS (Cell signalling, 32027), pPBK (Cell signalling, 4060), PKB (Cell signalling, 4691), pERK1/2 (Cell signalling, 9101), ERK1/2 (Cell signalling, 4695), GAPDH (Abcam, ab8245), α-tubulin(Sigma-Aldrich, T5168), p53 (Santa Cruz Biotechnology, sc-126), Bax (Cell signalling, 14796), Bcl2 (Cell signalling, 3498), caspase 3 (Cell signalling, 9662), cleaved caspase 3 (Cell signalling, 9664)**.** The membranes were developed with enhanced-chemiluminescence (ECL) detection reagent.

siRNA-mediated knockdown of gene expression

Lipofectamine™ LTX Reagent with PLUS™ Reagent (Invitrogen, cat 15338100) was employed to transfect siRNA into cardiomyocytes following manufacture's instruction. siRNA with transfection reagents was incubated with cardiomyocytes in 37^{o}C incubator for 6h to allow sufficient times for siRNA getting into cells, and then, the new media was changed to avoild reagents-induced toxicity. The cells were kept for 2 days to allow adequate knockdown of gene expression for futher analyses. siRNA for PRCP knockdown was ON-TARGETplus SMARTpool PRCP siRNA (Dharmacon, L-083294-02). siRNA was prepared following manufacture's instruction.

Immunofluorescent staining

Immunofluorescent staining H9C2 cells or primary cardiomyocytes were seeded onto sterile coverslips. After treatments, cells were fixed in 4% PFA. Cells were permeabilized in 0.2% triton X-solution and blocked with 10% normal donkey serum (Stratech). Fixed cells were incubated with primary antibody (1:200 dilution) against PRCP (biorbyt, orb538025) or α-actinin (Sigma-Aldrich, A7732). Secondary anti-rabbit antibody (Stratech, 1:1000) conjugated to Alexa Fluor®488 or Alexa Fluor®546 was used to detect PRCP or α-actinin. DAPI was used for nuclear visualization. Images were collected on an Olympus fluorescence microscope. To exclude the possibility of false positive by

the primary antibodies, IgG (Cell Signaling, 2729) was used as the control antibody. IgG did not detect any positive staining, which was not represented.

Lysosome isolation

Lysosomes were isolated using a lysosome isolation kit (ab234047, Abcam) according to the manufacturer's protocol. Briefly, ventricular tissues were homogenised in ice-cold lysosome isolation buffer for 2 min. The supernatant was collected by centrifugation (500g, 10 min) at 4°C followed by layering onto discontinuous density gradient. The lysosomes were then purified by ultracentrifuge at 145,000g at 4°C for 2 hours.

ELISA for detecting AngII and Ang1-7 levels

Serum was prepared by collecting blood in EDTA-precoated tubes. Blood cells were then removed from plasma by centrifugation for 10 minutes at 1,000–2,000xg using a refrigerated centrifuge. The ventricular tissues lysates were prepared as described above. AngII and Ang1-7 were detected by Angiotensin II EIA Kit (Sigma-Aldrich, RAB0010) and Angiotensin (1-7) ELISA Kit (Aviva Systems Biology, OKEH02599) according to the manufacturer's protocol.

Table S1. Details of human heart tissue.

		Age	Sex	Disease
control		n.a.	n.a.	Organ donor without known disease
		n.a.	n.a.	Organ donor without known disease
		n.a.	n.a.	Organ donor without known disease
		n.a.	n.a.	Organ donor without known disease
Patients with HTX		62	male	DCM, EF 20%, type 2 diabetes
		45	male	DCM, EF 20%, MR grade II, TR grade II
		18	male	DCM, EF 15-20%, MR grade III-IV, TR grade II
		43	male	DCM, EF 20%, MR grade II, TR grade III
		64	male	DCM, EF 20%, type 2 diabetes
		60	male	DCM, EF 20%, MR grade I, TR grade II, type 2 diabetes
		49	male	DCM, EF 20%, MR grade III, type 2 diabetes
		43	male	DCM, EF 15%, MR grade II-III, TR grade III, type 2 diabetes

DCM: dilated cardiomyopathy; EF: ejection fraction; HTX: heart transplantation; LV: left ventricle; MR: mitral regurgitation; TR: tricuspid regurgitation; n.a.: not available

