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

Mori et al: Angiotensin 1-7 Ameliorates Diabetic Cardiomyopathy

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Abstract

**Background**—The angiotensin converting enzyme 2 and Ang 1-7/MasR axis is 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 PKC levels and loss of phosphorylation of Erk1/2 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 (ATGL) expression. Changes in ATGL expression correlated with increased SIRT1 levels and deacetylation of FOXO1.

**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 ATGL. 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.

**Key Words:** angiotensin 1-7, diabetic cardiomyopathy, heart failure with preserved ejection fraction, diastolic dysfunction, ATGL, sirtuin
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. Diabetes 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 (RAS) 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 RAS. 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 (AT1R) axis. Loss of ACE2 exacerbates diabetic cardiomyopathy, while 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/MasR and the development of diabetic cardiomyopathy.

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 AMPK/SIRT1/FOXO1, PKC and PPARα pathways were restored and cardiac metabolism was partially corrected by Ang 1-7. These findings demonstrate that Ang 1-7 ameliorates diabetic cardiomyopathy due to 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 (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Micro-osmotic pumps (model 1002, Alza Corp, Palo Alto, California), 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 (NIH 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 LV pressure-volume relationship, 1.2F admittance 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 offline calculations were performed using LabScribe2 software (version 2.347000).

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

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

**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 mmHg and an aortic afterload of 50 mmHg, as previously reported. The perfusate contained 2.5 mM Ca$^{2+}$, 5 mM [$U^{-14}$C]glucose, and 1.2 mM [9, 10-$^3$H]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 db/db mice.\textsuperscript{23} 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 $^{14}$CO$_2$ and $^3$H$_2$O from [U-$^{14}$C]glucose and [9,10-$^3$H]palmitate, respectively. Glucose-derived and palmitate-derived ATP production rates were calculated from the rates of glucose oxidation and palmitate oxidation.\textsuperscript{19}

\textbf{Western Blot Analysis.} Western blot analyses were performed, as reported previously\textsuperscript{11,19} with the following antibodies: anti-phospho AMPK (Cell signaling Inc), anti-total AMPK (Cell signaling Inc), anti-phospho JAK2 (Millipore), anti-total JAK2 (Cell signaling Inc), anti-phospho Erk1/2 (Cell signaling Inc), anti-total Erk1/2 (Cell signaling Inc), anti-PKC\textsuperscript{α} (Santa Cruz), anti-PKC\textsuperscript{β1} (Santa Cruz), anti-SERCA2 (Thermo Scientific), anti-phospho phospholamban (Ser16) (Badrilla), anti-phospholamban (Badrilla), anti-α-tubulin (Cell Signaling Inc) or anti-β-actin (Santa Cruz).

\textbf{Biochemical Analyses.} Short-chain CoA analysis was determined, as reported previously.\textsuperscript{19} Tissue triacylglycerol (TAG) was extracted and plasma and tissue TAG were quantified using a colorimetric enzymatic assay (Wako Pure Chemical Industries, Osaka, Japan).\textsuperscript{24} Cardiac ceramide levels were determined by ultra performance liquid chromatography with modifications.\textsuperscript{25}
**Histology.** Picro-Sirius Red (PSR) staining, dihydroethidium (DHE) staining, F4/80 macrophage staining, Oil-O Red staining and, hematoxylin and eosin (H&E) staining were performed as previously reported. Images for PSR, DHE 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 H&E staining were captured by light microscope (DM4000B, Leica).

**NADPH oxidase activity assay.** NADPH oxidase activity assay was performed by using a lucigenin enhanced chemiluminescence assay as reported previously.27

