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

Jun Mori, MD, PhD; Vaibhav B. Patel, PhD; Osama Abo Alrob, BSc; Ratnadeep Basu, MD, PhD; Tariq Altamimi, BSc; Jessica DesAulniers, BSc; Cory S. Wagg; Zaneh Kassiri, PhD; Gary D. Lopaschuk, PhD; Gavin Y. Oudit, MD, PhD

**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.11,19 Mice received Ang 1–7 (0.5 mg kg⁻¹ d⁻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.11,19

Invasive Pressure–Volume Analysis

To measure left ventricular pressure–volume relationship, 1.2F ad- ditional catheter (Scisense Inc) was used as previously described. 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 of- 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 de- scribed 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 re- ported previously.22

Isolated Working Heart Perfusion

After 28 days of Ang 1–7 or saline infusion, isolated hearts were per- fused 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 [9,10-³H]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 μU/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 [¹⁴C]CO₂ and [³H]O from [U-¹⁴C]glucose and [9,10-³H]palmitate, respectively. Glucose-derived and palmitate- derived ATP production rates were calculated from the rates of glu- cose 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/endoplas- mic reticulum Ca²⁺ ATPase; Thermo Scientific), anti–phospho- phospholamban (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 report- ed previously.19 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.11,25,26 Images for picrosirius red, di- hydroethidium, 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 ac- tivity assay was performed by using a lucigenin-enhanced chemilu- minescence 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 compari- son 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 per- formed the nonparametric Kruskal–Wallis test for multiple compari- son 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 ventric- ular 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 mean±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.
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 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 epidydimal 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 IIB in the Data Supplement), but plasma insulin levels at 120 minutes (Figure III F) and area under the curve (Figure III G) 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 III H and III I). Consistent with these results, random plasma glucose levels (Figure III J) and plasma TAG levels (Figure III K) were decreased in response to Ang 1–7. All of these functional and structural changes in the db/db adipose tissue were accompanied by a marked increase in inflammatory cells (Figure IV A), with increased expression of inflammatory cytokines, tumor necrosis factor α, interleukin 1β, interleukin 6, and monocyte chemoattractant protein 1 (Figure IV B–IV E), which were reversed by Ang 1–7 (Figure IV F–IV J). In contrast, the myocardium from db/db mice did not display these inflammatory changes (Figure IV F–IV J). 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 β-oxidation play a key role in the pathogenesis of diabetic cardiomyopathy, including the db/db model. Although Ang II/Ang II type 1 receptor represents a key driver of metabolic perturbations and diastolic abnormalities, Ang 1–7/MasR is considered a physiological antagonist of Ang II action. Insulin stimulation of WT mice showed a marked increase in glucose oxidation, which was severely blunted in placebo-treated db/db mice (Figure V A). Ang 1–7 treatment resulted in a 120% increase in insulin-stimulated glucose oxidation in db/db hearts (Figure V A). Rates of palmitate oxidation in hearts from placebo-treated WT mice significantly decreased in response to insulin but not in db/db hearts (Figure V B). 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 β-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 V C), 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 V D). These changes occurred despite a similar reduction in phosphorylation of serine-473 (Figure V E) and threonine-308 (Figure V F) 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. 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. PKC α and PKCβ1, critical players in diabetic cardiomyopathy, were elevated in db/db hearts, whereas Ang 1–7 treatment completely reversed these elevations (Figures 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

**Figure 4.** Angiotensin-(1–7) (Ang 1–7) treatment reduced inflammation in adipose tissue. Hematoxylin and eosin staining showed increased inflammation cells in epididymal adipose tissue (EAT), which was reduced by Ang 1–7 treatment (A). TaqMan real-time polymerase chain reaction (RT-PCR) analysis revealed that mRNA of inflammation markers, tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), and monocyte chemoattractant protein 1 (MCP-1), was increased in EAT from db/db mice, which were reversed by Ang 1–7 treatment (B–E). F4/80 macrophage staining did not show increased macrophage infiltration in hearts from db/db mice (F). RT-PCR also showed no difference in mRNA of inflammation markers between the 3 groups (G–J). Values are the mean±SEM of n=6 in each group; *P<0.05 compared with placebo-treated wild-type (WT) mice. A.U. indicates arbitrary units.
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.39,40 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,41 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

Figure 6. Modulation of pathological signaling pathways by angiotensin-(1–7) (Ang 1–7) in db/db mice. Phosphorylation of myocardial Janus-activated kinase 2 (JAK2; A) and signal transducer and activator of transcription 3 (STAT3; B) was significantly increased and decreased, respectively, in db/db mice, which was not affected by Ang 1–7 treatment. The phosphorylation of myocardial extracellular signal-regulated kinase 1/2 (Erk1/2; C) and expression of myocardial protein kinase Cα (PKCα; D) and PKCβ1 (E) were decreased and increased in db/db mice, which were reversed by Ang 1–7 treatment. Adipose tissue adipose triglyceride lipase (ATGL) level was suppressed in db/db mice and partially rescued by Ang 1–7 (F). 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. A.U. indicates arbitrary units.
pathogenic role in this process. In diabetic states, myocardial glucose oxidation is reduced with increased reliance on fatty acid oxidation. Ang 1–7 partially ameliorates the cardiac metabolic perturbations in db/db mice by increasing insulin-stimulated glucose oxidation. Moreover, high myocardial levels of acetyl CoA, likely because of accelerated fatty acid β-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. The ability of Ang 1–7 to lower PKCα 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.

