Angiotensin 1–7 Ameliorates Diabetic Cardiomyopathy and Diastolic Dysfunction in db/db Mice by Reducing Lipotoxicity and Inflammation

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Background—The angiotensin-converting enzyme 2 and angiotensin-(1–7) (Ang 1–7)/MasR (Mas receptor) axis are emerging as a key pathway that can modulate the development of diabetic cardiomyopathy. We studied the effects of Ang 1–7 on diabetic cardiomyopathy in db/db diabetic mice to elucidate the therapeutic effects and mechanism of action.

Methods and Results—Ang 1–7 was administered to 5-month-old male db/db mice for 28 days via implanted micro-osmotic pumps. Ang 1–7 treatment ameliorated myocardial hypertrophy and fibrosis with normalization of diastolic dysfunction assessed by pressure–volume loop analysis and echocardiography. The functional improvement by Ang 1–7 was accompanied by a reduction in myocardial lipid accumulation and systemic fat mass and inflammation and increased insulin-stimulated myocardial glucose oxidation. Increased myocardial protein kinase C levels and loss of phosphorylation of extracellular signal-regulated kinase 1/2 were prevented by Ang 1–7. Furthermore, Ang 1–7 treatment decreased cardiac triacylglycerol and ceramide levels in db/db mice, concomitantly with an increase in myocardial adipose triglyceride lipase expression. Changes in adipose triglyceride lipase expression correlated with increased SIRT1 (silent mating type information regulation 2 homolog 1) levels and deacetylation of FOXO1 (forkhead box O1).

Conclusions—We identified a novel beneficial effect of Ang 1–7 on diabetic cardiomyopathy that involved a reduction in cardiac hypertrophy and lipotoxicity, adipose inflammation, and an upregulation of adipose triglyceride lipase. Ang 1–7 completely rescued the diastolic dysfunction in the db/db model. Ang 1–7 represents a promising therapy for diabetic cardiomyopathy associated with type 2 diabetes mellitus. (Circ Heart Fail. 2014;7:327-339.)

Key Word: diabetic cardiomyopathies

Diabetes mellitus (DM) is one of the most common public health problems in both developing and developed countries. The leading cause of mortality in patients with DM is cardiovascular disease. DM per se is an independent risk factor for the development of heart failure and is partly driven by diabetic cardiomyopathy. DM and inflammation is often linked to increased prevalence of heart failure and is known to increase the mortality in patients with heart failure with preserved ejection fraction (HFPEF). The myocardial renin–angiotensin system is locally activated, which contributes to the functional abnormalities and increased cardiovascular risk in DM. Indeed, elevated angiotensin II (Ang II) levels lead to cardiac hypertrophy, diastolic dysfunction, and cardiac insulin resistance. Angiotensin-converting enzyme 2 (ACE2) and its product, angiotensin 1–7 (Ang 1–7), are negative regulators of the renin–angiotensin system. ACE2 hydrolyzes Ang II into Ang 1–7, which promotes vasodilation and antifibrotic and antihypertrophic effects. As such ACE2 and its predominant peptide product, Ang 1–7, essentially oppose the Ang II/Ang II type 1 receptor axis. Loss of ACE2 exacerbates diabetic cardiomyopathy, whereas enhancing ACE2 action attenuates Ang II–induced cardiac dysfunction and streptozotocin-induced diabetic cardiomyopathy in rats. Collectively, these observations suggest a possible link between ACE2/Ang 1–7/Mas receptor (MasR) and the development of diabetic cardiomyopathy.

Clinical Perspective on p 339

We assessed the effects of Ang 1–7 treatment on diabetic cardiomyopathy in db/db mice. Ang 1–7 treatment corrected cardiac hypertrophy and diastolic dysfunction. Ang 1–7 treatment also reduced fat mass, adipose inflammation, cardiac triacylglycerol (TAG) levels, and cardiac lipotoxicity, with increased myocardial and adipose triglyceride lipase (ATGL)
expression. Ang 1–7 also completely suppressed myocardial oxidative stress. The AMP-activated protein kinase (AMPK)/silent mating type information regulation 2 homolog 1 (SIRT1)/forkhead box O1 (FOXO1), protein kinase C (PKC), and peroxisome proliferator-activated receptor α pathways were restored, and cardiac metabolism was partially corrected by Ang 1–7. These findings demonstrate that Ang 1–7 ameliorates diabetic cardiomyopathy because of decreased lipotoxicity, oxidative stress, and inflammation and provides important insight into a potential new therapy for HFPEF.

