Enhanced susceptibility to biomechanical stress in ACE2 null mice is prevented by loss of the p47phox NADPH oxidase subunit

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Aims

Angiotensin-converting enzyme 2 (ACE2) is an important negative regulator of the renin-angiotensin system. Loss of ACE2 enhances the susceptibility to heart disease but the mechanism remains elusive. We hypothesized that ACE2 deficiency activates the NADPH oxidase system in pressure overload-induced heart failure.

Methods and results

Using the aortic constriction model, we subjected wild-type (Ace2+/-), ACE2 knockout (ACE2KO, Ace22/2), p47phox knockout (p47phoxKO, p47phox2/2), and ACE2/p47phox double KO mice to pressure overload. We examined changes in peptide levels, NADPH oxidase activity, gene expression, matrix metalloproteinases (MMP) activity, pathological signalling, and heart function. Loss of ACE2 resulted in enhanced susceptibility to biomechanical stress leading to eccentric remodelling, increased pathological hypertrophy, and worsening of systolic performance. Myocardial angiotensin II (Ang II) levels were increased, whereas Ang 1–7 levels were lowered. Activation of Ang II-stimulated signalling pathways in the ACE2-deficient myocardium was associated with increased expression and phosphorylation of p47phox, NADPH oxidase activity, and superoxide generation, leading to enhanced MMP-mediated degradation of the extracellular matrix. Additional loss of p47phox in the ACE2KO mice normalized the increased NADPH oxidase activity, superoxide production, and systolic dysfunction following pressure overload. Ang 1–7 supplementation suppressed the increased NADPH oxidase and rescued the early dilated cardiomyopathy in pressure-overloaded ACE2KO mice.

Conclusion

In the absence of ACE2, biomechanical stress triggers activation of the myocardial NADPH oxidase system with a critical role of the p47phox subunit. Increased production of superoxide, activation of MMP, and pathological signalling leads to severe adverse myocardial remodelling and dysfunction in ACE2KO mice.

Keywords

Renin–angiotensin system • Angiotensin 1–7 • Angiotensin-converting enzyme 2 • NADPH oxidase • Signalling

1. Introduction

The renin–angiotensin system (RAS) plays a critical role in the pathophysiology of heart failure, and many of the key proven pharmacotherapies are antagonists of the RAS. Cardiac-specific elevation of angiotensin II (Ang II) leads to angiotensin 1 (AT1)-dependent ventricular hypertrophy and heart failure.1,2 Activation of NADPH oxidase is a major effector of Ang II-mediated adverse remodelling of the cardiovascular system.3–8 Heart failure, in experimental models and in patients, is associated with increased NADPH oxidase activity and up-regulation of the NADPH oxidase complex in the myocardium, thereby providing a critical link between elevated Ang II levels and the increased susceptibility to pathological stimuli.9–13 In the heart, kidneys, and lungs, angiotensin-converting enzyme 2 (ACE2) is a negative regulator of the RAS.14–19 In the heart, ACE2 is expressed in cardiomyocytes, fibroblasts, and endothelial cells.19–21
Loss of ACE2 is associated with age-dependent cardiomyopathy and enhances the adverse remodelling in response to myocardial infarction and pressure overload. Pressure overload is associated with early and progressive decrease in the myocardial ACE2 protein levels. In human heart failure, plasma ACE2 activity is increased and correlates with adverse clinical outcomes.

In this study, we investigated the mechanism of the adverse remodelling and increased propensity to develop heart failure in male ACE2KO mice. In response to pressure overload, ACE2-deficient mice develop accelerated adverse remodelling and worsened systolic function in association with increased NADPH oxidase activity and activation of pathological signalling pathways. Myocardial Ang II and Ang 1–7 levels were increased and decreased, respectively, in the pressure-overloaded ACE2-deficient heart. Loss of the p47phox NADPH oxidase subunit prevented the downstream adverse myocardial remodelling. Our study shows that in the absence of ACE2, biomechanical stress facilitates the activation of the NADPH oxidase system leading to early adverse myocardial remodelling.