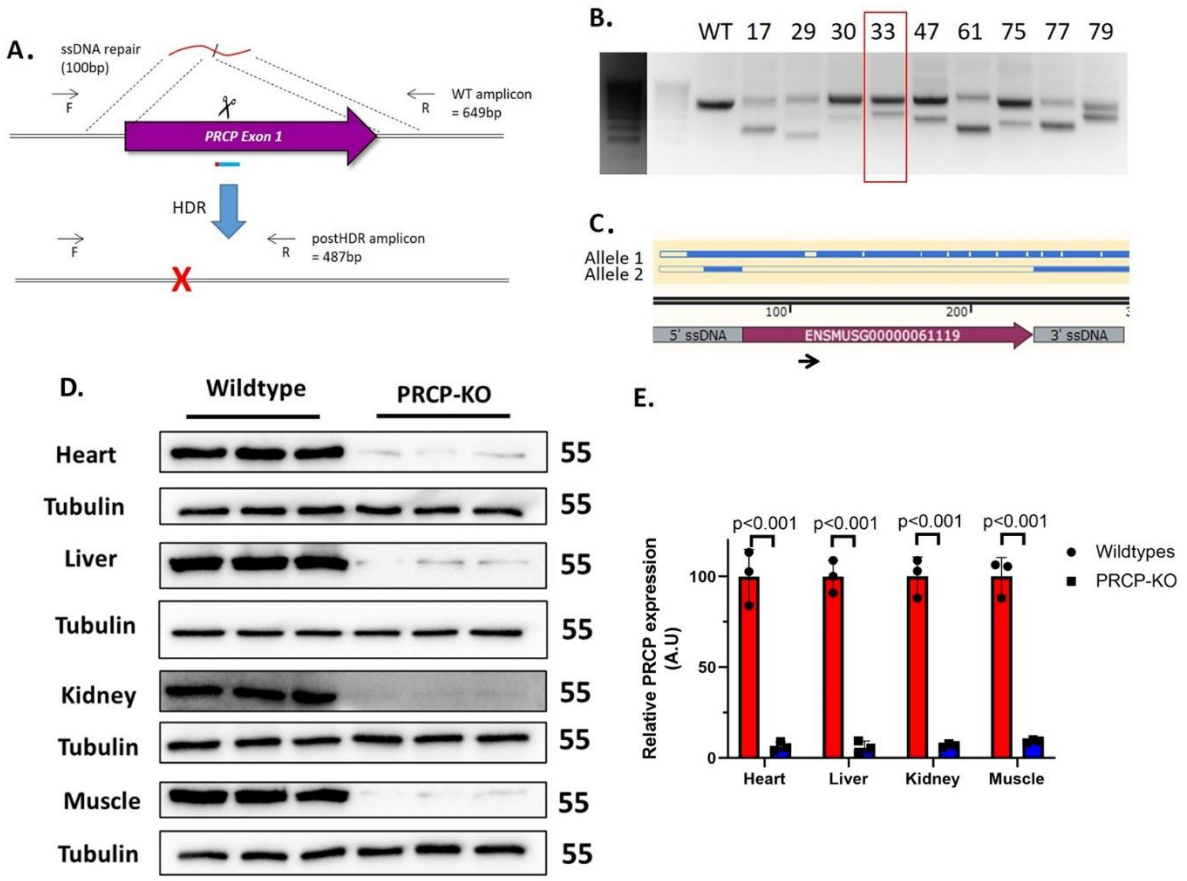


Figure S1: Generation of the PRCP knockout (PRCP-KO) mouse. (A) Targeting design. Schematic indicating the sgRNA specific to Exon 1 of the Prcp gene, and a ssDNA oligo repair template designed to excise the full exon by HDR. **(B)** PCR based genotyping of candidate pups reveals a range of editing outcomes. Pup 33 was carried forward for further analysis. **(C)** Sanger sequencing of the alleles of Pup 33, when aligned to WT sequences, reveals a small -6bp Deletion on allele 1 (discarded), and HDR mediated deletion of the full 162bp exon on allele 2. Mice harbouring the exon deletion allele 2 were bred forward and a colony established. **(D** and **E)** Western blot followed by quantifications shows global deletion of PRCP expression (>90%) of several organs in PRCP-KO mice including hearts, kidney, liver, and muscle. Tubulin served as the loading control. n=3 per group. Data are presented as means±SEM. Student t test was used for analysis. PRCP: Prolylcarboxylpeptidase, HDR: Homology-Directed Repair.