Methods

Experimental Animals and Protocol
Male C57BL/6J-lepr/lepr (db/db) and C57BL/6J (wild-type [WT]) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Micro-osmotic pumps (model 1002, Alza Corp, Palo Alto, CA), containing Ang 1–7 or saline, were implanted subcutaneously at the dorsum of the neck in 5-month-old male db/db mice as previously described.22 Mice received Ang 1–7 (0.5 mg kg⁻¹ day⁻¹) or saline for 28 days. Animal use conformed to the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996) and to the guidelines of the Canadian Council on Animal Care.

Echocardiography and Tissue Doppler Imaging
Cardiac function was assessed and analyzed in a blinded manner using a Vevo 770 high resolution imaging system equipped with a 30-MHz transducer (RMV-707B; VisualSonics, Toronto, Canada) as reported previously.19

Invasive Pressure–Volume Analysis
To measure left ventricular pressure–volume relationship, 1.2F ad- mittance catheter (Sciensce Inc) was used as previously described.20 The position of the catheter was monitored by pressure along with the magnitude and phase using ADvantage pressure-volume system (Sciensce Inc) and iworx (iWorx Systems Inc). Online as well as off-line calculations were performed using LabScribe2 software (version 2.347000).

Oral Glucose Tolerance and Insulin Tolerance Testing
Oral glucose tolerance test was performed to assess systemic insulin resistance as reported previously.19 Insulin tolerance test was performed after 6 hours fasting by using insulin (1 IU/kg, IP) as described previously.21

Quantitative Magnetic Resonance
Body composition, either fat mass or lean mass, was assessed by using an EchoMRI-900 (Echo Medical Systems, Houston, TX) as reported previously.22

Isolated Working Heart Perfusion
After 28 days of Ang 1–7 or saline infusion, isolated hearts were perfused in a working mode at a left atrial preload of 11.5 mm Hg and an aortic afterload of 50 mm Hg as previously reported.11,19 The perfusate contained 2.5 mmol/L Ca²⁺, 5 mmol/L [U⁻¹⁴C]glucose, and 1.2 mmol/L [9,10⁻³⁵H]palmitate prebound to 3% fatty acid free BSA. We used a higher concentration of palmitate to simulate the physiological fatty acid levels in db/db mice.23 Hearts underwent aerobic perfusion in the absence of insulin for the first 30 minutes, then 100 μM/mL insulin was added to the perfusate 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 [9,10⁻³⁵H]palmitate, respectively. Glucose-derived and palmitate-derived ATP production rates were calculated from the rates of glucose oxidation and palmitate oxidation.19

Western Blot Analysis
Western blot analyses were performed as reported previously11,19 with the following antibodies: anti–phospho-AMPK (Cell signaling Inc), anti-total AMPK (Cell signaling Inc), anti–phospho-Janus-activated kinase 2 (JAK2; Millipore), anti-total JAK2 (Cell signaling Inc), anti–phospho–extracellular signal-regulated kinase 1/2 (Erk1/2; Cell signaling Inc), anti-total Erk1/2 (Cell signaling Inc), anti–PKCα (Santa Cruz), anti–PKCβ1 (Santa Cruz), anti–SERCA2 (sarco/endoplasmic reticulum Ca²⁺-ATPase; Thermo Scientific), anti–phosphopholamban (Ser16; Badrilla), anti-phospholamban (Badrilla), anti–α-tubulin (Cell Signaling Inc), or anti–β-actin (Santa Cruz).

Biochemical Analyses
Short-chain coenzyme A (CoA) analysis was determined as reported previously.10 Tissue TAG was extracted, and plasma and tissue TAG were quantified using a colorimetric enzymatic assay (Wako Pure Chemical Industries, Osaka, Japan).24 Cardiac ceramide levels were determined by ultra performance liquid chromatography with modifications.25

Histology
Picrosirius red staining, dihydroethidium staining, F4/80 macrophage staining, Oil O red staining, and hematoxylin and eosin staining were performed as previously reported.11,12,26 Images for picrosirius red, dihydroethidium, and F4/80 macrophage staining and autofluorescence imaging of adipocyte were captured by fluorescence microscopy and analyzed using MetaMorph software (Olympus IX81, Center Valley, PA). Oil O red and hematoxylin and eosin staining were captured by light microscope (DM4000B, Leica).