2. Methods
Further methods are available in the Supplementary Material online.

2.1 Experimental animals and protocols

Ace2−/− mutant and p47phox knockout mice (p47phox−/−) mice back-crossed into the C57BL/6 background for at least eight generations were used in the present study. Only littermate controls were used. All experiments were performed in accordance with institutional guidelines, Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.2 Pressure overload

Young (8–9-week-old) male Ace2−/− wild-type (WT), Ace2−/− (ACE2KO), p47phox−/− (p47phoxKO), and Ace2−/−/p47phox−/− double mutant (ACE2/p47phox DKO) mice were subjected to pressure overload as previously described. Mice were anasthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), the descending aortic arch was accessed via a left thoracotomy, and the descending thoracic aorta was surgically constricted against a 26 gauge needle. The sham animals underwent the same procedure without the aortic banding. ACE2KO mice were also implanted with mini-osmotic pumps to deliver Ang 1–7 (Bachem, Germany) at 2.4 ng/mouse/day against a 26 gauge needle. The Sham animals underwent the same procedure without the aortic banding.

2.3 Echocardiographic and haemodynamic measurements

Mice were lightly anasthetized with isoflurane/oxygen (1%/99%), and measurements were made as previously described. Briefly, transthoracic echocardiography was carried out using an Acuson Sequoia C256 equipped with a 15 MHz linear transducer (Version 4.0, Acuson Corporation, Mountain View, CA, USA), and haemodynamic measurements were made using a 1.4 French Millar catheter (Millar Inc., Houston, which was advanced into the proximal aorta via the right carotid artery and then through the aortic valve into the left ventricle.

2.4 Superoxide assay and dihydroethidium staining

The chemiluminesence lucigenin assay was used to measure NADPH oxidase activity using a single-tube luminometer (Berthold FB12, Berthold Technologies, Germany) modified to maintain the sample temperature at 37°C as previously described. Lucigenin (5 μM) and NADPH (100 μM) were added to the samples and light emission was recorded every 2 min over an 8 min period. The superoxide scavenger, polyethylene glycol-superoxide dismutase (PEG-SOD; 500 U/mL), and the specific peptide inhibitor of NADPH oxidase, gp91phox ds tatt (50 μM), were used to confirm superoxide generation from NADPH oxidase. All measurements were performed in triplicates and results were normalized per 1 mg of protein. Dihydroethidium (DHE) fluorescence was performed on 20 μm frozen myocardial sections, which were washed with Hank’s balanced salt solution (HBBS), incubated at 37°C for 30 min with DHE (20 μM) in HBBS, and then imaged using confocal microscopy.

2.5 Histology and immunohistochemistry

For heart morphometry, hearts were arrested with 1 M KCl, perfuse-fixed with buffered 10% formalin, and embedded in paraffin. Trichrome and Picro-Sirius Red staining and visualization were carried out as previously described.

2.6 Myocardial and plasma peptide levels

Myocardial and plasma Ang II and Ang 1–7 levels were measured at the Hypertension Core Laboratory, Wake Forest University, Winston-Salem, North Carolina, as previously described. Frozen tissues were rapidly weighed and homogenized, and tissue homogenates were extracted using Sep-Pak columns. Ang II was measured using an Alpco Diagnostic kit. Ang-(1–7) was measured using an antibody produced by the Hypertension Core Laboratory, Wake Forest University.

2.7 Taqman real-time–PCR, western blot analysis, immunoprecipitation, and gelatin zymography

RNA levels of the indicated genes were determined as previously described with 18S rRNA as the internal standard (Supplementary Material online, Table S1). Western blot analysis of phospho- and total extracellular signal regulated kinase 1/2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3), Janus kinase 2 (JAK2), and β1D integrin, and membrane and cytosolic protein kinase C (PKCα) were carried out as previously described. Assessment of phosphorylated p47phox was measured by immunoprecipitation with a rabbit polyclonal anti-p47phox antibody (Santa Cruz) followed by western blot using a monoclonal anti-phospho-serine antibody (Abcam). Gelatin zymography was carried out as previously described. Following electrophoresis using 8% SDS-polyacrylamide gels copolymerized with gelatin (2 mg/mL, Sigma), gels were incubated for 48 h at 37°C in an incubation buffer, stained with 0.05% Coomassie Brilliant Blue (G-250; Sigma), and then destained in a mixture of methanol:acetic acid:water (2.5:1:6.5 v/v).

