Left ventricular diastolic dysfunction and myocardial stiffness in diabetic mice is attenuated by inhibition of dipeptidyl peptidase 4

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Aims

Obesity and Type 2 diabetes mellitus (DM) induce left ventricular (LV) diastolic dysfunction, which contributes to an increasing prevalence of heart failure with a preserved LV ejection fraction. We investigated the effects of sitagliptin (SITA), an inhibitor of dipeptidylpeptidase-4 (DPP-4) and anti-diabetic drug, on LV structure and function of obese mice with Type 2 DM.

Methods and results

Obese Type 2 diabetic mice (Leprdb/db, BKS.Cg-Dock7m+/+ Leprdb/J), displaying increased cardiomyocyte and LV stiffness at the age of 16 weeks, were treated with SITA (300 mg/kg/day) or vehicle for 8 weeks. SITA severely impaired serum DPP-4 activity, but had no effect on glycaemia. Invasive haemodynamic recordings showed that SITA reduced LV passive stiffness and increased LV stroke volume; LV end-systolic elastance remained unchanged. In addition, SITA reduced resting tension of isolated single cardiomyocytes and intensified phosphorylation of the sarcomeric protein titin. SITA also increased LV concentrations of cGMP and increased activity of protein kinase G (PKG). In vitro activation of PKG decreased resting tension of cardiomyocytes from vehicle-treated mice, but had no effect on resting tension of cardiomyocytes from SITA-treated mice.

Conclusions

In obese Type 2 diabetic mice, in the absence of hypoglycaemic effects, inhibition of DPP-4 decreases LV passive stiffness and improves global LV performance. These effects seem at least partially mediated by stimulatory effects on the myo-cardial cGMP–PKG pathway and, hence, on the phosphorylation status of titin and the hereto coupled cardiomyocyte stiffness modulus.

Keywords

Diabetes • Heart failure • Haemodynamics

1. Introduction

Type 2 diabetes mellitus (DM) is an important risk factor for heart failure, and is associated with high heart failure-related mortality and hospitalization rates.1–3 The pathogenesis of DM-induced left ventricular (LV) dysfunction is complex and includes both cellular and systemic abnormalities.4–9 LV diastolic dysfunction is an early manifestation of DM-induced LV dysfunction, and often leads to heart failure, even in the presence of a preserved LV ejection fraction.10–11 LV diastolic dysfunction in patients with DM has been linked to the development of interstitial myocardial fibrosis,12 and to increased passive cardiomyocyte stiffness.13 Although both processes are potential targets for drug therapy, to date there is no specific treatment for LV diastolic dysfunction.

Inhibitors of dipeptidylpeptidase-4 (DPP-4) are novel drugs for the treatment of Type 2 DM,13,14 but their potential effects on the structure and function of the heart in DM remain to be elucidated. DPP-4 inhibitors exert anti-diabetic effects by inhibiting the breakdown of incretins,
such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide, which stimulate insulin secretion from pancreatic β-cells.\textsuperscript{13–15} GLP-1 may also have direct effects on other organs via the GLP-1R.\textsuperscript{16,17}

DPP-4 is a serine exopeptidase that cleaves X-proline or (X)-alanine dipeptides from the N-terminus of polypeptides.\textsuperscript{18} DPP-4 is expressed on the surface of several cell types, including cardiac microvascular endothelial cells.\textsuperscript{19} DPP-4 catalyses the breakdown of several cardioactive peptides, including brain natriuretic peptide (BNP) and stromal cell-derived factor-1α (SDF-1α).\textsuperscript{20–22} There is emerging evidence that DPP-4 inhibition has direct cardiovascular effects, occurring independently of glycaemic control. For example, DPP-4-deficient mice show improved survival following myocardial infarction.\textsuperscript{23} Also, pharmacological DPP-4 inhibition attenuates myocardial stunning,\textsuperscript{24} protects the heart from ischaemia/reperfusion injury in obese pre-diabetic rats,\textsuperscript{25} and improves LV function during pacing-induced heart failure in pigs.\textsuperscript{26}

Recently, Shigeta et al.\textsuperscript{27} showed that pharmacological and genetic DPP-4 suppression attenuated intestinal myocardial fibrosis and LV diastolic dysfunction in streptozotocin-induced Type 1 diabetic rats.

