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Knockout of ACE-N facilitates improved cardiac function after myocardial infarction

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Abstract

Angiotensin-converting enzyme (ACE) hydrolyzes *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) into inactive fragments through its N-terminal site (ACE—N). We previously showed that Ac-SDKP mediates ACE inhibitors' cardiac effects. Whether increased bioavailability of endogenous Ac-SDKP caused by knocking out ACE-N also improves cardiac function in myocardial infarction (MI)-induced heart failure (HF) is unknown. Wild-type (WT) and ACE-N knockout (ACE-NKO) mice were subjected to MI by ligating the left anterior descending artery and treated with vehicle or Ac-SDKP (1.6 mg/kg/day, s.c.) for 5 weeks, after which echocardiography was performed and left ventricles (LV) were harvested for histology and molecular biology studies. ACE-NKO mice showed increased plasma Ac-SDKP concentrations in both sham and MI group compared to WT. Exogenous Ac-SDKP further increased its circulating concentrations in WT and ACE-NKO. Shortening (SF) and ejection (EF) fractions were significantly decreased in both WT and ACE-NKO mice post-MI, but ACE-NKO mice exhibited significantly lesser decrease. Exogenous Ac-SDKP ameliorated cardiac function post-MI only in WT but failed to show any additive improvement in ACE-NKO mice. Sarcoendoplasmic reticulum calcium transport ATPase (SERCA2), a marker of cardiac function and calcium homeostasis, was significantly decreased in WT post-MI but rescued with Ac-SDKP, whereas ACE-NKO mice displayed less loss of SERCA2 expression. Our study demonstrates that gene deletion of ACE-N resulted in improved LV cardiac function in mice post-MI, which is likely

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: None.

Appendix A. Supplementary data

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mediated by increased circulating Ac-SDKP and minimally reduced expression of SERCA2. Thus, future development of specific and selective inhibitors for ACE-N could represent a novel approach to increase endogenous Ac-SDKP toward protecting the heart from post-MI remodeling.

Keywords

Ac-SDKP; ACE-N; Myocardial infarction; SERCA2; Fibrosis; Cardiac function

1. Introduction

Angiotensin-converting enzyme (ACE) is a vital component of the renin-angiotensin system by not only converting inactive angiotensin (Ang) I to active Ang II but also degrading other endogenous peptides such as bradykinin [1,2]. ACE regulates blood pressure and inflammation, and it mediates other major biological functions such as renal development, hematopoiesis, immune responses, aberrant cell proliferation and migration, and fibrosis [3]. Two distinct domains of ACE, namely the N and C domains, play a significant role in several biological and pathological processes [4]. *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) is an endogenous tetrapeptide released from its precursor 43-amino acid thymosin β4 (Tβ4) by 2 successive enzymes meprin-α and prolyl oligopeptidase (POP) [5–8]. Ac-SDKP was initially reported to be produced by bone marrow and acts as a natural suppressor of cell proliferation [9]. However, recent *in vivo* studies have shown that both Ac-SDKP and its precursor Tβ4 are substantially found in the circulation and various organs, including the heart and kidneys [10,11]. Ac-SDKP is cleaved exclusively by the ACE-N domain with up to 40-fold selectivity toward the N domain of ACE; whilst Ang II generation from Ang I is preferentially catalyzed by the C-domain [12–14]. In diabetic mice, lack of functional ACE N-domain resulted in higher urinary Ac-SDKP with reduced renal inflammation (50 % lower renal expression of IL-1β and TNF-α) and albuminuria than diabetic wild-type mice, without altering renal Ang II contents [15,16]. Exogenous Ac-SDKP administration increases its plasma circulating concentrations 2- to 5-fold [17]. In this regard, plasma and urinary Ac-SDKP levels were increased in hypertensive patients treated with an ACE inhibitor (ACEi), and this finding was considered to be potentially valuable in assessing ACEi compliance [18].

Myocardial infarction (MI) is associated with high rates of acute death caused by arrhythmias or cardiogenic shock and long-term complications such as HF [19,20]. Both clinical and experimental studies provide strong evidence that maladaptive late-stage cardiac remodeling is associated with cardiomyocyte hypertrophy and reactive interstitial fibrosis, both of which contribute to progressive LV dysfunction culminating in overt HF [21,22]. Cardiac dysfunction is also mediated, in part, by reduced sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA2), resulting in an impaired cardiomyocyte Ca^{2+} homeostasis *i.e.* calcium cycling [23–25] and abnormal myocardial contractility/relaxation [26–30].

We have previously shown that Ac-SDKP improves LV function in mice with MI, likely through its anti-inflammatory, anti-fibrotic, and pro-angiogenic properties [31,32]. However, it remains unknown whether gene deletion of ACE-N would protect the heart post-MI and

improve cardiac function as a result of increased levels of Ac-SDKP. Thus, our study aims to 1) confirm that endogenous Ac-SDKP is increased in ACE-N knockout (ACE-NKO) mice *versus* WT mice and 2) test whether this condition confers cardiac protection and 3) that treatment with exogenous Ac-SDKP results in additive cardiac protective effect to ACE-N deletion.

2. Materials and methods

2.1. Animal model and experimental protocol

All experimental protocols were approved by the Henry Ford Hospital Institutional Animal Care and Use Committee (IACUC) and were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH Publication Eight edition, revised 2011). Briefly, ACE-NKO mice on WT background express a full-length ACE protein in which site-directed mutagenesis was used to replace histidine for lysine at positions 395 and 399 [12,33]. These specific amino acid changes inactivate the enzymatic activity of the ACE N-terminal catalytic site by mutating the two zinc-binding histidine [33]. Wild-type male C57BL/6J (WT) were purchased from Jackson Laboratories (Bar Harbor, ME) at 10–12 weeks of age (23–26 g). Both WT and ACE-NKO mice were maintained in a 12 h light/12 h dark cycling, had access to regular chow (0.5 % NaCl) and tap water *ad libitum*, and were set to adjust to the environment for at least one week.

