Dipeptidylpeptidase-4 Modulates Left-Ventricular Dysfunction in Chronic Heart Failure via Angiogenesis-Dependent and -Independent Actions

Running title: Shigeta et al.; Role of DPP4 in chronic heart failure

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Abstract:

**Background**— The inhibition of dipeptidylpeptidase-4 (DPP4) protects the heart from acute myocardial ischemia. However, the role of DPP4 in chronic heart failure independent of coronary artery disease remains unclear.

**Methods and Results**— We first localized the membrane-bound form of DPP4 (m-DPP4) to the capillary endothelia of rat and human heart tissue. Diabetes promoted m-DPP4 activation, leading to reduced myocardial stromal cell-derived factor-1α (SDF-1α) concentrations and resultant angiogenic impairment in rats. The diabetic rats exhibited diastolic left ventricular dysfunction (DHF) with enhanced interstitial fibrosis partly due to the increased matrix metalloproteinase 2 (MMP2)/tissue inhibitor of metalloproteinases 2 (TIMP2) ratios in a DPP4-dependent fashion. Both genetic and pharmacological DPP4 suppression reversed the SDF-1α-dependent microvasculopathy and DHF associated with diabetes. Pressure overload induced DHF which was reversed by DPP4 inhibition via a GLP-1/cyclic AMP-dependent distinct mechanism from that for diabetic heart. In patients with DHF, the circulating DPP4 (s-DPP4) activity in peripheral veins (PV) was associated with that in coronary sinus (CS) and with E/e’, an echocardiographic parameter representing DHF. Comorbid diabetes increased the s-DPP4 activities both in PV and CS.

**Conclusions**—DPP4 inhibition reverses DHF via m-DPP4/SDF-1α-dependent local actions on angiogenesis and s-DPP4/GLP-1-mediated inotropic actions. Myocardium-derived DPP4 activity in CS can be monitored using PV sampling which partly correlates with DHF index; thus, s-DPP4 may potentially serve as a biomarker for monitoring DHF.

**Key words**: diabetes mellitus; heart failure; microcirculation; GLP-1; dipeptidyl peptidase-4
Introduction

Dipeptidylpeptidase-4 (DPP4), also known as cell-surface antigen CD26, is a 110-kDa type-II integral membrane glycoprotein that exhibits protease activity and belongs to the prolyl oligopeptidase family. A primary function of DPP4 is to truncate various bioactive molecules such as stromal cell-derived factor-1α (SDF-1α) and glucagon-like peptide-1 (GLP-1), and there are several reports suggesting that DPP4 represents a subfamily of gelatinolytic serine proteases that selectively bind to denatured collagen; hence, DPP4 modulates pathological conditions such as diabetes, malignancy, and inflammation. DPP4 is widely distributed in mammalian tissues, including kidney, small intestine, liver, and heart tissues. A soluble form of DPP4 (s-DPP4), present in the circulatory system and body fluids, is thought to result from the proteolytic cleavage of the membrane-bound form (m-DPP4). The results of an early study using colorimetric enzyme histochemistry suggested that the DPP4 protease activity is localized in the venous capillary vessels of the heart; however, investigations of the precise distribution of DPP4 in the heart and the immunohistochemical identification of the cell types involved have not been undertaken.

In recent preclinical studies, pleiotropic effects of DPP4 inhibition were demonstrated to reduce mortality and cardiac remodeling after myocardial infarction; however, whether the pathophysiological role of DPP4 in chronic heart failure is independent of coronary artery
disease remains unclear. Diastolic left-ventricular (LV) dysfunction (DHF) is a feature of heart failure that is characterized by pathological cardiac remodeling such as LV hypertrophy (LVH) and fibrosis induced by comorbidity such as pressure overload or diabetes\textsuperscript{9,10}. In addition, a role has been postulated for chronic ischemia, which gradually develops in response to a mismatch between the coronary vasculature and cardiomyocyte growth associated with pathological remodeling\textsuperscript{11-13}; however, the existence of a link between chronic ischemia and the pathogenesis of DHF remains uncertain.

This study aimed to clarify the role of DPP4 in cardiac remodeling and DHF using distinct rodent models of induced diabetes or pressure overload. Additionally, we examined whether peripheral venous DPP4 activity may correlate with several factors related to DHF with the goal of uncovering a potential role for DPP4 as a diagnostic or therapeutic target for DHF in humans.

Methods

A detailed description of the methods is provided in the Supplemental Methods.

Animals. Male congenital DPP4-null Fischer 344 rats, their syngeneic counterparts and Wistar rats were used in this study. DM was induced by streptozotocin and pressure overload was generated by transaortic constriction (TAC).
Cardiac Function Analyses. Cardiac function in rodents was analyzed using an echocardiogram and cardiac catheterization.

In Situ Cardiac DPP4 Activity. Cardiac DPP4 proteolytic activities of rat and human heart specimens were detected by in situ colorimetry using Gly-Pro-NA as the substrate.

In Vitro Cardiac and Plasma DPP4 Activity. The DPP4 activity in each heart tissue extract and in plasma was measured using the DPP4-Glo assay™ (Promega).

Patient Selection. DHF was diagnosed using Doppler echocardiography based on elevated values for the ratio of early mitral inflow (Ev) to mitral annular velocity (E/e′) and the deceleration time of the Ev (Dct) with concomitant plasma brain natriuretic peptide (BNP) elevation. The circulating s-DPP4 activities of these patients were measured using blood samples from peripheral veins, the coronary sinus, and the aortic root.

Statistical Analysis. Values are expressed as the mean ± SD. The homogeneity of data variance was assessed using the F-test (2 groups) and the Bartlett test (multiple groups). The mean values of 2 groups were compared using the Student’s t test (when the variance was homogenous) or Welch’s t test (for heterogeneous variance). Comparisons of multiple means were performed using ANOVA (for homogenous variance) or Wilcoxon rank-sum test (for heterogeneous variance) if indicated. All group comparisons were followed by Bonferroni’s post-hoc test. Pearson correlation coefficients were estimated for the association between DPP4 activity and...
other variables in humans. Values of $P<0.05$ were considered to be statistically significant.

Results

The Capillary Endothelium Expresses DPP4 in the Heart.

Previous reports suggested that the DPP4-active regions localizes in capillary vessels using a colorimetric procedure$^{3,6,14}$. We sought to identify the subtype of DPP4-expressing cells in heart using immunohistochemistry. DPP4-positive sites overlapped with sites expressing the panendothelial marker PECAM/CD31 (Figure 1A). Immunoblot analysis revealed the DPP4 expression in both cultured venous (HUVEC) and microvascular (HMVEC) endothelial cells (Figure 1B).

We next performed in situ colorimetric staining to assess m-DPP4 activity in heart sections of Fischer rats (left panel, Figure 1C), their DPP4-null counterparts (right panel, Figure 1C), and humans (Figure 1D). DPP4-positive regions were colored in red in both rat and human heart specimens, and the pattern of the DPP4 positivity was consistent with the results obtained by immunohistochemistry (Figure 1A). We validated the specificity of the in situ DPP4 staining using an in vitro assay to detect the proteolytic activity of DPP4 in rat heart extracts (Figure 1F) and plasma specimens (Figure 1G).
Cardiac m-DPP4 Activity is Elevated in the Diabetic Heart.

Diabetes exacerbates heart failure\textsuperscript{15} and is associated with DHF\textsuperscript{9} and coronary microangiopathy\textsuperscript{16,17}. We investigated whether diabetes influences cardiac m-DPP4 activity (Figure 2A and B). Diabetic DP(+) rat [DP(+)/DM] hearts exhibited enhanced m-DPP4 activity in situ (Figure 2A); this trend was confirmed in vitro (Figure 2B). We next examined the role of m-DPP4 in cardiac function by cardiac catheterization (Figure 2C-2F) and echocardiography (Table 1). DP(+)/DM rats exhibited decreased dp/dt\textsubscript{min} (Figure 2D) and increased Tau (Figure 2E) and diastolic stiffness (Figure 2F), findings consistent with the prolonged Dct and reduced E/A value measured by echocardiography (Table 1). Immunohistochemistry (Figures 2G-2J) revealed that the cardiomyocyte surface area (CSA, Figure 2H) and vascularity (Figures 2I and 2J) were reduced in the DP(+)/DM rats. In contrast, diabetes had no influence on either cardiac function or histological remodeling in DP(−) rats. We further examined whether the reduced vascularity in DP(+)/DM rats promoted myocardial hypoxia using pimonidazole (Figure 2K). Diabetes increased pimonidazole-positive green spots in the myocardium (right upper panel), which were absent in DP(−)/DM rats (right lower panel). As an additional measure of hypoxia, HIF-1\textalpha \ expression (Figures 2L and 2M) was consistently higher in DP(+)/DM rats than in their nondiabetic counterparts [DP(+)/c]; however, this trend was absent in the DPP4-null groups [DP(−)/DM and DP(−)/c].
Elevated Cardiac DPP4 Activity Causes Impaired Angiogenesis and Fibrosis in Diabetes.