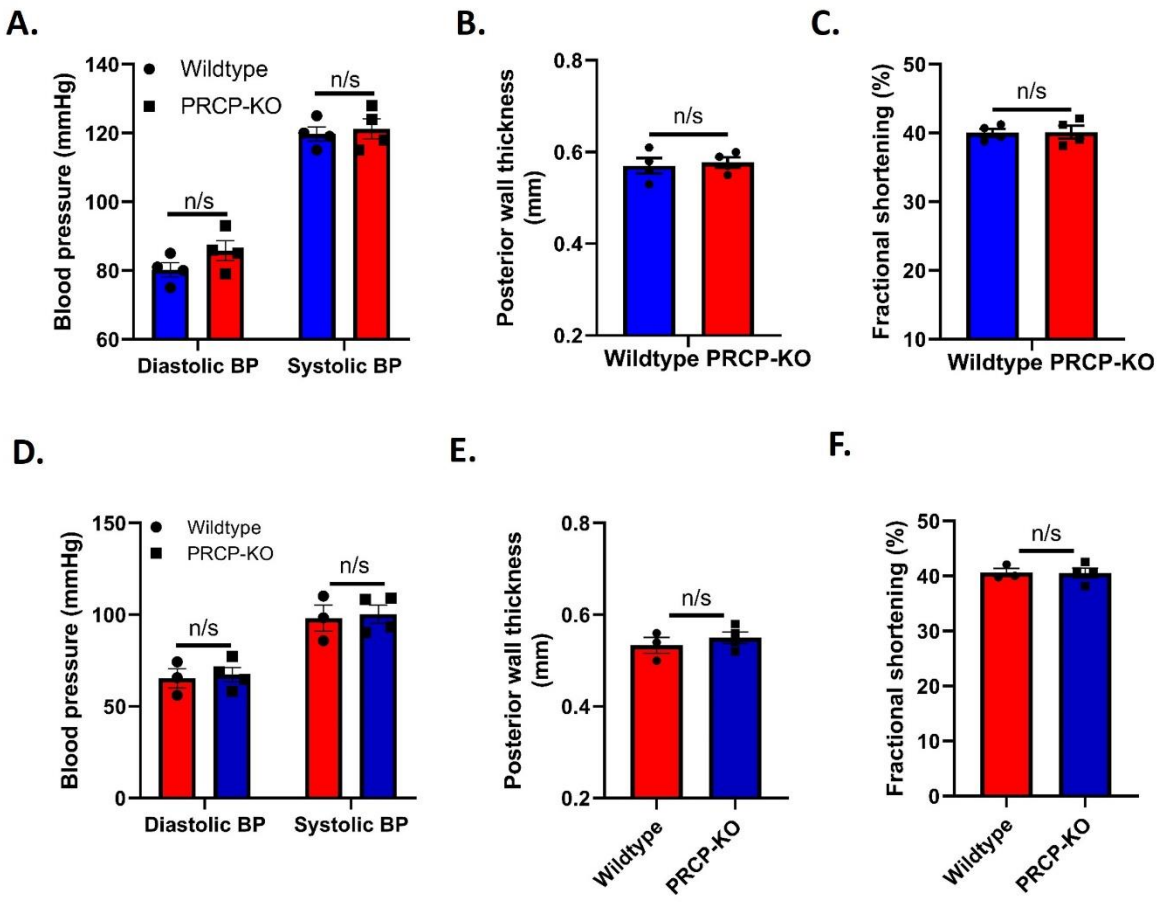


Figure S2: 2-month-old and 6-month-old PRCP-KO mice exhibited normal blood pressure and cardiac function at basal condition. (A) 2-month-old PRCP-KO mice showed comparable diastolic and systolic blood pressure to wildtype mice. Blood pressure was examined by the tail-cuff method. **(B and C)** Echocardiography shows insignificant changes in left ventricle end-diastolic posterior wall (PW) and fractional shortening between wildtype and PRCP-KO mice at 2-month-old. n=4 per group. **(D)** 6-month-old PRCP-KO mice showed comparable diastolic and systolic blood pressures to wildtype mice. Arterial blood pressure was examined by the invasive hemodynamic assessment. **(E and F)** Echocardiography shows insignificant changes in left ventricle end-diastolic posterior wall (PW) and fractional shortening between wildtype and PRCP-KO mice at 6-month-old. n=3 or 4 per group. Data are presented as means±SEM. Student t test was used for analysis. BP: blood pressure, PRCP-KO: Prolylcarboxylpeptidase knockout.

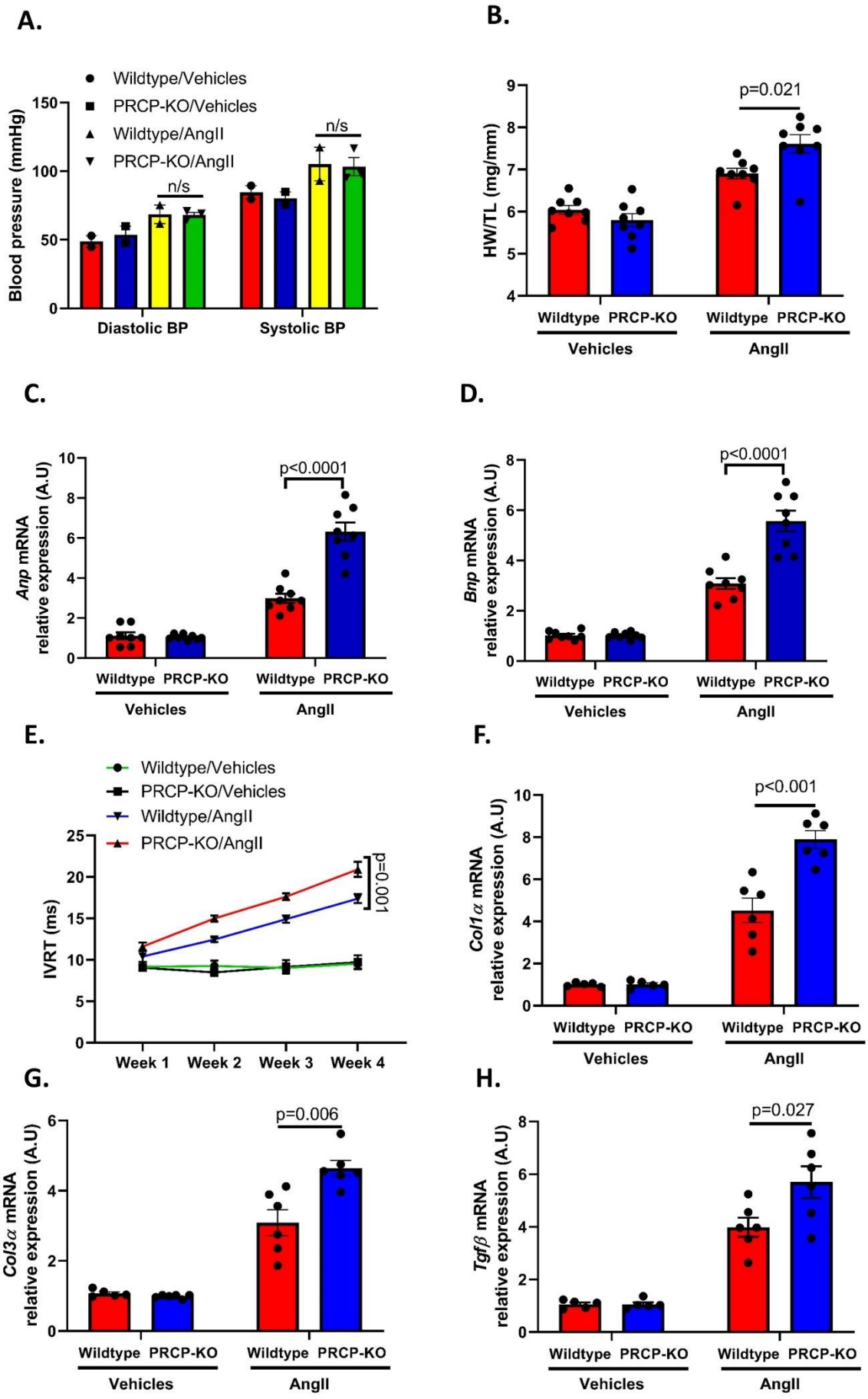

A.
Wildtype/Vehicles
PRCP-KO/Vehicles
Wildtype/AngII
PRCP-KO/AngII
Blood pressure (mmHg)
150
100
50
0
n/s
n/s
Diastolic BP
Systolic BP
B.
HW/TL (mg/mm)
9
8
7
6
5
4
p=0.021
Wildtype PRCP-KO
Wildtype PRCP-KO
Vehicles
AngII
C.
Anp mRNA relative expression (A.U)
10
8
6
4
2
0
p<0.0001
Wildtype PRCP-KO
Wildtype PRCP-KO
Vehicles
AngII
D.
Bnp mRNA relative expression (A.U)
8
6
4
2
0
p<0.0001
Wildtype PRCP-KO
Wildtype PRCP-KO
Vehicles
AngII
E.
Wildtype/Vehicles
PRCP-KO/Vehicles
Wildtype/AngII
PRCP-KO/AngII
IVRT (ms)
25
20
15
10
5
0
p=0.001
Week 1
Week 2
Week 3
Week 4
F.
Col1α mRNA relative expression (A.U)
10
8
6
4
2
0
p<0.001
Wildtype PRCP-KO
Wildtype PRCP-KO
Vehicles
AngII
G.
Col3α mRNA relative expression (A.U)
6
4
2
0
p=0.006
Wildtype PRCP-KO
Wildtype PRCP-KO
Vehicles
AngII
H.
Tgfβ mRNA relative expression (A.U)
8
6
4
2
0
p=0.027
Wildtype PRCP-KO
Wildtype PRCP-KO
Vehicles
AngII