We found that Ang 1–7 normalizes the increased cardiac lipotoxicity in db/db mice. Indeed, elevated cardiac TAG and ceramide levels and PKC signaling are normalized by Ang 1–7, consistent with a partial reduction in the improving in diastolic dysfunction in our murine model of HFPEF. The decrease in myocardial lipid accumulation and lipotoxicity in combination with suppression of NADPH oxidase activity resulted in the marked reduction in oxidative stress by Ang 1–7. The phosphorylation of myocardial AMP-activated protein kinase (AMPK; F) and expression of peroxisome proliferator-activated receptor α (PPARα; G) were significantly decreased in db/db hearts, which were prevented by Ang 1–7 treatment. Values are the mean±SEM of n=5 in each group; *P<0.05 compared with placebo-treated wild-type (WT) group, †P<0.05 compared with placebo-treated db/db group.

Figure 7. Angiotensin-(1–7) (Ang 1–7) reduced myocardial oxidative stress and induced adipose triacylglycerol lipase expression in association with deacetylation of forkhead box O1 (FOXO1) by SIRT1 in the heart. Ang 1–7 reduced superoxide production measured by dihydroethidium (DHE) staining (A) and NADPH oxidase activity (B), resulting in decreased myocardial nitrotyrosine levels (C) in db/db hearts. Immuno-precipitation and Western blot analysis showed increased myocardial acetylation of FOXO1 in db/db mice (D), concomitantly with decreased SIRT1 expression (E), which were completely reversed by Ang 1–7. The phosphorylation of myocardial AMP-activated protein kinase (AMPK; F) and expression of peroxisome proliferator-activated receptor α (PPARα; G) were significantly decreased in db/db hearts, which were prevented by Ang 1–7 treatment. Values are the mean±SEM of n=5 in each group; *P<0.05 compared with placebo-treated wild-type (WT) group, †P<0.05 compared with placebo-treated db/db group.
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, Angel 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 in which there is currently limited therapy. Although targeting the Ang II type 1 receptor pathway failed to improve the outcome of patients with HFPEF, targeting the other aspect of the renin–angiotensin system/ACE2 pathway such as enhancing Ang 1–7 action may represent a novel therapy for HFPEF.

Sources of Funding
Dr Mori is a fellow of the Mazankowski Alberta Heart Institute. Dr Oudit is an Alberta Innovates-Health Solution (AI-HS) Clinician Investigator and a Distinguished Clinician-Scientist of the Heart and Stroke Foundation of Canada and Canadian Institutes of Health Research (CIHR). Dr Lopaschuk is an AI-HS Scientist. Dr Kassiri is an AI-HS Scholar. Research was funded by grants to Drs Oudit and Lopaschuk from the CIHR and the Heart and Stroke Foundation of Canada.

Disclosures
None.

References


ANGIOTENSIN 1–7 AMELIORATES DIABETIC CARDIOMYOPATHY

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


_Circ Heart Fail._ 2014;7:327-339; originally published online January 3, 2014;
doi: 10.1161/CIRCHEARTFAILURE.113.000672

_Circulation: Heart Failure_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/7/2/327

Data Supplement (unedited) at:
http://circheartfailure.ahajournals.org/content/suppl/2014/01/03/CIRCHEARTFAILURE.113.000672.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation: Heart Failure_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation: Heart Failure_ is online at:
http://circheartfailure.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Angiotensin 1-7 ameliorates diabetic cardiomyopathy and diastolic dysfunction in db/db mice by reducing lipotoxicity and inflammation

by


Supplemental Table 1. Echocardiographic assessment of cardiac function in WT and db/db mice in response to Ang 1-7

<table>
<thead>
<tr>
<th></th>
<th>WT+Placebo</th>
<th>db/db+ Placebo</th>
<th>db/db+ Ang 1-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>492±11</td>
<td>497±12</td>
<td>501±14</td>
</tr>
<tr>
<td>E-wave (mm/s)</td>
<td>714±26</td>
<td>686±37</td>
<td>745±47</td>
</tr>
<tr>
<td>A-wave (mm/s)</td>
<td>443±16</td>
<td>474±40</td>
<td>480±29</td>
</tr>
<tr>
<td>E/A Ratio</td>
<td>1.61±0.12</td>
<td>1.45±0.08</td>
<td>1.55±0.10</td>
</tr>
<tr>
<td>DT (ms)</td>
<td>26.3±1.5</td>
<td>35.2±1.4*</td>
<td>25.9±1.9</td>
</tr>
<tr>
<td>EWDR (mm/s²)</td>
<td>27.1±1.6</td>
<td>19.4±1.7*</td>
<td>28.8±2.1</td>
</tr>
<tr>
<td>E' (mm/s)</td>
<td>26.8±1.8</td>
<td>22.1±1.9</td>
<td>28.9±2.5</td>
</tr>
<tr>
<td>E/E' Ratio</td>
<td>29.6±2.8</td>
<td>31.0±3.1</td>
<td>25.8±3.5</td>
</tr>
<tr>
<td>A' (mm/s)</td>
<td>18.2±1.2</td>
<td>28.6±1.4*</td>
<td>22.6±1.8</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.24±0.11</td>
<td>4.29±0.10</td>
<td>4.28±0.12</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.68±0.07</td>
<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>0.543±0.047</td>
<td>2.626±0.480*</td>
<td>1.724±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
Mori J et al.  Supplemental Figure 4