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Long-term administration of angiotensin (1-7) prevents heart and lung dysfunction in a mouse model of type 2 diabetes (*db/db*) by reducing oxidative stress, inflammation and pathological remodeling

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Abstract

Congestive heart failure is one of the most prevalent and deadly complications of type 2 diabetes that is frequently associated with pulmonary dysfunction. Among many factors that contribute to development and progression of diabetic complications is angiotensin II (Ang2). Activation of pathological arm of renin-angiotensin system results in increased levels of Ang2 and signaling through angiotensin type 1 receptor. This pathway is well recognized for its role in induction of oxidative stress (OS), inflammation, hypertrophy and fibrosis. Angiotensin (1-7) [A(1-7)], through activation of Mas receptor, opposes the actions of Ang2 which can result in the amelioration of diabetic complications; enhancing the overall welfare of diabetic patients. In this study, 8 week-old *db/db* mice were administered A(1-7) daily via subcutaneous injections. After 16 weeks of treatment, echocardiographic assessment of heart function demonstrated significant improvement in cardiac output, stroke volume and shortening fraction in diabetic animals. A(1-7) also prevented cardiomyocyte hypertrophy, apoptosis, lipid accumulation, and decreased diabetes-induced fibrosis and OS in the heart tissue. Treatment with A(1-7) reduced levels of circulating proinflammatory cytokines that contribute to the low grade inflammation observed in diabetes. In addition, lung pathologies associated with type 2 diabetes, including fibrosis and congestion, were decreased with treatment. OS and macrophage infiltration were also reduced in the lungs after treatment with A(1-7). Long-term administration of A(1-7) to *db/db* mice is effective in improving heart and lung function in *db/db* mice. Treatment prevented pathological remodeling of the tissues and reduced OS, fibrosis and inflammation.

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DECLARATION OF INTERESTS

No conflicts of interest have been identified.

AUTHOR CONTRIBUTION

AMP and MS designed and performed the experiments and wrote the manuscript, CJM performed the experiments, KER helped in research design and reviewed and edited the manuscript.

Graphical abstract

Keywords

diabetes mellitus; angiotensin (1-7); heart failure; lung congestion; remodeling

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disease that has reached epidemic proportions in the US and throughout the globe. One of the hallmarks of T2DM is dysregulation of the renin-angiotensin system (RAS). Angiotensin II (Ang2) is the central player in the pathological arm of the RAS. By interacting with the angiotensin type 1 receptor (AT1) receptor, it can cause hypertension (1,2), inflammation (3,4), OS (2,5), fibrosis (6), and insulin resistance (7,8); all factors that contribute to and exacerbate pathogenesis of diabetes. The protective arm of the RAS is comprised of the Mas receptor, activated by angiotensin (1-7) [A(1-7)] peptide (9). Dysregulation of the RAS in diabetic patients results in increased levels of Ang2 and chronic activation of AT1 contributing to many of the diabetic complications. In contrast, levels of circulating A(1-7) in diabetic patients are significantly lower than in non-diabetics and are considered an independent factor associated with decreased left ventricular function (10). A(1-7) through induction of vasodilation, reduction of OS and inflammation, can be highly beneficial in the treatment of diabetic complications such as heart disease and lung dysfunction, and may contribute to the enhancement of overall welfare of diabetic patients.

Two thirds of diabetic patients die due to cardiovascular dysfunction (11). Congestive heart failure (CHF) is a common cardiovascular complication associated with T2DM. Decreased heart function causes fluid build-up in different parts of the body, including the lung. CHF is frequently characterized with cardiac hypertrophy and fibrosis, reduced ventricular volume, and decreased contractility. Activation of pathological arm of the RAS, through the Ang2/AT1 axis, is well recognized for its role in heart disease progression (12). Increased inflammation and OS, which result from hyperglycemia and Ang2/AT1 signaling, add to tissue damage, abnormal remodeling, fibrosis and hypertrophy. Obesity also leads to

accumulation of fat in the heart, which may cause lipotoxicity and result in cell death and impaired cardiac function. Although diabetic heart disease is a well-recognized problem, the underlying mechanisms are still not completely understood.