The aim of the present study was to assess the effect of pharmacological DPP-4 inhibition on LV function and structure in obese Type 2 diabetic mice (Leprdb/db mice). Importantly, pharmacological DPP-4 inhibition did not reduce glycaemia in these mice. We hypothesized that DPP-4 inhibition would protect against DM-induced LV dysfunction, despite the absence of effects on glycaemia.

2. Methods

2.1 Experimental animals and study design

All study protocols were approved by the local animal care committee and conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, updated 2011).

Lepr\textsuperscript{db/db} mice, a leptin receptor-deficient model of obesity with secondary Type 2 diabetes, and their non-diabetic lean heterozygous Lepr\textsuperscript{db/+} littermates were purchased from Charles River Laboratories (BKS.Cg-Dock7m+/- Lepr\textsuperscript{m+}). Eight-week-old male Lepr\textsuperscript{db/db} and age-matched Lepr\textsuperscript{db/+} mice were randomized to drinking water supplemented with or without sitagliptin (SITA; 300 mg/kg/day). During the 8-week treatment, weight and fasting glycaemia were measured.

2.2 DPP-4 enzymatic activity

DPP-4 enzymatic activity was assayed by using glycyl-prolyl-4-methoxy-β-naphthylamide (Gly-Pro-4-Me-Na, Sigma-Aldrich) as fluorogenic substrate as described previously.\textsuperscript{28} In a 96-well plate, 10 μL of serum samples were mixed with 0.5 mM Gly-Pro-4-Me-Na in 50 mM Tris buffer (pH 8.3) in a final volume of 110 μL. DPP-4 activity was determined kinetically during 10 min at 37°C by measuring the velocities of 4-Me-β-NA release (λ\textsubscript{ex} = 340 nm, λ\textsubscript{em} = 430 nm) from the substrate using an Infinite 200 (Tecan Group Ltd, Switzerland). Fluorescence intensity was related to a 4-Me-β-NA standard curve in the same buffer. Because SITA is a reversible inhibitor, we corrected for the dilution of the serum in the assay to estimate in vivo percentage inhibition of the DPP-4 enzymatic activity in undiluted plasma. Percentage inhibition was calculated by comparing DPP-4 enzymatic activity of treated mice with non-treated mice, which were not enzymatically inhibited (defined as 100% activity).

2.3 Peritoneal glucose tolerance tests

After a 16-h fasting, blood glucose levels (mg/dL, OneTouch test strips, Accu-Chek Sensor, Roche, Germany) were determined. Next, a glucose bolus was injected intraperitoneally (1 mg/g body weight) and blood glucose levels were measured after 15, 30, 60, and 120 min.

2.4 Echocardiographic evaluation

Transthoracic echocardiograms were performed before sacrifice on lightly anaesthetized mice [inhalation of 4% (vol/vol) sevoflurane; Sevorane®, Abbott Laboratories, Wavre] using a Toshiba diagnostic ultrasound system (SSA-700A), equipped with a 15 MHz transducer. A 2D short-axis view of the mid-LV was obtained at the chordal level. LV anterior and posterior wall thickness (AWT and PWT) and end-diastolic and -systolic internal dimensions (end diastolic diameter (EDD) and ESD) were measured on three consecutive M-mode cycles and averaged by a single observer in a blinded fashion. Fractional shortening (FS) was calculated as %FS (EDD – ESD/EDD) × 100. LV mass was calculated using the following equation: LV mass = 1.055 × [(AWT + EDD + PWT)3 – EDD3], where 1.055 is the specific gravity of myocardium.

2.5 Haemodynamic measurements

Invasive haemodynamic measurements were recorded in anaesthetized mice with a mixture of urethane (1000 mg/kg), etomidate (10 mg/kg), and morphine (1 mg/kg), intraperitoneally. Anaesthetized mice were mechanically ventilated and fluid was administered via the right jugular vein. After thoracotomy, a four-electrode pressure conductance catheter (1.4 F, SPR-839, Millar Instruments, Houston, TX, USA) was inserted into the LV through the apex. After stabilization, steady-state measurements were recorded. Subsequently, LV preload was decreased by occlusion of the inferior vena cava for 5–10 s to derive load-independent parameters of contractility (Powerlab/4SP, ADInstruments, LaChart 7 Pro Software, Castle Hill, Australia). Both steady-state and load-independent parameters were determined offline in a blinded fashion using the PVAN 3.5 Software (Millar Instruments).