DPP4 truncates SDF-1α\(^2\).\(^7\), inactivating its angiogenic potential\(^18\).\(^19\). Accordingly, we hypothesized that the cardiac m-DPP4 activation observed in the DP(+)DM rats may suppress angiogenesis through the reduction of SDF-1α. Because electrophoresis (Tricine-SDS-PAGE) cannot separate the DPP4-cleaved biologically inactive form of SDF-1α protein from intact SDF-1α\(^7\).\(^20\), we performed preliminary experiments (Supplemental Figure 1)\(^21\) and found ELISA to be superior for the detection of biologically active SDF-1α. The cardiac SDF-1α levels were reduced in DP(+)DM rats compared with DP(+)c rats, whereas these levels remained unchanged in DP(−)/DM rats (Figure 3A). The cardiac concentration of vascular endothelial growth factor (VEGF)\(^16\).\(^17\) was reduced independently of DPP4 deficiency (Figure 3B).

SDF-1α activates Akt/eNOS signaling, which is essential for angiogenesis mediated by the trafficking of endothelial progenitor cells (EPCs)\(^22\)-\(^24\). We examined whether the cardiac SDF-1α gradient is related to Akt/eNOS signaling (Figure 3C and Supplemental Figure 2) and EPC homing (Figures 3D and Supplemental Figures 3 and 4). DP(+)DM rats exhibited decreased Akt/eNOS phosphorylation levels and CXCR4- and KDR-double-positive (CXCR4\(^+\)/KDR\(^+\)) cell numbers, a trend that was reversed in DP(−)/DM rats. Cardiac CXCR4 expression levels were unaffected by DPP4 deficiency or diabetes (Supplemental Figure 4).
DPP4 Inhibition Reverses Diabetes-induced Cardiac Fibrosis via the MMP2/TIMP2 Axis.

We examined the impact of DPP4 on cardiac remodeling (Figures 3E-3H). DP(+)/DM rats exhibited increased interstitial fibrosis of the heart, as detected by picro-Sirius Red staining, which was reversed in DP(-)/DM rats (Figures 3E and 3F). Real-time PCR analysis revealed a DPP4-dependent increase in the accumulation of collagen (collagen types 1 and 3), matrix metalloproteinase 2 (MMP2) with a concomitant decline in the level of tissue inhibitor of metalloproteinases 2 (TIMP2) and an elevation of connective tissue growth factor (CTGF, Supplemental Figure 5) in DP(+)/DM rats. The level of Beta-myosin heavy chain (β-MHC) of DP(+)/DM rats was reduced (Figure 3H), which was partially but significantly reversed in DP(-)/DM counterpart. MMP9, TIMP1, BNP, and ANP levels were elevated (Figures 3G and 3H) in DP(+)/DM rats independently of DPP4 activity.

The Pharmacological Inhibition of DPP4 Reverses Diabetic DHF.

Small-molecule inhibitors of DPP4 are widely used to treat diabetic patients. We examined whether vildagliptin could ameliorate diabetes-induced cardiac remodeling and DHF. We validated the inhibitory effect of vildagliptin on circulating DPP4 activity with an in vitro assay (Supplemental Figure 6). Vildagliptin ameliorated the diabetes-induced DHF without affecting systolic LV function (Figures 4A and 4B and Table 2) or the declines in CSA and vascularity induced by diabetes (Figures 4C and 4D). Diabetes suppressed cardiac SDF-1α levels (Figure
4E) and Akt/eNOS phosphorylation levels (Figure 4F and Supplemental Figure 7) with a concomitant increase in the level of HIF-1α (Supplemental Figure 8), and the MMP2/TIMP2 ratio (Figure 4G) with increased CTGF levels (Supplemental Figure 9); these changes were reversed in response to vildagliptin treatment.

**DPP4 Inhibition Protects the Heart from Nondiabetic Stress Independently of SDF-1α-mediated Angiogenesis.**

Pressure overload is nondiabetic intervention contributing to the development of DHF. We evaluated the effect of genetic DPP4 inhibition on DHF and cardiac remodeling induced by TAC (Figures 5 and 6 and Table 3). Echocardiograms revealed DHF with LVH and a mild systolic dysfunction in DP(+)/TAC rats. The DP(−)/TAC group exhibited LVH, but their cardiac function remained intact (Table 3). Both DP(+)/TAC and DP(−)/TAC rats exhibited increases in CSA (Figure 5B) without reduced angiogenesis (Figure 5C), leading to a proportional decrease in vascular density (Figure 5D). Consistently, Akt/mTOR phosphorylation levels were elevated in the TAC groups independently of DPP4 activity (Supplemental Figure 10). Pimonidazole staining and HIF1α expression levels indicated that TAC induced cardiac ischemia independently of DPP4 activity (Figure 5E-5G). Angiogenic chemokines, Akt/eNOS phosphorylation levels (Figures 5H-5J and Supplemental Figure 11), and CXCR4+/KDR+ EPC numbers (Supplemental Figure 12) were increased in both DP(+)/TAC and DP(−)/TAC rats.
Cardiac fibrosis (Figures 5K, 5L, and Supplemental Figure 13) and hypertrophic signaling (Figure 5M and Supplemental Figure 10) were DPP4-independently increased in response to TAC.

**DPP4 Inhibition Reverses Pressure-overload-induced LV Dysfunction through the GLP-1/Cyclic AMP Axis.**

Cardiac m-DPP4 activities (Figures 6A and 6B) were reduced in DP(+)/TAC rats both in situ and in vitro. We then hypothesized whether local hypoxia induced by TAC may modulate endothelial DPP4 expression. Hypoxic treatment reduced the levels of DPP4 of cultured endothelial cells (Figure 6C). Glucagon-like peptide-1 (GLP-1) is an incretin\(^{27,28}\) that is cleaved by s-DPP4\(^2\) and exhibits inotropic effects\(^8,29-31\). We next hypothesized that the s-DPP4 activity may increase in response to pressure overload and the resultant GLP-1 may contribute to the DPP4-mediated and SDF-1\(\alpha\)-independent amelioration of cardiac dysfunction in the TAC rats. The s-DPP4 activity was increased in DP(+)/TAC rats (Figure 6D) and their GLP-1 levels were decreased and were reversed in DP(−)/TAC rats (Figure 6E). Because GLP-1 activity is mediated by cyclic AMP (cAMP)\(^{27,31}\), we measured cardiac cAMP levels in each group (Figure 6F). Cardiac cAMP concentrations were reduced in DP(+)/TAC rats. We observed no changes in the cardiac cAMP concentrations in diabetic rats (Supplemental Figure 14). We then tested the impact of GLP-1 receptor activation on the pressure-overload-induced cardiac dysfunction by administering the
DPP4-resistant agonist exendin-4 using mouse TAC models (Supplemental Table 1). Exendin-4 improved diastolic and systolic LV function in TAC mice.

**The Peripheral Venous DPP4 Activity of DHF Patients Correlates with E/e’ and Increases with Comorbid Diabetes.**

The coronary sinus (CS) plasma is used to assess the myocardial release of bioactive molecules. We assumed that the CS plasma would be the best specimen in which to measure myocardium-derived s-DPP4 activity in human patients (Figure 7). Considering the ethical limitations on the repeated collection of blood specimens by invasive cardiac catheterization, we evaluated whether s-DPP4 activity in the peripheral veins (PV) is correlated with that in the CS (Figure 7A). We found that s-DPP4 activity in the CS was positively associated with that observed in PV. In contrast, the s-DPP4 activity in aortic arterial blood showed no correlation with the PV specimens (Figure 7B). The s-DPP4 activity of PV was positively correlated with E/e’ (Figure 7C and Tables 4 and 5). Comorbid diabetes increased s-DPP4 activity in both the PV (Figure 7D and 7E) and the CS (Figure 7E).