Nicotinamide Adenine Dinucleotide Phosphate Oxidase Activity Assay
Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity assay was performed by using a lucigenin-enhanced chemiluminescence assay as reported previously.27

Statistical Analysis
All data are presented as means±SEM. Statistical analysis of the data was performed using 1-way ANOVA followed by multiple comparison testing using Student–Neuman–Keuls testing (SPSS Statistics 19 software). For the oral glucose tolerance test and insulin tolerance test, 2-way ANOVA was performed. We first confirmed that the data were normally distributed (Shapiro–Wilk Statistic; P<0.05) and then performed statistical analyses as noted above. In addition, we performed the nonparametric Kruskal–Wallis test for multiple comparison and confirmed that the results were completely congruent with our 1-way ANOVA analysis. A P<0.05 was considered significant.

Results

Ang 1–7 Improves Diastolic Dysfunction, Cardiac Hypertrophy, and Lipotoxicity in Diabetic Cardiomyopathy
Invasive pressure–volume hemodynamic analysis showing preserved systolic function with diastolic dysfunction in db/db hearts, which was reversed by Ang 1–7 (Figure 1). Pressure–volume analysis showed preserved systolic function in db/db mice (Figure 1A–1D). In contrast, db/db hearts showed diastolic dysfunction characterized by elevated left ventricular end-diastolic pressure and end-diastolic pressure–volume...
Figure 1. Angiotensin-(1–7) (Ang 1–7) improved diastolic dysfunction associated with diabetic cardiomyopathy. Invasive pressure–volume hemodynamic analysis showing preserved systolic function with diastolic dysfunction in db/db hearts, which was reversed by Ang 1–7. Representative pressure–volume traces (A), heart rate (HR; B), +dP/dt max (C), and end-systolic elastance (D) showed no change in db/db mice. In contrast, left ventricular (LV) end-diastolic pressure (LVEDP; E) and slope of the end-diastolic pressure–volume relationship (EDPVR; F) were increased with reduced –dP/dt min (G) and prolongation of the LV relaxation time constant (τ; H), all of which were reversed by Ang 1–7 treatment. Echocardiographic assessment confirmed diastolic dysfunction characterized by prolongation of the isovolumetric relaxation time (IVRT; I) and decreased E'/A' ratio (J), which were reversed by Ang 1–7 treatment in the db/db mice (A–J). Values are means±SEM; n=8 for each group. *P<0.05 compared with all groups. A' indicates tissue Doppler velocity from atrial contraction; E', early diastolic tissue Doppler velocity; and WT, wild type.
relationship, reduced \( -\Delta P/\Delta t \min \), and prolongation of the left ventricular relaxation (Figure 1A, 1E–1H), all of which were reversed by Ang 1–7 treatment. Transthoracic echocardiography confirmed preserved systolic function in \( db/db \) mice (Table I in the Data Supplement), with elevated isovolumetric relaxation time and reduced E’/A’ ratio (Figure 1I and 1J) indicating diastolic dysfunction. Administration of Ang 1–7 to \( db/db \) mice resulted marked improvement in cardiac hypertrophy and diastolic dysfunction in \( db/db \) mice (Figure 1; Table I in the Data Supplement). The beneficial effect of Ang 1–7 treatment on cardiac hypertrophy was confirmed by morphometric and histological assessment (Figure 2A–2E). Left ventricular dry weight was significantly increased in \( db/db \) hearts compared with WT hearts (Figure 2A and 2B), with markedly increased cardiomyocyte cross-sectional area (Figure 2C), normalized by Ang 1–7. Picosirius red staining revealed that Ang 1–7 treatment also reversed the increased myocardial fibrosis in \( db/db \) hearts (Figure 2D and 2E). These data suggest that Ang 1–7 treatment improves diastolic dysfunction, cardiac hypertrophy, and fibrosis in the \( db/db \) murine model.

We next explored the mechanism for the profound beneficial effects of Ang 1–7 on diabetic cardiomyopathy. Lipotoxicity, including myocardial TAG accumulation, represents a key pathogenic factor in diabetic cardiomyopathy. Oil O red staining showed a predictable and marked increase in lipid droplets in \( db/db \) hearts, which was markedly decreased in response to Ang 1–7 (Figure 2F). Biochemical analysis of the hearts revealed that hearts in \( db/db \) mice accumulated high levels of cardiac TAG and ceramide, which was prevented by Ang 1–7 treatment (Figure 2G and 2H). This occurred without changes in either liver or skeletal muscle (gastrocnemius) TAG levels (Table II in the Data Supplement). We determined the expressions of CD36, ATGL, and diacylglycerol acyltransferase 2 that are the key mediators of fatty acid uptake, TAG degradation, and TAG synthesis, respectively. ATGL levels (Figure 2I) were decreased, whereas CD36 and diacylglycerol acyltransferase 2 levels were increased (Figure 1A and 1B in the Data Supplement) in \( db/db \) hearts. Ang 1–7 treatment upregulated ATGL (Figure 2I), without altering CD36, and diacylglycerol acyltransferase 2 levels (Figure 1A and 1B in the Data Supplement). The phosphorylation of hormone-sensitive lipase, which is cardioprotective against lipotoxicity, was not changed in \( db/db \) hearts (Figure IC in the Data Supplement). These results highlight a novel role of Ang 1–7 in decreasing cardiac lipotoxicity in association with upregulation of ATGL.

