ACE2 deficiency worsens epicardial adipose tissue inflammation and cardiac dysfunction in response to diet-induced obesity

By

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ABSTRACT

Obesity is increasing in prevalence and is strongly associated with metabolic and cardiovascular disorders. The renin-angiotensin system (RAS) has emerged as a key pathogenic mechanism for these disorders; angiotensin (Ang) converting enzyme 2 (ACE2) negatively regulates RAS by metabolizing Ang II into Ang 1-7. We studied the role of ACE2 in obesity-mediated cardiac dysfunction. ACE2-null (ACE2KO) and wildtype (WT) mice were fed a high-fat diet (HFD) or control diet and studied at 6-months of age. Loss of ACE2 resulted in decreased weight gain, but increased glucose intolerance, epicardial adipose tissue (EAT) inflammation and polarization of macrophages into a pro-inflammatory phenotype in response to HFD. Similarly, human EAT in patients with obesity and heart failure (HF) display a pro-inflammatory macrophage phenotype. Exacerbated EAT inflammation in ACE2KO-HFD mice was associated with decreased myocardial adiponectin, decreased phosphorylation of AMPK, increased cardiac steatosis and lipotoxicity and myocardial insulin resistance which worsened heart function. Ang 1-7 (24 µg/kg/hr) administered to ACE2KO-HFD mice resulted in ameliorated EAT inflammation, and reduced cardiac lipotoxicity and steatosis resulting in normalization of HF. In conclusion, ACE2 has a novel role in heart disease associated with obesity, where ACE2 negatively regulates obesity-induced EAT inflammation and cardiac insulin-resistance.

KEYWORDS:

Angiotensin 1-7, Angiotensin converting enzyme 2, diet-induced obesity, insulin resistance, heart disease
LIST OF ABBREVIATIONS

ACE2 – angiotensin converting enzyme 2
ACE2KO – ACE2 knockout
AMPK – AMP-activated protein kinase
ANF – atrial natriuretic factor
ANG – angiotensin
BNP – brain natriuretic peptide
CON – control diet
DIO – diet-induced obesity
EAT – epicardial adipose tissue
EDPVR – end-diastolic pressure-volume relationship
HFD – high-fat diet
HFPEF – heart failure with preserved ejection fraction
IF – immunofluorescence
IL – interleukin
IPGTT – intra-peritoneal glucose tolerance test
LVEDP – left ventricular end-diastolic pressure
Mφ – macrophage
MCP-1 – monocyte chemoattractant protein-1
β-MHC – β-myosin heavy chain
PDH – pyruvate dehydrogenase
PDK – pyruvate dehydrogenase kinase
RAS – renin-angiotensin system
SIRT – Sirtuin
TAG – triacylglycerol
TNF-α – Tumor necrosis factor-alpha
WT – wildtype
INTRODUCTION

Obesity is a growing worldwide health problem and results in increased health care burden and decreased life expectancy. Obesity itself is an independent risk factor for the development of heart failure with preserved ejection fraction (HFPEF) independent of other co-morbid conditions (1-4). Although a number of mechanisms including lipotoxicity, inflammation, mitochondrial dysfunction, endoplasmic reticulum stress and apoptosis are speculated to contribute to obesity-induced cardiac dysfunction, the ultimate cause and mechanisms remain elusive (5-7). Both clinical and experimental evidences have revealed a key role of excessive fat in the onset of obesity and the accompanied inflammation and cardiac dysfunction.

Components of the renin-angiotensin system (RAS) is present in white and brown adipose tissues where the local RAS can be pathogenic (8). The angiotensin type 1 (AT1R) and type 2 (AT2R) receptors may mediate the effect of Ang II and cause upregulation of adipose tissue lipogenesis (mediated via AT2R) and downregulation of lipolysis (mediated via AT1R) (9; 10). Angiotensin (Ang) converting enzyme (ACE) 2 is a central member of the RAS family which degrades Ang II into Ang 1-7 (11; 12). Ang 1-7 is a biologically active product of the Ang II degradation, which via the activation of Mas receptors leads to vasodilatory, anti-hypertrophic and anti-fibrotic effects (13-16). ACE2 has a widespread distribution in various organs and cell-types including adipocytes (17). ACE2 is a negative regulator of the activated RAS in various disease states including heart failure, diabetic nephropathy and cardiomyopathy, and vascular dysfunction (18-21). In this study, we determined a novel role of ACE2 in adipose tissue inflammation and its effects on cardiac function in diet-induced obesity (DIO).
RESEARCH DESIGN AND METHODS

Experimental animals and protocols. Ace2\textsuperscript{-/y} mutant mice (ACE2KO) backcrossed into the C57BL/6 background for at least 8 generations were used in the present study (18; 21; 22). All experiments were performed in accordance with University of Alberta institutional guidelines which conformed to guidelines published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (revised 2011). Male wildtype (WT) and ACE2KO mice were fed either a high-fat diet (HFD; 45 kcal%) or control diet (CON; 10 kcal%) from weaning until 6 months of age. Alzet micro-osmotic pumps (Model 1002, Durect Corp.) were implanted subcutaneously in ACE2KO-HFD mice to deliver Ang 1-7 (24 µg/kg/hr) or saline (control) for 4 weeks (21). All the mice were studied at 6 months of age. Epicardial adipose tissues (EAT) were collected under a stereo microscope following the removal of the pericardium and the pericardial fat.

Human EAT. Human EAT obtained from explanted non-obese non-failing hearts and diseased hearts from obese (BMI>30) patients with HFPEF secondary to hypertension or transplant vasculopathy, was studied as part of the Human Explanted Heart Program (HELP) at the Mazankowski Alberta Heart Institute and the Human Organ Procurement and Exchange (HOPE) program at the University of Alberta Hospital. All experiments were performed in accordance with the institutional guidelines and were approved by Institutional Ethics Committee. Informed consents were obtained from all participants.