2.8 Statistical analysis

All data are shown as mean ± SEM. All statistical analyses were performed using SPSS software (Chicago, IL, USA; Version 10.1). The effects of genotype and Ang 1–7 were evaluated using ANOVA followed by the Student Neuman–Keuls test for multiple comparison testing, and comparison between two groups were made using Student’s t-test.

3. Results

3.1 Loss of ACE2 enhances cardiac decompensation in response to biomechanical stress

We hypothesize that young Ace2−/− (ACE2KO) mutant mice will be more susceptible to pressure overload-mediated heart failure compared with Ace2−/− WT littermate controls. Aortic constriction led...
to a compensatory concentric hypertrophy in WT mice, whereas parallel ACE2KO mice developed very severe cardiac hypertrophy as indicated by the gross morphology (Figure 1A and B), LV weight-to-tibial length (Supplementary Material online, Figure S1A), and expression of hypertrophy markers, atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and α-skeletal actin (Supplementary Material online, Figure S1B–D). Non-invasive functional assessment by echocardiography and invasive haemodynamic measurements showed a marked decrease in systolic function and increased ventricular dilation in ACE2KO mice, while WT mice maintained preserved systolic function (Supplementary Material online, Figure S1E–I and Table 1).

While banded WT mice exhibited minimal alterations in the myocardial peptide levels for Ang II and Ang 1–7, ACE2-deficiency resulted in a significant increase in myocardial Ang II levels (Figure 1C) and a 25% decrease in myocardial Ang 1–7 levels (Figure 1D), leading to a marked lowering of the myocardial Ang 1–7/Ang II ratio (Figure 1E). In contrast, plasma Ang II and Ang 1–7 levels were similar at baseline and remained unaltered in response to pressure overload (Supplementary Material online, Table S2). Western blot analysis showed increased and decreased AT1 receptor levels in pressure-overloaded WT and ACE2KO myocardium, respectively (Figure 1F). These data show that ACE2 is required to suppress the early eccentric remodelling in response to biomechanical stress.
stress by maintaining an optimal balance of Ang 1–7 and Ang II peptides in the heart.

**3.2 Increased oxidative stress, pathological signalling, and activation of the matrix metalloproteinases axis in ACE2-deficient hearts**

The critical role of ACE2 as a negative regulator of Ang II-mediated signalling in the heart suggests that loss of ACE2 could facilitate the adverse effects of Ang II signalling.\(^1^9,23\) Activation of the NADPH oxidase complex is a central mediator of the pathological effects of Ang II.\(^6,8,11,12\) In addition, pressure overload triggers oxidative stress. In the heart, ACE2 has been shown to form a complex with the p47phox subunit of the NADPH oxidase system.\(^5,6,8,36\) In agreement with previous studies,\(^5–7\) we showed that the p47phox subunit also plays a critical role in the exaggerated oxidative stress response in pressure overloaded ACE2KO mice. (Figure 2B). Next, we examined the activation of various pathological signalling pathways known to be activated by Ang II and NADPH oxidase-derived reactive oxygen species.\(^8,13\) Western blot analysis showed increased phosphorylation of ERK1/2 (Figure 2F) and STAT3 (Figure 2G) without a differential impact on the activation of the JAK2 pathway (Figure 2H). Fractionation of PKCa showed that the membrane fraction decreased to a similar extent (Figure 2J) without changes in the cytosolic fraction (Figure 2I) in response to pressure overload.