Figure S3: PRCP-KO heart showed greater levels of cardiac hypertrophy and interstitial fibrosis in response to 4 weeks AngII treatment. (A) Invasive hemodynamic assessment showed no significant difference in diastolic and systolic blood pressure (BP) between wildtype and PRCP-KO mice after 4 weeks of AngII infusion. **(B)** Heart weight/Tibial length (HW/TL) ratio as a hypertrophic marker is significantly increased in PRCP-KO mice. (**C and D)** Quantification of mRNA levels of hypertrophic gene markers *Anp* and *Bnp* by qPCR. **(E)** Mitral Doppler showed isovolumic relaxation time (IVRT) indicated worsen diastolic function in PRCP-KO mice. **(F**, **G and H)** Quantitative qPCR of fibrotic marker genes including *Col1α*, *Col3α* and *Tgf-β*. (n=5 to 6 per group). Data are presented as means±SEM. Two-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. Anp: Atrial natriuretic peptide, Bnp: B-type natriuretic peptide, *Col1α*: collagen type I alpha 1 chain, *Col3α*: collagen type III alpha 1 chain, *Tgf-β*: transforming growth factor-beta, PRCP-KO: Prolylcarboxylpeptidase knockout.

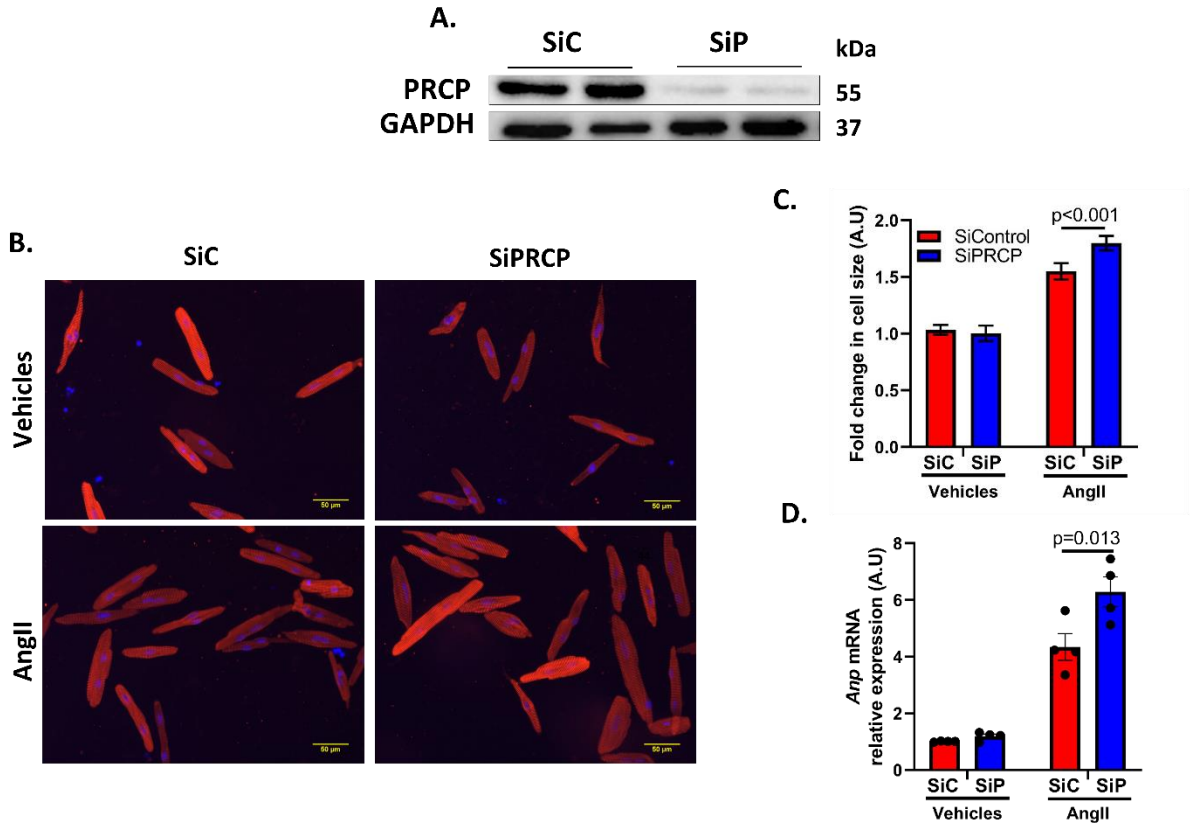


Figure S4: SiRNA-mediated PRCP deletion in adult rat cardiomyocytes (ARCM) led to the increase in cell sizes in response to AngII (100nM) for 48 hours. (A) Immunoblot analysis shows knockdown of PRCP expression using SiRNA in ARCM. **(B and C)** Representative images of α-actinin immunostaining of adult rat cardiomyocytes (scale bars = 50μm), followed by quantification of cell surface area.100 cells were used for quantification. **(D)** qPCR showed a significant increase of ANP level. n=4 per groups. Data are presented as means±SEM). Two-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. SiC: SiRNA control, SiP: SiRNA-PRCP. Anp: Atrial natriuretic peptide, AngII: Angiotensin II, GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