Intra-peritoneal glucose tolerance test. Intraperitoneal glucose tolerance test (IPGTT) was performed on overnight (16 hr) fasted mice. Briefly, mice were administered glucose (1 g/kg,
blood glucose levels were monitored at 0, 15, 30, 60, 90 and 120 minutes post glucose administration, and were used to plot the blood glucose v/s time curves to determine the glucose tolerance (13).

**Echocardiography, pressure-volume loop analysis, and quantitative magnetic resonance.** Transthoracic echocardiography was performed noninvasively to assess systolic and diastolic function, as described previously using a Vevo 770 high-resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada) (13; 18). Mice were placed on a heating pad and a nose cone with 1.5% isoflurane in 100% oxygen was applied. The temperature was maintained at 36.5 to 37.5°C. Ultrasound gel was placed on the chest of the anesthetized mouse. The temperature and heart rate (HR) were constantly monitored during the scanning. M-mode images were obtained for measurements of left ventricular ejection fraction (LVEF). Trans-mitral flow and tissue Doppler imaging were used to assess the E/A ratio and E/E’ ratios. LV pressure-volume relationships were assessed using a 1.2F admittance catheter (Scisense Inc.), as previously described (13). The position of the catheter was monitored by pressure along with the magnitude and phase using ADvantage pressure-volume system (Scisense Inc.) and iworx (iWorx Systems Inc.). Online as well as offline calculations were performed using LabScribe2 software (version 2.347000). Body composition, either fat mass or lean mass, was assessed using an EchoMRI-900 (Echo Medical Systems, Houston, TX, USA), as reported previously (13).

**Isolated working heart perfusion.** At the end of the protocol, isolated hearts were perfused in a working mode at a left atrial preload of 11.5 mmHg and an aortic afterload of 50 mmHg, as previously reported (13). The perfusate contained 2.5 mM Ca$^{2+}$, 5 mM [U-$^{14}$C]glucose, and 1.2
mM [9, 10-3H]palmitate prebound to 3% fatty acid free bovine serum albumin. We used a higher concentration of palmitate to simulate the physiological fatty acid levels in high-fat diet fed mice. Hearts underwent aerobic perfusion in the absence of insulin for the first 30 min, then 100 µU/mL insulin was added to the perfusate in order to examine the response to insulin. Glucose oxidation rates or palmitate oxidation rates were measured by quantitative collection of 14CO2 and 3H2O from [U-14C]glucose and [9,10-3H]palmitate, respectively. Glucose-derived and palmitate-derived ATP production rates were calculated from the rates of glucose oxidation and palmitate oxidation.

**Histological and immunofluorescence (IF) analyses, Oil O red staining, Western blot analysis and Taqman real time PCR.** Mouse and human EAT and mouse hearts were utilized to study the histology, immunofluorescence (IF) staining, WGA staining to delineate the cell membrane and confocal imaging as well as Western blot and Taqman real-time PCR molecular analyses, as previously described (13; 18; 20). Picro-sirius red staining was performed on the 10 µm thick formalin-fixed paraffin-embedded sections to assess the cardiac fibrosis as previously described (18; 23). Oil O red staining was carried out on the 5 µm thick OCT-embedded cryosections, as previously described (13).

**Tumor necrosis factor-alpha (TNF-α) ELISA assay.** ELISA assay was carried out in the EAT to assess TNF-α protein levels using a commercially available kit (R&D Systems.), as previously described (24). Briefly, 50 µg of the total proteins isolated from pooled EAT was utilized to assess the TNF-α levels using the murine recombinant TNF-α as standard. The plates were analyzed with the multi-plate reader (Spectramax).
Statistical analysis. All data are shown as mean ± SEM. All statistical analyses were performed using SPSS software (Chicago, Illinois; Version 22). Comparison between two groups was made using a non-paired Student t-test. The effects of genotype and HFD were evaluated using one-way ANOVA followed by the Student-Neuman-Keuls test for multiple-comparison testing. In experiments with multiple treatments, one-way ANOVA was followed by multiple comparisons using the Student Neuman-Keuls test. Statistical significance is recognized at P<0.05.

RESULTS

Loss of ACE2 increased epicardial adipose tissue inflammation in diet-induced obesity. A marked upregulation of ACE2 was observed in the EAT from WT mice subjected to DIO (Fig. 1A). Growth curves and assessment of body composition (analyzed by quantitative magnetic resonance) showed that ACE2KO-HFD mice had a smaller gain in the total body weight and total fat mass (Fig. 1B, Supp. Fig. 1A-B) without a differential effect on the body composition as illustrated by equivalent increase in fat (Supp. Fig. 1C) and lean (Supp. Fig. 1D) mass compositions compared with the WT-HFD mice. Despite the reduced obesity, ACE2KO-HFD mice showed a greater increase in fasting plasma glucose (Supp. Fig. 1E) and increased glucose intolerance (Fig. 1C; Supp. Fig. 1F) compared with the WT-HFD mice. Our results are in consistent with the previous observation of impaired glucose intolerance in the ACE2KO mice in response to high-calorie diet (25).