While the WT mice showed increased collagen deposition and relative preservation of the interstitial collagen network, the extracellular matrix (ECM) in the banded ACE2KO hearts was clearly disrupted (Figure 3A). Trichrome staining confirmed the increase in interstitial fibrosis in WT myocardium, which was absent in the ACE2KO hearts (Figure 3B). We hypothesized that the Ang II-mediated oxidative stress could activate matrix metalloproteinases (MMPs) leading to degradation of the ECM proteins. Expression analysis of key MMPs involved in heart disease showed equivalent elevation in the expression of MMP2 (Figure 3C), but a greater increase in the expression of MMP9 (Figure 3D) and the membrane-anchored collagenase membrane type 1 (MT1)-MMP (Figure 3E) in ACE2KO compared with WT mice post-thoracic aortic coarctation. Gelatin zymography showed greater activation of the MMP2 system (Figure 3F) with higher levels of the active MMP2 (Figure 3G), while the total amount of pro-MMP9 was also elevated in the absence of active MMP9 (Figure 3H) in ACE2-deficient myocardium. Integrins are important mediators of the adaptive response to biomechanical stress. In the heart, ACE2 has been shown to form a complex with β1 integrin.\(^18\) However, our analysis of the cardiac-specific β1D integrin showed no change in ACE2KO hearts compared with WT hearts (Figure 3I). These results show that loss of ACE2 results in greater activation of the NADPH oxidase system and pathological signalling in the heart with activation of the MMPs and ECM degradation.

**3.3 Increased oxidative stress with ACE2 deficiency is dependent on the p47phox NADPH oxidase subunit**

The p47phox subunit of the NADPH oxidase system is a critical determinant of the Ang II-mediated oxidative stress in the cardiovascular system.\(^5–8,36\) In agreement with previous studies,\(^5–7\) we showed that Ang II-mediated superoxide production and NADPH oxidase activity are p47phox-dependent (Figure 4A and B). Next, we examined whether the p47phox subunit also plays a critical role in the exaggerated oxidative stress response in pressure overloaded ACE2KO mice. We generated double mutant mice lacking Ace2 and p47phox genes (Ace2\(^−/−\)/p47phox\(^−/−\)) and subjected them to aortic constriction. We found that NADPH oxidase activity was significantly reduced in the banded Ace2\(^−/−\)/p47phox\(^−/−\) mice (Figure 4C). While the expression of the NOX2 subunit was not significantly lowered in the DKO

| Table 1 Worsened systolic function in Ace2\(^−/−\) mutant mice in response to pressure overload |
|------------------|------------------|------------------|------------------|------------------|
|                  | Ace2\(^−/−\) + SH | Ace2\(^−/−\) + AB | Ace2\(^−/−\) + AB | Ace2\(^−/−\) + SH | Ace2\(^−/−\) + AB | Ace2\(^−/−\) + AB |
| N                | 8                | 10               | 10               | 8                | 10               | 10               |
| HR (b.p.m.)      | 527 ± 14         | 534 ± 12         | 521 ± 15         | 524 ± 11         | 539 ± 17         | 536 ± 12         |
| PWT (mm)         | 0.59 ± 0.06      | 0.72 ± 0.07      | 0.84 ± 0.08\(^a\) | 0.60 ± 0.051     | 0.64 ± 0.08      | 0.79 ± 0.08\(^a\) |
| LVEDD (mm)       | 3.81 ± 0.07      | 3.83 ± 0.09      | 3.68 ± 0.08      | 3.83 ± 0.08      | 4.13 ± 0.14      | 4.98 ± 0.19\(^a\) |
| LVESD (mm)       | 1.72 ± 0.06      | 1.78 ± 0.09      | 1.73 ± 0.07      | 1.79 ± 0.08      | 2.58 ± 0.13      | 3.62 ± 0.16\(^a\) |
| FS (%)           | 54.8 ± 2.6       | 53.5 ± 3.1       | 52.8 ± 2.5       | 51.2 ± 3.1       | 41.6 ± 2.3\(^a\) | 26.9 ± 3.2\(^a\) |
| VCF (cirs/cm)    | 10.4 ± 0.3       | 10.1 ± 0.35      | 9.92 ± 0.26      | 10.1 ± 0.42      | 7.83 ± 0.41      | 6.71 ± 0.31\(^a\) |
| PAV (cm/s)       | 95.7 ± 2.4       | 92.6 ± 3.5       | 94.5 ± 3.2       | 93.4 ± 2.9       | 90.4 ± 4.8       | 78.3 ± 4.1\(^a\) |
| SBP (mm Hg)      | 114.4 ± 29       | NM               | 171.6 ± 4.9\(^a\) | 116.7 ± 3.8      | NM               | 149.6 ± 4.8\(^a\) |
| DBP (mm Hg)      | 71.5 ± 2.6       | NM               | 75.8 ± 3.1       | 70.2 ± 3.1       | NM               | 69.3 ± 3.6        |

\(^{a}\)P < 0.01 compared with all other groups.