2.2. Myocardial infarction-induced heart failure and Ac-SDKP delivery

Myocardial infarction (MI) was surgically induced by ligating the left anterior descending (LAD) coronary artery, as fully described previously [34]. Briefly, mice were anesthetized with sodium pentobarbital (50 mg/kg, *i.p.*), intubated, and ventilated with room air using a positive-pressure respirator. The LAD coronary artery was ligated with an 8–0 silk suture near its origin between the pulmonary outflow tract and the edge of the left atrium. Acute myocardial ischemia was deemed successful when the anterior wall of the LV became cyanotic. The lungs were then inflated by increasing positive end-expiratory pressure, and the thorax was closed. Sham-operated mice were prepared in the same manner but did not undergo coronary artery ligation. Osmotic minipumps (Alzet, Cupertino, CA) were implanted intraperitoneal under aseptic conditions to deliver either Ac-SDKP (1.6 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$; Genscript, Piscataway, NJ) or vehicle (0.01 N acetic acid in saline) as previously described [32]. The treatment for both WT and ACE-NKO groups were as follows: 1) Sham; 2) MI + Vehicle; 3) MI + Ac-SDKP. Treatment was continued for up to five weeks and was started right after surgery [32]. In the present study, we selected the dose of 1.6 mg/kg/day based on our previous studies and reported equivalent surface area dosing conversion factors [35–41].

All fully recovered mice were returned to their cages in a temperature and humidity-controlled environment with *ad libitum* access to food and water.

2.3. Cardiac remodeling and function by echocardiography

Cardiac geometry and function were examined in non-anesthetized mice at week 5 post-MI. Diastolic left ventricular dimensions (LVDd) and areas (LVAd), diastolic posterior wall thickness (PWTd), LV ejection fraction (EF), and shortening fraction (SF) were measured using M-mode echocardiography with a 15-MHz linear transducer (Acuson c256, Mountain View, CA), as described previously [42].

2.4. Plasma Ac-SDKP levels

At the end of each experimental protocol, mice were euthanized by an overdose of sodium pentobarbital (100 mg/kg, *i.p.*). Blood was withdrawn *via* the vena cava with a 1-ml syringe coated with heparin containing captopril (final concentration 10^{-5} M) and centrifuged at 1600 ×*g* for 20 min at 4 °C. Plasma was isolated and stored at −20 °C until assayed. Plasma Ac-SDKP concentrations at 5 weeks post-MI were quantified using an Ac-SDKP enzyme immunoassay kit (SPI-BIO, Massey Cedex, France) and expressed in nM, as previously described [38,40,43,44].

2.5. Histopathological analysis

At the end of the experiments, mice were weighed and euthanized with sodium pentobarbital (100 mg/kg, *i.p.*). The heart was stopped at diastole by intraventricular injection of 15 % KCl and rapidly excised and weighed. The LV, including the septum, was sectioned transversely into 3 slices from apex to base, and the slices were quickly frozen in isopentane precooled in liquid nitrogen and then stored at −70 °C until use. Cryosections of LV (6 μm) were cut from each frozen slice and stained with fluorescein-labeled peanut agglutinin (Vector Laboratories, Inc., Burlingame, CA) and rhodamine-labeled Griffonia simplicifolia lectin I (Vector Laboratories) to determine the interstitial collagen fraction (ICF), capillary density, and myocyte cross-sectional area (MCSA) as described previously [45]. Twelve fields were randomly chosen from 3 slices and photographed using a microscope (IX81; Olympus American, Melville, NY) equipped with a digital camera (DP70; Olympus American). ICF, capillary density and MCSA were quantified with Microsuite Biological imaging software (Olympus American).

2.6. Assessment of infarct size

A 6-μm LV cryosection was cut from each slice and stained with Masson's trichrome. The images of the whole LV sections were captured at low magnification and processed with Microsuite Biological imaging software. The endocardial and epicardial circumferences and the length of the scar were measured for each slice. The scar length to the ventricular circumference ratio of the endocardium and epicardium for each of the 3 slices was determined, and the 3 ratios were averaged and expressed as a percentage to define infarct size [46]. Mice with an infarct size <20 % due to technical problems were excluded from the analysis.

2.7. Western blot analysis

Electrophoretic separation of proteins and immunoblotting was performed as previously reported [36]. Briefly, about 20 mg of snap-frozen LV tissue from the base of the

heart was thawed in RIPA lysis buffer (Cell Signaling Technology, Danvers, MA) containing protease (Sigma Aldrich) and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and 1 mM phenylmethanesulfonyl fluoride (PMSF) that was added immediately before lysing the tissues. LV proteins (60 μg) were subjected to 10 % SDS-PAGE under reducing conditions and electro-transferred to nitrocellulose membrane. The primary antibodies for phospholamban (PLB) (Ser16/Thr17; Cell Signaling Technology, Cat# 8496S), Sarcoplasmic Ca-ATPase (SERCA2) and GAPDH were obtained from Cell Signaling Technology (Danvers, MA) and Abcam (Waltham, MA), respectively. The membrane was incubated with primary antibody at 4 °C overnight, followed by IgG, HRP-linked secondary antibody at room temperature for 1 h. ECL (Bio-Rad technologies, life science group) was used to visualize the bands. Band intensity was quantified using NIH Image J densitometry software and normalized with GAPDH. The results were expressed as a fold change *vs.* sham.

2.8. Statistical analysis

Binary data are expressed as proportions, and groups are compared using a Chi-squared test for two-by-two tables. Continuous data are defined as means ± standard errors, and groups were compared using the two-sample Wilcoxon test. A nonparametric method was chosen if the variances differed substantially between groups. A Hochberg's method was utilized to determine significance in all settings where multiple testing was used. An adjusted p-value <0.05 was considered significant.

3. Results

3.1. Effects of ACE-N gene deletion on plasma Ac-SDKP

MI was induced in both WT and ACE-NKO mice by ligating the left anterior descending (LAD) coronary artery, and Ac-SDKP plasma level was measured at the end of weeks 5 post-MI. In sham or MI + Vehicle groups, ACE-NKO mice showed significantly increased plasma Ac-SDKP concentrations compared to sham and post MI WT mice treated with vehicle ($p < 0.001$). Conversely, WT mice subjected to MI and treated with Ac-SDKP showed significantly higher plasma Ac-SDKP levels than sham or vehicle-treated WT mice ($p < 0.001$). The Ac-SDKP plasma concentrations were nearly 16-fold higher in the Ac-SDKP-treated ACE-NKO mice compared to the vehicle-treated ACE-NKO and WT mice Post-MI (Fig. 1; $p < 0.001$).

3.2. Effects of ACE-N gene deletion on infarction size and LV dimensions

At the end of 5 weeks of post-MI, infarct size was measured in MI + vehicle and MI + Ac-SDKP groups. Infarcted area did not differ among WT and ACE-NKO mice subjected to MI and treated with vehicle or Ac-SDKP (Fig. 2). LV posterior wall thickness (PWTd) at diastole did not change between mouse strain or treatment (Table 1). However, LV area (LVAd) and dimension at diastole were significantly increased in both mouse strains post-MI with no difference between the two strains. Increased LVAd was significantly diminished by administration of exogenous Ac-SDKP in WT but not ACE-NKO mice (Table 1).