**Discussion**

Studies investigating DPP4 are very common in the fields of hematology, oncology, and diabetology, however, little is known about the pathophysiological significance of DPP4 in
chronic heart failure. Our study presents experimental evidence illuminating the regulatory roles of DPP4 in chronic heart failure as mediated by its distinct forms and substrates, i.e., m-DPP4 for SDF-1α, and presumably, the MMP2/TIMP2 axis and s-DPP4 for GLP-1, which we validated in distinct rodent models (Figure 8). Local and systemic stresses presumably alter DPP4 activity via unknown mechanisms. In the present study, consistent with a previous report, diabetes promoted the activation of m-DPP4, thereby reducing cardiac SDF-1α concentrations (Figure 3A) and causing impaired angiogenesis, presumably via a reduction of CXCR4+/KDR+ EPC homing to the heart (Figure 3D and Supplemental Figure 3). In contrast, cardiac m-DPP4 activity of TAC was reduced (Figures 6A and 6B) with elevated cardiac SDF-1α levels (Figure 5H), suggesting that cardiac m-DPP4 activity may be a key determinant of cardiac angiogenesis by modulating the SDF-1α gradient in response to both diabetic and non-diabetic pathologies.

In contrast to that observed in diabetes, m-DPP4 activity was decreased in the pressure-overloaded heart (Figures 6A and 6B), presumably due to hypoxia-mediated decrease in the level of endothelial m-DPP4 protein (Figure 6C), which was unchanged by high glucose treatment (data not shown). One possible explanation for this reduction in m-DPP4 has been suggested by Lamers’s observation demonstrating the potential existence of regulatory system(s) for m-DPP4 release in response to pathological stimuli. In case of pressure overload, concomitant hypoxia may accelerate m-DPP4 release (in other words, the cleavage of m-DPP4)
from the capillary endothelium of the heart. In addition, our data also suggest that this regulatory system(s) may be impaired in diabetes, leading to the resultant DPP4 activation and microvasculopathy.

STZ-induced diabetes has been associated with myocardial atrophy and increased interstitial fibrosis. In addition, ample evidence indicates that STZ rat hearts exhibit diastolic abnormalities; both impaired relaxation and increased stiffness occur in the absence of hypertrophy and precede by weeks the onset of systolic dysfunction. However, the precise mechanism of the diabetes-induced maladaptive collagen metabolism remains unclear. We examined the impact of DPP4 on cardiac fibrosis in both diabetic (Figure 3E-3G, Figure 4F, and Supplemental Figure 5) and pressure-overload models (Figures 5K and 5L, and Supplemental Figure 9). The diabetic heart exhibited an increase in interstitial fibrosis, which was reversed in the DPP4-null group (Figure 3E and 3F). We next evaluated changes in collagen accumulation (types 1 and 3), collagen breakdown promoters (MMP2 and MMP9) and collagen breakdown inhibitors (TIMP1 and TIMP2). There are various regulators for collagen metabolism in pathological conditions (extensively reviewed elsewhere). In particular, clinical and experimental studies have reported the increased expression of MMP2 in diabetic subjects. MMP2 plays a primary role in the development of diabetic microvasculopathy (retinopathy) by enhancing MMP2 with a corresponding decrease in TIMP 2 in endothelial cells. Cardiac
MMP2-transgenic mice display increased collagen accumulation and exhibit biventricular
dilation and disruption of the myocardium\(^\text{16}\). In the present study, the trend of increasing MMP2
levels in the STZ rat heart was consonant with the atrophic changes in cardiomyocytes; in
contrast, we observed a decrease in MMP2 expression and a corresponding TIMP2 increase in
the TAC heart (Figure 5L). Our finding based on TAC models also supports previous reports that
TIMP2 is upregulated in patients with pressure-overload cardiomyopathy\(^\text{17,18}\). With regard to
angiogenesis, MMP2 is known as a protease that degrades SDF1\(\alpha\)\(^\text{7}\); thus, the trend of MMP2
expression in both diabetic and TAC models is consistent with our results regarding cardiac
SDF1\(\alpha\) contents (Figure 3A and Figure 5H) and capillary density (Figure 2I and Figure 5C).
DPP4 is a weak endopeptidase for denatured collagen\(^\text{4,5}\), preferentially degrading collagens I and
III. Although further evaluation is required, the DPP4 dependency of the MMP2/TIMP2 axis
observed in the diabetic heart may constitute a link to collagen metabolism mediated by DPP4 as
an “endopeptidase”. Taken together, our results and the results of previous studies suggest that
the MMP2/TIMP2 ratio should increase in a DPP4-dependent manner when cardiac remodeling
tends toward atrophy, i.e., under diabetic stress without insulin stimulus.

GLP-1 is an incretin\(^\text{27,28}\) that is easily cleaved by s-DPP4 in circulation\(^\text{2}\). GLP-1 exhibits
positive-inotropic effects via the endocrine system and the exogenous administration of its
receptor agonists (e.g., exendin-4) ameliorates heart failure in humans\(^\text{29}\) and in animals\(^\text{8,30,31}\).
DPP-4 is a principal determinant of the circulating level of bioactive GLP-1\textsuperscript{35,38}, suggesting that the balance between s-DPP4 and GLP-1 may affect LV contractility (Figure 8). In addition to intact GLP-1, the DPP-4-cleaved form, GLP-1(9-36)amide, exerts cardioprotective effects in rodents through mechanisms distinct from those of intact GLP-1\textsuperscript{31}. In the present study, we did not explore the difference between these cardioprotective molecules, and further investigation will be necessary to address this question.

In conclusion, the present study highlights a novel pathophysiological role for DPP4 in chronic heart failure and raises the possibility of using DPP4 as a diagnostic surrogate and/or a therapeutic target for chronic heart failure.

Acknowledgments: The authors wish to acknowledge Mr. Seiichi Kotoda for technical assistance with the hemodynamic studies. We thank Ms. Ikuyo Mizuguchi and Mr. Yoshikazu Fujita at the Division of Medical Research Engineering, Nagoya University Graduate School of Medicine, for their technical support. Vildagliptin was a generous gift from Novartis Inc.

Funding Sources: This work was supported in part by a Grant-in-Aid for Scientific Research No. 20249045 (to TM), No. 23390208 (to TM), No. 23591080 (to YKB), and No. 21790716 (to AH) from the Ministry of Education, Culture, Sports, Science, and Technology Japan and Suzuken Memorial Foundation (to YKB).

Conflict of Interest Disclosures: None.

References:


Table 1. Impact of DPP4 activity on cardiac functions in diabetic rats.

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<th>Parameter</th>
<th>DP(+)/c</th>
<th>DP(+)/DM</th>
<th>DP(−)/c</th>
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<tr>
<td>Body weight (g)</td>
<td>308 ± 17</td>
<td>157 ± 27**</td>
<td>318 ± 19</td>
<td>149 ± 20**</td>
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<td>Heart weight (mg)</td>
<td>823 ± 31</td>
<td>591 ± 39**</td>
<td>844 ± 34</td>
<td>558 ± 66**</td>
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<td>Heart weight/Body weight</td>
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<td>2.6 ± 0.1</td>
<td>3.8 ± 0.2**</td>
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<td>Fasting blood glucose (mg/dl)</td>
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<td>464 ± 41**</td>
<td>133 ± 16</td>
<td>421 ± 56**</td>
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<td>Glycated albumin (%)</td>
<td>0.8 ± 0.2</td>
<td>8.3 ± 1.2**</td>
<td>0.9 ± 0.1</td>
<td>11.4 ± 1.3**</td>
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<td>HR (min⁻¹)</td>
<td>329 ± 21</td>
<td>285 ± 18</td>
<td>310 ± 13</td>
<td>269 ± 17</td>
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<tr>
<td>SBP (mmHg)</td>
<td>122 ± 18</td>
<td>119 ± 12</td>
<td>126 ± 17</td>
<td>119 ± 13</td>
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<td>LVDd (mm)</td>
<td>6.2 ± 0.4</td>
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<td>IVST (mm)</td>
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<td>1.5 ± 0.1</td>
<td>1.4 ± 0.1†</td>
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<td>PWT (mm)</td>
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<td>1.3 ± 0.1†</td>
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<td><strong>Systolic function</strong></td>
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<tr>
<td>dp/dtmax (mmHg⁻¹ sec⁻¹)</td>
<td>9596 ± 1558</td>
<td>6430 ± 1619*</td>
<td>9617 ± 1998</td>
<td>7674 ± 1503</td>
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<td>LVET (%)</td>
<td>67.3 ± 3.7</td>
<td>68.9 ± 2.4</td>
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<td>FS (%)</td>
<td>32.8 ± 2.8</td>
<td>34.0 ± 1.8</td>
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<td><strong>Diastolic function</strong></td>
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<td>−dp/dtmin (mmHg⁻¹ sec⁻¹)</td>
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<td>6195 ± 1508*</td>
<td>9040 ± 2609</td>
<td>7673 ± 1198</td>
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<td>Tau (msec)</td>
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<td>LVEDP (mmHg)</td>
<td>7 ± 1</td>
<td>9 ± 2*</td>
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<td>Peak E (m⁻¹ sec⁻²)</td>
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<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1**</td>
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<td>E/A</td>
<td>2.1 ± 0.4</td>
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<td>IVRT−1HR (msec⁻¹ 300 bpm)²</td>
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<td>28.0 ± 6.9</td>
<td>38.7 ± 3.1††</td>
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Data were obtained from Fischer rats. Supplemental information was provided on-line. Values (mean ± SD) were obtained by ¹left-ventricular catheterization and ²transthoracic echocardiography; *, P<0.05; **, P<0.01 (Student’s t test); †, P<0.05 (Welch’s t test) versus nondiabetic counterpart (n=4–7); ††, P<0.01 versus DP(+)/DM.
Table 2. Effect of vildagliptin on cardiac functions of diabetic rats.