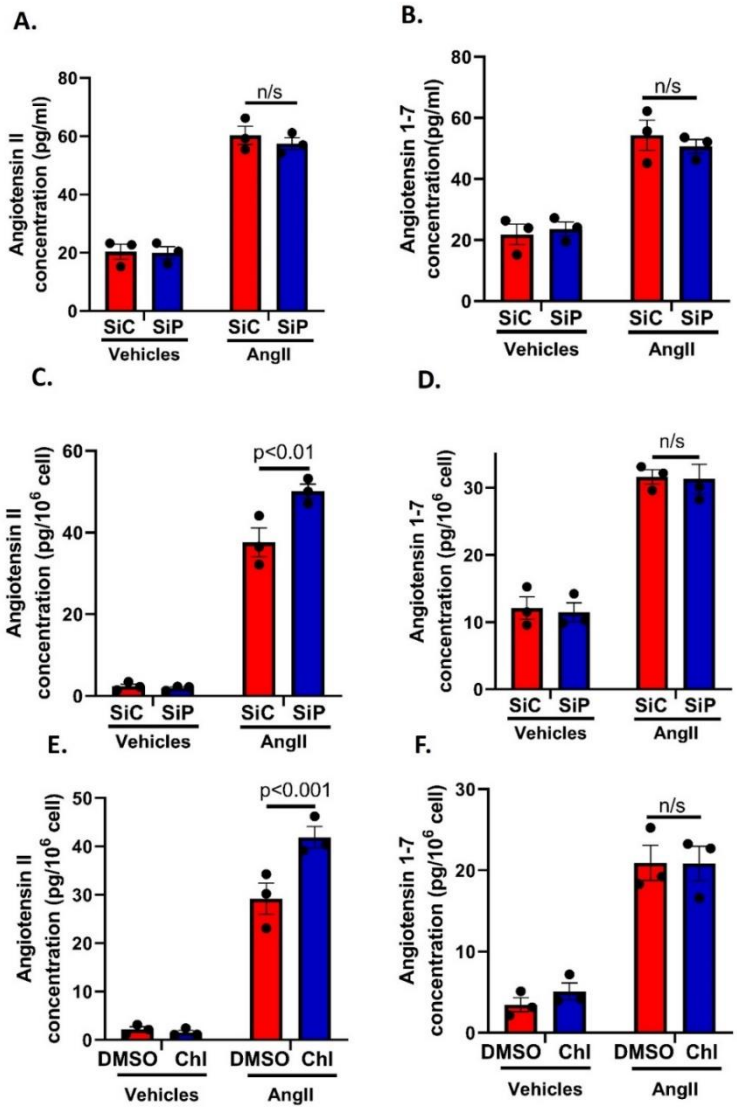


Figure S5: PRCP negatively regulates Angiotensin II in human induced Pluripotent Stem Cell (hiPSC)-derived cardiomyocytes in response to Angiotensin II treatment. (A and B) Levels of Angiotensin II in culture media and hiPSC-derived cardiomyocytes after knockdown of PRCP and followed by 48 hours of Angiotensin II treatment (100nM). **(C and D)** Levels of Angiotensin 1-7 in culture media and hiPSC-derived cardiomyocytes after knockdown of PRCP and followed by 48 hours of Angiotensin II treatment (100nM). **(E and F)** Levels of Angiotensin II and Angiotensin 1-7 in hiPSC-derived cardiomyocytes after 24 hours of chloroquine (Chl) treatment and followed by 48 hours of Angiotensin II treatment (100nM). Data are presented as means±SEM. n=3 per group. Two-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. SiC: SiRNA control, SiP: SiRNA-PRCP. Chl: chloroquine.

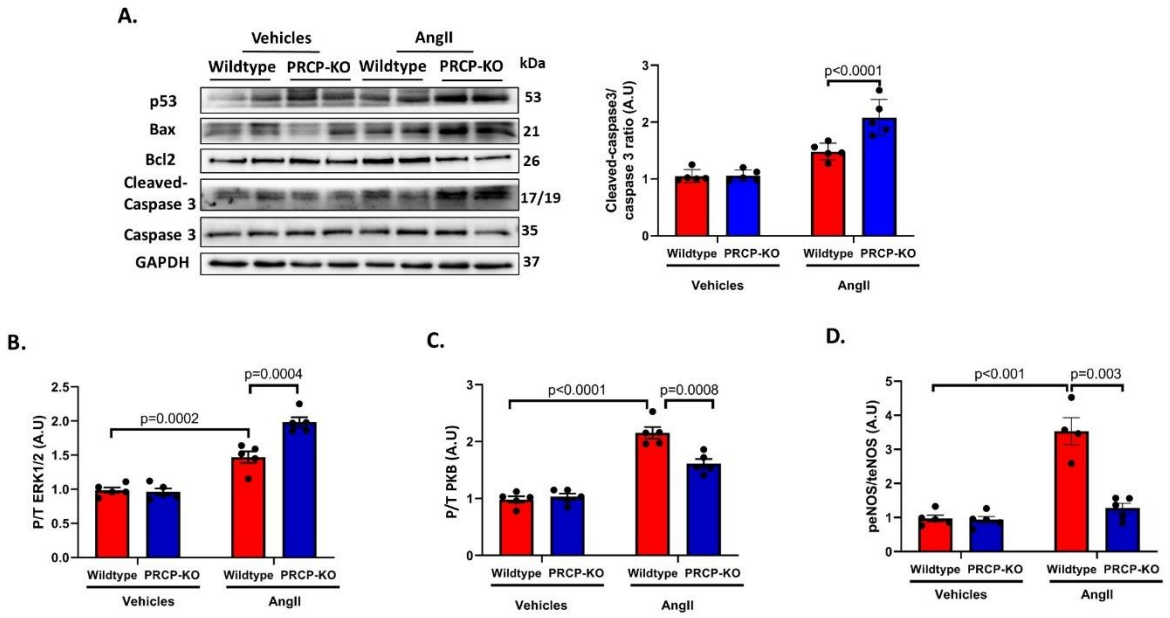


Figure S6: PRCP-KO hearts revealed more ROS production and apoptosis than wildtype hearts in response to 2 weeks of Angiotensin II infusion. (A) Immunoblot analysis of expression levels of various proteins involved in apoptotic signalling pathway and quantifications of caspase-3 activity (cleaved form/total caspase 3) (n=5 per group). **(B, C** and **D)** Quantifications of pERK1/2 / tERK1/2, pPKB/tPKB and peNOS/teNOS ratios are presented by bar graphs. n=5 per group. Data are presented as means±SEM. Two-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. AngII: Angiotensin II, Bax: Bcl-2 Associated X-protein, Bcl2: B-cell lymphoma 2, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, ERK1/2: Extracellular signal-regulated kinases1/2, PKB: Protein kinase B, eNOS: Endothelial NOS, PRCP-KO: Prolylcarboxylpeptidase knockout.