3.3. Effects of ACE-N gene deletion on cardiac function at 5 weeks post-MI

Cardiac function, as assessed by echocardiography in conscious mice (SF and EF) at the end of five weeks post-MI, was significantly better in mice with ACE-N gene deletion compared to WT mice. In contrast, no significant changes in SF or EF were observed in both sham-operated WT and ACE-NKO mice. As expected, exogenous supplementation of Ac—SDKP improved cardiac functions in WT mice but failed to exhibit any additional protective effects on cardiac function in ACE-NKO mice despite a substantial additional increase in circulating plasma Ac-SDKP level (Fig. 3A–C).

3.4. Effects of ACE-N gene deletion on cardiac remodeling and fibrosis at 5 weeks post-MI

After five weeks of post-MI, analysis of the LV section located remotely from the infarct zone shows that the capillary density in the myocardium was diminished and fibrosis was increased in the MI + vehicle group compared to the sham-MI group for both mouse strains (Fig. 4). ACE-NKO mice had significantly lower capillary density compared to WT mice (Fig. 4B). Capillary density was ameliorated considerably after treatment with Ac-SDKP in both WT and ACE-NKO mice. MI also caused cardiac fibrosis similarly in both mouse strains, as indicated by increased LV interstitial collagen fraction (ICF). Ac-SDKP significantly inhibited collagen deposition in the heart with a substantially better reduction in ACE-NKO *versus* WT mice (Fig. 4C).

Cardiomyocyte cross-sectional area (MCSA) was similar in shams from both strains but significantly increased similarly in both strains when subjected to MI (Fig. 4D). Ac-SDKP treatment significantly reduced MCSA with a more profound decrease in ACE-NKO MI mice.

3.5. Effects ACE-N gene deletion of myocardial expression of SERCA2 and phospholamban at 5 weeks post-M

SERCA2 and PLB protein expression was measured by Western blotting in both WT and ACE-NKO mice hearts after 5 weeks post-MI. In sham-operated mice, SERCA2 expression normalized to house-keeping protein GAPDH appears diminished in WT *versus* ACE-NKO mice. Furthermore, SERCA2 expression was significantly and intensely decreased in WT subjected to MI, but not in ACE-NKO mice. The loss of SERCA2 expression in WT mice post-MI was significantly recovered with exogenous supplementation of Ac-SDKP. However, in ACE-NKO mice, no additional improvement was observed when exogenous Ac-SDKP was administered, but rather and unexpectedly, SERCA2 expression seems to be diminished in Ac-SDKP-treated ACE-NKO mice post-MI (Fig. 5A, C).

Conversely, we observed a significantly higher expression of PLB in sham WT compared to ACE-NKO mice (Fig. 5B, D). Expression of PLB was slightly increased in ACE-NKO but decreased in WT mice when subjected to MI. Ac-SDKP did not change PLB expression at 5 weeks post-MI in both mouse strains. Hence, the PLB/SERCA2 was significantly higher in sham WT than in ACE-NKO mice (Fig. 5E). The ratio was further increased in WT mice but only slightly increased in ACE-NKO mice post-MI. Ac-SDKP significantly reduced PLB/SERCA2 ratio in WT subjected to MI but had no additive effect in ACE-NKO mice.

4. Discussion

Results from the present study indicate that gene silencing of the ACE N-terminal domain in mice protected the hearts when subjected to myocardial infarction (MI)-induced cardiac remodeling and dysfunction. Notably, ACE-NKO mice (sham and MI) had increased plasma Ac-SDKP concentrations compared to WT mice. Administration of exogenous Ac-SDKP increased its circulating concentrations in both WT and ACE-NKO, but the increase was substantially higher in the ACE-NKOs given that the ACE-NKO mice had already experienced augmented concentrations of endogenous Ac-SDKP. Cardiac function (SF and EF) was significantly diminished in both WT and ACE-NKO mice post-MI; however, ACE-NKO mice exhibited significantly less reduction in SF and EF following MI. Exogenous Ac-SDKP ameliorated cardiac function post-MI in WT but failed to show any additive improvement in ACE-NKO mice despite a substantial increase of plasma Ac-SDKP by >10-fold, likely the result of saturation at the site of action of Ac-SDKP due to exposure since mouse embryologic stage. Future identification, cloning and characterization of putative receptors for Ac-SDKP will surely help understand better the pharmacodynamic and mechanism(s) of action of Ac-SDKP. SERCA2, a hallmark protein for cardiac calcium homeostasis, was significantly decreased in WT post-MI but was rescued during treatment with Ac-SDKP. On the other hand, ACE-NKO mice displayed only a slight decrease in SERCA2 expression but was maintained by exogenous Ac-SDKP at the WT levels. Thus, the present study demonstrates that gene deletion of ACE-N resulted in improved cardiac function in mice post-MI, mediated, in part, by increased circulating Ac-SDKP as it has been demonstrated for other non-cardiac experimental models [15,47,48].

Previously, we and others have shown that 1) exogenous Ac-SDKP improves cardiac function and prevents/ameliorates myocardial fibrosis and inflammation in mice and/or rats [32,49,50], and 2) Tβ4, the source of Ac-SDKP, also mitigates myocardial inflammation and fibrosis and promote neo-angiogenesis and improves cardiac function post-MI [32,51–53]. Moreover, treatment with ACEi is often used to treat myocardial injury and dysfunction post-MI in patients and numerous experimental animal models [45,54–59]. More importantly, ACEi increase circulating and tissue levels of excreted Ac-SDKP multiple fold in humans and animal models [38,43, 60,61]. However, inhibiting ACE results not only in increased Ac-SDKP but also prevents degradation of other vasoactive peptides such as bradykinin (BK) [1,2] and the formation of Ang II. Previously, ACE was believed to have a single catalytic domain, until the cloning of the ACE gene has divulgated that the somatic ACE is made of two homologous protein domains with well-defined catalytic activity and zinc-dependent domains [33,62]; thus, gene silencing of the ACE N-terminal domain by mutation enabled investigation on the role of specific effect of ACE-N while maintaining the ACE-C intact [4]. Indeed, BK through B_2 receptors has been demonstrated to mediate part of the cardiac protective effects of ACE inhibition or Ang II receptor blockers in MI [45,63]. However, recent findings indicate that selective inhibition of ACE-C is more effective than ACE-N in protecting BK in agreement with the hypothesis that the effects seen with ACE-NKO mice are unlikely to be mediated by BK [64,65]. Others reported that BK is equally degraded by ACE-C and ACE-N [4,66]. Nevertheless, ACE-NKO mice subjected to MI failed to exhibit any additional improvement in cardiac function when they were treated