Adipose tissue inflammation is linked to obesity-induced insulin resistance (26). While high-fat diet feeding resulted in equivalent increase in EAT mass in WT and ACE2KO mice (Supp.
Fig. 1G), histological analysis by hematoxylin-eosin staining (Supp. Fig. 2A) and wheat-germ agglutinin staining (Supp. Fig. 2B) showed an uncoupling between obesity and adipose tissue inflammation in the ACE2KO-HFD EAT. Despite the reduced obesity in ACE2KO-HFD mice, EAT showed increased inflammatory cell infiltration (Supp. Fig. 2A), “crown-like structures” (Supp. Fig. 2C) and increased adipocyte area (Supp. Fig. 2B, 2D). The increased “crown-like structures” in the EAT of ACE2KO-HFD mice is suggestive of advanced inflammation and increased adipocyte necrosis (27). Immunofluorescence staining for macrophage (Mφ) cell markers including the F4/80 (Mφ cell-surface marker), CD11c (marker for pro-inflammatory phenotype of Mφ which resembles M(IFN-γ) Mφ) and CD206 (marker for anti-inflammatory phenotype of Mφ which resembles M(IL-4) Mφ) was carried out (Fig. 1D) to characterize the phenotypes of Mφ in the EAT (28; 29). The EAT of WT-HFD displayed a smaller increase in the CD11c+/F4/80+ and a greater increase in the CD206+/F4/80+ phenotypes of Mφ based on IF staining (Fig. 1D-1F). In contrast, EAT of ACE2KO-HFD showed polarization of the Mφ phenotype resulting in increased CD11c+/F4/80+ and decreased CD206+/F4/80+ phenotypes of Mφ (Fig. 1D-1F) compared to the WT-HFD. Correspondingly, mRNA expression profile of ACE2KO-HFD EAT showed a greater increase in the expression of pro-inflammatory cytokine, tumor necrosis factor (TNF)-α resulting in increased TNF-α protein levels in the ACE2KO-HFD EAT (Fig. 1G-1H). Gene expression analysis also showed greater increase in M(IFN-γ)-associated inducible nitric oxide synthase (iNOS), monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-1β and IL-6 (Fig. 1I, Supp Fig. 1E-1G) and a lesser increase in the M(IL-4)-associated anti-inflammatory cytokine IL-10 (Fig. 1J) mRNA expressions in the ACE2KO-HFD EAT compared with the WT-HFD EAT.
We also found increased ACE2 in the EAT obtained from the obese HFPEF patients compared with the non-obese NFC (Fig. 1K). The BMI was 25.3±1.6 (n=6) and 36.7±2.1 (n=6; p<0.05) in the non-obese NFC and obese patients with HFPEF, respectively. Immunofluorescence staining revealed increased resident Mφ in the EAT obtained from explanted diseased hearts from patients with obesity (Fig. 1L). Importantly, the Mφ in the EAT from obese patients displayed greater increase in CD11c+/F4/80+ phenotype of Mφ compared with CD206+/F4/80+ phenotype of Mφ (Fig. 1L-1N). These results illustrate ACE2 plays a dominant role in suppressing EAT inflammation and in maintaining glucose tolerance in the setting of obesity.

**ACE2 deficiency worsened cardiac insulin resistance resulting in heart failure with preserved ejection fraction.** We next examined the effects of increased EAT inflammation on HFPEF. Western blot analysis (Fig. 2A), IF (Fig. 2B) and activity assay (Fig. 2C) showed upregulation of myocardial ACE2 in WT mice in response to DIO. Diet-induced obesity resulted in equivalent pathological hypertrophy in WT and ACE2KO mice (Fig. 2D). Conversely, loss of ACE2 resulted in decreased cardiac work when assessed by ex vivo working heart perfusions (Fig. 2E). In WT-CON hearts, perfusion with insulin resulted in a marked increase in glucose oxidation (Fig. 2F). This increase was suppressed in the WT-HFD and ACE2KO-CON hearts, with ACE2KO-HFD hearts showing a severely blunted response to insulin. Myocardial palmitate oxidation in WT and ACE2KO hearts was similarly decreased in response to insulin (Fig. 2G) with an equivalent loss of sensitivity to insulin in both genotypes with DIO (Fig. 2G). Assessment of total ATP (Supp Fig. 3A) and %ATP production (Supp Fig. 3B) showed that ATP production originating from glucose oxidation was stimulated by insulin in WT-CON and ACE2KO-CON hearts, but was partially and
almost completely suppressed in WT-HFD and ACE2KO-HFD hearts, respectively. Left ventricular (LV) pressure-volume analysis (Fig. 2H) showed worsened diastolic dysfunction, as illustrated by a greater increase in LVEDP (Fig. 2I), with preserved systolic function (Supp Fig. 3C), in the ACE2KO-HFD mice compared with the WT-HFD mice. Worsened diastolic dysfunction in the ACE2KO-HFD mice was mainly due to the impaired active relaxation, as evidenced by a greater decrease in the LV dp/dt_{min} (Fig. 2J) and increased Tau (Fig. 2K; Supp Fig. 3D), the exponential time constant of the decay in LV pressure during isovolumic relaxation, coupled with equivalent increase in the passive stiffness as reflective in the end-diastolic pressure-volume relationship (EDPVR; Supp Fig. 3E). Trans-mitral flow and tissue Doppler imaging revealed markedly increased E/A and E/E’ ratios, consistent with diastolic dysfunction in the ACE2KO-HFD mice (Fig. 2L-2M). mRNA expression profile of molecular markers of cardiac diseases showed a greater increase in the expression of atrial natriuretic factor (ANF; Fig. 3A), brain natriuretic peptide (BNP; Fig. 3B), β-myosin heavy chain (β-MHC; Fig. 3C) and α-skeletal muscle actin (Fig. 3D) in the ACE2KO-HFD hearts compared with the WT-HFD hearts. These results clearly demonstrate that loss of ACE2 enhances the susceptibility of the heart to obesity-induced heart disease.

