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

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Background—The inhibition of dipeptidyl peptidase-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 to the capillary endothelia of rat and human heart tissue. Diabetes mellitus promoted the activation of the membrane-bound form of DPP4, leading to reduced myocardial stromal cell-derived factor-1α concentrations and resultant angiogenic impairment in rats. The diabetic rats exhibited diastolic left ventricular dysfunction (DHF) with enhanced interstitial fibrosis caused partly by the increased ratio of matrix metalloproteinase-2 to tissue inhibitor of metalloproteinase-2 in a DPP4-dependent fashion. Both genetic and pharmacological DPP4 suppression reversed the stromal cell-derived factor-1α-dependent microvascularopathy and DHF associated with diabetes mellitus. Pressure overload induced DHF, which was reversed by DPP4 inhibition via a glucagon-like peptide-1/cAMP-dependent mechanism distinct from that for diabetic heart. In patients with DHF, the circulating DPP4 activity in peripheral veins was associated with that in coronary sinus and with E/e’, an echocardiographic parameter representing DHF. Comorbid diabetes mellitus increased the circulating DPP4 activities in both peripheral veins and coronary sinus.

Conclusions—DPP4 inhibition reverses DHF via membrane-bound DPP4/stromal cell-derived factor-1α–dependent local actions on angiogenesis and circulating DPP4/glucagon-like peptide-1–mediated inotropic actions. Myocardium-derived DPP4 activity in coronary sinus can be monitored by peripheral vein sampling, which partly correlates with DHF index; thus, circulating DPP4 may potentially serve as a biomarker for monitoring DHF. (Circulation. 2012;126:1838-1851.)

Key Words: angiogenesis ■ diabetes mellitus ■ dipeptidyl peptidase 4 ■ glucagon-like peptide 1 ■ heart failure ■ microcirculation

Dipeptidyl peptidase-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.1–3 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 several reports have suggested that DPP4 represents a subfamily of gelatinolytic serine proteases that selectively bind to denatured collagen4,5; hence, DPP4 modulates pathological conditions such as diabetes mellitus (DM), malignancy, and inflammation. DPP4 is widely distributed in mammalian tissues, including kidney, small intestine, liver, and heart tissues.2 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).3 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 heart1,6; however, investigations of the precise distribution of DPP4 in the heart and the immunohistochemical identification of the cell types involved have not been undertaken.

Clinical Perspective on p 1851

In recent preclinical studies, pleiotropic effects of DPP4 inhibition were demonstrated to reduce mortality and cardiac
remodeling after myocardial infarction\textsuperscript{7,8}; 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 characterized by pathological cardiac remodeling such as LV hypertrophy and fibrosis induced by comorbidity such as pressure overload or DM.\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 DM 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 Methods in the online-only Data Supplement.

### 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 with 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 with Gly-Pro-NA used as the substrate.

### In Vitro Cardiac and Plasma DPP4 Activity

The DPP4 activity in each heart tissue extract and in plasma was measured with the DPP4-Glo assay (Promega).
Figure 2. Effect of diabetes mellitus (DM) on cardiac dipeptidyl peptidase-4 (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 bottom [DP(−)/DM and DP(−)/c]. Scale bar=50 μm. B, In vitro cardiac DPP4 activity. Closed bars indicate 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. Top, LVP; bottom, dP/dt. D through F, A summary of changes in −dp/dtmin (D), τ (E), and left ventricular diastolic stiffness estimated by the ratio of end-diastolic pressure (LVEDP) to end-diastolic diameter (LVDd; n=5–8; F). 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 CMCs, 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 hypoxia-inducible factor-1α (HIF-1α) expression levels. *P<0.05; **P<0.01; #P<0.05 (Welch and Wilcoxon).
Table 1. Impact of Dipeptidyl Peptidase-4 Activity on Cardiac Functions in Diabetic Rats

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DM indicates diabetes mellitus; HR, heart rate; SBP, systolic blood pressure; LVDd, end-diastolic left ventricular diameter; LVEDp, end-diastolic left ventricular diameter; IVST, interventricular septal thickness; PWT, posterior wall thickness; LVEF, left ventricular ejection fraction; FS, fractional shortening; IVRT, isovolumic relaxation time. Data were obtained from Fischer rats. Additional information is provided in the online-only Data Supplement.

Values (mean±SD) were obtained by *left-ventricular catheterization and †transthoracic echocardiography.

†P<0.05.
§P<0.01 (Student t test).
¶P<0.05 (Welch t test) versus nondiabetic counterpart (n=4–7).
#P<0.01 versus DP(+/c)/DM.