**Statistical Analysis.** All data are presented as mean±SEM. Statistical analysis of the data was performed using one-way ANOVA followed by multiple comparison testing using Student Neuman-Keuls (SNK) testing (SPSS Statistics 19 software). For the OGTT and ITT, two-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 non-parametric Kruskal-Wallis test for multiple comparison and confirmed that the results were completely congruent with our one-way ANOVA analysis. A probability value of <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 (Fig. 1). Pressure-volume analysis showed preserved systolic function in db/db mice (Fig. 1A-D). In contrast, db/db hearts showed diastolic dysfunction characterized by elevated LV end-diastolic pressure and end-diastolic pressure volume relationship (EDPVR), reduced –dP/dtmin and prolongation of the LV relaxation (Fig. 1A, E-H) all of which were reversed by Ang 1-7 treatment. Transthoracic echocardiography confirmed preserved systolic function in db/db mice (Supplemental Table 1) with elevated isovolumetric relaxation time and reduced E'/A' ratio (Fig. 1I-J) 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 (Fig. 1, Supplemental Table 1). The beneficial effect of Ang 1-7 treatment on cardiac hypertrophy was confirmed by morphometric and histological assessment (Fig. 2A-E). LV dry weight was significantly increased in db/db hearts compared to WT hearts (Fig. 2A-B), with markedly increased cardiomyocyte cross-sectional area (Fig. 2C), normalized by Ang 1-7. Picro-sirius red staining revealed that Ang 1-7 treatment also reversed the increased myocardial fibrosis in db/db hearts (Fig. 2D-E). 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 \textit{db/db} hearts, which was markedly decreased in response to Ang 1-7 (Fig. 2F). Biochemical analysis of the hearts revealed that hearts in \textit{db/db} mice accumulated high levels of cardiac TAG and ceramide, which was prevented by Ang 1-7 treatment (Fig. 2G-H). This occurred without changes in either liver or skeletal muscle (gastrocnemius) TAG levels (Supplemental Table 2). We determined the expressions of CD36, ATGL and diacylglycerol acyltransferase 2 (DGAT2), which are the key mediators of fatty acid uptake, TAG degradation and TAG synthesis, respectively. ATGL levels (Fig. 2I) were decreased, while CD36 and DGAT2 levels were increased (Supplemental Fig. 1A-B) in \textit{db/db} hearts; Ang 1-7 treatment upregulated ATGL (Fig. 2I), without altering CD36, and DGAT2 levels (Supplemental Fig. 1A-B). The phosphorylation of hormone-sensitive lipase (HSL), which is cardioprotective against lipotoxicity, was not changed in \textit{db/db} hearts (Supplemental Fig. 1C). These results highlight a novel role of Ang 1-7 in decreasing cardiac lipotoxicity in association with upregulation of ATGL.

\textbf{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\textsuperscript{15}, 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 (Fig. 3A) but not lean mass (Fig. 3B), in response to Ang 1-7 without a differential effect on total body weight of the db/db mice (Fig. 3C). Adipocyte cross-sectional area was increased in db/db mice and Ang 1-7 treatment resulted in a decrement in adipocyte size (Fig. 3D-E) and epididymal adipose tissue independent of food intake (Supplemental Table 2). Using the oral glucose tolerance test (OGTT), we showed that Ang 1-7 treatment did not alter the hyperglycemic response (Supplemental Fig. 2A-B), but plasma insulin levels at 120 min (Fig. 3F) and area under the curve (Fig. 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 (Fig. 3H-I). Consistent with these results, random plasma glucose levels (Fig. 3J) and plasma TAG levels (Fig. 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 (Fig. 4A) with increased expression of inflammatory cytokines, TNFα, interleukin-1β, interleukin-6 and monocyte chemoattractant protein-1 (Fig. 4B-E) which were reversed by Ang 1-7 (Fig. 4A-E). In contrast, the myocardium from db/db mice did not display these inflammatory changes (Fig. 4F-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, plays a key role in the pathogenesis of
diabetic cardiomyopathy, including the db/db model.\textsuperscript{28, 29} While Ang II/AT1R represent a key driver of metabolic perturbations and diastolic abnormalities\textsuperscript{11, 19}, Ang 1-7/MasR is considered a physiological antagonist of Ang II action.\textsuperscript{30} Insulin stimulation of WT mice showed a marked increase in glucose oxidation, which was severely blunted in placebo-treated db/db mice (Fig. 5A). Ang 1-7 treatment resulted in a 120\% increase in insulin-stimulated glucose oxidation in db/db hearts (Fig. 5A). Rates of palmitate oxidation in hearts from placebo-treated WT mice significantly decreased in response to insulin, but not in db/db hearts (Fig. 5B). A non-significant 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 due to increased fatty acid β-oxidation rates, with Ang 1-7 treatment reducing cardiac acetyl CoA levels (Supplemental Table 3). While the total ATP production was not altered in the absence and presence of insulin (Fig. 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 to placebo-treated db/db hearts (Fig. 5D). These changes occurred despite a similar reduction in phosphorylation of serine-473 (Fig. 5E) and threonine-308 (Fig. 5F) residues of Akt in placebo and Ang 1-7 treated db/db mice. These results highlight a key impact 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 Janus-activated kinase 2 (JAK2) (Fig. 6A), while phosphorylation of signal transducer and activator of transcription 3 (STAT3) (Fig. 6B) and extracellular signal-regulated kinase 1/2 (ERK1/2) (Fig. 6C) were reduced. Ang 1-7 treatment did not affect the phosphorylation of JAK2 and STAT3 (Fig. 6A-B), but did reverse the lower phosphorylation levels of ERK1/2 in \( db/db \) hearts (Fig. 6C) consistent with the cardioprotective effect of ERK1/2.31 Protein kinase C (PKC) \( \alpha \) and PKC\( \beta \)1, critical players in diabetic cardiomyopathy 32, were elevated in \( db/db \) hearts, while Ang 1-7 treatment completely reversed these elevations (Fig. 6D-E). In line with the reduction of adipose tissue mass, ATGL expression in epididymal adipose tissue was also increased in response to Ang 1-7 treatment (Fig. 6F). We also determined the expressions of SERCA2 and phospholamban (PLN), key determinants of intracellular Ca\(^{2+} \) signaling which are altered in diabetic cardiomyopathy. The myocardial level of SERCA2 and PLN was significantly decreased or unchanged in \( db/db \) hearts, respectively, which was unaffected by Ang 1-7 treatment (Supplemental Fig. 3).