\(^{b}\)P < 0.05 compared with corresponding sham-operated group.
Loss of ACE2 leads to increased NADPH oxidase activity and activation of pathological hypertrophy signalling pathways in response to pressure overload. (A–E) Representative DHE fluorescence images (A) and NADPH oxidase activity quantified by lucigenin-enhanced chemiluminescence (B) showing increased superoxide production with elevation in NADPH oxidase activity and increased expression of NADPH oxidase subunits NOX2 (gp91phox) (C), NOX4 (D) and p47phox (E) in ACE2KO mice in response to pressure overload. (F–J) Western blot analysis showing increased phosphorylation of ERK1/2 (F) and STAT3 (G) without differential phosphorylation of the JAK2 pathway (H) while the membrane levels of PKCα (I) was decreased to the same extent with no change in cytosolic PKCα level (J). Sh, sham-operated; AB, aortic banded; R.E., relative expression; PEG-SOD, polyethylene glycol-superoxide dismutase (500 U/mL), gp91 tat, gp91phox ds tat peptide (50 μM) and Scr tat, scrphox ds tat peptide (50 μM). ERK1/2, extracellular signal-regulated kinase 1/2; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription 3 and PKC, Protein kinase C. n = 6 for sham-operated; n = 8 for aortic-banded. *P < 0.05 compared with corresponding sham-operated group; #P < 0.05 compared with WT + AB group.
Figure 3 Adverse remodelling of the ECM in pressure overload ACE2-deficient myocardium due to increased activation of the matrix metalloproteinase axis. (A and B) Histological assessment of the ECM using picrosirius red staining with confocal imaging (A) and trichrome staining (B) showing disruption of the ECM and less collagen deposition in the ACE2KO hearts. (C–E) Expression analysis of the key matrix metalloproteinases (MMPs) in the heart, MMP2 (C), MMP9 (D), and membrane type 1 (MT1)-MMP (E) revealing equivalent increase in MMP2 levels with a greater increase in MMP9 and MT1-MMP levels in the banded ACE2KO hearts. (F–H) Gelatin zymography showing greater cleavage and activation of MMP2 (F), which is quantified and shown (G) with greater pro-MMP9 levels (H) in the ACE2 knockout myocardium. (I) Western blot analysis and quantification of myocardial β1D integrin levels showed no difference between WT and ACE2KO hearts. Sh, sham-operated; AB, aortic banded; R.E., relative expression; 18S rRNA was used as an endogenous control. n = 6 for sham-operated; n = 8 for aortic-banded. *p < 0.05 compared with the corresponding sham-operated group; **p < 0.05 compared with WT + AB group.
mice (Figure 4D), expression of the NOX4 subunit was reduced by 50% (Figure 4E) in the pressure overloaded DKO mice. The generation of reactive oxygen species is sufficient to activate various downstream pathways resulting in pathological hypertrophy. Consistent with the suppressed oxidative stress in the DKO mice, phosphorylation of ERK1/2 and STAT3 was significantly reduced in response to pressure overload compared with the ACE2KO group (Figure 4F and G). Interestingly, aortic constriction of mice lacking the p47phox subunit (p47phoxKO) was also associated with reduced NADPH oxidase activity and expression of NOX2 and NOX4 subunits with lack of activation of the pathological signalling pathways (Figure 4C–G).