<table>
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<tr>
<th>Parameter</th>
<th>NonDM +vehicle</th>
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<td><strong>Baseline characteristics</strong></td>
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<tr>
<td>Body weight (g)</td>
<td>398 ± 11</td>
<td>309 ± 55**</td>
<td>391 ± 16</td>
<td>279 ± 24</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>953 ± 29</td>
<td>1000 ± 119*</td>
<td>934 ± 55</td>
<td>924 ± 94</td>
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<tr>
<td>Heart weight/Body weight</td>
<td>2.4 ± 0.1</td>
<td>3.3 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>3.2 ± 0.2</td>
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<tr>
<td>Fasting blood glucose (mg⁻¹ dl)</td>
<td>124 ± 28</td>
<td>206 ± 47##</td>
<td>110 ± 11</td>
<td>205 ± 26**</td>
</tr>
<tr>
<td>Glycated albumin (%)</td>
<td>1.6 ± 0.1</td>
<td>6.0 ± 0.8##</td>
<td>1.6 ± 0.3</td>
<td>6.8 ± 0.9**</td>
</tr>
<tr>
<td>HR (min⁻¹)</td>
<td>298 ± 9</td>
<td>256 ± 24*</td>
<td>301 ± 44</td>
<td>276 ± 24*</td>
</tr>
<tr>
<td>SBP (mmHg⁻¹)</td>
<td>120 ± 7</td>
<td>127 ± 14</td>
<td>120 ± 6</td>
<td>131 ± 5</td>
</tr>
<tr>
<td>LVDd (mm)²</td>
<td>6.7 ± 0.4</td>
<td>6.9 ± 0.5</td>
<td>6.5 ± 0.2</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>LVDs (mm)²</td>
<td>4.2 ± 0.4</td>
<td>4.4 ± 0.4</td>
<td>4.2 ± 0.3</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>IVST (mm)²</td>
<td>1.7 ± 0.1</td>
<td>1.5 ± 0.1**</td>
<td>1.8 ± 0.3</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>PWT (mm)²</td>
<td>1.79 ± 0.1</td>
<td>1.5 ± 0.1**</td>
<td>1.7 ± 0.2</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td><strong>Systolic function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dp/dt max (mmHg⁻¹ sec⁻¹)</td>
<td>8650 ± 364</td>
<td>7248 ± 1169*</td>
<td>9576 ± 899</td>
<td>9558 ± 865</td>
</tr>
<tr>
<td>LVEF(%)²</td>
<td>72.7 ± 6.0</td>
<td>70.5 ± 1.4</td>
<td>74.0 ± 4.9</td>
<td>69.2 ± 2.9</td>
</tr>
<tr>
<td>FS (%)²</td>
<td>37.2 ± 4.6</td>
<td>36.2 ± 1.0</td>
<td>40.6 ± 4.4</td>
<td>34.3 ± 2.2</td>
</tr>
<tr>
<td><strong>Diastolic function</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-dp/dt min (mmHg⁻¹ sec⁻¹)</td>
<td>9024 ± 1784</td>
<td>6375 ± 202##</td>
<td>8502 ± 1020</td>
<td>9493 ± 1115*</td>
</tr>
<tr>
<td>Tau (msec⁻¹)</td>
<td>14.3 ± 3.2</td>
<td>20.0 ± 1.7*</td>
<td>14.2 ± 1.4</td>
<td>15.5 ± 3.2*</td>
</tr>
<tr>
<td>LVEDP (mmHg⁻¹)</td>
<td>7 ± 1</td>
<td>10 ± 2*</td>
<td>6 ± 1</td>
<td>6 ± 0*</td>
</tr>
<tr>
<td>Peak E (m⁻¹ sec⁻¹)</td>
<td>1.3 ± 0.1</td>
<td>1.0 ± 0.1**</td>
<td>1.2 ± 0.3</td>
<td>1.3 ± 0.1††</td>
</tr>
<tr>
<td>E/A</td>
<td>2.1 ± 0.4</td>
<td>2.1 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>DcT (msec⁻¹)</td>
<td>35.3 ± 2.2</td>
<td>48.0 ± 3.4**</td>
<td>36.5 ± 4.7</td>
<td>42.6 ± 1.8**††</td>
</tr>
<tr>
<td>IVRT⁻¹HR (msec⁻¹300 bpm)²</td>
<td>29.4 ± 2.0</td>
<td>51.4 ± 6.6**</td>
<td>30.7 ± 2.7</td>
<td>38.8 ± 2.0††</td>
</tr>
</tbody>
</table>

Data were obtained from Wistar rats with or without vildagliptin treatment (30 mg kg⁻¹ day⁻¹). Values (mean ± SD) were obtained by ¹left ventricular catheterization and ²echocardiography; *, P<0.05; **, P<0.01 (Student’s t test); #, P<0.05; ##, P<0.01 (Welch’s t test) versus the nondiabetic control (nonDM; n=4–7); † P<0.05; ††, P<0.01 versus DM+vehicle.
Table 3. Effect of DPP4 activity on cardiac functions of pressure-overloaded rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DP(+)/c</th>
<th>DP(+)/TAC</th>
<th>DP(−)/c</th>
<th>DP(−)/TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>223 ± 10</td>
<td>190 ± 6</td>
<td>216 ± 13</td>
<td>215 ± 5</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>610 ± 10</td>
<td>680 ± 40</td>
<td>620 ± 20</td>
<td>760 ± 60**</td>
</tr>
<tr>
<td>Heart weight /Body weight</td>
<td>2.8 ± 0.1</td>
<td>3.5 ± 0.4**</td>
<td>2.9 ± 0.2</td>
<td>3.5 ± 0.3**</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (min⁻¹)</td>
<td>345 ± 18</td>
<td>322 ± 25</td>
<td>347 ± 22</td>
<td>319 ± 19</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>5.6 ± 0.5</td>
<td>5.9 ± 0.6</td>
<td>5.9 ± 0.3</td>
<td>5.8 ± 0.9</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>3.5 ± 0.4</td>
<td>4.0 ± 0.6</td>
<td>3.7 ± 0.2</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>IVST(mm)</td>
<td>1.5 ± 0.1</td>
<td>1.6 ± 0.0**</td>
<td>1.4 ± 0.2</td>
<td>1.7 ± 0.1**</td>
</tr>
<tr>
<td>PWT(mm)</td>
<td>1.4 ± 0.1</td>
<td>1.6 ± 0.1*</td>
<td>1.3 ± 0.2</td>
<td>1.7 ± 0.1**</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>74.2 ± 3.9</td>
<td>64.9 ± 7.1†</td>
<td>74.3 ± 1.5</td>
<td>73.3 ± 2.3††</td>
</tr>
<tr>
<td>FS (%)</td>
<td>38.0 ± 3.4</td>
<td>31.0 ± 4.7**</td>
<td>38.1 ± 1.2</td>
<td>37.3 ± 1.7††</td>
</tr>
<tr>
<td>Peak E (m¹ sec)</td>
<td>0.9 ± 0.2</td>
<td>0.6 ± 0.1*</td>
<td>1.0 ± 0.3</td>
<td>0.6 ± 0.2*</td>
</tr>
<tr>
<td>E/A</td>
<td>2.2 ± 0.5</td>
<td>1.6 ± 0.3*</td>
<td>2.3 ± 0.6</td>
<td>2.1 ± 0.3†</td>
</tr>
<tr>
<td>Dc1 (msec)</td>
<td>34.5 ± 2.0</td>
<td>40.8 ± 5.5‖ †‖</td>
<td>36.4 ± 1.1</td>
<td>34.6 ± 3.9†</td>
</tr>
<tr>
<td>IVRT⁻¹ HR (msec⁻¹ 300 bpm)</td>
<td>23.8 ± 4.6</td>
<td>57.9 ± 7.6\ †‖</td>
<td>22.7 ± 2.6</td>
<td>40.5 ± 11.1††</td>
</tr>
</tbody>
</table>