Failing heart is associated with fluid accumulation and may lead to pulmonary edema. Lung disease has long been an understudied complication of T2DM. Forced vital capacity (FVC) and forced expiratory volume (FEV) are negatively affected by both hyperglycemia and diabetic heart disease (13,14). Diabetes is also associated with an increased risk for other lung dysfunctions such as asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, pneumonia, and idiopathic pulmonary fibrosis (15,16).

The goals of this study were to further delineate heart and lung dysfunctions associated with T2DM, as well as to evaluate the pharmacological role of A(1-7) in the amelioration of these pathologies. Using a mouse model of severe T2DM (*db/db*) we investigated protective effects of long-term administration of A(1-7) on the heart and lung function and characterized the structural and molecular changes, such as inflammation and OS, in these organs.

2. METHODS

2.1. Animal procedures

Eight-week old male BKS.Cg-Dock7^m *+/+* Lepr^{db}/J (*db/db*) mice and age-matched heterozygous controls (non-diabetic) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were randomized into four treatment groups (n=6/group). Animals were kept on a 12h light/dark cycle and food and water were available *ad libitum*.

Animals were administered either vehicle (saline) or A(1-7) (0.5 mg/kg/day) subcutaneously, daily for 16 weeks. Previous dose finding studies performed in this laboratory revealed the optimal dosing to be 0.5 mg/kg/day with no further benefit at 1 mg/kg/day (17,18). Pharmaceutical grade A(1-7) was purchased from Bachem (Torrance, CA, USA).

Body weight was assessed at necropsy. Blood glucose level was measured using a hand-held blood glucose meter from a drop of blood obtained from the saphenous vein. At the necropsy mice were overdosed with ketamine/xylazine and the blood was collected by cardiac puncture into EDTA coated tubes. Immediately after collection plasma was isolated by centrifugation and stored at -80°C until analysis.

2.2 Echocardiography

Heart function was assessed noninvasively using a high frequency, high-resolution two-dimensional echocardiography system consisting of Vivid 7 Dimension ultrasound machine equipped with a 6–13MHz linear transducer (GE Healthcare, Little Chalfont, UK) after 16 weeks of treatment. Anesthesia was induced with 3% isoflurane in an induction chamber. The mouse was then placed in a supine position on a heating pad to maintain body temperature at 36.5–37°C. Anesthesia was maintained through a nosecone and adjusted to maintain heart rate at 450–550 beats per minute. The images of the left ventricle were

acquired and the shortening fraction (SF), stroke volume (SV), and cardiac output (CO) were calculated in accordance with the American Society of Echocardiography guidelines (19). CO was calculated using a modified Simpson's rule. To facilitate measurements, mice were injected with a microbubble contrast agent through the tail vein as described previously (20).

2.3 Histological analysis

At the necropsy hearts were rapidly excised and weighed. The weights were normalized to tibia length. The heart tissue was cut in half; one half was formalin-fixed and paraffin-embedded and cut at 5 μ m, and other half was embedded in OCT and cut at 7 μ m. The lungs were isolated, embedded in OCT and stored at -80°C . Lung sections were cut at 7 μ m and stored at -80°C until use.

Cardiomyocyte hypertrophy—Heart sections were stained using hematoxylin & eosin (H&E). Cross-area of 30–40 cardiomyocytes located in the left ventricle was measured using freehand selection in ImageJ (1.47v, NIH, USA).

Apoptosis—Apoptosis in the heart was evaluated using Click-iT TUNEL Alexa Fluor from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA) with modifications: tissue was permeabilized using proteinase K solution (Thermo Fisher Scientific, Waltham, MA, USA) at 20 $\mu\text{g}/\text{ml}$ for 12 minutes and washed thrice with PBS for 5 minutes each. Number of apoptotic cells was quantified in the left ventricles.

Fibrosis—Fibrosis was evaluated in sections stained using standard Masson's trichrome method. Ten pictures at 40 $\times$ magnification were acquired for each slide and the collagen was quantified using color-deconvolution plugin and threshold function in ImageJ (1.47v, NIH, USA). Results are expressed as percentage of total tissue area stained for collagen.