The reduction in pathological signalling resulted in reduced hypertrophic response based on morphometry (Figure 5A) and expression of hypertrophy markers BNP (Figure 5B), ANF (Figure 5C), and α-skeletal actin (Figure 5D) in the p47phoxKO and DKO mice. Cardiac systolic function was dramatically preserved in the banded DKO based on fractional shortening (Figure 5E) and haemodynamic assessment of +dP/dt max (Figure 5F). The reversal of pathological hypertrophy and systolic dysfunction in the DKO mice indicates that the adverse remodelling of the myocardial ECM was minimized. While expression analysis showed reduced expression of MT1-MMP (Figure 5G), MMP2 (Figure 5H), and MMP9 (Figure 5I) expression was not altered by the loss of p47phox in ACE2 null mice, suggesting that up-regulation of these MMPs is p47phox-independent. In contrast, activation of MMP2 as shown by gelatin zymography (Figure 5J) and quantification (Figure 5K) showed reduced active MMP2 levels and reduced pro-MMP9 protein levels (Figure 5L) in banded DKO mice compared with the banded ACE2KO mice. These results show that
Figure 5 Loss of p47phox subunit rescues the ACE2 knockout mice in response to pressure overload. (A–D) Reduced pathological hypertrophy based on morphometry (A) and expression of BNP (B), ANF (C), and α-skeletal muscle actin (D) in the double knockout (ACE2KO/p47phoxKO) mice. (E–F) Marked improvement in left ventricular function in double mutant (ACE2KO/p47phoxKO) mice based on echocardiographic (E) and haemodynamic (F) measurements. (G–I) Expression levels of MT1-MMP (G), MMP2 (H), and MMP9 (I) showing a marked reduction in MT1-MMP levels without lowering of the MMP2 and MMP9 mRNA levels. (J–L) Gelatin zymography (J) and quantification of active and pro-MMP2 levels (K) and pro-MMP9 protein levels (L) showing a drastic reduction in the activation of MMP2 and lowered pro-MMP9 levels in response to loss of the p47phox subunit. HW, heart weight; LVW, left ventricular weight; TL, tibial length; Sh, sham-operated; AB, aortic banded; R.E., relative expression; 18S rRNA was used as an endogenous control. ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; FS, fractional shortening. n = 6 for sham-operated; n = 8 for aortic-banded. *P < 0.01 compared with corresponding sham-operated; #P < 0.01 compared with ACE2KO + AB group.
Figure 6  Ang 1–7 supplementation reduces pathological myocardial hypertrophy, restores cardiac systolic function, and suppressed superoxide production in pressure-overloaded ACE2KO mice. (A–D) Ang 1–7 supplementation resulted in normalization of the increased hypertrophy based on morphometry (A) and expression analysis of ANF (B), BNP (C), and α-skeletal actin (D). (E–I) Functional assessment based on echocardiography and invasive haemodynamics revealed restoration of the fractional shortening (E) and LV dilation (F) with reduction in the elevated left ventricular end diastolic pressure (G) and partial restoration of myocardial contractility as determined by $+\Delta P/\Delta t_{\text{max}}$ (H) and $-\Delta P/\Delta t_{\text{min}}$ (I). (J–L) Representative DHE fluorescence images (J) and NADPH oxidase activity (K) showing reduced superoxide production with reversal of the elevated phospho-p47phox levels (L) in response to Ang 1–7 supplementation. See Supplementary Material online, Figure S1 for list of abbreviations. $n = 8$ for all aortic-banded groups. *P < 0.01 compared with corresponding sham-operated; **P < 0.01 compared with ACE2KO + AB group.
the adverse myocardial remodelling in ACE2KO mice in response to biomechanical stress is clearly NADPH oxidase-dependent with a dominant role of the p47phox subunit.

3.4 Ang 1–7 supplementation rescues the early dilated cardiomyopathy in pressure-overloaded ACE2 knockout mice

Loss of ACE2 reduces the myocardial steady-state levels of Ang 1–7. Ang 1–7 mediates important direct cardioprotective effects.37,38 We treated pressure-overloaded ACE2KO mice with exogenous Ang 1–7 to test whether Ang 1–7 deficiency played an important role in the adverse myocardial remodelling in ACE2KO mice. Ang 1–7 supplementation resulted in normalization of the increased hypertrophy based on morphometry (Figure 6A) and expression analysis of ANF (Figure 6B), BNP (Figure 6C), and α-skeletal actin (Figure 6D). Echocardiographic assessment revealed restoration of the fractional shortening (Figure 6E) and ventricular dilation (Figure 6F) with reduction in the elevated left ventricular end diastolic pressure (Figure 6G) and partial restoration of myocardial contractility as determined by $+\Delta P/\Delta t_{\text{max}}$ (Figure 6H) and $-\Delta P/\Delta t_{\text{min}}$ (Figure 6I). This functional rescue was associated with reduced superoxide production as shown by DHE staining (Figure 6J) and NADPH oxidase activity (Figure 6K) with reversal of the elevated phospho-p47phox levels (Figure 6L). These results show that in diseased ACE2-deficient myocardium, Ang 1–7 supplementation suppresses the elevated NADPH oxidase-mediated superoxide production and restores heart function.