Data were obtained from Fischer rats. Values (mean ± SD) were obtained by echocardiography; *, P<0.05; **, P<0.01 (Student’s t test); †, P<0.05 (Welch’s t test) versus DP(+)/c (n=6); ††, P<0.01 versus DP(+)/TAC.
Table 4. Clinical characteristics of HFPEF patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HFPEF with DM</th>
<th>HFPEF without DM</th>
<th>All HFPEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (% male)</td>
<td>53.3</td>
<td>73.3</td>
<td>63.3</td>
</tr>
<tr>
<td>BMI (kg⁻¹ m²)</td>
<td>24.2 ± 2.9</td>
<td>23.9 ± 3.7</td>
<td>24.1 ± 3.3</td>
</tr>
<tr>
<td>HbA1C (%)</td>
<td>6.7 ± 0.5</td>
<td>5.4 ± 0.4</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td>Fasting glucose (mg⁻¹ dl)</td>
<td>124 ± 34</td>
<td>97 ± 13</td>
<td>110 ± 29</td>
</tr>
<tr>
<td>BNP (pg⁻¹ ml)</td>
<td>102 ± 130</td>
<td>132 ± 142</td>
<td>118 ± 134</td>
</tr>
<tr>
<td>Cardiac function</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>69 ± 13</td>
<td>65 ± 12</td>
<td>67 ± 14</td>
</tr>
<tr>
<td>SBP (mmHg)¹</td>
<td>134 ± 24</td>
<td>132 ± 22</td>
<td>133 ± 23</td>
</tr>
<tr>
<td>DBP (mmHg)¹</td>
<td>72 ± 13</td>
<td>78 ± 13</td>
<td>75 ± 13</td>
</tr>
<tr>
<td>LVDd (cm)²</td>
<td>4.9 ± 0.8</td>
<td>4.7 ± 0.6</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>LVDs (cm)²</td>
<td>3.1 ± 0.7</td>
<td>2.7 ± 0.6</td>
<td>2.9 ± 0.7</td>
</tr>
<tr>
<td>LVPW (cm)²</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>IVS (cm)²</td>
<td>1.4 ± 0.4</td>
<td>1.2 ± 0.3</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Systolic indices</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dp/dt_max (mmHg⁻¹ sec)¹</td>
<td>1507 ± 274</td>
<td>1495 ± 274</td>
<td>1501 ± 269</td>
</tr>
<tr>
<td>LVESV¹</td>
<td>39 ± 28</td>
<td>44 ± 15</td>
<td>42 ± 22</td>
</tr>
<tr>
<td>EF (%)²</td>
<td>65.7 ± 9.7</td>
<td>70.8 ± 9.1</td>
<td>68.2 ± 9.6</td>
</tr>
<tr>
<td>Diastolic indices</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-dp/dt_min (mmHg⁻¹ sec)¹</td>
<td>1395 ± 397</td>
<td>1474 ± 316</td>
<td>1434 ± 354</td>
</tr>
<tr>
<td>T 1/2 (msec)¹</td>
<td>36.4 ± 6.5</td>
<td>46 ± 15.0</td>
<td>41.0 ± 12.2</td>
</tr>
<tr>
<td>LVEDP (mmHg)¹</td>
<td>16 ± 7</td>
<td>14 ± 6</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>LVEDV¹</td>
<td>115 ± 34</td>
<td>126 ± 29</td>
<td>121 ± 32</td>
</tr>
<tr>
<td>Peak E (m⁻¹ sec)²</td>
<td>62.9 ± 17.4</td>
<td>73.9 ± 24.6</td>
<td>68.4 ± 21.7</td>
</tr>
<tr>
<td>E/e' ratio²</td>
<td>13.8 ± 5.0</td>
<td>18.6 ± 11.2</td>
<td>16.4 ± 9.2</td>
</tr>
<tr>
<td>DcT (msec)²</td>
<td>231 ± 90</td>
<td>246 ± 82</td>
<td>239 ± 84</td>
</tr>
</tbody>
</table>

¹; See Supplemental information for definitions.

Data (mean ± SD) were obtained by ¹cardiac catheterization and ²echocardiography in HFPEF patients with DM (n=15) and without DM (n=15).
Table 5. Correlation coefficients for E/e’.

<table>
<thead>
<tr>
<th>Variable</th>
<th>r (versus E/e’)²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-DPP4 activity&lt;sup&gt;1&lt;/sup&gt; (peripheral vein)</td>
<td>0.38</td>
<td>0.04</td>
</tr>
<tr>
<td>BNP&lt;sup&gt;1&lt;/sup&gt; (peripheral vein)</td>
<td>0.64</td>
<td>0.0002</td>
</tr>
<tr>
<td>LVEDP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-0.13</td>
<td>0.50</td>
</tr>
<tr>
<td>–dp/dt&lt;sub&gt;min&lt;/sub&gt;&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-0.32</td>
<td>0.11</td>
</tr>
<tr>
<td>T1/2&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.41</td>
<td>0.035</td>
</tr>
<tr>
<td>Peak E&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.16</td>
<td>0.006</td>
</tr>
<tr>
<td>Dct&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.12</td>
<td>0.54</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values indicate each level in peripheral vein. Data were obtained by <sup>2</sup>left ventricular catheterization and by <sup>3</sup>transthoracic echocardiography (n=26-30).

Figure Legends:

Figure 1. DPP4 localization in rat and human hearts. A, Representative confocal microscopic images of DPP4 (CD26, red) co-localized with CD31-positive endothelial regions (green) in the rat heart. Scale bar; 20 μm. B, DPP4 expression in venous (HUVEC) and microvascular (HMVEC) endothelial cells. C and D, The in situ DPP4 activities of DPP4-positive Fischer rat heart tissue [C, left panel: DPP4(+)], heart tissue from a DPP4-deficient rat [C, right panel: DPP4(-)], and a human heart (D). DPP4 positivity is colored in red. Scale bar: 50 μm. E and F, In vitro DPP4 activity in rat heart extracts (E) and plasma specimens (F): *, P<0.05; **, P<0.01 (n=6-7).

Figure 2. Effect of diabetes on cardiac DPP4 activity and the impact of DPP4 deficiency on cardiac function and remodeling in diabetic rats. A, The in situ cardiac DPP4 activity (red spots) in diabetic [DP(+)/DM] and nondiabetic [DP(+)/c] Fischer rats. Typical images obtained from
DPP4-null counterparts (as negative controls) are shown in the lower panels [DP(-)/DM and DP(-)/c]. Scale bar: 50 μm. B, In vitro cardiac DPP4 activity. Closed bars: DP(+) rats; Open bars: DP(-) rats. Values are reported as the ratio of each relative light unit of DPP4 activity to that found in DP(+)/c (n=5-8). C, Representative left-ventricular pressure (LVP) recordings. The upper panels represent LVP and the lower panels represent dp/dt. D, E, and F, A summary of changes in the –dp/dt_{min} (D), Tau (E), and left-ventricular diastolic stiffness estimated by the ratio of end-diastolic pressure (LVEDP) to end-diastolic diameter (LVDd) (F) (n=5-8). G, Changes in cardiac capillary density (green) and cardiomyocyte (CMC) size (red). A summary of changes in the CMC surface area (CSA in μm², H), vascular proportion (I), and vascular density (J). The vascular proportion was calculated as the number of capillary vessels/number of CMC and the vascular density was estimated as the vascular proportion per CSA. K, Cardiac ischemia estimated with pimonidazole (green). L and M, Representative immunoblots (L) and graphs (M) indicate the changes in cardiac HIF-1α expression levels. *, P<0.05; **, P<0.01; #, P<0.05 (Welch and Wilcoxon).