with exogenous Ac-SDKP, indicating that BK is unlikely to be involved in the protective effects of ACE-N gene deletion post-MI. Additionally, we and others have previously shown that the protective effects of ACE inhibition are in part mediated by Ac-SDKP [38,44,67]. Inhibition of POP-mediated release of Ac-SDKP from thymosinβ4 fragments has been shown to significantly blunt the effects of ACE-N gene deletion or ACE inhibitors in other different experimental conditions [8,15], indicating an important role of Ac-SDKP in ACE inhibition therapeutic effects. Nevertheless, the remaining factors mediating the protective effects of ACE inhibition could be through AT_2, mas (Ang 1–7) or B_1/B_2 kinin receptors [34,45,58,68,69]. It is possible that administering exogenous Ac-SDKP into ACE-NKO mice resulted in full protection of the peptide from enzymatic degradation, and very concentrations of Ac-SDKP, in turn, inhibits ACE-C as well as previously reported [70]. Thus, there remains more rigorous studies to be performed once the Ac-SDKP receptors and respective antagonists are identified and characterized to evaluate the contribution of Ac-SDKP and BK in the protective effects of ACE inhibition.

The sarcoplasmic reticulum (SR) is well known as a key regulator of cytosolic calcium and, therefore, a key modulator of myocardial contractility and relaxation *via* SERCA2 and in combination with ryanodine calcium release channels [32,71–74]. SERCA2, a hallmark of myocardial function, is under the control of the inhibitory protein PLB [73,75]. In the present study, this concept is further confirmed in WT mice subjected to MI in which SERCA2 expression was deeply decreased with a slightly increased PLB, resulting in significantly high SERCA2/PLB ratio similar to recent observations made in human cardiomyocyte from human heart post-MI [75] or in mice [76] in which SERCA2 expression is deeply depressed with increased or no changes in the PLB expression. These changes are paralleled by the dramatic decrease in cardiac function (EF and SF). However, PLB/SERCA2 ratio was conserved in ACE-NKO mice post-MI compared to sham. Interestingly, Ac-SDKP reduced PLB/SERCA2 ratio to levels very close to sham hearts in WT but did not provide additional decrease in ACE-NKO mice further supporting the hypothesis that Ac-SDKP is almost exclusively degraded by ACE-N [14,33,38].

The uptake of cytosolic Ca^{2+} into the SR is an essential mechanism for regulating Ca^{2+} signaling and muscle contraction and relaxation, and this is controlled in part by SERCA2 and its inhibitor PLB. Increased Ac-SDKP *via* exogenously administered the tetrapeptide or inhibition of ACE-N likely preserved SERCA2 expression without affecting much PLB expression post-MI.

Thus, we conclude that increased bioavailability of endogenous Ac-SDKP by gene silencing ACE-N is sufficient to provide cardiac protection in MI-induced cardiac remodeling and dysfunction. The results suggest that novel specific and selective ACE-N inhibitors may represent a novel approach for the management of post-MI structural and functional remodeling.

4.1. Limitations of the study

Overall, the study showed that ACE-N gene deletion promoted improved cardiac function in a mouse model of MI-induced heart failure with reduced ejection fraction. This effect is not further improved by administration of exogenous Ac-SDKP despite substantial increases

in the circulating concentrations of the tetrapeptide. An unexpected feature of our data was the dissociation between angiogenesis (capillary density) and fibrosis in one hand and the improved cardiac function in the other hand as seen in ACE-NKO mice with MI. We saw an improved EF/SF in ACE-NKO but not capillary density or fibrosis; the latters are only improved after exogenous Ac-SDKP was administered. At present, we could not explain this phenomenon but with future identification, cloning and characterization of the putative Ac-SDKP receptors the mechanism(s) will be better dissected. Also, the higher expression of SERCA2 with decreased PLB expression in sham-operated ACE-NKO await deep molecular studies to understand the relationship between ACE-N and the dynamism of the components of the sarcoplasmic reticulum components in regulating intracellular calcium and contraction relaxation coupling of the cardiomyocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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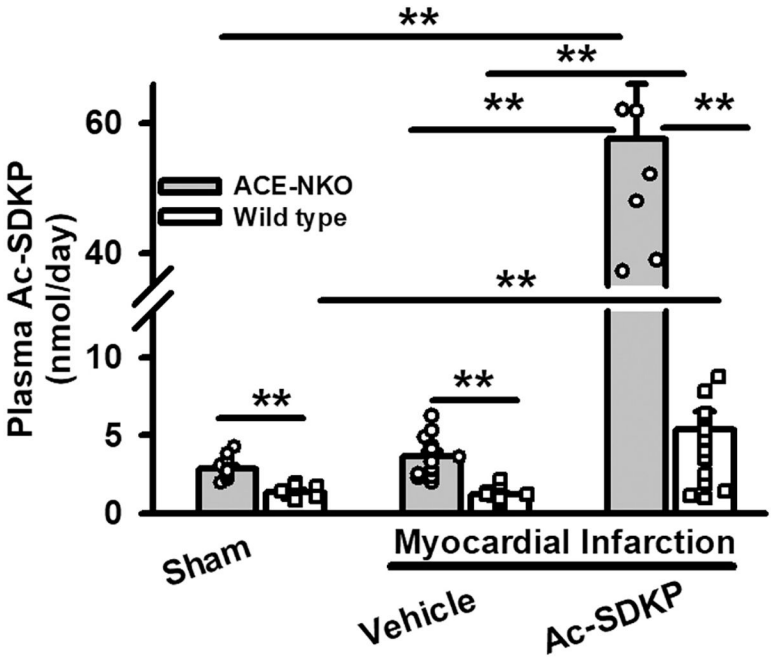


Fig. 1.