4. Discussion

Inhibition of the RAS has lead to important pharmacological tools to treat a wide variety of cardiovascular and renal disorders. ACE2 has emerged as a central negative regulator of Ang II signalling,14,15,18,19 and strategies aimed at enhancing ACE2 action may have important therapeutic potential for cardiovascular disorders.18,19 In this study, we showed that absence of ACE2 leads to greater Ang II and lowered Ang 1–7 levels, leading to an amplification of Ang II-mediated pathological hypertrophy, ventricular remodelling, and systolic dysfunction. We provided definitive evidence that ACE2 deficiency leads to an Ang II-mediated activation of the NADPH oxidase system and exacerbated oxidative stress leading to pressure overload-induced heart failure. These results are compatible with the exacerbation of Ang II-induced myocardial remodelling and dysfunction by the loss of ACE2 and the ability of human recombinant ACE2 to suppress Ang II-induced heart disease and pressure overload-induced heart failure.19 In pressure overloaded ACE2-deficient myocardium, relative loss of Ang 1–7 facilitated this adverse remodelling given the ability of Ang 1–7 to mediate direct cardioprotective effects.37,38

Ang II-mediated NADPH oxidase activation is mediated by Ang II-induced expression of several NADPH oxidase subunits including p47phox39 and/or Ang II-induced serine phosphorylation of the p47phox subunit.61 In response to pressure overload, ACE2KO hearts showed increased expression and phosphorylation of the p47phox subunit, indicative of NADPH oxidase activation. These data further substantiate the increased DHE fluorescence and NADPH oxidase activity in the banded ACE2KO hearts. The rescue strategy using the p47phoxKO mice provides definitive evidence for a key role of the p47phox subunit in Ang II-mediated NADPH oxidase activation in the absence of ACE2. Loss of p47phox resulted in reduced activation of major signalling cascades, which is consistent with a major role of p47phox subunit in mediating the adverse effects of Ang II in the cardiovascular system.4,7,11 Our results are consistent with the reduced ventricular dilation and improved ejection fraction in p47phoxKO mice subjected to myocardial infarction.30 Moreover, human recombinant ACE2 suppresses Ang II-induced NADPH oxidase activation and reactive oxygen species (ROS) production in cardiomyocytes19 and mesangial cells,18 which is partially dependent on Ang 1–7 signalling. Given the ability of ACE2 to metabolize Ang II into Ang 1–7, loss of ACE2 likely resulted in enhanced Ang II-mediated activation of the AT1 receptor.19,23,24

Mechanical stress and Ang II-mediated generation of ROS activates MMP2 in a p47phox-dependent manner.40–42 In addition to the enzymatic cleavage and activation of MMP2 by cell surface-expressed MT1-MMP,43 ROS have also been shown to directly activate MMP2.44 Indeed, while loss of p47phox did not prevent up-regulation of MMP2 expression, activation of MMP2 was drastically reduced in the ACE2/p47phox DKO mice in response to biomechanical stress. In summary, loss of ACE2 results in enhanced Ang II-mediated pathological hypertrophy and ventricular dilation in response to acute biomechanical stress due primarily to increased activation of the NADPH oxidase system. Enhancing Ang 1–7 action in the pressure overloaded ACE2KO mice prevented the NADPH oxidase activation, resulting in reduction of the adverse myocardial remodelling and preservation of systolic function. ACE2 negatively regulates the RAS in the setting of heart disease by metabolizing Ang II into Ang 1–7. Further studies are needed to better define the changes in myocardial Ang 1–7 levels and the precise mechanism of the suppression of NADPH oxidase activity associated with Ang 1–7 supplementation in pressure overloaded ACE2KO mice.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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