Patient Selection

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

Statistical Analysis

Values are expressed as mean±SD. The homogeneity of data variance was assessed with the F test (2 groups) and the Bartlett test (multiple groups). The mean values of 2 groups were compared by use of the Student t test (when the variance was homogenous) or the Welch t test (for heterogeneous variance). Comparisons of multiple means were performed with ANOVA (for homogeneous variance) or the Wilcoxon rank-sum test (for heterogeneous variance) if indicated. All group comparisons were followed by the Bonferroni 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 statistically significant.

Results

The Capillary Endothelium Expresses DPP4 in the Heart

Using a colorimetric procedure, previous reports suggested that the DPP4-active regions localize in capillary vessels.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 platelet endothelial cell adhesion molecule/CD31 (Figure 1A). Immunoblot analysis revealed the DPP4 expression in both cultured venous and microvascular endothelial cells (Figure 1B).

We next performed in situ colorimetric staining to assess m-DPP4 activity in heart sections of Fischer rats (Figure 1C, left), their DPP4-null counterparts (Figure 1C, right), and humans (Figure 1D). DPP4-positive regions were red in both rat and human heart specimens, and the pattern of the DPP4 positivity was consistent with the results obtained by immu-
nohistochemistry (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 1E) and plasma specimens (Figure 1F).

Cardiac m-DPP4 Activity Is Elevated in the Diabetic Heart

DM exacerbates heart failure and is associated with DHF and coronary microangiopathy. We investigated whether DM influences cardiac m-DPP4 activity (Figure 2A and 2B).

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/dtmin (Figure 2D) and increased τ (Figure 2E) and diastolic stiffness (Figure 2F), findings consistent with the prolonged deceleration time of the early mitral inflow and reduced E/A value measured by echocardiography (Table 1). Immunohistochemistry (Figure 2G–2J) revealed that the car-
diomyocyte surface area (Figure 2H) and vascularity (Figure 2I and 2J) were reduced in the DP(+)/DM rats. In contrast, DM had no influence on either cardiac function or histological remodeling in DP(−)/c rats. We further examined whether the reduced vascularity in DP(+)/DM rats promoted myocardial hypoxia using pimonidazole (Figure 2K). DM increased pimonidazole-positive green spots in the myocardium (top right), which were absent in DP(−)/DM rats (bottom right). As an additional measure of hypoxia, hypoxia-inducible factor-1α expression (Figure 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 Mellitus

DPP4 truncates SDF-1α, inactivating its angiogenic potential. Accordingly, we hypothesized that the cardiac m-DPP4 activation observed in the DP(+) rats may suppress angiogenesis through the reduction of SDF-1α. Because electropho-
DPP4 Inhibition Reverses DM-Induced Cardiac Fibrosis via the Matrix Metalloproteinase-2/Tissue Inhibitor of Metalloproteinase-2 Axis

We examined the impact of DPP4 on cardiac remodeling (Figure 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 (Figure 3E and 3F). Real-time polymerase chain reaction analysis revealed a DPP4-dependent increase in the accumulation of collagen (collagen types 1 and 3) and matrix metalloproteinase-2 (MMP2) with a concomitant decline in the level of tissue inhibitor of metalloproteinase-2 (TIMP2) and an elevation of connective tissue growth factor (Figure V in the online-only Data Supplement) in DP(−)/DM rats. The level of β-miosin heavy chain of DP(+)/DM rats was reduced (Figure 3H), which was partially

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DM indicates diabetes mellitus; HR, heart rate; SBP, systolic blood pressure; LVDd, end-diastolic left ventricular diameter; LVDs, end-systolic left ventricular diameter; IVST, interventricular septal thickness; PWT, posterior wall thickness; LVEF, left ventricular ejection fraction; FS, fractional shortening; LVEDP, left ventricular end-diastolic pressure; DcT, deceleration time of the early mitral valve; and IVRT, isovolumic relaxation time. Data were obtained from Wistar rats with or without vildagliptin treatment (30 mg · kg⁻¹ · d⁻¹).

| Values (mean±SD) were obtained by *left ventricular catheterization and †echocardiography. |
|§P<0.05. †P<0.01 (Student t test). |
|¶P<0.01 (Welch t test) versus the non-DM control (n=4–7). |
|#P<0.01 versus DM+vehicle. |