Plasma Ac-SDKP in WT and ACE-NKO mice after 5 weeks post-MI. The bar graph shows that ACE-NKO mice have significantly higher plasma Ac-SDKP either under sham or MI + vehicle conditions. Plasma Ac-SDKP was significantly higher in both mouse strains that were subjected to MI and exogenous Ac-SDKP administration; however, Ac-SDKP increase was more than ten-fold higher in ACE-NKO compared to WT mice. The number of experiments represents was as follows: WT-Sham ($n = 13$); WT- MI+ vehicle ($n = 15$); WT- MI + Ac-SDKP ($n = 20$); ACE-NKO-Sham ($n = 12$); ACE-NKO- MI+ vehicle ($n = 17$); ACE-NKO- MI + Ac-SDKP ($n = 9$); *$p \leq 0.05$; **$p < 0.001$ (By Student's t-tests with Satterthwaite correction for unequal variance).

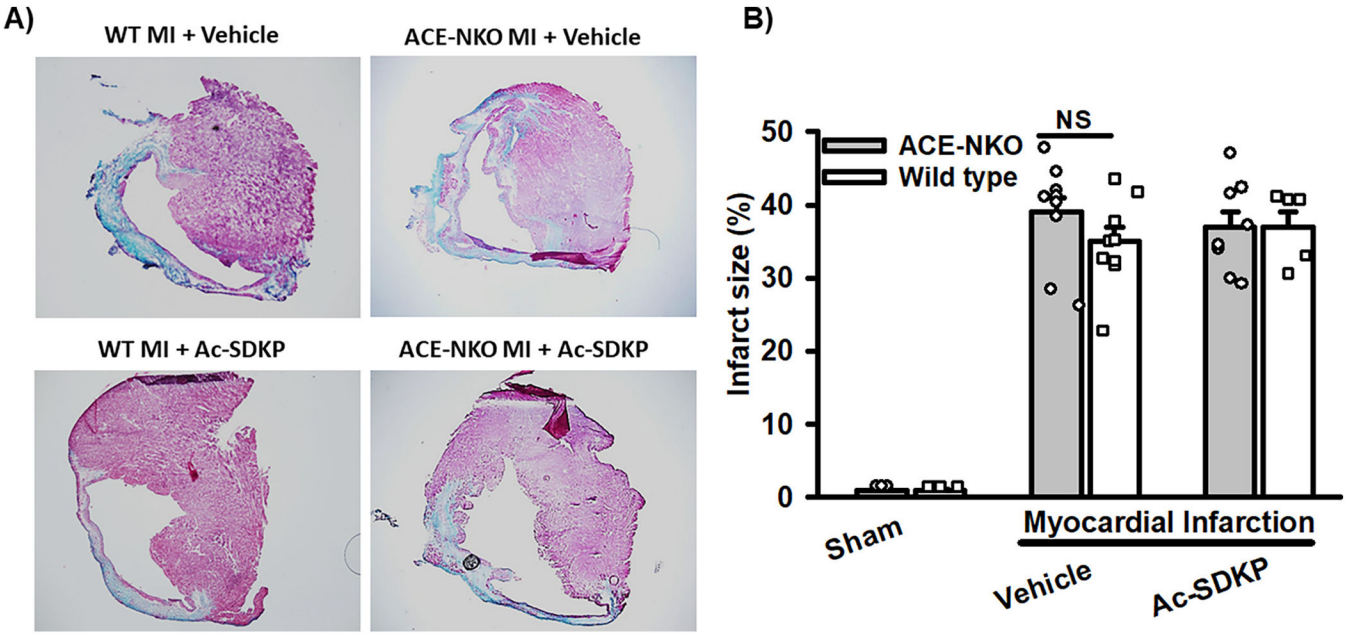


Fig. 2.

A) representative Masson's trichrome staining, showing infarcted portion of the LV; B) quantitative analysis of infarct size shows that ACE-N deletion and/or treatment with Ac-SDKP did not affect the size of infarction post-MI. The number of experiments was as follows: WT-Sham (n = 9), WT- MI+ vehicle (n = 9), WT- MI + Ac-SDKP (*n* = 8), ACE-NKO-Sham (n = 9), ACE-NKO- MI+ vehicle (*n* = 10), ACE-NKO- MI + Ac-SDKP (n = 8).