Ang 1–7 Reduces Adipose Tissue Mass and Inflammation, Improves Insulin Sensitivity, and Increases Myocardial Glucose Oxidation

The global delivery of Ang 1–7 and its potential ability to regulate adipogenesis and fat metabolism\(^{30}\) suggest that extra-cardiac effects of Ang 1–7 action occur in the \( db/db \) model. Body composition was analyzed by quantitative magnetic resonance, which showed a significant reduction in fat mass (Figure 3A) but not lean mass (Figure 3B) in response to Ang 1–7 without a differential effect on total body weight of the \( db/db \) mice (Figure 3C). Adipocyte cross-sectional area was increased in \( db/db \) mice, and Ang 1–7 treatment resulted in a decrement in adipocyte size (Figure 3D and 3E) and epididymal adipose tissue independent of food intake (Table II in the Data Supplement). Using the oral glucose tolerance test, we showed that Ang 1–7 treatment did not alter the hyperglycemic response (Figure II A and II B in the Data Supplement), but plasma insulin levels at 120 minutes (Figure 3F) and area under the curve (Figure 3G) were significantly decreased, suggesting that Ang 1–7 increased insulin sensitivity. Indeed, insulin tolerance test clearly demonstrates insulin resistance in the \( db/db \) mice which was markedly improved by Ang 1–7 (Figure 3H and 3I). Consistent with these results, random plasma glucose levels (Figure 3J) and plasma TAG levels (Figure 3K) were decreased in response to Ang 1–7. These functional and structural changes in the \( db/db \) adipose tissue were accompanied by a marked increase in inflammatory cells (Figure 4A), with increased expression of inflammatory cytokines, tumor necrosis factor \( \alpha \), interleukin 1\( \beta \), interleukin 6, and monocyte chemoattractant protein 1 (Figure 4B–4E), which were reversed by Ang 1–7 (Figure 4A–4E). In contrast, the myocardium from \( db/db \) mice did not display these inflammatory changes (Figure 4F–4J). We conclude that Ang 1–7 treatment reduces adipose tissue mass and inflammation and improves insulin sensitivity in an obese type 2 diabetic preclinical model.

High rates of myocardial fatty acid \( \beta \)-oxidation play a key role in the pathogenesis of diabetic cardiomyopathy, including the \( db/db \) model.\(^{24,29}\) Although Ang II/Ang II type 1 receptor represents a key driver of metabolic perturbations and diastolic abnormalities,\(^{10,16}\) Ang 1–7/MasR is considered a physiological antagonist of Ang II action.\(^{30}\) Insulin stimulation of WT mice showed a marked increase in glucose oxidation, which was severely blunted in placebo-treated \( db/db \) mice (Figure 5A). Ang 1–7 treatment resulted in a 120% increase in insulin-stimulated glucose oxidation in \( db/db \) hearts (Figure 5A). Rates of palmitate oxidation in hearts from placebo-treated WT mice significantly decreased in response to insulin but not in \( db/db \) hearts (Figure 5B). A nonsignificant reduction in palmitate oxidation rates were seen in \( db/db \) mice treated with Ang 1–7. Short-chain CoA analysis showed high cardiac acetyl CoA and succinyl CoA levels in \( db/db \) mice, which is likely because of increased fatty acid \( \beta \)-oxidation rates, with Ang 1–7 treatment reducing cardiac acetyl CoA levels (Table III in the Data Supplement). Although the total ATP production was not altered in the absence and presence of insulin (Figure 5C), the percent of ATP derived from glucose and palmitate oxidation was increased and decreased, respectively, in \( db/db \) hearts exposed to Ang 1–7 compared with placebo-treated \( db/db \) hearts (Figure 5D). These changes occurred despite a similar reduction in phosphorylation of serine-473 (Figure 5E) and threonine-308 (Figure 5F) residues of Akt in placebo and Ang 1–7-treated \( db/db \) mice. These results highlight a key effect of Ang 1–7 in suppressing adipose tissue accumulation, improving insulin sensitivity and myocardial glucose oxidation.