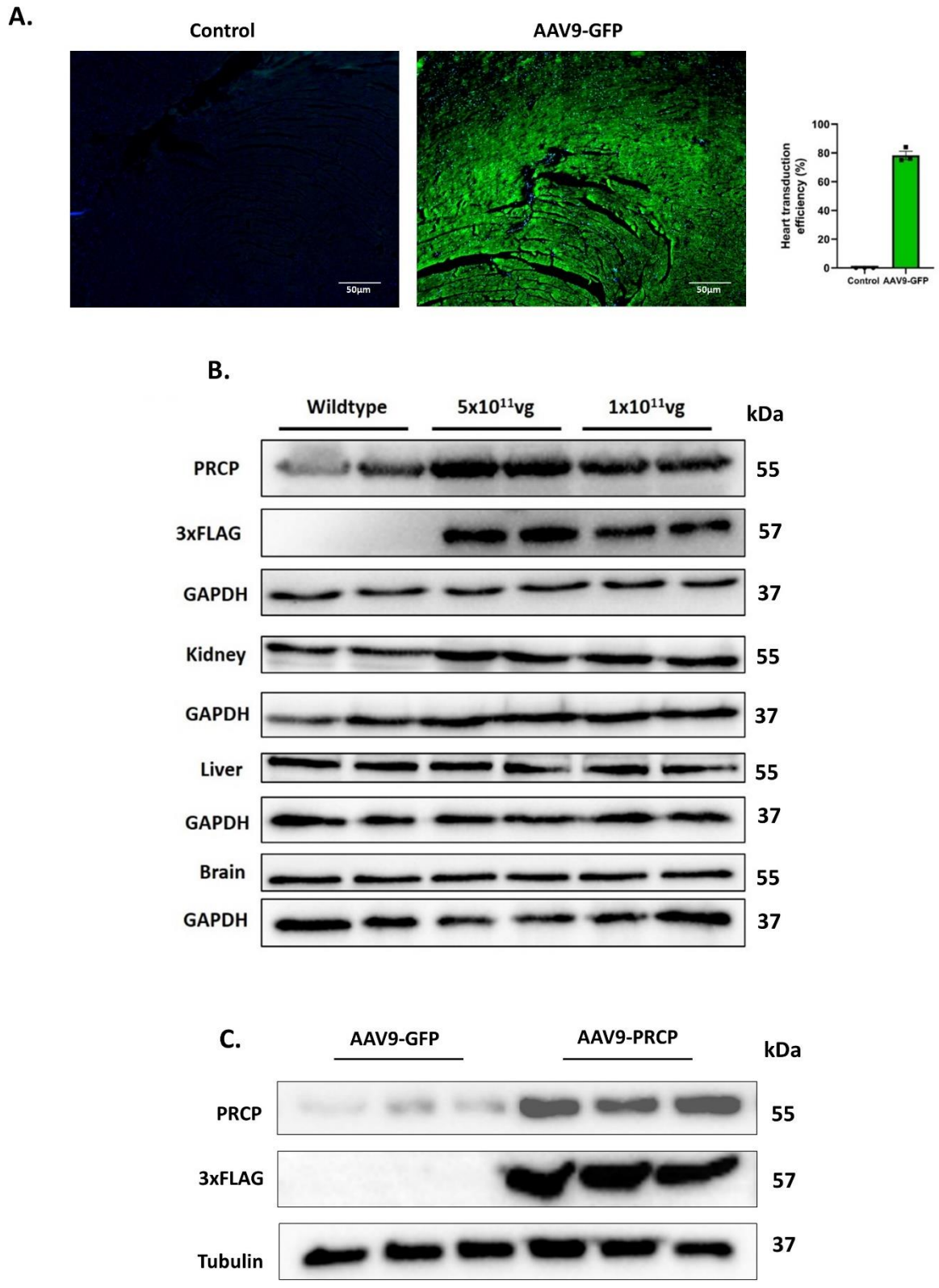
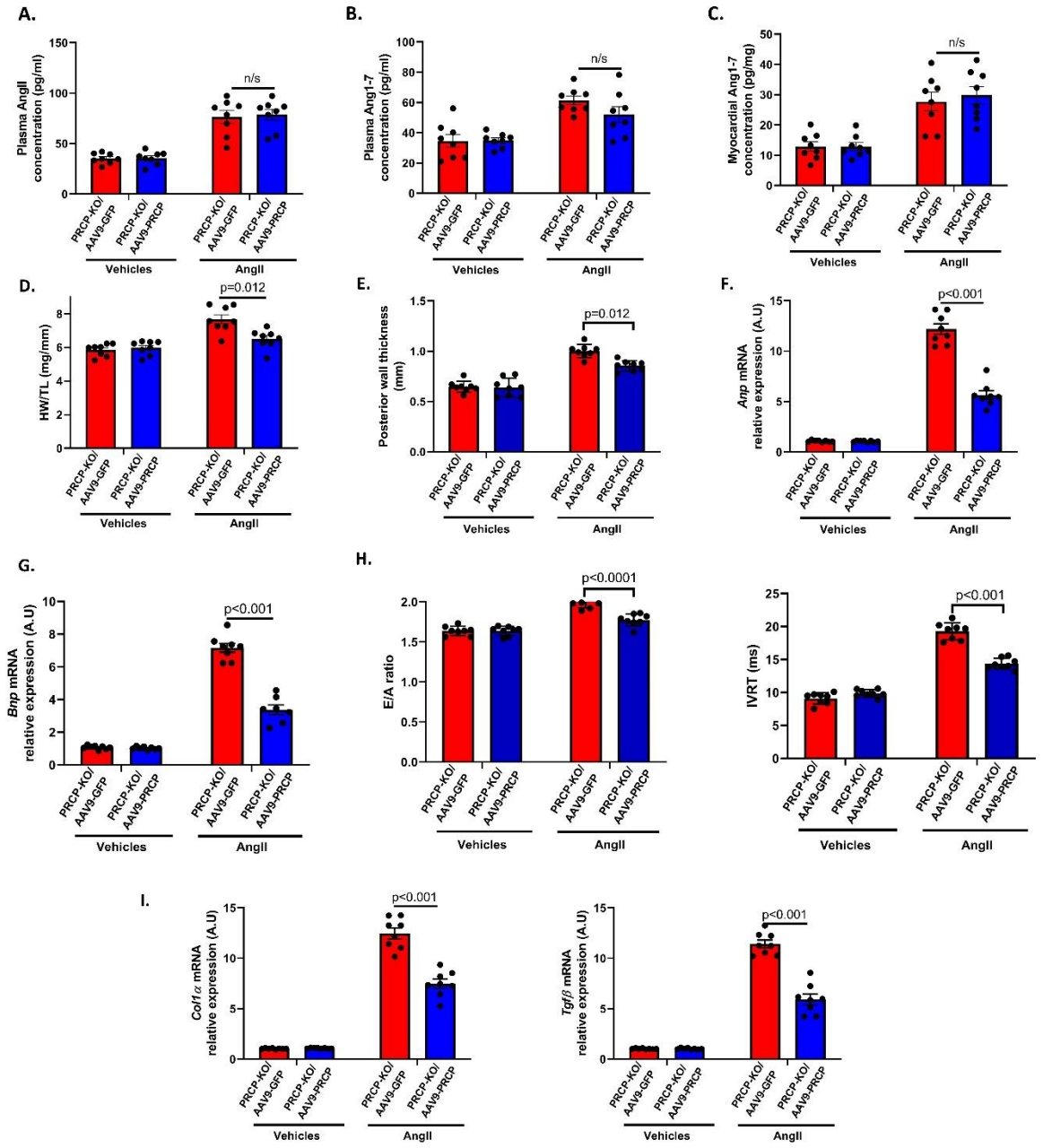