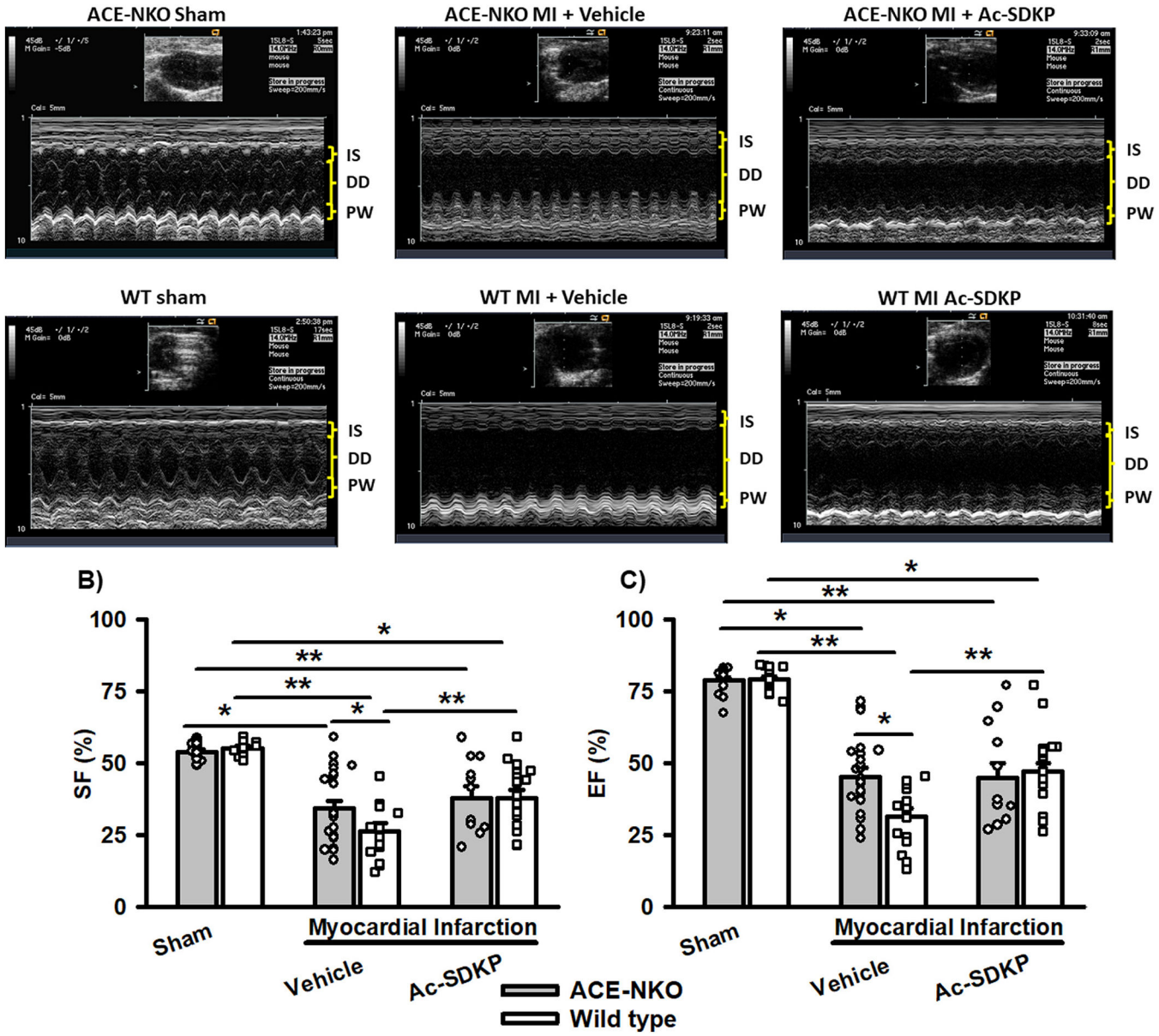
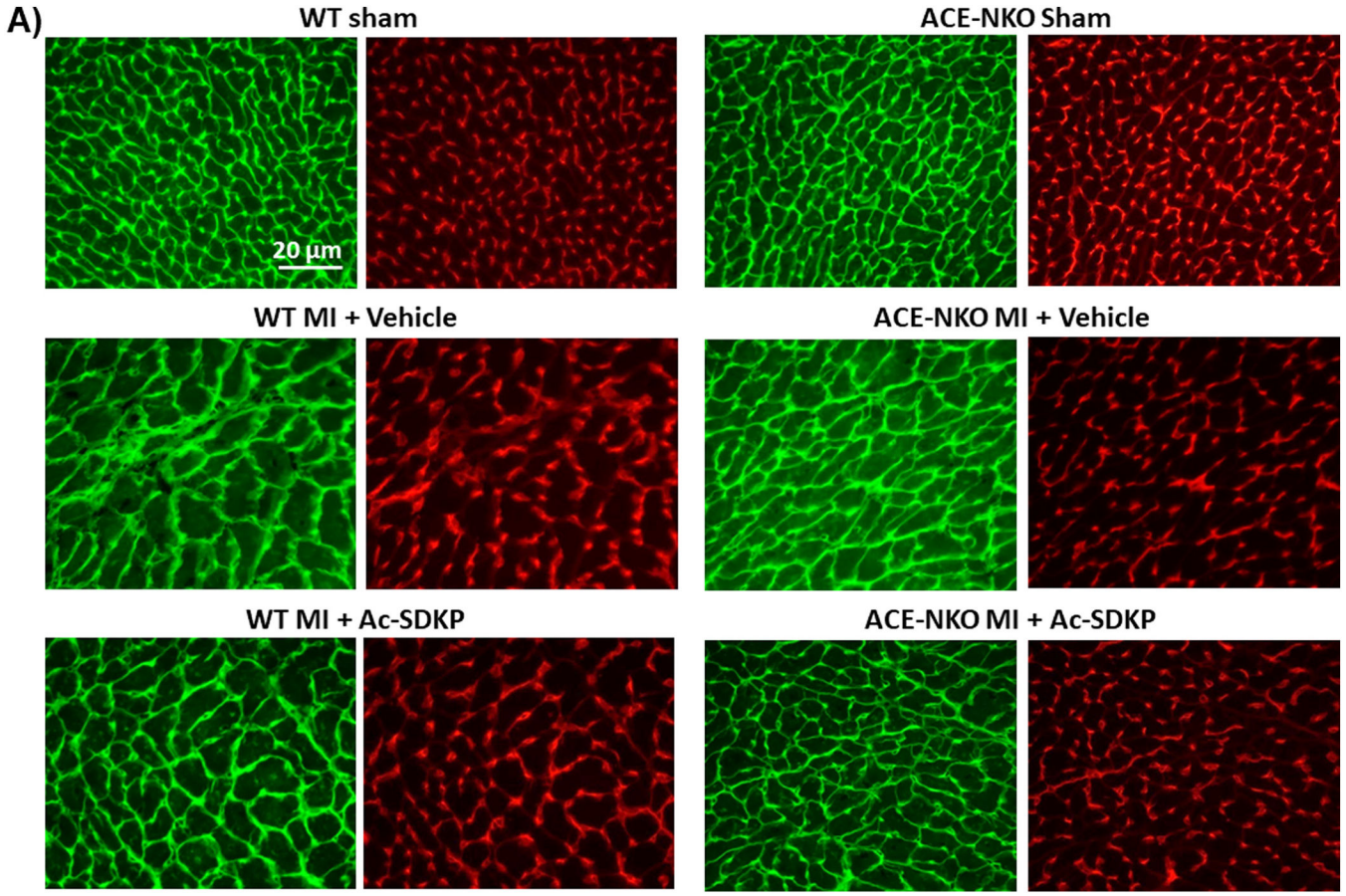


Fig. 3.

Echocardiography measurement in conscious mice 5 weeks post-MI. A) Representative M-mode echocardiography on conscious mice given either vehicle or Ac-SDKP for 5 weeks after MI. IS indicates interventricular septum; DD, LV diastolic dimension; and PW, LV posterior wall. B) and C) Left ventricular shortening fraction (SF) and ejection fraction (EF) in mice treated with or without Ac-SDKP, respectively. Left ventricular chamber dilation and markedly reduced EF and SF were exhibited in mice with MI, ACE-NKO mice had significantly better cardiac performances. Both EF and SF were improved by Ac-SDKP treatment in WT mice but were not further improved in ACE-NKO mice. The number of experiments was as follows: SF; WT-Sham (n = 13), WT- MI+ vehicle (n = 16), WT-MI + Ac-SDKP (n = 21), ACE-NKO-Sham (n = 16), ACE-NKO- MI+ vehicle (n = 26), ACE-NKO- MI + Ac-SDKP (n = 12); EF; WT-Sham (n = 14), WT- MI+ vehicle (n = 15),

WT- MI + Ac-SDKP (n = 20), ACE-NKO-Sham (n = 12), ACE-NKO- MI+ vehicle (n = 17), ACE-NKO- MI + Ac-SDKP (n = 12). *p < 0.05 vehicle *vs* sham; **p < 0.001 Ac-SDKP *vs* vehicle (By Student's t-tests with Satterthwaite correction for unequal variance).