Fat accumulation—Heart and lung samples embedded in OCT were cut at 7 μ m and stored at -80°C until used. Lipid accumulation was measured using standard Oil-Red-O staining method. Five random views of the tissue were acquired at 60 $\times$ magnification for each slide. Oil-Red-O staining was quantified using color-deconvolution plugin and threshold function in ImageJ (1.47v, NIH, USA). Results are expressed as percentage of total tissue area stained for lipids.

Immunohistochemistry staining of nitrotyrosine (N-Tyr)—The paraffin-embedded heart sections were treated using a standard heat-induced antigen retrieval procedure. Lung sections did not require this step. The slides were incubated with rabbit anti-mouse polyclonal antibody directed against nitrated tyrosine residues (EMD Millipore, Billerica, MA, USA) at 1:250. After incubation with a proper secondary antibody, an avidin-biotin complex method of detection was used. Heart sections were scanned using an Aperio CS2 slide scanner (Leica Biosystem, Wetzlar, Germany). Vessels positive and negative for N-Tyr were quantified in left ventricle. Only vessels larger than 10 μ m in diameter were counted. For lungs, 10–15 pictures of each slide were taken at 40 $\times$ magnification. ImageJ software (1.47v, NIH, USA) was used to quantify the percentage of total area positive for N-Tyr.

Lung congestion—Frozen lung sections were fixed for 30 min in 10% formalin and then stained using H&E. In order to quantify the open alveolar area, 10 pictures per slide were taken at 40× magnification and analyzed using ImageJ (1.47v, NIH, USA) to measure the unstained area.

Macrophage infiltration in the lung—Frozen sections were stained per standard immunofluorescent protocol. Briefly, sections were probed with a rabbit anti-mouse F4/80 antibody conjugated to FITC (eBioscience Inc., San Diego, CA) at a 1:250 dilution. Propidium Iodide was used as a counter stain. Five to ten pictures were taken at 10× magnification for each slide and F4/80 positive cells were counted.

2.4 Concentration of cytokines in plasma

Multi-Array technology by Meso Scale Diagnostics (Rockville, MD, USA) allows quantitative assessment of expression levels of proteins using electrochemiluminescence. To evaluate concentration of proinflammatory cytokines, a Mouse Proinflammatory 7-Plex kit (Meso Scale Diagnostics, Rockville, MD, USA) was used. Assay was performed in accordance to manufacturer's manual. The plate was read using a Sector S 600 analyzer (Meso Scale Diagnostics, Rockville, MD, USA).

2.5 Statistical analysis

GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, San Diego, CA, USA) was used to analyze the data. One-way ANOVA followed by Dunnett's multiple comparisons test were used to compare data. The level of statistical significance was set at 5%. Data are expressed as mean value $\pm$ standard error of the mean (SEM).

3. RESULTS

3.1 A(1-7) has no effect on body mass and hyperglycemia in *db/db* mice

db/db mice develop hyperglycemia at age of 6–8 week-old primarily due to obesity. Both blood glucose levels and body mass were highly increased in diabetic animals compared to heterozygotes (Fig. 1). Administration of A(1-7) did not have any effect on the body mass and hyperglycemia in *db/db* mice.

3.2 Treatment with A(1-7) improved heart function as measured by echocardiography

One of the hallmarks of heart disease is decreased CO that results in decreased volume of blood pumped from the left ventricle and contributes to pulmonary edema. Diabetic mice had significantly decreased CO compared to heterozygous controls. Treatment with A(1-7) partially prevented the reduction in CO in *db/db* mice (Fig. 2A). Like CO, SV is a measurement of ability of the heart to effectively circulate the blood. Similar to CO, SV was decreased in diabetic animals compared to non-diabetics. Treatment with A(1-7) improved this parameter, which suggests a cardio-protective role of the peptide in this model of T2DM (Fig. 2B). SF of the left ventricle in diabetic mice was decreased compared to age-matched, non-diabetic animals suggesting impaired contractility (Fig. 2C). Daily administration of A(1-7) improved this measurement in diabetic mice.