**Molecular mechanism of the cardiac insulin resistance and HFPEF in the ACE2KO-HFD hearts.**

Western blot analyses of various metabolic enzymes and metabolic signaling pathways (30; 31) was carried out in the insulin-perfused hearts to elucidate the mechanism of myocardial insulin resistance in the ACE2KO-HFD hearts. Importantly, insulin-mediated phosphorylation of Akt was decreased in the WT-HFD hearts, a response further exacerbated in the ACE2KO-HFD hearts suggesting a marked cardiac insulin resistance in DIO in ACE2 deficient mice (Fig. 3E). Decreased
phosphorylation of Akt in the ACE2KO-HFD hearts was associated with increased pyruvate dehydrogenase kinase (PDK) 4 protein levels (Supp Fig. 4A), with no difference in PDK2 levels (Supp Fig. 4B), and increased phosphorylation of pyruvate dehydrogenase (PDH; Fig. 3F), a rate-limiting enzyme in carbohydrate oxidation. Phosphorylation of AMP-activated protein kinase (AMPK) was increased in the WT-HFD hearts, whereas the ACE2KO-HFD hearts showed decreased phosphorylation of AMPK (Fig. 3G). We assessed myocardial levels of adiponectin, an adipokine which regulates inflammation and cardiac metabolism (32; 33). Western blot and IF analyses showed decreased myocardial adiponectin levels in the WT hearts in response to DIO (Fig. 3H-I). ACE2KO-CON hearts showed reduced basal levels of myocardial adiponectin which remained low in response to DIO (Fig. 3H-I) while the increase in cardiomyocyte cross-sectional area was equivalent in the WT and ACE2KO mice in response to HFD feeding (Fig. 3J).

There was no difference in the protein levels of sirtuin (SIRT)-1 and SIRT-4 (Supp Fig. 4C-4D), while SIRT-3 protein levels were decreased (Supp Fig. 4E) and PGC-1α protein levels were increased (Supp Fig. 4F) in the WT-HFD, ACE2KO-CON and ACE2KO-HFD hearts compared with the WT-CON hearts. In addition, other than reduction in the ACE levels in the WT hearts in response to DIO, there was no noticeable difference in the protein levels of AT1R, ACE and the Mas receptor in WT and ACE2KO hearts (Supp Fig. 4G-4I) Obesity is closely linked to cardiac steatosis and lipotoxicity which are key pathogenic events in driving heart disease in obese states (26; 34). Myocardial triacylglycerol (TAG) levels showed marked increase in the ACE2KO-HFD hearts compared with the WT-HFD hearts (Fig. 3K) confirmed by Oil O red staining which showed markedly increased myocardial lipid accumulation in ACE2KO-HFD hearts (Fig. 3L). Increased cardiac steatosis was associated with increased oxidative stress, as shown by
increased NADPH oxidase activity (Fig. 3M) and DHE fluorescence (Fig. 3N-3O), predisposing the ACE2KO-HFD hearts to lipotoxicity. There was no difference in the mRNA expression of pro-inflammatory cytokines including TNF-α, MCP-1 and IL-6 and equivalent increase in IL-1β in between the ACE2KO-HFD and WT-HFD hearts (Supp Fig. 5A-D). Picro-sirius red staining showed equivalent increase in myocardial fibrosis in the ACE2KO-HFD hearts compared with the WT-HFD hearts, which was consistent with the equivalent increase in passive stiffness (EDPVR) (Supp Fig. 5E-5F).

**Ang 1-7 treatment decreased epicardial adipose tissue inflammation, corrected signaling pathways and lipotoxicity, and rescued the heart disease in ACE2KO-HFD mice.** Ang 1-7 treatment for 4 weeks reduced the DIO-induced glucose intolerance in the ACE2KO mice (Supp 6A-6B) without affecting body weight (Supp Fig. 6C). Body composition was analyzed by quantitative magnetic resonance, which showed reduction in fat mass (Supp Fig. 6D) but not lean mass (Supp Fig. 6E) in the HFD-fed ACE2KO mice in response to Ang 1–7. Adverse remodeling of the EAT in the ACE2KO-HFD group was markedly corrected in response to Ang 1-7 leading to reduced inflammatory cell infiltration and adipocyte size (Supp Fig. 4F-4I) concomitant with reduced CD11c+/F4/80+ and CD206+/F4/80+ Mϕ (Fig. 4A-4D) and decreased expression of pro- and anti-inflammatory cytokines, TNF-α (mRNA and protein levels; Fig. 4E-4F), iNOS, MCP-1, IL-1β, IL-6 and IL-10 (Fig. 4G-4K). The anti-inflammatory effects closely linked to Ang 1-7 treatment reversed the changes in phosphorylation of Akt (Fig. 4L), PDH (Fig. 4M) and AMPK (Fig. 4N), consistent with improved myocardial response to insulin. Interestingly, Ang 1-7 treatment markedly increased the myocardial adiponectin levels confirmed by Western blot analysis (Fig. 4O) and IF staining (Fig. 4P). Assessment of cardiomyocyte cross-sectional area
confirmed Ang 1-7 treatment mediated reduction in cardiac hypertrophy in the HFD-fed ACE2KO mice (Fig. 4Q)

Oil O red staining (Fig. 4R) and biochemical analysis of TAG (Fig. 4S) showed that Ang 1-7 treatment also attenuated myocardial lipid accumulation in the ACE2KO-HFD hearts leading to reduced cardiac steatosis (Fig. 4R-4S). Ang 1-7 treatment reduced the myocardial oxidative stress and myocardial fibrosis as evident by decreased NADPH oxidase activity and DHE fluorescence along with reduced myocardial collagen fraction (Fig. 4T; Supp Fig. 7A-7D). Ang 1-7 mediated attenuation of EAT inflammation, correction of altered signaling and reduced lipotoxicity prevented DIO-induced heart failure in the ACE2KO mice and reduced the mRNA expressions of cardiac disease markers including ANF, BNP, α-skeletal actin and β-MHC (Supp Fig. 7E-7H). LV pressure-volume loop analysis (Supp Fig. 4I) showed reduced LVEDP (Supp Fig. 7J), suggesting improved diastolic function in the Ang 1-7 treated ACE2KO-HFD mice which was associated with improved active relaxation (Tau, Supp Fig. 7K) and passive stiffness (EDPVR, Supp Fig. 7L).