2.6 Morphometric analysis, tissue preparation, and western blotting

After haemodynamic data collection, anaesthetized animals were sacrificed by cardiectomy. Lungs were dissected and weighed. The apex of the LV was cut, formalin-fixed, and embedded in paraffin for histology, and the remaining portion of the LV was snap-frozen and stored at −80°C for protein analysis. LV samples were homogenized with a Polytron homogenizer (Pt 2100; Kinematica, Littau, Switzerland) in lysis buffer supplemented with protease and phosphatase inhibitors (Complete; Roche and Sigma, respectively). Lysates were heat-denatured for 4 min and loaded on 4–12% NuPage gels (Invitrogen). After electrophoresis, proteins were electrotransferred to polyvinylidene fluoride membranes overnight at 4°C. Membranes were blocked with 5% BSA and incubated with primary antibodies overnight at 4°C whereafter secondary horseradish peroxidase-conjugated antibody was applied for 1 h at room temperature. Blots were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Antibodies to phospho-endothelial nitric oxide synthase (eNOS) (pS1177) and total-eNos were from BD Transduction Laboratories, and anti-phospho-Akt (Ser473) and anti-total-Akt antibodies were purchased from Cell Signaling Technologies. Western blots were subjected to densitometric analysis using the ImageJ 1.42 software, and quantifications were statistically analysed using the Bonferroni-adjusted t-test.

2.7 Histochemochemical analysis

Paraffin-embedded LV sections were cut and stained with laminin, Sirius red, Trichrome Masson, and collagen types I and III, according to the manufacturer’s instructions. Section images were acquired with a microscope (Olympus U-TU1X-2, Japan) and analysed with the ImageJ software to determine cardiomyocyte diameter and myocardial fibrosis. Cardiomyocyte diameter was determined by the measurement of transnuclear widths of random, longitudinally oriented myocytes. About 130–150 myocytes per
animal were measured by an investigator blinded for the origin of the samples. Nine predefined fields of a \( \times 20 \) magnification were photographed and analysed. The number of counted myocytes per field varied between 15 and 25.

Myocardial fibrosis was quantified as the percentage of fibrosis compared with the total amount of tissue within an image. This percentage was assessed by determining the total tissue areas occupied by collagen and excluding the lumen (i.e. empty spaces), as well as excluding annotations of staining artefacts.

2.8 Titin isoform composition and phosphorylation

To determine titin isoform composition, all tissue samples were solubilized in 50 mM Tris–sodium dodecyl sulfate buffer (pH 6.8) containing 8 \( \mu \)g/ml of leupeptin (Peptin Institute, Japan) and phosphatase inhibitor cocktail (PIC [P2880], 10 \( \mu \)g/mL; Sigma). After heating and centrifugation, samples (20 \( \mu \)g, triplicate) were separated on agarose-strengthened 1.8% sodium dodecyl sulfate–polyacrylamide gels. The gel was run at 5 mA constant current and stained with SYPRO Ruby (Molecular Probes). Staining was visualized using the Fuji LAS-4000 Image Reader (460 nm/605 nm Ex/EEm; 2 s illumination), and signals were analysed with the Multi Gauge V3.2 and AIDA software.\(^{29}\) Mean values for each sample were calculated and the ‘mean of means’ was determined for all groups. Titin isoform composition is presented in relative values (N2B + N2BA = 100%). Titin isoform phosphorylation was determined using the same samples and was handled as for titin isoform composition. The gels were subsequently stained with Pro-Q Diamond and analysed as described above.

2.9 Force measurements in isolated cardiomyocytes

Force measurements were performed in single, mechanically isolated cardiomyocytes as described previously.\(^{29}\) Samples were incubated in a relaxing solution (free Mg\( ^{2+} \), KCl 100, EGTA 2, Mg-ATP 4, and imidazole 10 mM; pH 7.0), mechanically disrupted, and transfected in a similar solution supplemented with 0.2% Triton X-100 to remove all membrane structures. Single cardiomyocytes were subsequently attached with silicone adhesive between a force transducer and a piezoelectric motor. \( F_{\text{passive}} \) was measured at sarcomere lengths ranging from 1.8 to 2.4 \( \mu m \). To assess the reversibility of elevated \( F_{\text{passive}} \), myocytes were also incubated in relaxing solution supplemented with of protein kinase G-1 inhibitor (PKG1\( \alpha \), 0.1 U/mL; Sigma, batch034K1336). After 40 min of incubation with PKG1\( \alpha \), \( F_{\text{passive}} \) measurements were repeated. Force values were normalized for myocyte cross-sectional area.