**Figure 3.** Cardiac remodeling in the diabetic heart was SDF-1α- and DPP4-dependent.

A-D, The impaired angiogenesis observed in the diabetic heart was SDF-1α- and DPP4-dependent. Cardiac SDF-1α (A) and VEGF (B) concentrations determined by ELISA. Closed bars: DP(+) rats; Open bars: DP(−) rats. C, Phosphorylated Akt (p-Akt) and eNOS (p-eNOS) levels in rat heart extracts. D, A summary of CXCR4^{+}KDR^{+} cell counts in each heart section by immunohistochemistry. Data were presented as percentages of CXCR4^{+}KDR^{+} cells among all cells as detected by Hoechst staining. Typical confocal microscopy images were shown in Supplemental Figure 3. E-H, Changes in cardiac fibrosis assessed by picro-Sirius-Red staining (E and F) and related gene expression as detected by real-time PCR (G and H); **, P<0.01; n.s., not
significant; #, P<0.01 (Wilcoxon) (n=6-8).

**Figure 4.** Pharmacological DPP4 inhibition reverses diabetes-induced cardiac remodeling. A, Representative recordings of LVP (upper) and dp/dt (lower). Vilda: vildagliptin-treated, nonDM: nondiabetic. B, A summary of changes in the hemodynamic indices for diastolic function. C and D, Changes in cardiac capillary density (green) and CSA (red). The CSA, vascular proportion, and vascular density of each group are summarized in bar graphs. Scale bar: 20 μm. E, Effect of vildagliptin on cardiac SDF-1α levels (E), cardiac HIF-1α expression, p-eNOS and p-Akt (F), and cardiac fibrosis-related gene expression as detected by real-time PCR (G); *, P<0.05; **, P<0.01; #, P<0.05 (Wilcoxon); n.s., not significant (n=5-8).

**Figure 5.** Effects of DPP4 activity on cardiac remodeling in pressure-overload-induced heart failure. A-E, Typical immnohistological images (A) and summarized data (B-D) of the changes in capillary density (green: CD31) and CSA (red: dystrophin) of the pressure-overloaded rat hearts. Closed bars: DP(+) rats; Open bars: DP(−) rats. Scale bar: 50 μm. E-G, Cardiac hypoxia detected by pimonidazole (green, E) and HIF-1α expression (F and G; n=6-8). Scale bar: 50 μm. H-J, Cardiac SDF-1α (H) and VEGF (I) concentrations and an immunoblot analysis of p-Akt and p-eNOS levels (J). K-M, Changes in cardiac fibrosis assessed by picro-Sirius-Red staining (K) and related gene expression detected by real-time PCR (L and M). *, P<0.05; **, P<0.01; #, P<0.01 (Wilcoxon).

**Figure 6.** Impact of pressure overload on cardiac and circulating DPP4 activities in rats.

A and B, Effects of pressure overload on the in situ (A) and in vitro (B) cardiac activity of DPP4.
Closed bars: DP(+) rats; Open bars: DP(−) rats. Scale bar: 50 μm. C, Effect of hypoxia on DPP4 protein levels in cultured endothelium. HUVEC and HMVEC were exposed to hypoxia for 24 hours and subjected to immunoblotting. Ten micrograms of protein was loaded into each lane. Changes in circulating activities of DPP4 (D) and GLP-1 (E), and cardiac cyclic AMP concentrations (F) in rats with pressure overload; *, P<0.05; **, P<0.01 (n=5-8).

**Figure 7.** The in vitro DPP4 activity in heart failure patients with diastolic left-ventricular function.

A summary of patient characteristics is provided in Table 4. A, DPP4 activity in the coronary sinus (CS, vertical axis) was positively correlated with the DPP4 activity in the peripheral vein (PV, A), but not with that in the coronary artery (B) in patients with DHF (n=30). C, DPP4 activity was negatively correlated with diastolic function (E/e’: the ratio of early mitral inflow to mitral annular velocity). D and E, DPP4 activities of PV (D) and CS (E) in diabetic (black bar) and non-diabetic (white bar) patients with DHF. Data are shown as mean ± SD; *, P<0.05; **, P<0.01 (n=15 per each group).

**Figure 8.** A suggested working model. DPP4 activity modulates cardiac dysfunction via m-DPP4-/SDF-1α-dependent and MMP2/TIMP2-dependent mechanisms. Circulating DPP4 activity determines the GLP-1 level, which may modulate LV contractility.
Dipeptidylpeptidase-4 Modulates Left-Ventricular Dysfunction in Chronic Heart Failure via Angiogenesis-Dependent and -Independent Actions
Toshimasa Shigeta, Morihiko Aoyama, Yasuko K. Bando, Akio Monji, Toko Mitsui, Miwa Takatsu, Xiang-Wu Cheng, Takahiro Okumura, Akihiro Hirashiki, Kohzo Nagata and Toyoaki Murohara

Circulation. published online October 3, 2012;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL

Dipeptidylpeptidase-4 Modulates Left-Ventricular Dysfunction in Chronic Heart Failure via Angiogenesis-dependent and -Independent Actions

Toshimasa Shigeta, MD†; Morihiko Aoyama, MD†; Yasuko K Bando, MD, PhD, FAHA†; Akio Monji, MD†; Toko Mitsui, MD†; Miwa Takatsu, BSc†; Xiang-Wu Cheng, MD, PhD†; Takahiro Okumura, MD†; Akihiro Hirashiki, MD, PhD†; Kohzo Nagata, MD, PhD†; Toyoaki Murohara, MD, PhD†

1Department of Cardiology, Nagoya University Graduate School of Medicine,
2Department of Pathophysiology Laboratory Sciences, Nagoya University Graduate School of Medicine,
3Department of Medical Technology, Nagoya University School of Health Sciences,
Supplemental Figure 1. The effect of DPP4-mediated truncation on the detection of SDF-1α.
A. Changes in the molecular weight of the recombinant SDF-1α (rSDF-1α) truncated by recombinant DPP4 (rDPP4) could not be distinguished from changes in intact SDF-1α by immunoblottings.
B. In vitro truncation by rDPP4 caused rSDF-1α to be mostly undetectable by ELISA. Recombinant SDF-1α was truncated at various concentrations (in ng/ml: 1.25, 2.5, 5.0, and 10.0) with different concentration of recombinant DPP4 (in units/liter; 0, 25, 100, 250). Vertical axis indicates the percent changes of rSDF-1α concentrations after truncation by rDPP4 at each concentration to the corresponding concentration of intact rSDF-1α.
Supplemental Figure 2. Changes in the Akt/eNOS phosphorylation levels in streptozotocin-induced diabetic heart. A summary of densitometry was shown below. Black bars: DPP4-positive groups. White bars: DPP4-null groups. DP(+)/c: DPP4-positive nondiabetic group; **, P<0.01; n.s., not significant (n=6).
Supplemental Figure 3.
A, Single typical images representing immunohistochemical staining of the control rat heart section are shown. CXCR4-positive cells (green) were merged with KDR (red)- and Hoechst (blue)-positive spots. Scale bar: 50 μm.
B, Diabetes reduces the CXCR4- and KDR- double-positive cell counts in a DPP4-dependent fashion. Arrows indicate the CXCR4- and KDR- double-positive cells. Summarized data were demonstrated in Figure 3D. Scale bar: 50 μm.
Supplemental Figure 4. A representative immunoblotting of cardiac CXCR4 expression. The CXCR4 expression levels remained unchanged in the hearts of DPP4-null rats [DPP(-)] and their control counterparts [DPP(+) with or without diabetes.]
Supplemental Figure 5. Changes in the mRNA expression levels of the connective tissue growth factor (CTGF) in streptozotocin-induced diabetic rat heart. Black bars: DPP4-positive control groups, white bars: DPP4-null groups. DP(+)c: DPP4-positive nondiabetic group; *, P<0.05; n.s., not significant; #, P<0.01 (Wilcoxon rank test) (n=8).
Supplemental Figure 6. Vildagliptin (Vilda) suppressed diabetes (DM)-induced increase in the circulating DPP4 activity in Wistar rats; *, P<0.05; **, P<0.01 (n=4-8).
Supplemental Figure 7. A summary of the effect of vildagliptin on the Akt/eNOS phosphorylation levels in streptozotocin-induced diabetic (DM) rat heart. Representative immunoblot images were shown in Figure 4F; **, P<0.01; *, P<0.05; n.s., not significant (n=4-8).
Supplemental Figure 8. A summary of the effect of vildagliptin (Vilda) on the HIF1α protein expression levels in streptozotocin-induced diabetic (DM) rat heart. Each heart extract was obtained from the experimental groups as demonstrated in Figure 4. Ten microgram proteins were loaded to each lanes. Lower graph indicates a summary of densitometry; *, P<0.05; n.s., not significant (n=4-8).
Supplemental Figure 9. A summary of real-time PCR demonstrating the effects of vildagliptin (Vilda) on the mRNA expression levels of the connective tissue growth factor (CTGF) in streptozotocin-induced diabetic (DM) heart; *, P<0.05; n.s., not significant; #, P<0.01 (Wilcoxon rank test) (n=8).
Supplemental Figure 10. Changes in the hypertrophic signaling in pressure-overloaded (left) and diabetic hearts (right). Each heart extract of Fischer rat were obtained by the same specimens used in the Figures 2, 3, and 5. p-mTOR; phosphorylated mTOR, p-S6K; phosphorylated S6-kinase, p-Akt; phosphorylated Akt. Equal amounts (30 μg/lane) were loaded and subjected to SDS-PAGE and following immunoblots.
Supplemental Figure 11. A summary of changes in the Akt/eNOS phosphorylation levels in pressure-overloaded (TAC) heart. Representative immunoblot images were shown in Figure 5J. Lower graph indicates a summary of densitometry: black bars; DPP4-positive groups, white bars: DPP4-null groups; **, P<0.01; *, P<0.05; n.s., not significant (n=8).
Supplemental Figure 12. Changes in the CXCR4⁺KDR⁺ cell number in the pressure-overloaded heart.