Furthermore, we hypothesized that Ang 1-7 treatment ameliorates reactive oxygen species (ROS)-derived damage in hearts from \( db/db \) mice, as lipotoxicity triggers oxidative stress, and Ang 1-7 reduces NADPH-stimulated superoxide production. 33-35 Superoxide levels was increased in \( db/db \) hearts, driven by NADPH oxidase activity, leading to increased nitrotyrosine
levels (Fig. 7A-C; Supplemental Fig. 4). Ang 1-7 treatment prevented ROS production in db/db hearts in association with lowered NADPH oxidase activity and nitrotyrosine levels (Fig. 7A-C; Supplemental Fig. 4). 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 (Fig. 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 (Fig. 7E). Furthermore, we checked the expression of AMPK, which interacts with SIRT1 in regulating FOXO1 activity, and PPARα, the downstream target of the AMPK/SIRT1 pathway. The expression of PPARα and phosphorylation of AMPK were lowered in db/db hearts (Fig. 7F-G). Importantly, Ang 1-7 treatment increased phosphorylation of AMPK, concomitant with increased PPARα expression in the db/db hearts (Fig. 7F-G). 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 diabetes is rising rapidly worldwide. Type 2 diabetes 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 \textit{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, \textit{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, due to an increased ATGL activity, and reduced oxidative stress. These data clearly demonstrate the protective role of Ang 1-7 in obesity and diabetic cardiomyopathy (Fig. 8).

Ang 1-7 treatment improved cardiac hypertrophy and myocardial fibrosis, and reduced cardiac TAG accumulation and lipotoxicity in \textit{db/db} mice, which could contribute to the improvement of diastolic dysfunction\textsuperscript{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\textsuperscript{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 RAS plays a key 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 due to 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 ROS 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. In Zucker Diabetic Fatty (ZDF) rats, ventricular dysfunction improves when cardiac TAG and ceramide levels are normalized. We show that ATGL expression is significantly decreased in db/db mice, with similar findings in ob/ob mice and human obese subjects with insulin resistance. 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. 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 in 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 suggests 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 (Fig. 8). Increased circulating Ang 1-7 levels improves lipid metabolism and reduces visceral fat mass while the Mas knockout mice showed increased visceral adipose tissue accumulation. These data are consistent with the protective role of the ACE2/Ang 1-7/MasR axis in cardiovascular disease including diabetic cardiomyopathy.

SIRTs play a critical role in the control of energy metabolism. SIRT1 is a well-studied SIRT member, and controls FOXO1 activity via deacetylation. Acetylation of FOXO1 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, in which there is currently limited therapy. While targeting the AT1R pathway failed to improve the outcome of patients with HFPEF, targeting the other aspect of the RAS/ACE2 pathway such as enhancing Ang 1-7 action may represent a novel therapy for HFPEF.
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Disclosures

None.

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Figure Legends

Figure 1. Angiotensin 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 (Ees) (D) showed no change in db/db mice. In contrast, LV end-diastolic pressure (LVEDP) (E) and slope of the end-diastolic pressure volume relationship (EDPVR) (F) were increased with reduced –dP/dtmin (G) and prolongation of the LV relaxation time constant (Tau) (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). E’, early diastolic tissue Doppler velocity and A’, tissue Doppler velocity from atrial contraction. Values are mean±SEM; n=8 for each group. *p<0.05 compared with all groups.