2.10 Myocardial PKG activity

Myocardial PKG activity was assessed in homogenized tissue samples. Supernatants containing equal amounts of total protein were analysed for PKG activity. The reaction mixture containing 40 mMol/L of Tris–HCl (pH 7.4), 20 mMol/L of magnesium acetate, 0.2 mMol/L of \( ^{32} \)P[3P]ATP (500–1000 c.p.m./pmol), 113 mg/mL of heptapeptide (RRKRSRAE), 3 \( \mu \)mol/L of cyclic guanosine monophosphate (cGMP), and a highly specific inhibitor of cAMP-dependent protein kinase was incubated at 30°C for 10 min. Next, 70 \( \mu \)L of the reaction mix was spotted onto Whatman P-81 filters, which were then washed with 75 mMol/L of \( \text{H}_2\text{PO}_4 \) for 5 min and washed. To quantification of PKG activity, counts were taken in a Wallac 1409 Liquid Scintillation Counter. Specific activity of PKG was expressed as pmol of \( ^{32} \)P incorporated into the substrate (pmol/min/mg protein).

2.11 Myocardial cGMP concentration

Myocardial cGMP was determined in homogenates by use of the parameter cGMP assay immunoassay kit (R&D systems). Results of duplicate determinations were averaged and expressed as pmol/mL.\(^{39}\)

2.12 Quantification of serum atrial natriuretic peptide

Mouse serum samples were analysed with the Raybio human/mouse/rat atrial natriuretic peptide (ANP) EIA kit (Raybiotech, Inc.). Before analysis, samples were five-fold diluted in the supplied buffer. The readout was performed with a Tecan infinite M200 microtiterplate reader and concentrations were determined using a four-parameter logistic curve with the Graphpad prism software.

2.13 Data analysis and statistics

Data are expressed as means ± SEM. Data were tested for statistically significant differences using the Bonferroni-adjusted t-test. Statistical significance was defined as \( p < 0.05 \). All statistical analyses were done using the GraphPad Prism software (version 6.01).

3. Results

3.1 Lepr\(^{db/db}\) mice display reduced LV diastolic compliance

The phenotypic characteristics of 16-week-old Lepr\(^{db/db}\) and Lepr\(^{db/+}\) mice are listed in Table 1 and Figure 1, showing clinical, echocardiographic, histological, molecular, and invasive haemodynamic recordings. In summary, compared with Lepr\(^{db/+}\) mice and apart from increased body weight and increased fasting glycaemia, Lepr\(^{db/db}\) mice displayed no change in LV contractility [slope of the end-systolic pressure–volume relationship (slope ESPVR) and preload recruitable stroke work (PRSVW)], a reduced diastolic LV compliance [an increased slope of slope of the end-diastolic pressure–volume relationship (EDPVR); Figure 1A], and a reduced arterial elastance (Ea). In addition, Lepr\(^{db/db}\) mice displayed lower levels of myocardial cGMP, lower activity of myocardial PKG, and reduced levels of phosphorylated titin N2B isoform (Figure 1B).

Histological analyses of the LV wall could not reveal any change in the diameter of cardiomyocytes (Table 1) and in the expression of collagen type 1 or 3 (Figure 1C), suggesting no prominent cardiomyocyte hypertrophy or myocardial fibrosis in the diabetic hearts. Similarly, stainings with Trichrome Masson stains and mRNA expression of fibronectin and procollagen type 1 (data not shown) showed no difference between Lepr\(^{db/+}\) and Lepr\(^{db/db}\) mice. In contrast, Sirius red stains of the LV wall revealed increased interstitial myocardial LV fibrosis in Lepr\(^{db/db}\) mice (Figure 1C).