Figure S7: Evaluation of cardiac-specific overexpression of PRCP using AAV9. (A) Representative image of GFP expression in the myocardium mediated by AAV9-GFP and quantification of GFP expression to show gene transduction efficiency in the heart. **(B)** To test the efficacy of the AAV9-PRCP virus, wildtype mice are subjected to different doses of AAV9-PRCP virus, which are $1x10^{11}$ vg (low dose) and $5x10^{11}$ vg (high dose) through tail-vein injection. Levels of PRCP expression in the heart and other organs including kidney, liver, brain were checked by Western blot. **(C)** Restoration of PRCP expression in PRCP-KO hearts. GAPDH: glyceraldehyde-3-phosphate dehydrogenase, AAV9: Adeno-associated virus 9.


A.
Plasma AngII concentration (pg/ml)
n/s
B.
Plasma Ang1-7 concentration (pg/ml)
n/s
C.
Myocardial Ang1-7 concentration (pg/mg)
n/s
D.
HW/TL (mg/mm)
p=0.012
E.
Posterior wall thickness (mm)
p=0.012
F.
Anp mRNA relative expression (A.U)
p<0.001
G.
Bnp mRNA relative expression (A.U)
p<0.001
H.
E/A ratio
p<0.0001
IVRT (ms)
p<0.001
I.
Col1α mRNA relative expression (A.U)
p<0.001
Tgfβ mRNA relative expression (A.U)
p<0.001
PRCP-KO/ AAV9-GFP
PRCP-KO/ AAV9-PRCP
Vehicles
AngII