DISCUSSION

Obesity is strongly associated with HFPEF, a condition with high mortality and morbidity, and limited therapy (1-4). Rodents exposed to DIO are generally accepted as a valid model to mimic human obesity (5-7). We showed that ACE2 was upregulated in murine and human EAT in association with obesity and cardiac dysfunction. Loss of ACE2 resulted in multifactorial alterations resulting in pathological hypertrophy, lipotoxicity, and altered cardiac metabolism in
the setting of epicardial adipose tissue inflammation (Supp. Fig. 8). We found increased glucose intolerance, which has been linked to pathological cardiac hypertrophy (35; 36), in the HFD-fed ACE2KO mice despite the reduced obesity. Diet-induced obesity in the WT mice resulted in increased CD206+/F4/80+ resident Mφ in the EAT which was associated with gene expression linked to the M(IL-4) Mφ phenotype. In contrast, ACE2KO-HFD showed polarization of Mφ phenotype resulting in increased resident CD11c+/F4/80+ Mφ, which resulted in increased mRNA expression of pro-inflammatory cytokines linked with the M(IFN-γ) Mφ phenotype (Supp Fig. 8). While ACE2 expression in the bone marrow can regulate macrophage polarization and adipose tissue inflammation (37), we showed a novel role of ACE2-regulated macrophage polarization and EAT inflammation in the progression of HFPEF. Importantly, human EAT obtained from obese HFPEF patients also showed marked increase in the EAT inflammation and resident CD11c+/F4/80+. These results illustrate a fundamental role of Mφ in adipose tissue inflammation and regulation of insulin sensitivity (28) as illustrated by the increased in insulin sensitivity associated with deletion of M1 mediated inflammatory marker gene (e.g. TNF-α) and the ablation of CD11c+ cells (38; 39).

Ang 1-7 treatment prevented the DIO mediated EAT inflammation and cardiac dysfunction in the ACE2-null background. Ang 1-7 effects are predominantly mediated by the activation of its endogenous G-protein coupled receptor receptor, Mas, which is widely expressed (14; 40). Our results showed a critical role of ACE2 in the regulation of macrophage phenotypes. Ang II binding and activation of the AT1R polarizes macrophages via the AT1R activation (41), whereas Ang 1-7/MasR axis activation decreases the expression of the pro-inflammatory cytokines including TNF-α and IL-6 (42). By regulating the balance of the RAS
towards the Ang 1-7/MasR axis activation, ACE2 is expected to decrease the polarization of macrophages. However the exact mechanism of the role of RAS in macrophage polarization remains uncertain and warrants detailed investigation.

Epicardial adipose tissue thickness and inflammation in obesity is associated with the progression of cardiac dysfunction (26; 43-45). Common pathways involved in the pathogenesis of obesity and cardiovascular disease include insulin resistance and lipotoxicity. ACE2KO-HFD mice showed increased cardiac steatosis and lipotoxicity in response to DIO. Cardiac steatosis and lipotoxicity are associated with the worsening of heart failure in non-diabetic obese men (26; 34). We also found increased myocardial insulin resistance in the ACE2KO-HFD hearts which was associated with the worsened global insulin signaling, decreased myocardial adiponectin levels and decreased phosphorylation of AMPK with associated diastolic dysfunction and heart disease. Myocardial insulin resistance is well-known to be associated with the heart failure, though the cause and effect relationship in the present study needs further investigation (46). Insulin-perfused ACE2KO-HFD hearts showed decreased phosphorylation of Akt, increased PDK4, and phosphorylation and inactivation of PDH indicative of impaired myocardial insulin signaling resulting in decreased glucose oxidation (47). AMPK acts as a metabolic master switch regulating several intracellular systems including the cellular uptake of glucose, the β-oxidation of fatty acids and the biogenesis of GLUT4 and thus decreased phosphorylation of AMPK (inactivation) may contribute to the cardiac insulin resistance in the ACE2KO-HFD hearts. Phosphorylation of AMPK in the healthy heart is partly regulated by adiponectin, an adipokine exclusively produced in the adipocytes (32; 48). Adiponectin is important for maintaining heart function in the setting of DIO (33); our
observation of reduced myocardial adiponectin in the ACE2KO-HFD model may be an important link between the pathological remodeling of EAT and adverse effects on the heart. Importantly, ACE2KO-HFD hearts showed greater increase in impaired active relaxation with equivalent passive stiffness compared with the WT-HFD hearts, which could be attributed to the enhanced metabolic dysfunction and equivalent fibrosis in these hearts. Importantly, Ang 1-7 treatment reversed pathological changes observed in the EAT and heart of ACE2KO-HFD mice. These results are consistent with a pivotal role of the ACE2/Ang 1-7 axis in cardiovascular (13; 21) and diabetic kidney diseases (49; 50).