3.2 SITA improves LV diastolic compliance in Lepr\(^{db/db}\) mice

The effect of an 8-week SITA treatment on above recordings in Lepr\(^{db/db}\) mice is summarized in Table 2 and Figure 2. Importantly, SITA impaired >85% of serum DPP-4 activity, but it had no effect on fasting glycaemia or body weight. SITA also failed to change glycaemia of Lepr\(^{db/+}\) and Lepr\(^{db/db}\) mice. In contrast, Sirius red stains of the LV wall revealed increased interstitial myocardial LV fibrosis in Lepr\(^{db/db}\) mice (Figure 2D).
and PKG activity in LV tissue of Leprdb/db mice and increases cGMP

3.3 SITA reduces cardiomyocyte stiffness of Leprdb/db mice and increases cGMP and PKG activity in LV tissue

Above observations indicate that SITA improves LV diastolic compliance of Leprdb/db. In the absence of significant interstitial myocardial fibrosis, these improvements were unlikely explained by changes at the level of the extracellular matrix. We therefore hypothesized that SITA reduced cardiomyocyte stiffness of Leprdb/db mice. Figure 3A shows that, over a wide range of sarcomere lengths, \( F_{\text{passive}} \) of isolated skinned cardiomyocytes of SITA-treated Leprdb/db mice was significantly lower than \( F_{\text{passive}} \) of cardiomyocytes from untreated Leprdb/db mice. This difference was not explained by SITA-induced changes in titin isoform composition (Leprdb/db N2BA: 11.5 \( \pm \) 0.8%; N2B: 88.5 \( \pm \) 0.8% vs. SITA-treated Leprdb/db N2BA: 10.49 \( \pm \) 1.02; N2B: 89.5 \( \pm \) 1.0%).

However, as also shown in Figure 3A, the high \( F_{\text{passive}} \) of cardiomyocytes from untreated Leprdb/db mice fell to levels of SITA-treated mice after in vitro administration of PKG, whereas it remained unchanged for the samples from SITA-treated mice. This observation suggests that SITA treatment reduced cardiomyocyte stiffness through a cGMP–PKG–titin phosphorylation pathway. Consistent with this hypothesis, SITA significantly increased total levels of titin phosphorylation (Figure 3B), which resulted from elevated phosphorylation of the N2B isoform (Figure 3C). In addition, SITA significantly increased LV myocardial cGMP concentration and PKG activity in LV tissue homogenates (Figure 3D and E).

### 3.4 SITA does not increase serum ANP concentrations or LV levels of phosphorylated eNOS in Leprdb/db mice

In an attempt to explain LV myocardial cGMP concentrations after SITA treatment, we measured serum ANP levels and LV levels of phosphorylated eNOS in vehicle- and SITA-treated Leprdb/db mice. These experiments showed, however, that SITA did not increase serum ANP levels, remaining below 0.5 pg/mL in both vehicle and SITA-treated mice (data not shown), and that SITA did not increase LV myocardial levels of phosphorylated eNOS, despite significantly increased levels of phosphorylated Akt (Figure 3F). Also, SITA did not change mRNA expression of BNP in Leprdb/db mice, and did not change mRNA expression of inflammation-associated genes IL-6, tumour necrosis factor-\( \alpha \), and metalloproteinase-9 as assessed by quantitative RT-PCR (data not shown).

### 4. Discussion

An 8-week pharmacological DPP-4 inhibition with SITA decreased LV stiffness in a mouse model of Type 2 DM-induced LV dysfunction. This resulted in improved LV stroke volume and cardiac output. Importantly, these effects emerged in the absence of significant effects on serum glycaemia. Stimulatory effects of DPP-4 inhibition on myocardial cGMP, which is known to prevent myocardial hypertrophy and reduce cardiomyocyte stiffness,\(^ {31,32} \) most likely underlie these effects. Consistently, DPP-4 inhibition increased myocardial PKG activity and induced phosphorylation of N2B titin in this study. Of note, DPP-4 inhibition also reduced arterial stiffness in the diabetic mice.

These observations add further evidence to the molecular understanding of cardiac mechanotransduction, and underscore its potential for translation into heart failure treatment. Titin, a protein anchored to the sarcomere Z-line that serves as a major determinant of myocardial passive tension and stiffness, can be modulated via phosphorylation. Although the ability of cAMP-dependent protein kinase A to phosphorylate titin had been well known, it was more recently discovered that the cGMP-dependent enzyme PKG can mediate similar phosphorylation in both dog and human hearts.\(^ {32,33} \) As confirmed in this study, this
posttranslational modification reduces titin-based passive tension and thus may represent a promising therapeutic target for reducing myocardial stiffness. The observation in this study of the in vitro reversibility of high $F_r$ of cardiomyocytes of Lepr$^{db/db}$ mice by in vitro administration of PKG to the isolated cardiomyocytes (Figure 3A) indicates that the high $F_r$ of cardiomyocytes of Lepr$^{db/db}$ mice results more from altered phosphorylation status than from structural changes of titin, such as isoforms shift or oxidative damage, and that increasing myocardial cGMP-dependent PKG activity, as here induced by SITA, may suffice to correct for increased cardiomyocyte stiffness in these mice.