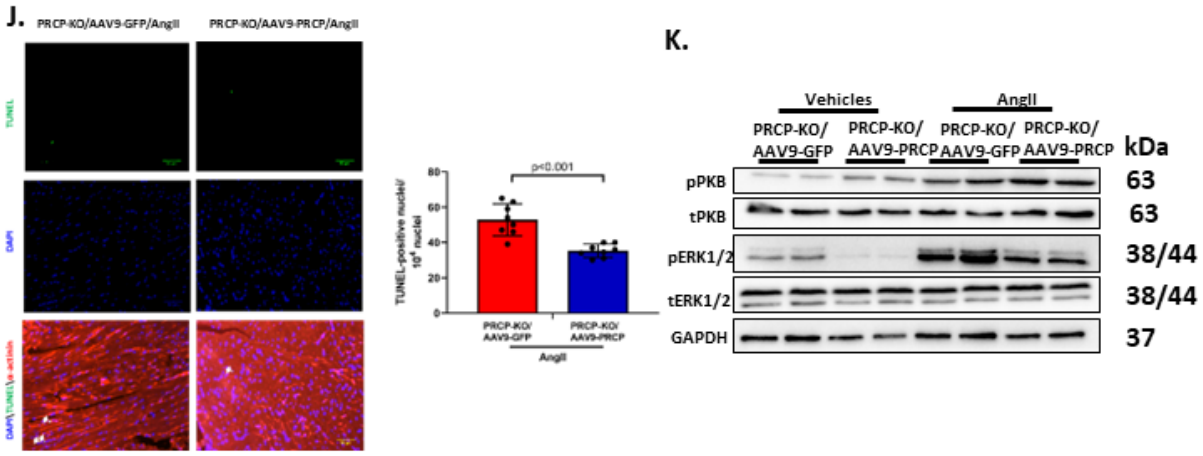


Figure S8: AAV9-mediated restoration of PRCP expression in the PRCP-KO heart alleviated AngII-mediated cardiac remodelling. A-C ELISA quantifications show comparable levels of plasma AngII (**A**) plasma Ang1-7 (**B**) and myocardial Ang1-7 **(C**) between PRCP-KO/AAV9-GFP and PRCP-KO/AAV9-PRCP groups. **(D)** Heart weight/tibial length (HW/TL) ratio. **(E)** Left ventricle end-diastolic posterior wall. **(F** and **G)** mRNA levels of hypertrophic gene markers *Anp* and *Bnp* were dramatically decreased in the PRCP-KO/AAV9-PRCP group. **(H)** Mitral valve Doppler flow showing E/A ratio and IVRT to assess diastolic function. **(I)** qPCR of fibrotic marker genes including *Col1α*, and *Tgfβ*. n= 8 per group. **(J)** TUNEL assay to detect levels of apoptotic cells (Scale bar: 30µm). TUNEL: Green, Nuclei: Blue/DAPI, a-actinin: Red. Arrows indicate TUNEL-positive nuclei. The quantification of TUNEL positive nuclei was shown in bar graphs. n=8 mice per group. Data are presented as means±SEM. Student t test or two-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. **(K)** Immunoblot analysis demonstrates a significant increase and a significant decrease in pPKB and pERK1/2 expressions, respectively, in the PRCP-KO/AAV9-PRCP mice. Data are presented as means±SEM. Two-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. PRCP-KO: Prolylcarboxylpeptidase knockout, GFP: Green Fluorescent Protein, AAV9: Adeno-associated virus 9, HW: Heart weight, TL: Tibial length, Anp: Atrial natriuretic peptide, Bnp: B-type natriuretic peptide, IVRT: isovolumic relaxation time, *Col1α*: collagen type I alpha 1 chain, *Col3α*: collagen type III alpha 1 chain, *Tgf-β*: transforming growth factor-beta, ERK1/2: Extracellular signal-regulated kinases1/2, PKB: Protein kinase B, DAPI: 4',6-diamidino-2-phenylindole, TUNEL: Transferase dUTP nick end labelling.

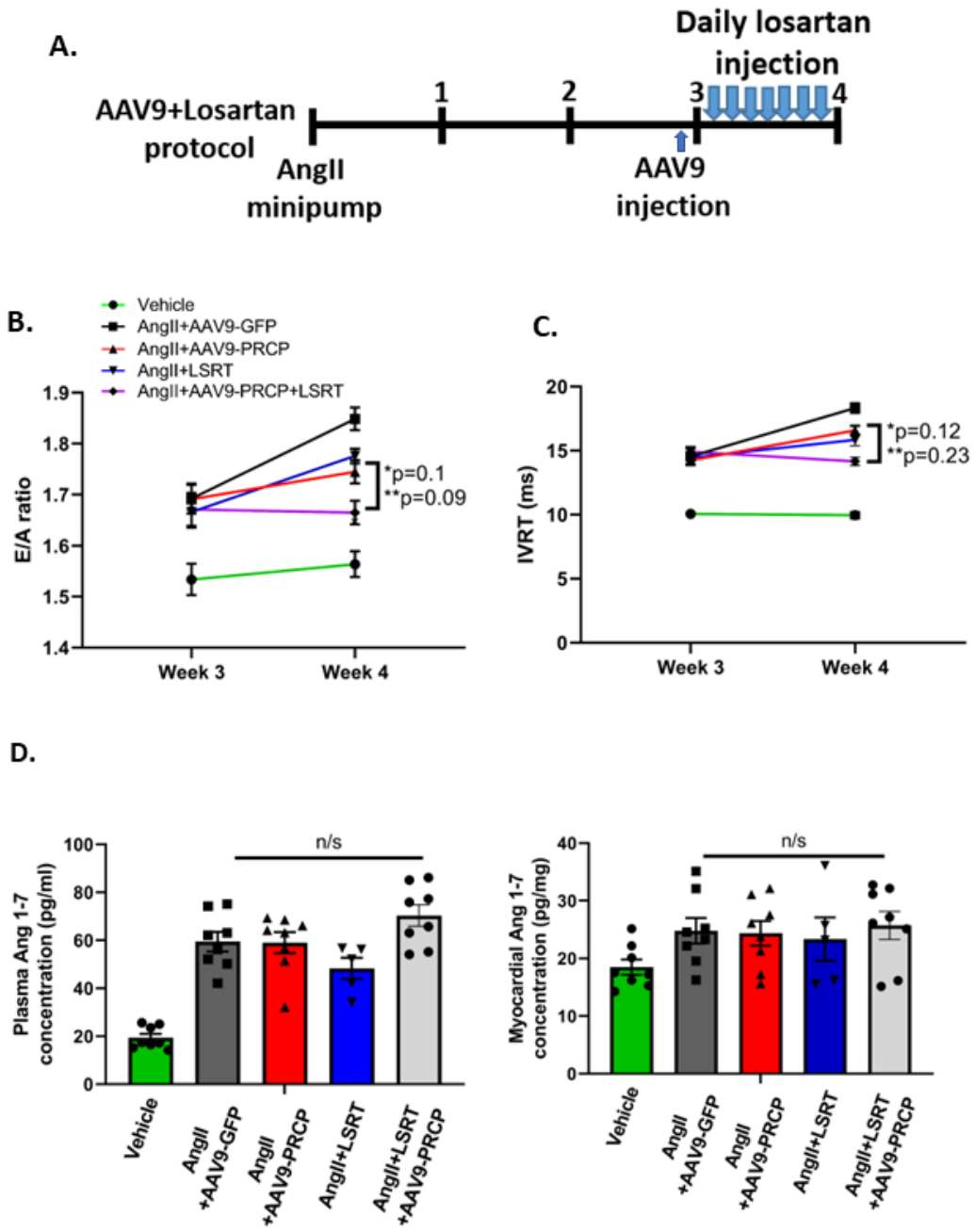


Figure S9: The combination therapy of AAV9-mediated PRCP overexpression and losartan (LSRT) improved the diastolic function. (A) Schematic figure describes experimental strategy. **(B** and **C)** Mitral valve Doppler flow showing E/A ratio and IVRT to assess diastolic function. **(D)** Ang1-7 levels in plasma and myocardial tissues. n=5 for AngII+LSRT group and n=8 for other groups. Data are presented as means±SEM. One-way or two-way ANOVA with Bonferroni correction for post hoc comparisons was used for analysis. *p value: AngII+AAV9-PRCP vs AngII+AAV9-PRCP+losartan; **p value: AngII+losartan vs AngII+AAV9-PRCP+losartan. PRCP: Prolylcarboxylpeptidase, GFP: Green Fluorescent Protein, AAV9: Adeno-associated virus 9, IVRT: isovolumic relaxation time, AngII: Angiotensin II, LSRT: Losartan.