Our data shows that Ang 1-7 mediated cardioprotection against obesity-induced cardiac dysfunction is multifactorial and mediated by improved molecular signaling, and decreased myocardial fibrosis, lipotoxicity and EAT inflammation. Ang 1-7 reduces the glucose intolerance and insulin resistance, and prevented diabetic cardiomyopathy in the obese type 2 diabetic model (13; 51). Our results illustrate novel effects of Ang 1-7 in increasing myocardial adiponectin levels and mediating anti-inflammatory effects on EAT and beneficial effects on heart function. In conclusion, we found a novel role of ACE2 in obesity, where ACE2 negatively regulates obesity induced EAT inflammation, cardiac insulin-resistance and alterations in cardiac metabolism. Importantly, Ang 1-7 treatment improves glucose intolerance, EAT inflammation, cardiac insulin resistance and also prevents the HFPEF phenotype in DIO. Enhancing ACE2 or Ang 1-7 action represents a potential therapeutic option for obesity and its associated heart disease.
AUTHOR CONTRIBUTIONS

VBP and GYO designed the research, analyzed the data and performed the statistical analysis. VBP, JM, BAM, RB, SKD, TR and NM conducted the experiments. JP, MGB and GL provided new reagents/analytical tools/mice and analyzed data. VBP, GL and GYO wrote the manuscript. GYO is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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DUALITY OF INTEREST

Vaibhav B. Patel – NONE
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Ratnadeep Basu – NONE
Subhash K. Das – NONE
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References


FIGURE LEGENDS

Figure 1. ACE2 is upregulated in the epicardial adipose tissue (EAT) in response to the diet-induced obesity (DIO), whereas loss of ACE2 results in increased DIO-induced EAT inflammation. Representative IF staining images showing increased adipocyte ACE2 expression in response to DIO (A). Body weight changes in the WT and ACE2KO mice in response to control diet (CON) or high-fat diet (HFD) showing decreased body weight gain in the ACE2KO-HFD mice compared with the WT-HFD mice (B). Intra-peritoneal glucose tolerance test in fasted mice showing increased glucose intolerance in the ACE2KO-HFD mice (C). Representative IF images for F4/80 and CD11c or CD206 (D) showing markedly increased inflammation in the ACE2KO-HFD EAT. WT-HFD showing increased resident CD206+ anti-inflammatory Mφ, whereas ACE2KO-HFD showing polarization in the Mφ phenotype resulting in increased CD11c+ pro-inflammatory Mφ (D-F). Gene expression analysis and ELISA assay showing greater increase in TNF-α [mRNA (G) and protein levels (H)] and iNOS mRNA expression (I) and lesser increase in IL-10 (J) mRNA expressions in the ACE2KO-HFD EAT compared with WT-HFD. Representative IF staining images showing increased adipocyte ACE2 expression in obese HFPEF patients compared with non-obese non-failing controls (K). Representative IF images for F4/80 and CD11c or CD206 in the human adipose tissue showing markedly increased CD11c+ Mφ with smaller increase in CD206+ Mφ in the EAT of human explanted hearts from obese HFPEF patients (L-N). n=4 (A, D-F, K-L); n=20 (B); n=8 (C, H); n=12 (G, I-J). *P<0.05 compared with the respective CON groups. #P<0.05 compared with WT-HFD group. $P<0.05 compared with the non-obese NFC. Scale bar indicates 25 µm. CLS: crown-like structure; Mφ: macrophage; IL-10: interleukin-10; TNF-α: tumor necrosis factor-α; WGA: wheat-germ agglutinin.
Figure 2. ACE2 is upregulated in the myocardium in response to diet-induced obesity (DIO), but loss of ACE2 results in worsened cardiac insulin resistance and heart failure. Western blot analysis (A), representative IF staining images and quantification (B) and ACE2 activity assay (C) showing myocardial ACE2 upregulation in response to DIO. Dry heart weight-to-tibial length ratio (D) showing equivalent cardiac hypertrophy in the WT-HFD and ACE2KO-HFD groups. Ex vivo working heart perfusions showing decreased cardiac work (E) in the ACE2KO-HFD hearts compared with WT-HFD hearts. Rates of glucose oxidation (F) determined by ex vivo working heart perfusions showing insulin-induced increase in glucose oxidation in the WT-CON hearts. WT-HFD and ACE2KO-CON hearts showed decreased effect of insulin on glucose oxidation which was entirely blunted in the ACE2KO-HFD hearts (F). Ex vivo working heart perfusions showing decreased palmitate oxidation rate (G) in response to insulin perfusion in WT-CON and ACE2KO-CON hearts with equivalent further reductions in the WT-HFD and ACE2KO-HFD hearts (G). Pressure-volume loop (H) analysis showing greater increase in LVEDP (I) in ACE2KO-HFD mice which is associated with greater slowing of cardiac relaxation shown by decreased LV dP/dt_{min} (J) and Tau (Glantz; K). Echocardiographic analysis of trans-mitral flow pattern and tissue Doppler imaging showing greater increase in E/A ratio (L) and E/E’ ratio (M) in the ACE2KO-HFD mice compared with the WT-HFD mice. n=6 (A); n=4 (B); n=8 (C); n=12 (D-G, L-M); n=10 (H-K). *P<0.05 compared with the respective CON groups. †P<0.05 compared with WT-HFD group. ‡P<0.05 compared with the WT-CON. Scale bar indicates 25 µm. LV dP/dt_{min}: rate of LV pressure decrease; LVEDP: left ventricular end-diastolic pressure; Tau (Glantz): exponential decay of the ventricular pressure during isovolumic relaxation.
Figure 3. Loss of ACE2 results cardiac steatosis, oxidative stress and modulation of molecular signaling pathways in response to diet induced obesity (DIO). Increased mRNA expression of ANF (A), BNP (B), β-MHC (C) and α-skeletal actin (D) showing heart failure progression in ACE2KO-HFD hearts in response to DIO. Western blot analysis showing greater decrease in phosphorylation of Akt in the ACE2KO-HFD hearts compared with the WT-HFD hearts (E). Phosphorylation of PDH was increased in the WT-HFD hearts which was further increased in the ACE2KO-HFD hearts (F). Phosphorylation of AMPK was increased in the WT-HFD hearts, whereas the ACE2KO-HFD displayed decreased phosphorylation of AMPK (G). Western blot analysis (H) and representative IF images (I) showing decreased adiponectin levels in the WT-HFD hearts. ACE2KO-CON hearts showed reduced basal levels of myocardial adiponectin which remained low in response to DIO (H-I). Representative WGA-stained images and myocyte cross-sectional area showing equivalent cardiac hypertrophy in WT and ACE2KO mice in response to HFD feeding (I-J). Biochemical analysis showing increased cardiac TAG levels (K) and the Oil O Red staining (L) showing increased intra-myocardial lipid accumulation in the ACE2KO hearts in response to DIO. NADPH oxidase activity (M), representative DHE staining images (N) and quantification of DHE fluorescence (O) showing increased oxidative stress in the ACE2KO-HFD hearts compared with WT-HFD hearts. n=12 (A-D); n=6 (E-H); n=4 (I-J, L, N-O); n=10 (K, M). *P<0.05 compared with the respective CON groups. #P<0.05 compared with WT-HFD group. Scalebar indicates 25 µm (I) and 100 µm (N). AMPK: AMP-activated protein kinase; ANF: atrial natriuretic factor; BNP: brain natriuretic peptide; DHE: dihydroethidium; β-MHC: beta-myosin heavy chain; PDH: pyruvate dehydrogenase; TAG: triacylglycerol.
Figure 4. Angiotensin 1-7 treatment ameliorates epicardial adipose tissue (EAT) inflammation, modulates molecular signaling pathways and decreases cardiac steatosis and oxidative stress resulting in prevention of heart failure in high fat diet-fed ACE2KO mice. Representative IF images for F4/80 and CD11c (A) or CD206 (B) and M1 and M2 phenotypes of Mφ (C-D) showing markedly increased inflammation in the ACE2KO-HFD EAT, where Ang 1-7 treatment entirely prevented the EAT inflammation (A-D). Gene expression analysis and ELISA assay showing increased expression of TNF-α [mRNA (E) and protein (F)], iNOS (G), MCP-1 (H), IL-1β (I), IL-6 (J) and IL-10 (K) mRNA in the ACE2KO-HFD EAT, which were reversed by the Ang 1-7 treatment. Ang 1-7 treatment reversed the changes in the phosphorylation of Akt (L), PDH (M) and AMPK (N) and also markedly increased myocardial adiponectin levels (O-P) shown by Western blot analysis (O) and representative IF images for adiponectin (P). Representative WGA-staining images and myocyte cross-sectional area showing Ang 1-7 mediated attenuation of cardiac hypertrophy in ACE2KO-HFD mice (P-Q). Oil O Red staining (R) revealing decreased intra-myocardial lipid accumulation and the biochemical analysis showing decreased cardiac TAG levels (S) in the Ang 1-7 treated ACE2KO-HFD hearts. NADPH oxidase activity (T) showing decreased oxidative stress in the Ang 1-7 treated ACE2KO-HFD hearts. n=4 (A-D; P-R); n=12 (E, G-K); n=8 (F); n=6 (L-O); n=10 (S-T). *P<0.05 compared with all the other groups. **P<0.05 compared with ACE2KO-CON group. Scale bar indicates 25 µm (A-B, P). AMPK: AMP-activated protein kinase; CLS: crown-like structure; IL-1β: interleukin-1β; IL-10: interleukin-10; IL-6: interleukin-6; Mφ: macrophage; MCP-1: Monocyte Chemoattractant Protein-1; PDH: pyruvate dehydrogenase; TAG: triacylglycerol; TNF-α: tumor necrosis factor-α; WGA: wheat-germ agglutinin.
SUPPLEMENTARY DATA