Above observations are in agreement with recent studies in Type 2 DM human patients with heart failure. According to these studies, increased cardiomyocyte resting tension is a determinant of DM-induced LV dysfunction, especially when LV ejection is preserved. In fact, within the population of patients with heart failure and preserved LV fraction (HFPEF), increased cardiomyocyte resting tension due to low myocardial cGMP, low PKG activity, and hypophosphorylation of titin appears a common theme. Attempts to counteract this defect by interventions to increase myocardial cGMP, such as treatment with sildenafil and BNP, are ongoing but have not been successful in HFPEF patients yet. Recently, sildenafil, a PDE5 inhibitor which reduces the breakdown of cGMP, failed to improve LV diastolic function and exercise capacity of HFPEF patients in the RELAX trial, suggesting that boosting cGMP production may be the necessary way to go.

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/104/3/423/2930917)
Our observations, showing that myocardial cGMP levels increased three-fold by SITA treatment, suggest that pharmacological DPP-4 inhibition may fulfill this objective.

Table 2: Clinical, echocardiographic, and invasive haemodynamic cardiac characteristics in vehicle-treated Lepr^db/db mice and SITA-treated Lepr^db/db mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lepr^db/db + vehicle</th>
<th>Lepr^db/db + SITA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW (g)</td>
<td>44 ± 2</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>HW/BW (n = 5/group)</td>
<td>2.7 ± 0.09</td>
<td>2.5 ± 0.06</td>
</tr>
<tr>
<td>HW/TTL (mg/cm)</td>
<td>713 ± 1.3</td>
<td>688 ± 2.3</td>
</tr>
<tr>
<td>(n = 5/group)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LW/TTL (mg/cm)</td>
<td>100.8 ± 4.7</td>
<td>76.79 ± 6.9***</td>
</tr>
<tr>
<td>(n = 5/group)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dL)</td>
<td>428 ± 31</td>
<td>461 ± 35</td>
</tr>
<tr>
<td>Cardiomyocyte diameter (µm)</td>
<td>15.1 ± 0.6</td>
<td>15.7 ± 0.3</td>
</tr>
<tr>
<td>DPP-4 serum activity (U/L)</td>
<td>6.6 ± 0.2</td>
<td>± 0.7***</td>
</tr>
<tr>
<td>Echo parameters (n = 17/group)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>519 ± 32</td>
<td>530 ± 21</td>
</tr>
<tr>
<td>AVT (mm)</td>
<td>1.03 ± 0.20</td>
<td>1.07 ± 0.13</td>
</tr>
<tr>
<td>PWV (mm)</td>
<td>1.10 ± 0.04</td>
<td>1.00 ± 0.15</td>
</tr>
<tr>
<td>EDD (mm)</td>
<td>3.14 ± 0.62</td>
<td>3.29 ± 0.23</td>
</tr>
<tr>
<td>Calculated LV mass (mg)</td>
<td>136 ± 46</td>
<td>126 ± 27</td>
</tr>
<tr>
<td>Invasive parameters</td>
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<td></td>
</tr>
<tr>
<td>Steady state</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>426 ± 11</td>
<td>458 ± 12</td>
</tr>
<tr>
<td>V_e (µL)</td>
<td>10.3 ± 2.9</td>
<td>7.1 ± 1.3</td>
</tr>
<tr>
<td>V_ed (µL)</td>
<td>17.3 ± 2.6</td>
<td>20.9 ± 2.4</td>
</tr>
<tr>
<td>P_sa (mmHg)</td>
<td>795 ± 52</td>
<td>831 ± 4.7</td>
</tr>
<tr>
<td>P_ee (mmHg)</td>
<td>3.9 ± 0.8</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>SV (µL)</td>
<td>7.9 ± 1.3</td>
<td>15.2 ± 1.6**</td>
</tr>
<tr>
<td>EF (%)</td>
<td>51.4 ± 9.4</td>
<td>71.3 ± 4.3*</td>
</tr>
<tr>
<td>CO (µL/min)</td>
<td>3419 ± 597</td>
<td>6964 ± 694***</td>
</tr>
<tr>
<td>SW (mmHg/µL)</td>
<td>547 ± 91</td>
<td>1169 ± 95***</td>
</tr>
<tr>
<td>Es (mmHg/µL)</td>
<td>129 ± 25</td>
<td>65 ± 1.2*</td>
</tr>
<tr>
<td>dP/dt_max (mmHg/s)</td>
<td>8193 ± 914</td>
<td>10540 ± 784*</td>
</tr>
<tr>
<td>dP/dt_min (mmHg/s)</td>
<td>-2629 ± 750</td>
<td>-7455 ± 750</td>
</tr>
<tr>
<td>Tau, Glantz (ms)</td>
<td>145 ± 1.4</td>
<td>± 0.8*</td>
</tr>
<tr>
<td>After preload reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope ESPVR (mmHg/µL)</td>
<td>13.75 ± 2.31</td>
<td>8.74 ± 0.93</td>
</tr>
<tr>
<td>PRSW (mmHg)</td>
<td>780 ± 9.2</td>
<td>82.9 ± 7.9</td>
</tr>
<tr>
<td>EDPRV (mmHg/µL)</td>
<td>0.53 ± 0.11</td>
<td>0.30 ± 0.03*</td>
</tr>
<tr>
<td>EDPRV-β (µL−1)</td>
<td>0.23 ± 0.05</td>
<td>0.12 ± 0.01*</td>
</tr>
</tbody>
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n = 12/group, unless otherwise indicated.
BW, body weight; HW/BW, heart weight/body weight; LW/TTL, lung weight to tibia length ratio; HR, heart rate; AVT, anterior wall thickness; PWV, posterior wall thickness; EDD, end diastolic diameter; V_ed, end systolic volume; V_sa, end systolic volume; P_ee, end systolic pressure; P_sa, end systolic pressure; SV, stroke volume; EF, ejection fraction; CO, cardiac output; SW, stroke work; Es, arterial elastance; slope ESPVR, slope of the end-systolic pressure–volume relationship; PRSW, preload recruitable stroke work; slope EDPRVR, slope of the end-diastolic pressure–volume relationship.

