Circulating Dipeptidyl Peptidase IV Activity Correlates with Cardiac Dysfunction in Human and Experimental Heart Failure

dos Santos et al: DPPIV and Cardiovascular Outcomes in HF

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Abstract

**Background**—The present study addressed the hypothesis that the activity of dipeptidyl peptidase IV (DPPIV), an enzyme that inactivates peptides that possess cardioprotective actions, correlates with adverse outcomes in heart failure (HF). The therapeutic potential of DPPIV inhibition in preventing cardiac dysfunction was also investigated.

**Methods and Results**—Measurements of DPPIV activity in blood samples obtained from 190 HF patients and 42 controls demonstrated that patients with HF exhibited an increase of approximately 130% in circulating DPPIV activity compared with healthy subjects. Furthermore, an inverse correlation was observed between serum DPPIV activity and left ventricular LV ejection fraction in HF patients. Similarly, radiofrequency LV ablation-induced HF rats displayed higher DPPIV activity in the plasma (~50%) and heart tissue (~3.5 fold) compared with sham-operated rats. Moreover, positive correlations were observed between the plasma DPPIV activity and LV end-diastolic pressure (LVEDP) and lung congestion. Two days after surgery, one group of LV ablation-induced HF rats was treated with the DPPIV inhibitor sitagliptin (40 mg/kg, twice daily) for six weeks, whereas the remaining rats were administered water. Hemodynamic measurements demonstrated that radiofrequency LV-ablated rats treated with sitagliptin exhibited a significant attenuation of HF-related cardiac dysfunction, including LVEDP, systolic performance and chamber stiffness. Sitagliptin treatment also attenuated cardiac remodeling and cardiomyocyte apoptosis and minimized pulmonary congestion.

**Conclusions**—Collectively, the results presented herein associate circulating DPPIV activity with poorer cardiovascular outcomes in human and experimental HF. Moreover, the results demonstrate that long-term DPPIV inhibition mitigates the development and/or progression of HF in rats.

**Key Words**: sitagliptin, myocardial injury, congestion, cardiac remodeling, glucagon-like peptide 1
Dipeptidyl peptidase IV (DPPIV) is a serine peptidase that exists on the surface of various cell types, including epithelial cells, endothelial cells, and lymphocytes.\textsuperscript{1} Moreover, a soluble form of DPPIV that lacks the cytoplasmic and transmembrane domains is present in plasma and other body fluids.\textsuperscript{1} The wide distribution of DPPIV in different tissues, including heart, kidney and vascular beds, combined with the fact that this peptidase inactivates glucagon-like peptide-1 (GLP-1)\textsuperscript{2} and brain natriuretic peptide (BNP),\textsuperscript{3} suggest a potential role for this peptidase in the pathophysiology of metabolic and cardiovascular diseases.

The incretin hormone GLP-1 plays an important role in the maintenance of systemic glucose homeostasis. The pharmacological inhibition of DPPIV, which enhances in vivo GLP-1 biological activity,\textsuperscript{4} is an efficient and safe therapy for patients with type 2 diabetes mellitus.\textsuperscript{5} GLP-1 is capable of decreasing cardiomyocyte apoptosis,\textsuperscript{6} activating cardioprotective signaling pathways,\textsuperscript{7} reducing infarct size after coronary occlusion and improving coronary flow.\textsuperscript{8} Therefore, incretin-based agents may also constitute a promising approach in the treatment and/or prevention of cardiovascular diseases.

Elevated plasma BNP is a strong indicator of heart failure (HF) severity.\textsuperscript{9} Paradoxically, despite exceedingly high circulating BNP, HF patients often display blunted BNP-mediated renal effects. This endocrine paradox may be partially explained by the finding that truncated forms of BNP with reduced physiological activity exist in the plasma of HF patients.\textsuperscript{10, 11} Notably, emerging evidence from in vitro and in vivo studies has demonstrated that DPPIV cleaves the N-terminal dipeptide from intact BNP(1-32), yielding BNP(3-32),\textsuperscript{3} which displays remarkably reduced renal and vascular activities.\textsuperscript{12} Thus, one of the possible mechanisms for the development of natriuretic peptide resistance in HF is the increased inactivation of BNP(1-32)
mediated by enhanced catalytic activity of DPPIV. To date, however, the hypothesis that DPPIV activity is increased in HF remains untested.

In this context, the present study tested the hypothesis that circulating DPPIV activity is increased and associated with adverse cardiovascular outcomes in patients and in a rat model of experimental HF. The potential use of the DPPIV inhibitor sitagliptin for preventing cardiac dysfunction and the possibility that the effect of DPPIV inhibition is, at least in part, mediated by cardiac GLP-1 receptor stimulation were also investigated.

**Methods**

An expanded Methods section is available in the online-only Data Supplement.