A. Representative images of confocal microscopic images of CXCR4⁺ (green)- and KDR⁺ (red)-double-positive cells (arrow). Blue color indicates nuclear staining by Hoechst 33342.

B. A summary of CXCR4⁺KDR⁺ cell counts per high power field in each heart section. Data are represented as the percentage of CXCR4⁺KDR⁺ cells by total cell number detected by Hoechst staining. Black bars: DPP4-positive groups, white bars: DPP4-null groups; *, P<0.05; **, P<0.01 (n=6).
Supplemental Figure 13. Changes in the mRNA expression levels of the connective tissue growth factor (CTGF) in pressure-overloaded heart. Black bars: DPP4-positive groups, white bars: DPP4-null groups; **, P<0.01; *, P<0.05; #, P<0.01 (Wilcoxon rank test) (n=8).
Supplemental Figure 14. Effects of diabetes (DM) on the changes in cardiac cyclic AMP concentrations (nM) in rats. Black bars: DPP4-positive groups, white bars: DPP4-null groups; n.s., not significant (n=4).
## Supplemental Table 1. Effect of GLP-1 receptor agonist on echocardiographic indices of pressure overload-induced cardiac dysfunction.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham/vehicle</th>
<th>TAC/vehicle</th>
<th>TAC/Ex-4</th>
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<tbody>
<tr>
<td><strong>Baseline characteristics</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Body weight (g)</td>
<td>27.8 ± 1.1</td>
<td>26.9 ± 0.9</td>
<td>25.0 ± 0.6</td>
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<tr>
<td>Heart weight (mg)</td>
<td>114.2 ± 83.5</td>
<td>162.0 ± 32.6</td>
<td>182.0 ± 62.8</td>
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<tr>
<td>Heart weight /Body weight</td>
<td>4.1 ± 0.4</td>
<td>6.0 ± 1.2*</td>
<td>7.9 ± 2.5*</td>
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<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (min⁻¹)</td>
<td>303.2 ± 44.5</td>
<td>329.6 ± 23.8</td>
<td>309.2 ± 43.1</td>
</tr>
<tr>
<td>LVDd (mm)</td>
<td>3.6 ± 0.4</td>
<td>3.4 ± 0.3</td>
<td>3.6 ± 0.2</td>
</tr>
<tr>
<td>LVDs (mm)</td>
<td>2.4 ± 0.3</td>
<td>2.5 ± 0.1</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>IVST(mm)</td>
<td>0.7 ± 0.0</td>
<td>1.2 ± 0.1**</td>
<td>1.0 ± 0.1***</td>
</tr>
<tr>
<td>PWT(mm)</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.1**</td>
<td>1.0 ± 0.1***</td>
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<td>LVEF (%)</td>
<td>68.0 ± 1.4</td>
<td>58.7 ± 3.4**</td>
<td>74.6 ± 5.5***</td>
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<td>FS (%)</td>
<td>32.6 ± 1.0</td>
<td>26.4 ± 2.1**</td>
<td>37.8 ± 4.5***</td>
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<tr>
<td>peakE (m⁻¹ sec)</td>
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<td>0.9 ± 0.2</td>
<td>1.1 ± 0.3</td>
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<tr>
<td>E/A</td>
<td>2.6 ± 0.8</td>
<td>2.2 ± 0.5</td>
<td>3.3 ± 1.1</td>
</tr>
<tr>
<td>DcT (msec)</td>
<td>35.2 ± 1.8</td>
<td>44.8 ± 4.2**</td>
<td>37.7 ± 5.1*</td>
</tr>
<tr>
<td>IVRT⁻¹ HR (msec⁻¹ 300 bpm)</td>
<td>18.1 ± 5.0</td>
<td>35.3 ± 4.0**</td>
<td>26.5 ± 5.5***</td>
</tr>
</tbody>
</table>

Data were obtained from male C57BL6 mice (12 week-old) operated with transverse aortic constriction (TAC) with or without treatment of exendin-4 (GLP-1 receptor agonist; Ex-4). **, P<0.01; *, P<0.05 versus sham; ##, P < 0.01 versus TAC/vehicle (n=4-6).
Supplemental information for Tables

1) Definitions and abbreviations of hemodynamic and echocardiographic parameters in rodents.

HR = heart rate; SBP = systolic arterial blood pressure; MBP = mean arterial blood pressure; LVDd = LV diastolic diameter; LVDs = LV systolic diameter; IVS = intraventricular septal thickness diameter; PWT = posterior wall thickness; dp/dtmax = peak rate of left ventricular pressure rise; EF = ejection fraction; FS = fraction shortening; –dp/dtmin = peak rate of pressure decline; Tau = relaxation time constant; LVEDP = end-diastolic left ventricular pressure; peak E; the early diastolic filling velocity of mitral inflow; DcT = deceleration time of early filling velocity of mitral inflow. IVRT = isovolumic relaxation time (the interval between the aortic closure click and the start of mitral flow). bpm = beats per minute.

2) Normal ranges of echocardiographic parameters in HFPEF patients.

LVDd [normal range; male (M): 4.8 ± 0.4 cm, female (F): 4.4 ± 0.3 cm ]; LVDs [normal range; (M): 3.0 ± 0.4 cm, (F): 2.8 ± 0.3 cm]; EF [normal range; (M): 64 ± 5, (F): 66 ± 5%]; LVPW [normal range; (M): 0.9 ± 0.1, (F): 0.8 ± 0.1 cm]; IVS [normal range; (M): 0.9 ± 0.1 and (F): 0.8 ± 0.1 cm ]; peak E; [normal range;(M): 0.70 ± 0.15, (F): 0.80 ± 0.16 ]; E/e’ = the ratio of early mitral inflow to mitral annular velocity [normal range;(M): 7.4 ± 2.2, (F): 7.9 ± 2.2 ]; DcT [normal range; (M):195 ± 40 msec, (F): 185 ± 34 msec].
Supplemental Methods

Animals.

All procedures involving animals were approved by the institutional animal care and use committee of Nagoya University. Male congenital DPP4-null Fischer 344 rats [DP(-) rats], their syngeneic counterparts [DP(+) rats], male Wistar rats, and male C57BL6 mice were purchased from SLC Japan. To induce diabetes, rats received 50 mg/kg streptozotocin (STZ) at the age of 10 weeks, as previously described. Nine weeks after the induction of hyperglycemia (at the age of 19 weeks), rats were subjected to investigation. In the case of vildagliptin treatment, vildagliptin was given orally at a concentration of 30 mg kg\(^{-1}\) day\(^{-1}\) for 4 weeks after induction of diabetes by STZ treatment and rats were analyzed at the age of 19 weeks. The transaortic constriction procedure to generate pressure overload (TAC) was performed as previously described. Animals were exposed to pressure overload for 2 weeks and were subjected to analysis before the development of systolic LV dysfunction [ejection fraction (EF) < 50%].