Our data showed that SITA treatment decreased LV stiffness and improved stroke volume and LV ejection fraction, but did not reduce LV end-diastolic pressure during open chest haemodynamic evaluation. In closed chest recordings during echocardiography, SITA failed to increase LV diameters and LV FS. The most likely explanation for these findings is the prompt fall in LV filling pressures during thoracotomy and anaesthesia, which reduced venous return to the heart, thereby making the LV operating at the flat portion of the diastolic pressure–volume relationship. Under these conditions, improved diastolic LV compliance manifests itself more by an increase in end-diastolic volume—which allows for the observed increase in stroke volume and ejection fraction—than by a fall in end-diastolic pressure. In closed chest conditions, the opposite occurs, explaining why FS during echocardiography did not change.

How did DPP-4 inhibition increase myocardial cGMP content? Since DPP-4 is a pleiotropic enzyme which truncates a large number of cardioactive peptides that may increase cGMP (either through natriuretic peptide receptors, or through ligand-induced eNOS activation), several mechanisms should be considered. First, DPP-4 inhibition prevents the breakdown of GLP-1, which may increase the serum levels of ANP through activation of the GLP-1 receptor in cardiac atria, and hence indirectly increase myocardial cGMP level. We could not demonstrate, however, any increase in serum ANP concentrations during DPP-4 inhibition with SITA. Secondly, DPP-4 inhibition has been shown to prevent the truncation of BNP(3–32) to the less bioactive BNP(3–28). This event potentially may increase myocardial and vascular cGMP. However, opposite to humans, rodent BNP(1–45) lacks a proline in the N-terminal penultimate position, which means that rodent BNP(1–45) is not a substrate for DPP-4. Thus, this pathway can be excluded in mice. Thirdly, DPP-4 inhibition has been shown to prevent the cleavage of several cardioactive peptides that may activate eNOS in cardiac cells upon receptor activation. These peptides include SDF-1α, bradykinin, and substance P. Given our observation that levels of phosphorylated eNOS were not increased in LV samples of SITA-treated Lepr^db/db mice (despite increased levels of phosphorylated Akt), peptides inducing a calcium-dependent activation of eNOS (such as bradykinin and substance P) are the most likely candidates.