ACE2 deficiency worsens epicardial adipose tissue inflammation and cardiac dysfunction in response to diet-induced obesity

by

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Supplemental Figure 1. Loss of ACE2 results in reduced obesity but increased glucose intolerance. Body weight at 6 months of age (A) showing reduced obesity in the ACE2KO-HFD mice compared with the WT-HFD mice. Body mass composition showing decreased total fat mass (B) in the ACE2KO-HFD mice with no difference in the %fat (C) and %lean (D) mass composition in the ACE2KO-HFD mice compared with the WT-HFD mice. Fasting plasma glucose (E) and area under curve (AUC; F) from the IPGTT showing increased hyperglycemia and glucose intolerance in the ACE2KO-HFD mice compared with the WT-HFD mice. Epicardial adipose tissue (EAT) mass showing equivalent increase in the WT and ACE2KO mice in response to HFD feeding (G). *P<0.05 compared with the respective CON groups. #P<0.05 compared with WT-HFD group.
SUPPLEMENTARY DATA

Supplemental Figure 2. Loss of ACE2 worsened EAT inflammation in response to diet-induced obesity. Representative hematoxylin & eosin staining (A) and wheat-germ agglutinin staining (B) showing increased adipose tissue inflammation (arrows; A), crown-like structures (CLS; arrowheads; A and C) and increased adipocyte cross-sectional area (ACSA) in the ACE2KO-HFD EAT (D). Taqman mRNA expression analysis showing increased expression of pro-inflammatory cytokines including MCP-1 (E), IL-1β (F) and IL-6 (F) in the EAT from ACE2KO-HFD compared with WT-HFD. *P<0.05 compared with the respective CON groups. #P<0.05 compared with WT-HFD group. Scale bar indicates 25 µm (B).
SUPPLEMENTARY DATA