Molecular Analysis of the Beneficial Effect of Ang 1–7 Treatment

We next studied potential pathological signaling pathways known to be involved in diabetic cardiomyopathy and lipotoxicity.\(^{25,27}\) Consistent with the increased cardiac
hypertrophy, db/db hearts showed increased phosphorylation of JAK2 (Figure 6A), whereas phosphorylation of signal transducer and activator of transcription 3 (Figure 6B) and ERK1/2 (Figure 6C) were reduced. Ang 1–7 treatment did not affect the phosphorylation of JAK2 and signal transducer and activator of transcription 3 (Figure 6A and 6B) but did reverse the lower phosphorylation levels of ERK1/2 in db/db hearts (Figure 6C), consistent with the cardioprotective effect of ERK1/2. PKCα and PKCβ1, critical players in diabetic cardiomyopathy, were elevated in db/db hearts, whereas Ang 1–7 treatment completely reversed these elevations (Figure 6D and 6E). In line with the reduction of adipose tissue mass, ATGL expression in epididymal adipose tissue was also increased in response to Ang 1–7 (Figure 6F). We also determined the expressions of SERCA2 and phospholamban, key determinants of intracellular Ca²⁺ signaling, which are altered in diabetic cardiomyopathy. The myocardial level of SERCA2 and phospholamban was
significantly decreased or unchanged in db/db hearts, respectively, which was unaffected by Ang 1–7 treatment (Figure III in the Data Supplement).

Furthermore, we hypothesized that Ang 1–7 treatment ameliorates reactive oxygen species–derived damage in hearts from db/db mice because lipotoxicity triggers oxidative stress...
and Ang 1–7 reduces NADPH-stimulated superoxide production.

Superoxide levels were increased in db/db hearts, driven by NADPH oxidase activity, leading to increased nitrotyrosine levels (Figure 7A and 7C; Figure IV in the Data Supplement). Ang 1–7 treatment prevented reactive oxygen species production in db/db hearts in association with lowered NADPH oxidase activity and nitrotyrosine levels (Figure 7A–7C; Figure IV in the Data Supplement). Next, we explored the mechanism of Ang 1–7–induced upregulation of ATGL by examining the AMPK/SIRT1/FOXO1 pathway. FOXO1, a key transcriptional factor involved in controlling energy metabolism, is regulated by SIRT1 via acetylation. Acetylation of FOXO1 was significantly increased in db/db hearts, and Ang 1–7 treatment deacetylated FOXO1 (Figure 7D). In line with the alterations of acetylation of FOXO1, SIRT1 expression was significantly decreased in db/db hearts, which was increased in response to Ang 1–7 (Figure 7E). Furthermore, we checked the expression of AMPK, which interacts with SIRT1 in regulating FOXO1 activity, and peroxisome proliferator-activated receptor α, the downstream
target of the AMPK/SIRT1 pathway. The expression of peroxisome proliferator-activated receptor α and phosphorylation of AMPK were lowered in db/db hearts (Figure 7F and 7G). Importantly, Ang 1–7 treatment increased phosphorylation of AMPK, concomitant with increased peroxisome proliferator-activated receptor α expression in the db/db hearts (Figure 7F and 7G). These results demonstrate that Ang 1–7 corrects the aberrant signaling pathways, ameliorates myocardial oxidative stress, and induces expression of ATGL possibly via deacetylation of FOXO1 by SIRT1.

Discussion
The prevalence of obesity and type 2 DM is rising rapidly worldwide. Type 2 DM results from insulin resistance, often associated with obesity, and inadequate insulin secretion to overcome the insulin resistance. Diabetic cardiomyopathy is characterized by cardiac hypertrophy and diastolic dysfunction with preserved systolic function leading to HFPEF. We randomized 5-month-old db/db mice into placebo or Ang 1–7 groups, thereby allowing the elucidation of the therapeutic effects of Ang 1–7 in a well-established model of type 2 diabetic cardiomyopathy, db/db mice. We showed that (1) Ang 1–7 improved the structural and functional abnormalities of diabetic cardiomyopathy (cardiac hypertrophy and diastolic dysfunction), (2) Ang 1–7 reduced body fat mass, inflammation, and enhanced systemic insulin sensitivity, (3) Ang 1–7 improved cardiac energy metabolism characterized by increased insulin-stimulated glucose oxidation, (4) Ang 1–7...
ameliorated cardiac lipotoxicity, because of an increased ATGL activity, and reduced oxidative stress. These data clearly demonstrate the protective role of Ang 1–7 in obesity and diabetic cardiomyopathy (Figure 8).