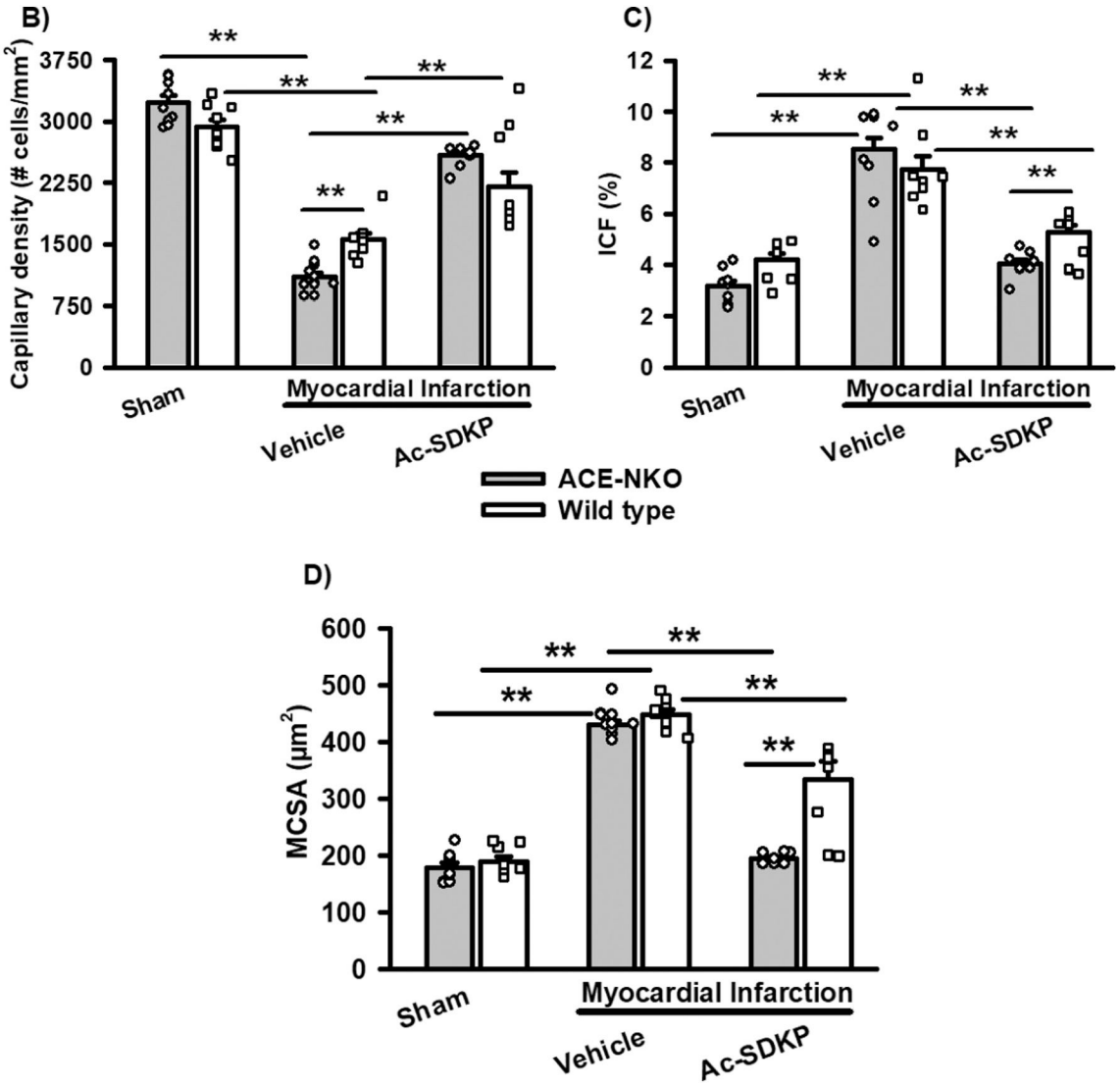


Fig. 4.

A) Representative images of capillaries (red stained) and interstitial collagen (green stained) in the LV 5 weeks after MI; B) Quantitative analysis of capillary density, interstitial collagen fraction (ICF; C) and cardiomyocyte size (MCSA; D) shows that MI caused a decrease in capillary density, an increase in ICF, and cardiomyocyte size in both mouse strains. Ac-SDKP significantly prevented these adverse effects. The increased MCSA was significantly reduced when Ac-SDKP was given exogenously in both the groups, with a pronounced effect in ACE-NKO mice compared to WT. The number of experiments was as follows: (A-C): WT-Sham ($n = 9$), WT- MI+ vehicle (n = 9), WT- MI + Ac-SDKP ($n = 11$), ACE-NKO-Sham (n = 9), ACE-NKO- MI+ vehicle ($n = 15$), ACE-NKO- MI + Ac-SDKP ($n = 8$). **$p < 0.001$ (By Student's t-tests with Satterthwaite correction for unequal variance).