3.3 A(1-7) reduced cardiomyocyte hypertrophy, number of apoptotic cells, and fat accumulation in hearts from diabetic animals

Heart hypertrophy is often associated with increased myocyte size. Cardiomyocytes become enlarged in order to keep up with increased functional demand. Even though no changes were observed in absolute (data not shown) and normalized heart weight between any of the treatment groups (Fig. 3A), significant increases in cardiomyocyte area were seen in hearts from diabetic animals, compared to non-diabetic controls. Treatment with A(1-7) normalized the cardiomyocyte size in *db/db* mice (Fig. 3B, 4A). The increase of myocyte size without increased organ weight suggests that growth of some of the cells might be associated with loss of others. An increased number of apoptotic cells was observed in *db/db* mice. A(1-7) reduced the number of cells undergoing apoptosis in hearts of diabetic mice (Fig. 3C). Number of apoptotic cells correlated with cardiomyocyte hypertrophy (Fig. 3D). Treatment with A(1-7) also decreased accumulation of lipids in the hearts from diabetic animals (Fig. 3E, 4B), which may contribute to reduced cell death and tissue remodeling. No change in lipid accumulation in the lungs was seen between any of the treatment groups (data not shown).

3.4 Administration of A(1-7) to diabetic animals results in reduced OS and fibrosis in the heart and decreased levels of circulating proinflammatory cytokines

Fibrosis not only compromises elasticity of the heart but also interferes with excitation-contraction mechanisms and may result in impaired contractility. Interstitial fibrosis was significantly increased in hearts of diabetic animals compared to non-diabetic controls. Daily treatment with A(1-7) reduced fibrotic area in hearts of *db/db* mice (Fig 3E, 4C). Two factors that contribute to enhanced scarring and fibrosis are increased inflammation and OS. OS was assessed in heart sections stained for N-Tyr, a peptide modification that is commonly used as an indirect marker of superoxide production contributing to the formation of peroxynitrite. N-Tyr levels in the cardiomyocytes were virtually undetectable. However, significant levels of staining were observed in the vessels (Fig. 3G). Number of vessels that were positive for N-Tyr was higher in *db/db* mice, indicating increased OS, which may result in endothelial dysfunction. A(1-7) reduced the fraction of positively stained vessels in hearts of diabetic mice (Fig. 3F). Increased OS contributes to activation of proinflammatory mechanisms that may in turn lead to excessive fibrosis and remodeling. Increased levels of TNF- α and IL-1 β were observed in plasma of diabetic animals, whereas no changes were seen in levels of IL-6 in any treatment group (Fig. 5). A(1-7) significantly reduced levels of circulating IL-1 β . The reduction of TNF- α levels was not as profound and has not reached statistical significance levels, possibly because the overall increase in the diseased animals is minimal. This data suggests reduction of systemic inflammation after administration of A(1-7).

3.5 Lung fibrosis and exudate accumulation were normalized by treatment with A(1-7)

Reduced FEV and FVC, as seen in diabetic patients, can be caused by structural changes, such as fibrosis, or fluid accumulation in alveolar spaces. Both pathologies impact the effectiveness of oxygen exchange in the lung and can have negative consequences on patient health. Diabetic animals had increased levels of collagen in the lungs, consistent with other

findings in both animal models and human studies (21,22). Treatment with A(1-7) decreased the total amount of collagen in the alveolar space back to normal levels (Fig. 6A). The area of non-stained alveolar space was used as a surrogate marker for open-air alveolar space. Diabetic mice had significantly lower open-air alveolar space when compared to the age-matched non-diabetic animals. Treatment with A(1-7) increased the open alveolar area (Fig. 6B). Overall, both pathological arms that contribute to alveolar obstruction and reduced oxygen exchange in *db/db* mice were alleviated by A(1-7) treatment.

3.6 Oxidative damage and macrophage infiltration in the lungs of diabetic mice were attenuated by A(1-7)

OS in the lungs of diabetic mice was assessed by measuring the extent of N-Tyr staining. *db/db* mice had a significantly larger area of tissue damage caused by OS than the heterozygous controls. This was normalized after treatment with A(1-7) (Fig. 4D, 6C). Tissue damage resulting from OS leads to increased inflammation and may result in fibrosis. Pulmonary macrophage infiltration, identified by F4/80 staining, was higher in the lungs of *db/db* mice compared to the non-diabetic controls (Fig. 4E, 6D). A(1-7) treatment decreased the number of macrophages in the lungs of diabetic mice. Prolonged macrophage infiltration can be a consequence or a cause of chronic inflammation and may result in lung fibrosis. The damage to the epithelial lining of the lung may increase the susceptibility of the host to pulmonary infections, which is an observation made in epidemiological studies (22).