Ang 1–7 treatment improved cardiac hypertrophy and myocardial fibrosis and reduced cardiac TAG accumulation and lipotoxicity in db/db mice, which could contribute to the improvement of diastolic dysfunction. These changes were associated with abrogation of pathological signaling pathways, such as PKC signaling. Phosphorylation of Erk1/2 was reduced, consistent with the ability of leptin to stimulate the phosphorylation of mitogen-activated protein kinase, and Ang 1–7 treatment prevented the loss of phosphorylation of Erk1/2, which is likely to be cardioprotective. Metabolic perturbations represent a key driver of diastolic abnormalities, and activation of the renin–angiotensin system plays a key role.
pathogenic role in this process.\textsuperscript{11,19} In diabetic states, myocardial glucose oxidation is reduced with increased reliance on fatty acid oxidation.\textsuperscript{24,42} Ang 1–7 partially ameliorates the cardiac metabolic perturbations in \textit{db/db} mice by increasing insulin-stimulated glucose oxidation. In addition, the high myocardial levels of acetyl CoA, likely because of accelerated fatty acid \(\beta\)-oxidation, was significantly reduced to within WT levels by Ang 1–7. However, insulin-mediated metabolic control was only partially corrected by Ang 1–7, consistent with a partial reduction in TAG and ceramide levels and partial rescue of insulin resistance. In addition, the sarcomeric protein, titin, is a key contributor to myocardial passive stiffness and contributes to the diastolic dysfunction in a metabolic model of HFPEF.\textsuperscript{43} The ability of Ang 1–7 to lower PKC\(\alpha\) and reactive oxygen species levels can also lower the stiffness of titin resulting in the improvement in diastolic dysfunction in our murine model of HFPEF.\textsuperscript{7,44}

We found that Ang 1–7 normalizes the increased cardiac lipotoxicity in \textit{db/db} mice. Indeed, elevated cardiac TAG and ceramide levels and PKC signaling are normalized by Ang 1–7, consistent with the protective role of myocardial ATGL in diabetic cardiomyopathy.\textsuperscript{46} The decrease in myocardial lipid accumulation and lipotoxicity in combination with suppression of NADPH oxidase activity\textsuperscript{34} resulted in the marked reduction in oxidative stress by Ang 1–7 in \textit{db/db} hearts. Ang 1–7 treatment also reduced systemic and epididymal adipose tissue burden without affecting TAG accumulation in the liver and skeletal muscle. These data suggest specific organs, such as heart and adipose tissue, have a preferential response to Ang 1–7, which may be related to the high expression of the Ang 1–7 Mas receptor in these tissues (Figure 8).\textsuperscript{15,30} Increased circulating Ang 1–7 levels improve lipid metabolism and reduce visceral fat mass,\textsuperscript{49} whereas the Mas knockout mice showed increased visceral adipose tissue accumulation.\textsuperscript{15} These data are consistent with the protective role of the ACE2/Ang 1–7/MasR axis in cardiovascular disease including diabetic cardiomyopathy.\textsuperscript{15,16,33,49}

SIRTs play a critical role in the control of energy metabolism. SIRT1 is a well-studied SIRT member and controls FOXO1 activity via deacetylation.\textsuperscript{36,38} Acetylation of FOXO1 mice, with similar findings in \textit{ob/ob} mice\textsuperscript{48} and human obese subjects with insulin resistance.\textsuperscript{47} Ang 1–7 treatment enhances cardiac ATGL activity, leading to increased TAG hydrolysis and decreased cardiac TAG accumulation, consistent with the protective role of myocardial ATGL in diabetic cardiomyopathy.\textsuperscript{46} The decrease in myocardial lipid accumulation and lipotoxicity in combination with suppression of NADPH oxidase activity\textsuperscript{34} resulted in the marked reduction in oxidative stress by Ang 1–7 in \textit{db/db} hearts. Ang 1–7 treatment also reduced systemic and epididymal adipose tissue burden without affecting TAG accumulation in the liver and skeletal muscle. These data suggest specific organs, such as heart and adipose tissue, have a preferential response to Ang 1–7, which may be related to the high expression of the Ang 1–7 Mas receptor in these tissues (Figure 8).\textsuperscript{15,30} Increased circulating Ang 1–7 levels improve lipid metabolism and reduce visceral fat mass,\textsuperscript{49} whereas the Mas knockout mice showed increased visceral adipose tissue accumulation.\textsuperscript{15} These data are consistent with the protective role of the ACE2/Ang 1–7/MasR axis in cardiovascular disease including diabetic cardiomyopathy.\textsuperscript{15,16,33,49}
was significantly increased in hearts from db/db mice, which is likely induced by SIRT1 downregulation. In accordance with the upregulation of SIRT1, acetylation of FOXO1 was decreased in response to Ang 1–7 treatment. SIRT1 is also known to control ATGL activity via acetylation of FOXO1. The parallel change in AMPK/FOXO1/ATGL might explain the mechanism of the beneficial effect of Ang 1–7 treatment. In conclusion, Ang 1–7 treatment improves cardiac hypertrophy and diastolic dysfunction in diabetic cardiomyopathy. Ang 1–7–induced ATGL expression leads to a decrease in lipotoxicity, with ATGL expression correlating with deacetylation of FOXO1 via SIRT1 upregulation. Ang 1–7 treatment represents a potential new therapeutic tool for the treatment of diabetic cardiomyopathy and HFPEF.