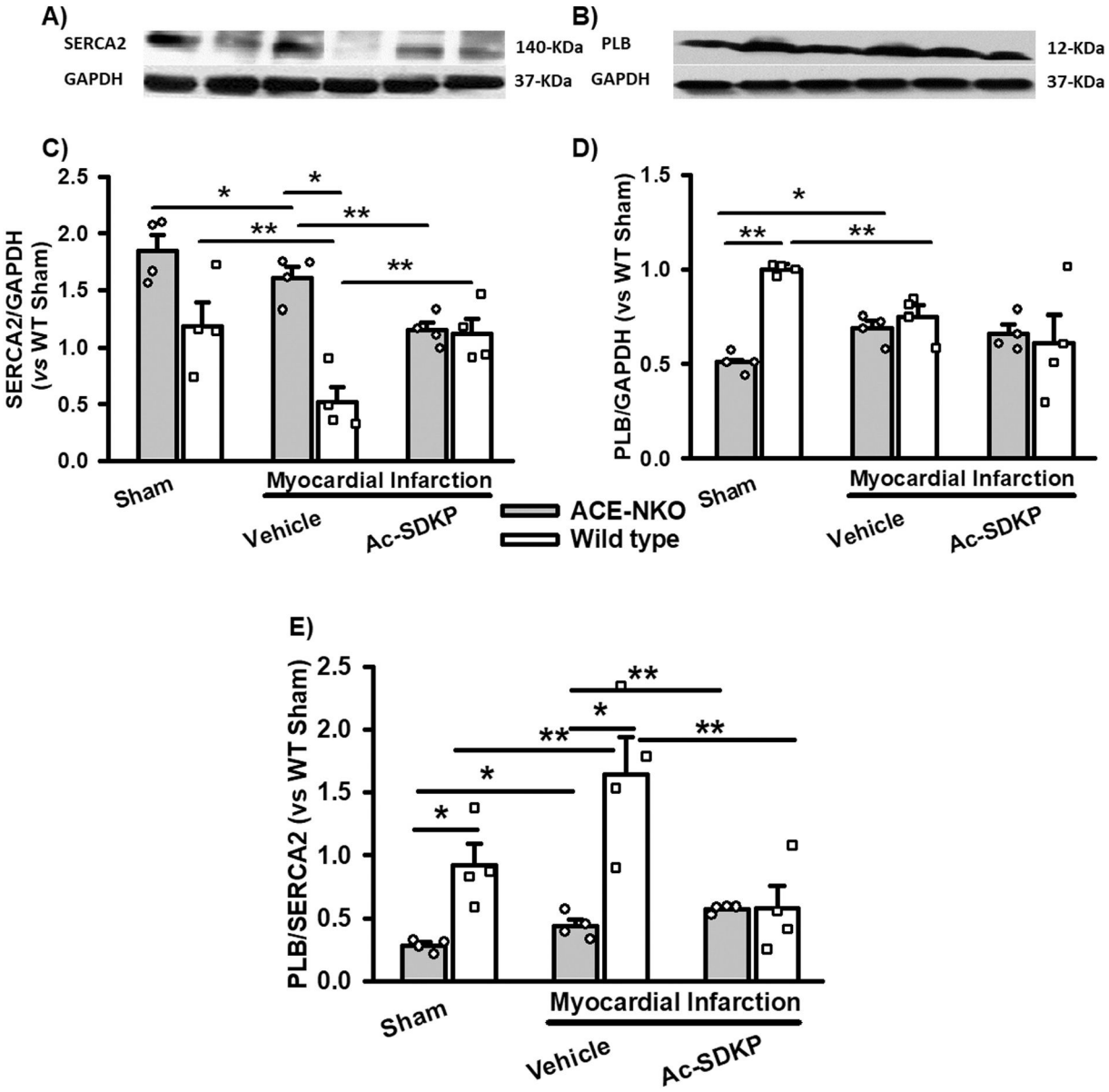


Fig. 5.

The protein expression analyzed by immunoblot in left ventricular (LV) tissue homogenates in WT *vs.* ACE-NKO in mice after 5 weeks post-MI for SERCA2 (A & C) and PLB (B & D). ACE-NKO mice protected the loss of SERCA2 expression compared to WT mice subjected to MI. The loss of SERCA2 expression in WT mice after 5 weeks post-MI, was significantly recovered with exogenous supplementation of Ac-SDKP. In contrast, PLB expression in sham was significantly higher in WT compared to ACE-NKO without any changes after 5 weeks of post-MI in between the two groups. (E) The ratio PLB/SERCA2 shows a significant increase in both sham and MI WT groups compared to ACE-NKO mice; administration of Ac-SDKP significantly normalized PLB/SERCA2 ratio. The number of

experiments repeated was $n = 4$/group; *$p \leq 0.05$, **$p < 0.001$ (By Student's t-tests with Satterthwaite correction for unequal variance).

Table 1

Echocardiographie and body weight measurements 5 weeks post-MI.

Parameters	WT			ACE-N KO		
	Sham	MI + Vehicle	MI + Ac-SDKP	Sham	MI + Vehicle	MI + Ac-SDKP
BW, g	26.75 ± 0.54	26.63 ± 0.67	27.74 ± 0.52	25.07 ± 0.66	27.09 ± 0.36 *	25.11 ± 0.79
LVM, mg/10 g BW	21.36 ± 0.83	46.77 ± 3.67 *	40.09 ± 3.76 *	24.66 ± 0.66	48.82 ± 3.14 *	54.14 ± 7.00 *
LVW, mg/10 g BW	34.48 ± 1.73	47.69 ± 3.23 *	49.10 ± 3.19	38.88 ± 0.89	50.73 ± 1.70 *	54.88 ± 1.51
Infarcted area (%)	None	35.0 ± 0.020	37.0 ± 0.023	None	39.0 ± 0.021	37.0 ± 0.022
PWTd, mm	0.83 ± 0.02	0.85 ± 0.02	0.87 ± 0.02	0.85 ± 0.01	0.90 ± 0.01 *	0.92 ± 0.02 *
LVDd, mm	2.47 ± 0.03	4.04 ± 0.16 *	3.45 ± 0.14 *,†	2.50 ± 0.03	3.95 ± 0.17 *	4.11 ± 0.29 *
LVAd, mm^2	4.11 ± 0.14	17.95 ± 1.96 *	12.84 ± 1.76 †	4.23 ± 0.14	15.00 ± 1.68 *	18.11 ± 2.99
HR, beats/min	684 ± 9	650 ± 16	658 ± 8	665 ± 12 $^{¥}$	614 ± 14	576 ± 23 ‡

Values are means ± standard error. MI, myocardial infarction; LVDd, left ventricular diastolic dimension; LVAd, left ventricular diastolic area; PWTd, diastolic posterior wall thickness; LVW, left ventricular weight, measured by gravimetry; LVM, left ventricular mass, measured by echocardiography; HR, heart rate; BW, body weight.

* $p < 0.005$, *vs* sham within WT or ACE-N KO.

† $p < 0.05$ *vs* MI within WT.

$^{¥}$ $p = 0.018$ *vs* MI within ACE-N KO.

‡ $p < 0.005$

WT *vs* ACE-NKO for MI + Ac-SDKP. The number of experiments was as follows: BW, LVM, PWTd, LVDd, LVAd and HR: (WT/ACE-NKO - Sham; $n = 13$), (WT/ACE-NKO - MI+ Vehicle; $n = 15$), (WT/ACE-NKO - MI + Ac-SDKP; $n = 20$); LVW: (WT - Sham; $n = 9$), (WT - MI+ Vehicle; n = 13), (WT - MI + Ac-SDKP; n = 15); LVW: (ACE-NKO - Sham; n = 15), (ACE-NKO - MI+ Vehicle; $n = 16$), (ACE-NKO - MI + Ac-SDKP; $n = 26$).