4. DISCUSSION

In this study we showed that daily subcutaneous administration of A(1-7) prevented progression of heart and lung dysfunction in a mouse model of severe T2DM. Heart disease has been well described in various mouse models of T2DM, including *ob/ob* mice, Zucker fatty rats and *db/db* mice (18,23–25). *db/db* mice develop more severe hyperglycemia than *ob/ob* mice early in life (6–8 weeks old), which results in more profound changes in heart function. In addition, strain used in this study also known as BKS db (BKS.Cg-Dock7^m +/+ Lepr^{db}/J) is characterized with more severe pathologies than similar B6 db strain (B6.BKS(D)-Lepr^{db}/J) and is described by the supplier to survive only up to 40 weeks. Animal models of diabetic pulmonary dysfunction focus on models of type 1 diabetes (T1D); these include the streptozotocin-induced diabetes and the OVE26 transgenic mouse model. Although these animal models indeed show histological evidence of increased pulmonary fibrosis they do not truly represent the effect of obesity-induced diabetes on lung pathologies. Here, we showed that 24 week-old *db/db* mice develop severe heart disease and lung dysfunction due to increased inflammation, OS and fibrosis.

Ang2 and AT1 have been long considered targets for new antihypertensive therapies. Three classes of drugs that modulate RAS have been developed: ACE inhibitors (ACEi), angiotensin receptor blockers (ARBs) and direct renin inhibitors, of which ACEi and ARBs are the most commonly used. It has been shown that ACEi and ARBs improve heart function in T2DM animal models as well as in patients (7,26). Treatment with losartan, a commercially available ARB, reduced lung fibrosis in a STZ-induced diabetic-mouse model (27). A(1-7) counteracts effects of Ang2 and has been shown to improve cardiovascular

function in some models of type 1 and type 2 diabetes (18,28–31). However, the exact mechanism of its protective action is not entirely understood. Safety studies showed that A(1-7) is not associated with any severe adverse effects. A(1-7) analog is currently in phase III clinical studies for the amelioration of side effects of chemotherapy. In this study, in contrast to profound effects observed in diabetic animals, A(1-7) did not affect any of the studied markers in heterozygous mice. This is consistent with safety studies, where no severe adverse effects of high dose A(1-7) were observed in patients (32).

First observations that A(1-7) is locally expressed in the cardiac tissue of animal models (33,34), and in intact human heart (35), suggested a role in controlling cardiovascular function. Since then, several animal studies have shown beneficial effect of A(1-7) on heart function in models of diabetes. A recent study by Oudit group showed improved diastolic dysfunction in 5 month-old B6 *db/db* strain (B6.BKS(D)-Lepr^{db}/J) implanted with micro-osmotic pump containing A(1-7) (31). The strain of *db/db* mice used in this study is characterized with less severe diabetes than the model used in studies described here. In addition, we show that daily subcutaneous injections are sufficient to prevent diabetes-related heart and lung dysfunction, which has a better translational potential than constant infusion using micropumps. Data collected in this study also shows that treatment with A(1-7) has a protective effect on the kidneys (unpublished results).