Supplemental Figure 3. Diet-induced obesity worsened cardiac insulin resistance and heart failure with preserved ejection fraction (HFPEF) in the ACE2KO mice. Total ATP (A) and %ATP production (B) showing ATP production via glucose oxidation was stimulated by insulin in WT-CON and ACE2KO-CON hearts which was suppressed in WT-HFD hearts and was markedly blunted in ACE2KO-HFD hearts. Cardiac function analysis from pressure-volume loops showing preserved ejection fraction (C), greater impairment in active relaxation with markedly increased Tau (Weiss; D) and equivalent increase in passive stiffness (EDPVR; E) in the ACE2KO-HFD mice compared with the WT-HFD mice. *P<0.05 compared with the respective CON groups. #P<0.05 compared with WT-HFD group.
SUPPLEMENTARY DATA

Supplemental Figure 4. Assessment of molecular signaling pathways and components of the RAS involved in the metabolic modulation in the heart. Western blots greater increase in myocardial PDK4 levels (A) with no difference in the PDK2 protein expression (B) in the ACE2KO-HFD hearts compared with the WT-HFD hearts. No difference in the SIRT1 (C) and SIRT4 (D) protein expressions in between all the 4 groups were observed. Western blots analyses showing equivalent decrease in SIRT3 (E) and equivalent increase in PGC-1α (F) levels in the WT-HFD, ACE2KO-CON and ACE2KO-HFD hearts compared with the WT-CON hearts. Western blot analysis showing no change in AT1 receptor (G), angiotensin converting enzyme (ACE) (H) and Mas receptor (I), except for a significant reduction in ACE levels in WT mice in response to DIO (H). *P<0.05 compared with the respective CON groups. #P<0.05 compared with WT-HFD group. $P<0.05 compared with the WT-CON group.
SUPPLEMENTARY DATA

Supplemental Figure 5. Lack of an effect of ACE2 deficiency on cardiac inflammation and myocardial fibrosis. Taqman mRNA expression analysis showing increased no change in the expression of pro-inflammatory cytokines including TNF-α (A), MCP-1 (B) and IL-6 (C) with equivalent increase in the IL-1β (D) in the hearts from ACE2KO-HFD compared with WT-HFD. Representative picro-sirius red (PSR) staining images (E) and quantification of myocardial collagen fraction (F) showing equivalent increase in myocardial fibrosis in the hearts from ACE2KO-HFD compared with WT-HFD. *P<0.05 compared with the respective CON groups. Scale bar indicates 25 µm (B).
SUPPLEMENTARY DATA

Supplemental Figure 6. Angiotensin (Ang) 1-7 treatment reduced the glucose intolerance and reversed the pro-inflammatory changes in the ACE2KO-HFD mice. Intra-peritoneal glucose tolerance test (A-B) and body weight measurements (C) showing suppression of DIO-induced glucose intolerance without effects on body weight in Ang 1-7 treated ACE2KO-HFD mice. Body mass measurement showing reduced %fat mass (D) with no change in the %lean mass (E) in Ang 1-7 treated ACE2KO-HFD mice. Histological analysis by H&E staining (F) and wheat-germ agglutinin staining (G) showing significantly decreased crown-like structures (H) and adipocyte area (I) in the Ang 1-7 treated ACE2KO-HFD EAT. *P<0.05 compared with all the other groups. $P<0.05$ compared with the ACE2KO-CON group. Scale bar indicates 25 µm (B).

![Graphs and images](image-url)
Supplemental Figure 7. Angiotensin (Ang) 1-7 treatment reduced the oxidative stress and myocardial fibrosis, and prevented the obesity-induced HFPEF in ACE2KO mice. DHE staining images (A) and the quantification of DHE fluorescence (B) showing the Ang 1-7 treatment mediated reduction in the oxidative stress in the hearts from ACE2KO-HFD mice. Representative picro-sirius red (PSR) staining images (C) and quantification of myocardial collagen fraction (D) showing anti-fibrotic effect of Ang 1-7 in the ACE2KO-HFD hearts. Decreased mRNA expression of cardiac disease markers ANF (E), BNP (F), β-MHC (G) and α-skeletal actin (H) in the Ang 1-7 treated ACE2KO-HFD hearts showing attenuation of cardiac disease progression in ACE2KO-HFD hearts in response to DIO. Pressure-volume loops (I) showing cardioprotective effects of Ang 1-7 treatment on DIO-induced HFPEF in the ACE2KO-HFD hearts. Quantification of diastolic indices from pressure-volume loop analysis showing Ang 1-7 treatment mediated decrease in LVEDP (J), Tau (Glantz; K) and EDPVR (L) in the ACE2KO-HFD mice. *P<0.05 compared with all the other groups. $P<0.05 compared with the ACE2KO-CON group. Scale bar indicates 100 µm (A) and 25 µm (C).
Supplemental Figure 8. Schematic representation of the effect of diet-induced obesity on the ACE2KO mice. Diet-induced obesity (DIO) leads to increased epicardial adipose tissue inflammation in the ACE2KO mice which is associated with increased resident CD11c+ Mφ. Increased EAT inflammation in ACE2KO-HFD mice is also associated with increased adipocyte necrosis and “crown-like structures” reflective of advanced inflammation. DIO also resulted in increased myocardial insulin resistance, altered metabolic signaling, increased lipotoxicity and decreased myocardial adiponectin levels culminating in worsened heart failure with preserved ejection fraction (HFPEF). Ang 1-7 treatment prevents EAT inflammation, alterations in cardiac metabolic signaling and lipotoxicity resulting in the prevention of DIO-induced heart failure.