resis (Tricine–SDS-PAGE) cannot separate the DPP4-cleaved biologically inactive form of SDF-1α protein from intact SDF-1α,23–25 we performed preliminary experiments (Figure I in the online-only Data Supplement)21 and found ELISA to be superior for the detection of biologically active SDF-1α. We examined whether 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 factor16–17 was reduced independently of DPP4 deficiency (Figure 3B). SDF-1α activates Akt/endothelial nitric oxide synthase (eNOS) signaling, which is essential for angiogenesis mediated by the trafficking of endothelial progenitor cells.22–24 We examined whether the cardiac SDF-1α gradient is related to Akt/eNOS signaling (Figure 3C and Figure II in the online-only Data Supplement) and endothelial progenitor cell homing (Figure 3D and Figures III and IV in the online-only Data Supplement). 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 DM (Figure IV in the online-only Data Supplement).
but significantly reversed in the DP(−)/DM counterparts. MMP9, TIMP1, brain natriuretic peptide, and atrial natriuretic peptide levels were elevated (Figure 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 DM-induced cardiac remodeling and DHF. We validated the inhibitory effect of vildagliptin on circulating DPP4 activity with an in vitro assay (Figure VI in the online-supplement). Vildagliptin ameliorated the DM-induced DHF without affecting systolic LV function (Figure 4A and 4B and Table 2) or the declines in cardiomyocyte surface area and vascularity induced by DM (Figure 4C and 4D). DM suppressed cardiac SDF-1α levels (Figure 4E) and Akt/eNOS phosphorylation levels (Figure 4F and Figure VII in the online-only Data Supplement) with a concomitant increase in the level of hypoxia-inducible factor-1α (Figure VIII in the online-only Data Supplement) and the MMP2/TIMP2 ratio (Figure 4G) and increased connective tissue growth factor levels (Figure IX in the online-only Data Supplement); these changes were reversed in response to vildagliptin treatment.

DPP4 Inhibition Protects the Heart Against 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 LV hypertrophy and a mild systolic dysfunction in DP(+)/TAC rats. The DP(−)/TAC group exhibited LV hypertrophy, but their cardiac function remained intact (Table 3). Both DP(+)/TAC and DP(−)/TAC rats exhibited increases in cardiomyocyte surface area (Figure 5B) without reduced angiogenesis (Figure 5C), leading to a

Figure 6. Impact of pressure overload on cardiac and circulating dipeptidyl peptidase-4 (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 indicate DP(+) rats; open bars, DP(−) rats. Scale bar=50 μm. C. Effect of hypoxia on DPP4 protein levels in cultured endothelium. Human umbilical venous (HUVECs) and microvascular (HMVECs) endothelial cells were exposed to hypoxia for 24 hours and subjected to immunoblotting. Protein (10 μg) was loaded into each lane. Changes in circulating activities of DPP4 (D), glucagon-like peptide-1 (GLP-1; E), and cardiac cAMP concentrations (F) in rats with pressure overload. *P<0.05; **P<0.01 (n=5–8).
functions of pressure-overloaded rats

Table 3. Effect of Dipeptidyl Peptidase-4 Activity on Cardiac Functions of Pressure-Overloaded Rats

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<td>5.9±0.3</td>
<td>5.8±0.9</td>
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<td>LVDs, mm</td>
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<td>FS, %</td>
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<td>34.0±4.7†</td>
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<td>37.3±1.7†</td>
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<td>DcT, ms</td>
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<tr>
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<td>57.9±7.6†</td>
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<td>40.5±11.1†</td>
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</table>

TAC indicates transaortic constriction; HR, heart rate; LVd, end-diastolic left ventricular diameter; LVDs, end-systolic left ventricular diameter; IVRT, isovolumic relaxation time.

Discussion

Studies investigating DPP4 are very common in the fields of hematology, oncology, and diabetes.32 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, ie, 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.34,35 In the present study, consistent with a previous report,23 DM 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+ endothelial progenitor cell homing to the heart (Figure 3D and Figure III in the online-only Data Supplement). In contrast, cardiac m-DPP4 activity of TAC was reduced.
with elevated cardiac SDF-1 levels (Figure 5H), suggesting that cardiac m-DPP4 activity may be a key determinant of cardiac angiogenesis by modulating the SDF-1 gradient in response to both diabetic and nondiabetic pathologies.

In contrast to that observed in DM, m-DPP4 activity was decreased in the pressure-overloaded heart (Figure 6A and 6B), presumably as a result of the 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 the observation of Lamers et al 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 the regulatory system(s) may be impaired in DM, leading to the resultant DPP4 activation and microvasculopathy.