Echocardiography, revealed significantly improved heart function in diabetic animals treated with A(1-7), which is consistent with our previous findings in younger animals (18). Longer treatment resulted in even more profound changes in CO and SF in *db/db* mice treated with A(1-7). T2DM has been previously described as an independent factor associated with cardiac hypertrophy in patients (36). Even though no change was seen in heart weight between treatment groups, increased cardiomyocyte size suggests presence of cardiac hypertrophy in diabetic mice. In addition, myocyte cross-area correlates with number of apoptotic cells. Activation of hypertrophic signals may be one of the mechanisms compensating for increased numbers of dying cells in diabetic hearts. We also demonstrated that A(1-7) reduces fat accumulation in the cardiac tissue from diabetic animals, which may contribute to reduction of lipotoxicity. Lipid accumulation is known to induce apoptosis, ER stress, mitochondrial dysfunction and overproduction of ROS not only in animal models of obesity but also in patients (37). Increased OS and levels of circulating proinflammatory cytokines also contribute to activation of fibrotic and hypertrophic signals. Tissue damage due to OS may be one of the factors leading to increased cell apoptosis in the hearts of *db/db* mice. Oxidative damage can activate immune response and partially contribute to systemically elevated inflammation. Epidemiological studies suggest strong correlation between systemic inflammation and cardiac hypertrophy (38). We observed increased concentration of TNF- α and IL-1 β in plasma from diabetic animals, whereas no changes in expression of IL-6 were seen. This suggests presence of a low grade of chronic inflammation that was attenuated by treatment with A(1-7) in diabetic animals. Both OS and inflammation can activate growth and fibrotic signals. Extensive fibrosis observed in diabetic hearts contributes not only to impairment of overall cardiac function but might also directly affect the elasticity and contractility of the heart. In this study we observed significant effects of A(1-7) on all of the discussed parameters. We hypothesize that A(1-7) antagonizes actions of Ang2 and therefore contributes to decreased OS, inflammation,

hypertrophy, lipid accumulation and fibrosis in the heart, and results in improved cardiac function *db/db* mice. Further, improvement of lung function may be directly associated with enhanced heart function. Cellular mechanisms that were investigated in this study may aid in understanding of the underlying pathologies in diabetic patients and support transition of A(1-7) to clinic.

A(1-7) and the enzyme that cleaves Ang2 to produce A(1-7), the angiotensin-converting enzyme 2 (ACE2), also play a significant role in some models of pulmonary distress. ACE2 KO mice show significantly more lymphocyte infiltration and fibrosis in acid wash, bleomycin and influenza induced lung failure (39). Treatment with A(1-7) in bleomycin models of lung fibrosis, a model dependent on pathological levels of OS and inflammation, has shown protective effects by inhibiting both the NOX4-derived ROS-mediated RhoA/Rho kinase and the MAPK/NF- κ B pathways (40). In this study we demonstrated that lungs of *db/db* animals have increased N-Tyr staining, macrophage infiltration, and collagen deposition but not lipid accumulation (data not shown). We hypothesize that OS-induced inflammation leading to fibrosis is indeed an important chain of events that results in organ damage. Treatment with A(1-7) inhibits all of the contributing factors that lead to structural remodeling of the lungs of *db/db* animals.

In recent years there has been an increase in epidemiological evidence that suggests a strong correlation between pulmonary dysfunction and cardiovascular health. Results from the Atherosclerosis Risk in Communities (ARIC) study show a significant and independent link between low FEV and risk of heart failure incidents (41). Similarly, CHF is associated with changes in pulmonary health, further complicating the disease state. One of the symptoms that link heart failure to pulmonary dysfunction is pulmonary edema. Heart disease, together with other diabetes-related problems such as hyperglycemia and hypertension, can eventually lead to CHF, which results in widespread edema. Sustained rise in blood pressure in these veins eventually leads to excretion of fluid into the alveoli, which decreases the ability of the lung to exchange gasses and is considered the main cause of heart failure-related pulmonary dysfunction. In addition, it is hypothesized that OS and inflammation may lead to abnormal remodeling and scarring. All of this plays a key role in the pathologies associated with both organs.

Reducing hyperglycemia in diabetic patients can decrease some of the complications but does not fully eliminate them. Targeting other mechanisms that contribute to the disease state may further improve the outcomes for these patients. Here we showed that daily administration of A(1-7) improved heart and lung function even in the face of uncontrolled hyperglycemia and obesity. We hypothesize that A(1-7) used in conjunction with a blood glucose lowering agent might have even more profound effects.

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Fig. 1. Hyperglycemia and body weight

Diabetic animals from both treatment groups demonstrated increased hyperglycemia (A) and body weight (B) compared to non-diabetic controls. Daily treatment with A(1-7) did not have any effect on these parameters. (hzg – heterozygous; **** $p < 0.001$ compared to hzg saline); calculated using one-way ANOVA; plotted as mean with SEM.