Cells.

Primary cultured human microvascular endothelial cells (HMVECs) and human umbilical venous cells (HUVECs) were purchased from Lonza Walkersville, Inc.
Cells were grown (used up to 4 passages) and then exposed to normoxic or hypoxic (24 hours) conditions using a hypoxia chamber system (Anaeropack™, MGC, Inc).

**Echocardiographic Physiological Analysis.**

Transmitral left ventricular (LV) inflow was measured by Doppler echocardiography to assess diastolic LV functions. To avoid any influence of the type of anesthesia on heart rate or LV function, we used a combination of ketamine (75 mg kg⁻¹) and xylazine (10 mg kg⁻¹) anesthesia to maintain the heart rate at approximately 300 beats per minute, which allowed us to assess cardiac function using a complete 2-dimensional and M-mode and Doppler echocardiogram [ACUSON Sequoia 512 system with a 15-MHz high-frequency transducer (Microson 15L8), Siemens].

**Hemodynamic Analyses.**

Rats were anesthetized via inhalation of isoflurane (2% in oxygen) and subjected to hemodynamic analysis. A 2.0 Fr micromanometer-tipped catheter (SPR-320; Millar Instruments) that had been calibrated relative to the atmospheric pressure was inserted through the right carotid artery into the left ventricle. Changes in hemodynamic parameters were monitored using the Power Lab™ system (AD Instruments). We evaluated the maximum first derivative of LV pressure (dp/dt$_{\text{max}}$) as an index of contractility and the minimal rate of LV pressure changes (dp/dt$_{\text{min}}$) and the LV relaxation time constant (Tau) as indices of diastolic
function. Each LV end-diastolic pressure (EDP) was recorded and used to assess myocardial stiffness as previously described\(^7\).

**In Situ Cardiac DPP4 Activity.**

Cardiac DPP4 proteolytic activity was detected in situ as described previously\(^8\). In brief, the DPP4 substrate (Gly-Pro-NA) was dissolved in dimethylformamide and mixed with Fast Blue BN. The reaction mixture was then applied to each frozen section of heart (8 \(\mu\)m). Each specimen was subjected to Carrazzi's hematoxylin staining and mounted in an aqueous medium.

**In Vitro Cardiac and Plasma DPP4 Activity.**

Small samples of heart tissue (approximately 10 mg) were snap-frozen in liquid nitrogen, and then subjected to frost shattering using a Cryopress™ (Mictotech Nichion) without heat denaturation. Proteins were extracted from each powdered tissue sample in cell lysis buffer (100 mM Tris-HCl (pH 8.0)). Equal amounts of protein (1 \(\mu\)g) and plasma (100 \(\mu\)l) were subjected to a DPP4-Glo assay™ (Promega) in the presence or absence of the DPP4 inhibitor, diprotin A (20 \(\mu\)mol/l). The DPP4-Glo™ assay utilizes Gly-Pro-aminoluciferin as a substrate and is more sensitive than assays based on fluorescence substrates (Gly-Pro-AMC fluorescent substrate). The luminescence intensity was measured using a luminometer and the diprotin-A sensitive value in relative light units (RLU) per \(\mu\)g of protein (heart extract) or per \(\mu\)l of plasma.
was used to represent the DPP4 activity.

**Immunoblotting.**

Each heart tissue sample was subjected to frost shattering using Cryopress™. Proteins were extracted in RIPA buffer containing protease and phosphatase inhibitor cocktails. Equal amounts (10 µg) of protein from each group were electrophoresed and subjected to immunoblotting. Protein bands were detected using the following specific antibodies: HIF-1α (Novus), GAPDH (Cell Signaling Technology), phospho-Akt (Ser473) (Cell Signaling Technology), total Akt1 (Santa Cruz Biotechnology), phospho-eNOS (Ser1177) (BD), total eNOS (BD), DPP4/CD26 (Abcam), and SDF-1α (Torrey Pines). The density of each protein band was analyzed using image analysis software (Image J).

**Immunohistochemistry And Confocal Microscopic Analysis.**

Frozen sections of heart tissue (8 µm) were subjected to immunohistochemistry and examined under a fluorescence microscope (Axio Observer Z1, Carl Zeiss MicroImaging) and a confocal microscope (A1Rsi, Nikon). Each protein was detected using the following specific antibodies: CD26 (clone OX-61, BD), CD31 (Santa Cruz), EphrinB4 (R&D), LYVE-1 (Acris), dystrophin (Novocastra), vascular endothelial growth factor receptor-2 (VEGFR-2/kinase insert domain–containing receptor [KDR]) (Abcam), and CXCR4 (Abcam). To measure the cardiomyocyte surface area (CSA) and the number of CD31-positive vessels, we measured
randomly selected ventricular cross-sections using NIH images as described previously.

Pimonidazole (Hypoxyprobe-1™) was used in accordance with the manufacturer’s instructions to detect cardiac tissue hypoxia.

**ELISA and Truncation of Recombinant SDF-1α.**

SDF-1α and VEGF levels in cardiac soluble protein specimens prepared by frost shattering were determined using commercially available kits (Quantikine ELISA™ series; R&D Systems) according to the manufacturers’ protocols and the techniques used in a previous report. To confirm the effect of DPP4-mediated protein truncation on biochemical characteristics, in vitro truncation of recombinant SDF-1α (Peprotec) was performed, as previously described, using recombinant DPP4 protein (Sigma). In brief, recombinant SDF-1α was incubated at various concentrations (in ng/ml; 1.25, 2.5, 5.0, and 10.0) with different concentration of recombinant DPP4 (in units/liter; 0, 25, 100, 250) in 50 mM Tris/HCl buffer, pH 7.5, 1 mM EDTA at 37 °C 10 min. The truncated protein was subjected to ELISA or immunoblotting using anti-SDF-1α antibody (CST). The circulating active GLP-1 concentration was determined using a commercially available ELISA kit (Millipore). For GLP-1 measurements, blood samples were treated with DPP4 inhibitor diprotin A immediately after each collection to prevent unwanted degradation.

**Cardiac Cyclic AMP Concentration.**
Frost-shattered heart samples were subjected to cyclic AMP concentration measurement according to the manufacturer’s protocols (Promega).

**Quantitation of gene expression**

Gene expression levels were determined by 2-step Real-time PCR. Total RNA was extracted from left-ventricular tissue using RNeasy fibrous tissues Mini Kit (Qiagen). cDNA was produced using a SuperScript RT-PCR system (Invitrogen). PCR procedure was performed with a Bio-Rad real-time PCR detection system using SYBR Green I as a double-standard DNA-specific dye. Bioinformatically validated primers specific for the collagen types I or III, Matrix Metalloprotinase 9 or 2, TIMP1 or 2, β-myosin heavy chain (β-MHC), ANP, BNP, CTGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from QIAGEN (QuantiTect primer assays).

**Patient Selection.**

The study protocol was approved by the ethics committee of the Nagoya University School of Medicine and all subjects provided written informed consent. This was an observational study of 30 consecutive patients with DHF and a normal systolic LV function who were entered into our heart failure registry. DHF was diagnosed in accordance with ACC/AHA guidelines based on the previously established normal values for echocardiographic measurements in the healthy Japanese population\(^\text{11-13}\). In brief, DHF was diagnosed by a slowed rate of ventricular relaxation.
as detected by Doppler echocardiography [a ratio of early mitral inflow to mitral annular
velocity (E/e’) > 8.0 for males and > 10.0 for females and a deceleration time of the early filling
velocity of mitral inflow (Dct) > 200 msec] and concomitant plasma BNP elevation (> 18.4 pg/ml). We excluded patients with valvular disease, coronary artery disease, or atrial fibrillation.

Circulating s-DPP4 activities in HFPEF patients were measured by using the blood samples
simultaneously obtained from the peripheral vein, the coronary sinus (CS; as a measure for the
myocardial excretion of DPP4), and the aortic root (as a measure for DPP4 activity at the
coronary arterial level) via a 6F multipurpose catheter at the time of biventricular catheterization,
followed by cardiac tissue biopsy as previously described. Blood samples were centrifuged,
and the plasma samples were stored in aliquots at −80 °C. Frozen heart specimens obtained by
cardiac biopsy were subjected to the in situ DPP4 colorimetric staining as described in the
above section of Supplemental Methods.
References for Supplemental Methods


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