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Disclosures
None.

References


Diabetic cardiomyopathy is an important cause of morbidity and mortality in patients with diabetic mellitus and may be increasing in prevalence because of the rise in obesity. The renin–angiotensin system is known as a critical regulator of diabetic cardiomyopathy. Angiotensin-converting enzyme 2 metabolizes angiotensin II into angiotensin-(1–7) (Ang 1–7), thereby negatively regulating the renin–angiotensin system. Several studies in animals and humans have shown that the myocardial renin–angiotensin system can be locally activated, and loss of angiotensin-converting enzyme 2 exacerbates diabetic cardiomyopathy. We showed that Ang 1–7/MasR axis has beneficial effects on diabetic cardiomyopathy and heart failure with preserved ejection fraction. By using db/db mice, well-established genetic model of type 2 diabetes mellitus, we showed that Ang 1–7 treatment corrected the diastolic dysfunction, a characteristic abnormality of diabetic cardiomyopathy. Ang 1–7 also reduced the pathological hypertrophy and myocardial fibrosis, decreased systemic fat mass, adipose inflammation, and cardiac triacylglycerol levels, which was accompanied by increased adipose triacylglycerol lipase expression in the heart and adipose tissue. There was only partial correction of the perturbation in cardiac metabolism in response to Ang 1–7. We demonstrated that AMP-activated protein kinase/SIRT1 (silent mating type information regulation 2 homolog 1)/FOXO1 (forkhead box O1) pathway plays a critical role in mediating Ang 1–7-induced adipose triacylglycerol lipase expression. Although targeting the angiotensin II type 1 receptor pathway failed to improve the outcome of patients with heart failure with preserved ejection fraction, our results imply that targeting the other aspect of the renin–angiotensin system such as enhancing Ang 1–7 can serve as a potential new therapeutic tool for the treatment of diabetic cardiomyopathy and heart failure with preserved ejection fraction.
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SUPPLEMENTAL MATERIAL

Angiotensin 1-7 ameliorates diabetic cardiomyopathy and diastolic
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by

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<td>LVESD (mm)</td>
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<td>2.77±0.07</td>
<td>2.67±0.09</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>36.8±2.2</td>
<td>35.3±2.3</td>
<td>37.6±2.1</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>63.8±2.8</td>
<td>64.8±2.9</td>
<td>67.4±3.2</td>
</tr>
<tr>
<td>VCFc (circ/s)</td>
<td>6.48±0.19</td>
<td>6.57±0.23</td>
<td>6.71±0.31</td>
</tr>
<tr>
<td>LVPWT (mm)</td>
<td>0.68±0.06</td>
<td>0.92±0.08†</td>
<td>0.73±0.07</td>
</tr>
</tbody>
</table>

n=8 for WT and n=10 for the db/db groups; values are mean±SEM. HR, heart rate; E-wave, peak early transmitral inflow mitral E velocity; A-wave, transmitral inflow velocity due to atrial contraction; DT, deceleration time; EWDR, E-wave deceleration rate (=E-wave/DT); E’, early diastolic tissue Doppler velocity; A’, late diastolic tissue Doppler velocity; LVEDD, left ventricular (LV) end diastolic diameter, LVESD, LV end systolic diameter; LVFS, LV fractional shortening; LVEF, LV ejection fraction; VCFc, Velocity of circumferential shortening corrected for heart rate; LVPWT, LV posterior wall thickness. *p<0.05 compared with all other groups, †p<0.05 compared with the WT+Placebo group.
### Supplemental Table 2. Food Intake and metabolic parameters.