Figure 7. The in vitro dipeptidyl peptidase-4 (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 diastolic left-ventricular dysfunction (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 nondiabetic (white bar) patients with DHF. Data are shown as mean±SD. *P<0.05; **P<0.01 (n=15 per each group).
Streptozotocin-induced DM has been associated with myocardial atrophy and increased interstitial fibrosis.\(^{36}\) In addition, ample evidence indicates that streptozotocin rat hearts exhibit diastolic abnormalities; both impaired relaxation and increased stiffness occur in the absence of hypertrophy and exhibit diastolic abnormalities; both impaired relaxation and hypertrophy (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 (reviewed extensively elsewhere\(^{37}\)). In particular, clinical and experimental studies have reported the increased expression of MMP2 in diabetic subjects.\(^{12-15}\) MMP2 plays a primary role in the development of diabetic microvascularopathy (retinopathy) by enhancing MMP2 with a corresponding decrease in TIMP2 in endothelial cells.\(^{15}\) Cardiac MMP2 transgenic mice display increased collagen accumulation and exhibit biventricular dilation and disruption of the myocardium.\(^{16}\) In the present study, the trend of increasing MMP2 levels in the streptozotocin rat heart was consonant with the atrophic changes in cardiomyocytes; in contrast, we observed a decrease in MMP2 expression and a corresponding increase in TIMP2 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.\(^{17,18}\) With regard to angiogenesis, MMP2 is known as a protease that degrades SDF1\(^{16}\); thus, the trend of MMP2 expression in both diabetic and TAC models is consistent with our results concerning cardiac SDF1\(\alpha\) contents (Figure 3A and Figure 5H) and capillary density (Figure 5I and Figure 5C). DPP4 is a weak endopeptidase for denatured collagen,\(^{43}\) 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, ie, under diabetic stress without insulin stimulus.

GLP-1 is an incretin\(^{27,28}\) that is easily cleaved by s-DPP4 in circulation.\(^{2}\) GLP-1 exhibits positive-inotropic effects via the endocrine system, and the exogenous administration of its receptor agonists (eg, exendin-4) ameliorates heart failure in humans\(^{29}\) and in animals.\(^{8,30,31}\) DPP-4 is a principal determinant of the circulating level of bioactive GLP-1,\(^{35,38}\) 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\textsuperscript{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 is necessary to address this question.

**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 or a therapeutic target for chronic heart failure.

**Acknowledgments**

We wish to acknowledge Seiichi Kotoda for technical assistance with the hemodynamic studies. We thank Ikuyo Mizuguchi and 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.

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**Disclosures**

None.

**References**


**Figure 8.** A suggested working model. Dipeptidyl peptidase-4 (DPP4) activity modulates cardiac dysfunction via membrane-bound form of DPP4 (m-DPP4)/stromal-derived factor-1\textsuperscript{1alpha} (SDF-1\textsuperscript{1alpha})–dependent and matrix metalloproteinase-2 (MMP2)/tissue inhibitor of metalloproteinase-2 (TIMP2)–dependent mechanisms. Circulating DPP4 activity determines the glucagon-like peptide-1 (GLP-1) level, which may modulate left ventricular (LV) contractility.


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**Clinical Perspective**

Dipeptidyl peptidase-4 (DPP4) is a serine protease with a primary function of truncating various bioactive molecules such as incretin hormones, angiogenic chemokines, inflammatory cytokines, denatured collagen, and brain natriuretic peptide. To date, the pathophysiological significance of DPP4 in the processes of diabetes mellitus, malignancy, and inflammation has been well recognized and well established; however, the role of DPP4 in the pathogenesis of cardiovascular disease remained unclear until as recently as several years ago, when the first report demonstrated the protective role of DPP4 inhibition in acute myocardial infarction. The present study highlights a novel pathophysiological role for DPP4 in diastolic left ventricular dysfunction and raises the possibility of using DPP4 as a diagnostic surrogate and a therapeutic target for chronic heart failure. One of the primary interpretations drawn from the present study is that the coronary microcirculation is essential for diastolic left ventricular dysfunction development, in which DPP4 may play a pathophysiological role by regulating coronary angiogenesis. Diabetes mellitus has been postulated to exacerbate heart failure, and the comorbidity of diabetic retinopathy, ie, diabetic microvasculopathy, has been suggested to be associated with the increased incidence of heart failure in patients with diabetes mellitus. Thus, the present study proposes another therapeutic benefit of the small-molecule DPP4 inhibitors in reducing the incidence of heart failure in patients with diabetes mellitus.
Dipeptidyl Peptidase-4 Modulates Left Ventricular Dysfunction in Chronic Heart Failure via Angiogenesis-Dependent and -Independent Actions

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SUPPLEMENTAL MATERIAL

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

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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>
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<td>FS (%)</td>
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<td>DcT (msec)</td>
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<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>
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</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).

3 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\(^5,6\), 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].

11 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.\(^7\)

3 **In Situ Cardiac DPP4 Activity.**

Cardiac DPP4 proteolytic activity was detected in situ as described previously. 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.

4 **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\textsuperscript{4}.

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

ELISA and Truncation of Recombinant SDF-1\(\alpha\).

SDF-1\(\alpha\) 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\textsuperscript{9}. To confirm the effect of DPP4-mediated protein truncation on biochemical characteristics, in vitro truncation of recombinant SDF-1\(\alpha\) (Peprotec) was performed, as previously described\textsuperscript{10}, using recombinant DPP4 protein (Sigma). In brief, recombinant SDF-1\(\alpha\) 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-1\(\alpha\) 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 from 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. 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\textsuperscript{14}. 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


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 ± 0.4cm, 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].