Figure 2. Angiotensin 1-7 treatment improved cardiac hypertrophy and myocardial fibrosis, while decreasing lipotoxicity. Left ventricular dry weight (A), corrected to tibial length (B), and cardiomyocyte cross-sectional area (MCA) (C) were significantly increased in db/db mice. Picrosirius red staining (D, E) showed increased myocardial fibrosis, which was normalized by Ang 1-7. Oil O red staining showed a marked decrease in myocardial lipid accumulation in the db/db hearts in response to Ang 1-7 (F) while cardiac TAG (G) and ceramide (H) levels were
significantly increased in $db/db$ mice, accompanied by decreased ATGL expression (I), which were all normalized by Ang 1-7. A.U. indicates arbitrary units. Values are the mean±SEM of n=6 in each group; *p<0.05 compared with placebo-treated WT group, †p<0.05 compared with placebo-treated $db/db$ group. TAG=triacylglycerol; ATGL=adipose triglyceride lipase.

**Figure 3.** Angiotensin 1-7 reduced fat mass, ameliorates systemic insulin sensitivity and abnormal plasma lipid profiles. Magnetic resonance imaging showed decreased percent fat mass/BW (A) with no change in percent lean mass/BW (B) and body weight (BW) (C) during the course of treatment in $db/db$ mice. Adipocyte cross-sectional area in epididymal adipose tissue was increased in $db/db$ mice and Ang 1-7 reduced adipocyte size (D, E). Plasma insulin level during the oral glucose tolerance test (OGTT) (F, G) and insulin tolerance test (ITT) (H, I) showed Ang 1-7 improved insulin resistance in $db/db$ mice. Increased random plasma glucose (J) and plasma TAG (K) levels were reduced in response to Ang 1-7 treatment. Values are the mean±SEM of n=8 in each group; *p<0.05 compared with placebo-treated WT mice, †p<0.05 compared with placebo-treated $db/db$ mice. AUC=area under the curve; TAG=triacylglycerol.

**Figure 4.** Angiotensin 1-7 treatment reduced inflammation in adipose tissue. Hematoxylin and eosin (H&E) 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 $\alpha$
(TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6) and monocyte chemoattractant protein 1 (MCP1), were 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 WT mice.

**Figure 5.** Angiotensin 1-7 treatment resulted in mild improvement in insulin-stimulated glucose oxidation in db/db mice. Glucose oxidation determined using ex vivo working heart perfusion in hearts from placebo-treated WT mice increased in response to insulin, whereas those from db/db mice showed a blunted response to insulin, which was significantly improved by Ang 1-7 treatment (A). Palmitate oxidation rate in hearts from placebo-treated WT mice was decreased in response to insulin with a non-significant reduction seen in Ang 1-7 treated db/db hearts (B). Total ATP production (C) and %ATP (D) showing ATP production via glucose oxidation is stimulated by insulin which is markedly suppressed in placebo-treated db/db hearts and partially restored in db/db hearts treated with Ang 1-7. Decreased phosphorylation of myocardial Akt, phospho-serine (Ser)-473 (E) and threonine (Thr)-307 (F), in db/db mice were not altered in response to Ang 1-7 treatment. Values are the mean±SEM of n=6-8 in each group; A.U. indicates arbitrary units; *p<0.05 compared to its corresponding group without insulin; †p<0.05 compared with placebo-treated WT mice.
**Figure 6.** Modulation of pathological signaling pathways by angiotensin 1-7 in db/db mice. Phosphorylation of myocardial JAK2 (A) and STAT3 (B) were significantly increased and decreased, respectively, in db/db mice, which was not affected by Ang 1-7 treatment. The phosphorylation of myocardial Erk1/2 (C), and expression of myocardial PKCa (D) and PKCβ1 (E) were decreased and increased in db/db mice, which were reversed by Ang 1-7 treatment. Adipose tissue ATGL level was suppressed in db/db mice and partially rescued by Ang 1-7 (F). 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. JAK2=Janus-activated kinase 2 (JAK2); STAT3=signal transducer and activator of transcription 3; PKC=protein kinase C; ATGL=adipose triglyceride lipase.

**Figure 7.** Angiotensin 1-7 reduced myocardial oxidative stress and induces ATGL expression in association with deacetylation of 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. Immunoprecipitation (IP) 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 AMPK (F) and expression of 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 WT group,
†p<0.05 compared with placebo-treated db/db group. AMPK=5' AMP-activated protein kinase; PPARα=peroxisome proliferator-activated receptors alpha.