Fig. 2. Physiological heart function

Treatment with A(1-7) prevented progression of heart dysfunction in diabetic animals. Parameters of heart function were assessed using echocardiography. Daily administration of A(1-7) prevented loss in cardiac output (A), stroke volume (B), and shortening fraction (C) in diabetic animals, which suggests protection against progressive heart dysfunction (hzg – heterozygous; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001); calculated using one-way ANOVA; plotted as mean with SEM.

Fig. 3. Remodeling of heart tissue

No significant changes were detected in heart weights between the treatment groups (A). Cardiomyocyte cross-area was increased in *db/db* mice compared to non-diabetic controls. Treatment with A(1-7) reduced myocyte hypertrophy in diabetic animals (B). Activation of hypertrophic signals is one of the mechanisms compensating for cell loss. Daily administration of A(1-7) reduced number of apoptotic cells in hearts from diabetic animals (C). Number of apoptotic cells correlated with the size of cardiomyocytes (D) ($r^2=0.52$, $p=0.001$). Accumulation of lipids in hearts may also contribute to increased apoptosis and remodeling of the tissue. Fat deposition was highly increased in hearts from *db/db* mice, compared to healthy controls. Treatment with A(1-7) reduced lipid accumulation in diabetic animals (E). Fibrosis in the heart was assessed using Masson's trichrome staining. Hearts from diabetic mice had significantly higher levels of collagen (blue) than hearts from heterozygous animals. A(1-7) reduced fibrosis in hearts of *db/db* mice (F). One of the factors that contribute to activation of fibrotic signals is oxidative stress. Staining positive for nitrated tyrosine residues was used as a marker of oxidative stress. Nitrotyrosine was detected in vessels but not in the cardiomyocytes (H). Fraction of vessels positively stained for nitrotyrosine was significantly higher in diabetic mice compared to non-diabetics. A(1-7) treatment prevented oxidative stress damage in the vessels (G). (hzg – heterozygous;

* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$); calculated using one-way ANOVA; plotted as mean with SEM.

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Fig. 4. Representative images

Representative images of H&E staining in the heart (A), Oil-Red-O staining in the heart (B), Masson's trichrome staining in the heart (C), immunohistochemistry staining for nitrotyrosine in the lung (D), and immunofluorescent staining for macrophages in the lung (E).

Fig. 5. Levels of circulating cytokines

Levels of circulating cytokines were measured using electrochemiluminescence assay.

Levels of two proinflammatory cytokines, TNF- α and IL- β , were significantly increased in diabetic mice (A, B). Treatment with A(1-7) reduced concentration of IL- β in plasma of *db/db* mice. The reduction of TNF- α levels was not as profound and has not reached statistical significance levels. No significant changes in IL-6 concentration were detected between any of the groups (C). (hzg – heterozygous; *p < 0.05; **p < 0.01; ***p < 0.001); calculated using one-way ANOVA; plotted as mean with SEM.

Fig. 6. Remodeling of the lung tissue

Pulmonary fibrosis was detected in lung sections stained with Masson's trichrome and expressed as percentage of total area stained for collagen (blue). Fibrosis was increased in diabetic mice compared to non-diabetic controls (A). Administration of A(1-7) reduced levels of collagen in lungs of *db/db* mice. Lung congestion was evaluated in sections stained with H&E. Area of open-air alveolar space was decreased in diabetic animals compared to non-diabetics (B). Treatment with A(1-7) increased the overall area that participates in oxygen-carbon dioxide exchange. Oxidative stress and inflammation contribute to lung pathologies in type 2 diabetes. Oxidative stress damage was assessed by quantifying staining for nitrotyrosine and inflammation was evaluated by enumerating macrophages positively stained with anti-F4/80 antibody in lung sections. Levels of nitrotyrosine were significantly increased in diabetic animals compared to heterozygous mice (C). Treatment with A(1-7) reduced the extent of staining in lungs from diabetic mice. Similarly, number of macrophages was increased in lungs from diabetic animals and A(1-7) prevented macrophage infiltration in these mice (D). (hzg – heterozygous; * $p < 0.05$; **** $p < 0.0001$); calculated using one-way ANOVA; plotted as mean with SEM.