<table>
<thead>
<tr>
<th></th>
<th>WT+Placebo</th>
<th>db/db+Placebo</th>
<th>db/db+Ang1-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake (g/day)</td>
<td>4.1±0.21</td>
<td>6.5±0.19</td>
<td>6.1±0.17</td>
</tr>
<tr>
<td>Epididymal adipose tissue (wet g)</td>
<td>0.54±0.047</td>
<td>2.62±0.480*</td>
<td>1.72±0.115*†</td>
</tr>
<tr>
<td>Plasma NEFA (mM)</td>
<td>0.56±0.03</td>
<td>0.77±0.17</td>
<td>0.68±0.16</td>
</tr>
<tr>
<td>Liver TAG (μmol/g wet wt)</td>
<td>78.0±15.1</td>
<td>139.7±19.0*</td>
<td>144.5±23.7*</td>
</tr>
<tr>
<td>Gastrocnemius TAG (μmol/g wet wt)</td>
<td>27.5±1.3</td>
<td>98.6±9.8*</td>
<td>106.9±4.7*</td>
</tr>
</tbody>
</table>

n=8 for WT and n=10 for the db/db groups; values are mean±SEM. NEFA, non esterified fatty acid; TAG, triacylglycerol. *p<0.05 compared with placebo-treated WT group, †p<0.05 compared with placebo-treated db/db group.

### Supplemental Table 3. Acetyl CoA, malonyl CoA, free CoA and succinyl CoA levels in WT, db/db, and db/db/+ Ang1-7 hearts

<table>
<thead>
<tr>
<th></th>
<th>WT+Placebo</th>
<th>db/db+Placebo</th>
<th>db/db+Ang1-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>free CoA</td>
<td>147.2 ± 7.1</td>
<td>157.2 ± 10.1</td>
<td>147.3 ± 10.1</td>
</tr>
<tr>
<td>Malonyl CoA</td>
<td>2.11 ± 0.15</td>
<td>2.43 ± 0.10</td>
<td>2.11 ± 0.13</td>
</tr>
<tr>
<td>Acetyl CoA</td>
<td>23.2 ± 2.8</td>
<td>39.5 ± 2.9*</td>
<td>24.0 ± 1.3</td>
</tr>
<tr>
<td>Succinyl CoA</td>
<td>13.4 ± 2.1*</td>
<td>21.4 ± 1.6</td>
<td>22.1 ± 3.0</td>
</tr>
</tbody>
</table>

n=6 for all experimental groups; values are mean ± SEM. *p<0.05 compared with all other groups.
Supplemental Figure Legends

**Supplemental Figure 1.** Angiotensin 1-7 (Ang1-7) does not affect fatty acid uptake and TAG synthesis. CD36 (A) and sn-1,2-diacylglycerol acyltransferase 2 (DGAT2) (B) expressions were not significantly changed in response to Ang 1-7 treatment. The phosphorylation of hormone-sensitive lipase (HSL) expression was also not affected by Ang 1-7 treatment (C). A.U. indicates arbitrary units. Values are the mean±SEM of n=5 in each group; *p<0.05 compared with placebo-treated WT group, †p<0.05 compared with placebo-treated db/db group.

**Supplemental Figure 2.** The oral glucose tolerance test (OGTT) revealed impaired glucose tolerance in the placebo-treated db/db mice which was not significantly changed in the Ang 1-7 group (A and B). Values are the mean±SEM of n=8 in each group; *p<0.05 compared with placebo-treated WT mice. AUC=area under the curve.

**Supplemental Figure 3.** Ang1-7 does not affect key Ca$^{2+}$ signaling proteins. Downregulation of sarco/endoplasmic reticulum Ca$^{2+}$-ATPase 2 (SERCA2) (A), and phosphorylation of phospholamban (PLN) (B) expression were not significantly changed in response to Ang 1-7 treatment. A.U. indicates arbitrary units. Values are the mean±SEM of n=5 in each group; *p<0.05 compared with placebo-treated WT group.

**Supplemental Figure 4.** Ang 1-7 reduces reactive oxygen species (ROS) production. Dihydroethidium (DHE) (A) and nitrotyrosine (B) staining showed increased ROS production in db/db hearts which was markedly reduced by Ang 1-7 treatment (A and B). n=3 in each group.
Mori J et al. Supplemental Figure 1
Mori J et al.  Supplemental Figure 2
A

SERCA2
α-tubulin

B

P-PLN (Ser16)
T-PLN

Mori J et al.  Supplemental Figure 3
Supplemental Figure 4

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