**Figure 8.** Schematic representation of the beneficial effects of Ang 1-7 in the heart and adipose tissue in the db/db model. In the heart, local activation of Ang II/AT1R pathway induced mitochondrial damage and impaired titin spring via reactive oxygen species (ROS) production, and loss of leptin signaling compromises this cardioprotective effect against heart failure. In adipocytes, either local activation of Ang II/AT1R pathway or lack of leptin signaling induce obesity, leading to influx of macrophage (MØ) into adipose tissue and secretion of proinflammatory mediators, eventually resulting in insulin resistance, altered metabolism and HFPEF. Ang1-7 binding to MasR in cardiomyocytes and adipocytes inhibits Ang II/AT1R pathway and is protective against HFPEF.
**Figure 2**

A. Dry LV Wt (mg)
- WT + Placebo
- db/db + Placebo
- db/db + Ang 1-7

B. Dry LV WT/TL (g/cm)
- WT + Placebo
- db/db + Placebo
- db/db + Ang 1-7

C. MCA (μm²)
- WT + Placebo
- db/db + Placebo
- db/db + Ang 1-7

D. Collagen Content (A.U.)
- WT + Placebo
- db/db + Placebo
- db/db + Ang 1-7

E. Images showing myocardial collagen content for different groups.

F. Images showing myocardial collagen content at higher magnification.

G. Cardiac TAG (μmol/g wet wt)
- WT + Placebo
- db/db + Placebo
- db/db + Ang 1-7

H. Ceramide (nmol/g wet wt)
- WT + Placebo
- db/db + Placebo
- db/db + Ang 1-7

I. ATGL α-tubulin (A.U.)
- WT + Placebo
- db/db + Placebo
- db/db + Ang 1-7

Mori J et al.
Figure 3

A. %Fat Mass/BW

B. %Lean Mass/BW

C. WT (g)

D. Random Plasma Glucose (mmol/l)

E. Plasma TAG (mg/dl)

F. Plasma Glucose (mmol/l)

G. Cell Surface Area (µm²)

H. AUC (ITT)

I. Plasma Insulin (ng/ml)

J. OGTT

K. Plasma TAG (mg/dl)

Mori J et al.
Figure 5

**A** Glucose Oxidation (nmol/g dry wt/min)

<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>insulin(-)</td>
<td></td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>insulin(+)</td>
<td>*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B** Palmitate Oxidation (nmol/dry wt/min)

<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>insulin(-)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>insulin(+)</td>
<td>*</td>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

**C** ATP Production (μmol/g dry wt/min)

<table>
<thead>
<tr>
<th></th>
<th>Insulin (-)</th>
<th>Insulin (+)</th>
<th>db/db Placebo</th>
<th>db/db+ Ang1-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT+ Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>db/db Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>db/db+ Ang1-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**D** %ATP

<table>
<thead>
<tr>
<th></th>
<th>Insulin (-)</th>
<th>Insulin (+)</th>
<th>db/db Placebo</th>
<th>db/db+ Ang1-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT+ Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>db/db Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>db/db+ Ang1-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**E**

- **P-Akt (Ser473)**
- **T-Akt**

**F**

- **P-Akt (Thr308)**
- **T-Akt**

Mori J et al.
Figure 6

**A**
P-JAK2
T-JAK2

**B**
P-STAT3
T-STAT3

**C**
P-Erk1/2
T-Erk1/2

**D**
PKCa
α-tubulin

**E**
PKCβ1
α-tubulin

**F**
ATGL
GAPDH

(Figure 6 from Mori J et al.)
Figure 7

Mori J et al.

**A**

DHE fluorescence (A.U.)

WT+Placebo vs db/db+Placebo vs db/db+Ang1-7

**B**

NADPH oxidase activity (A.U.)

WT+Placebo vs db/db+Placebo vs db/db+Ang1-7

**C**

Nitrotyrosine (A.U.)

WT+Placebo vs db/db+Placebo vs db/db+Ang1-7

**D**

IP: Ac-lys

Input

WB: FOXO1

WT+Placebo vs db/db+Placebo vs db/db+Ang1-7

**E**

SIRT1

α-tubulin

WT+Placebo vs db/db+Placebo vs db/db+Ang1-7

**F**

P-AMPK

T-AMPK

WT+Placebo vs db/db+Placebo vs db/db+Ang1-7

**G**

PPARα

β-Actin

WT+Placebo vs db/db+Placebo vs db/db+Ang1-7
Angiotensin 1-7 Ameliorates Diabetic Cardiomyopathy and Diastolic Dysfunction in db/db Mice by Reducing Lipotoxicity and Inflammation


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**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±0.16</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.
CD36/α-tubulin

DGAT2/α-tubulin

P-HSL (Ser660)/T-HSL

Mori J et al.  Supplemental Figure 1
A

**Supplemental Figure 2**

Mori J *et al.*
**A**

SERCA2

α-tubulin

![Bar chart](chartA.png)

**B**

P-PLN (Ser16)

T-PLN

![Bar chart](chartB.png)

Mori J et al. Supplemental Figure 3
Mori J et al.  Supplemental Figure 4