In this study, 16-week-old Lepr^db/db mice on a C57BLKSJ background displayed a normal or slightly reduced global LV contractility and a reduced LV diastolic compliance. It is known that the cardiac phenotype of Lepr^db/db mice is highly dependent on age and genetic background, which leads to variable cardiac profiles of Lepr^db/db mice in the literature. In this study, we also observed conflicting results in the presence of myocardial fibrosis depending on which method was applied. In particular, consistent with previous reports, but in contrast with immunohistochemical stainings for collagen types I and III, Trichrome Masson’s stains, as well as molecular analyses for procollagen type I and fibronectin, the more sensitive method of Sirius red staining revealed increased interstitial fibrosis in Lepr^db/db mice. SITA attenuated this signal. Therefore, we cannot exclude that myocardial interstitial fibrosis contributes to reduced LV compliance in Lepr^db/db mice, and that effects of SITA on LV compliance may encompass some effects on the myocardial interstitium.

Nevertheless, the phenotype characterized in this study reinforces the emerging concept that LV passive stiffening can occur in conditions of rather modest LV fibrosis. In obesity and Type 2 diabetes, this phenotype is most likely caused by a complex cascade involving risk factor-associated endothelial dysfunction, reduced PKG activity, and titin hypophosphorylation.
Previous studies have addressed the effect of DPP-4 inhibition on cardiac function in various heart failure models and models of diabetes.27,45–47 Lenski et al.46 showed that a 4-week pharmacological DPP-4 inhibition with SITA in Leprdb/db had beneficial effects on myocardial metabolism, including the uptake and oxidation of fatty acids. In contrast to our data, pressure–volume diagrams were not affected by DPP-4 inhibition. The use of younger animals, a shorter treatment period, and pressure–volume recording in excised heart Langedorff preparations most likely explain the discrepancy of this study with our results. Shigeta et al.27 investigated the cardiac effects of DPP-4 inhibition in streptozotocin-induced Type 1 diabetic rats. Consistent with our results, DPP-4 inhibition prevented diabetes-induced LV diastolic dysfunction. However, in this study, prevention of myocardial interstitial fibrosis and SDF-1α-mediated myocardial angiogenesis were the proposed mechanisms of actions.27 Effects of DPP-4 inhibition on cGMP and cardiomyocyte stiffness were not analysed. Similarly, Aroor et al.47 showed that DPP-4 inhibition improved LV diastolic function in Zucker obese rats, but again, effects on cardiomyocyte stiffness or titin were not studied.

In conclusion, in obese Type 2 DM mice, DPP-4 inhibition by SITA improves LV compliance and even global LV performance without affecting glycaemia. These effects seem at least partially mediated by stimulatory effects of SITA on the myocardial cGMP–PKG pathway.

Figure 2 SITA improves LV diastolic compliance in Leprdb/db mice. (A) Representative pressure–volume loops of vehicle-treated and SITA-treated Leprdb/db mice. (B) Bar graphs showing mean values of stroke volume (SV), cardiac output (CO), slope of end-systolic pressure–volume relation (slope ESPVR), and slope of end-diastolic pressure–volume relation (slope EDPVR) in both groups (n = 12/group). (C) Mean peritoneal glucose tolerance test (pGTT) in 8-week vehicle-treated and SITA-treated Leprdb/db mice (n = 5/group). (D) Representative Sirius red-stained LV sections. Scale bar = 100 μm. *P < 0.05, **P < 0.01.
Figure 3  SITA reduces cardiomyocyte stiffness of Lepr<sup>db/db</sup> mice and increases cGMP and PKG activity in LV tissue. (A) Cardiomyocyte $F_{\text{passive}}$ and effect of in vitro incubation with PKG. $F_{\text{passive}}$ at sarcomere length 1.8–2.4 μm recorded on isolated myocytes in non-activating buffer (solid curves) or following incubation with PKG (dotted curves); (n = 5 mice/group, with 4–6 cardiomyocytes/mice) *<i>p</i> < 0.05 vehicle vs. vehicle after PKG, **<i>p</i> < 0.05 vehicle vs. SITA. (B) Total titin phosphorylation (n = 5/group). (C) Titin isoform phosphorylation (n = 5/group). (D) LV cGMP concentration (n = 5/group). (E) LV PKG enzymatic activity (n = 5/group). (F) Relative eNos (left) and Akt (right) expression. Blots are representative of three experiments. *<i>p</i> < 0.01, **<i>p</i> < 0.005.
and, hence, on the phosphorylation status of titin and the heretofore coupled cardiomyocyte stiffness modulus. This mode of action of SITA may open new avenues for the treatment of LV diastolic dysfunction in diabetes.

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