*Selection of patients with HF* – All patients signed an informed consent form, and the study was approved by the Ethics Committee of the Heart Institute of the University of São Paulo (USP), São Paulo, Brazil. One hundred ninety HF patients from an ongoing inception cohort from the General Outpatient Clinic of the Heart Institute of University of São Paulo were included in this study. The ascertainment period was from 2005 to 2010. After enrollment, the serum samples were frozen at -80°C until analysis.

*Animal protocols, surgical procedures and drug treatment* – Experimental procedures were conducted in accordance with the guidelines established by the Brazilian College for Animal Experimentation (COBEA) and were approved by the institutional animal care and use committee. Experimental HF was induced in male Wistar rats (200-250 g, n = 58) via left ventricular (LV) myocardial injury using radiofrequency catheter ablation, as previously described.13 Sham-operated rats underwent left thoracotomy and were mock-ablated (n = 20).

*Determinant of DPPIV activity and abundance* – DPPIV activity was assayed by measuring the release of p-nitroaniline resulting from the hydrolysis of glycylproline p-
nitroanilide tosylate, as previously described. The abundance of DPPIV was evaluated by immunoblotting.

_Evaluation of cardiac function_ - Hemodynamic assessment was performed using a pressure-volume conductance catheter, as previously described.

_Determination of the plasma concentrations of active GLP-1 and total BNP_ – The plasma levels of intact GLP-1(7-36 amide) and total BNP were measured using enzyme-linked immunosorbent assays from Linco Research (St. Charles, MO, USA) and Bachem (Torrance, CA), respectively, in accordance with the manufacturer’s instructions.

_Statistical analysis_ – Data are expressed as the mean ± standard error of the mean (SEM) unless stated otherwise. For each data set, the Levene’s test was performed to examine the homogeneity of variances. If Levene’s test revealed unequal variance, then Welch’s ANOVA was used. If variance was equal, then a Student’s t-test or one-way ANOVA followed by Bonferroni’s post-hoc tests was performed. The relationships between the plasma DPPIV activity and the parameters of HF were assessed by Pearson’s correlation test. \( P < 0.05 \) was considered statistically significant.

**Results**

**Circulating DPPIV activity in patients with HF**

The general demographic characteristics of the studied population are shown in Supplementary Table 1. In the patients with HF, the measured DPPIV activity followed a normal distribution (Figure 1A). The mean (±SD) serum DPPIV activity in the 190 selected HF patients was significantly higher than that of the normal subjects (\( n = 42, \ P < 0.001 \)) (Figure 1B).
A significant negative correlation was found between serum DPPIV activity and LV ejection fraction in HF patients \((r = -0.20; P = 0.009)\). Interestingly, we also observed statistically significant correlations between serum DPPIV activity and age \((r = -0.19; P = 0.02)\), serum sodium \((r = 0.22; P = 0.004)\), hemoglobin \((r = 0.20; P = 0.01)\), and triglycerides \((r = 0.23; P = 0.005)\) (Supplementary Figure 1). The serum DPPIV activity in HF individuals did not significantly correlate with body weight index, heart rate, systolic or diastolic blood pressure, serum potassium, total cholesterol, serum creatinine, or serum glucose (data not shown).

**Experimental model, biometric and cardiac parameters**

Radiofrequency LV ablation-induced HF rats\(^{13}\) displayed typical cardiac dysfunction, including elevated LV end-diastolic pressure and time constant of relaxation, with decreased dP/dt, ejection fraction, cardiac output and stroke work, although there was no change in the heart rate (Table). The indexed lung mass and the percent of water content were significantly elevated in the HF animals compared with the sham group (Table), suggesting that our experimental model of LV ablation-induced HF exhibited pulmonary congestion secondary to cardiac decompensation.

**Determination of DPPIV activity and abundance in sham-operated and experimental HF rats treated or not with the DPPIV inhibitor sitagliptin.**

Figure 2 demonstrates that rats with HF displayed higher DPPIV activity in the plasma (Figure 2A) and heart (Figure 2B) compared with sham animals. The average plasma DPPIV activity in radiofrequency LV-ablated rats treated with 40 mg/kg bid sitagliptin for six weeks was 6.77 ± 1.05 nmol/ml/min, which corresponded to approximately 90% inhibition compared with HF rats.
and 85% inhibition compared with sham rats (Figure 2A). Moreover, treatment with sitagliptin significantly inhibited DPPIV heart activity in HF rats (Figure 2B). In agreement with the pattern of enzymatic activity, the abundance of DPPIV, as assessed by immunoblotting, increased both in the plasma (Figure 2C) and in the heart (Figure 2D) from the HF rats compared with the sham rats. As depicted in Figure 2C-D, sitagliptin not only inhibited DPPIV catalytic activity but also decreased the abundance of the enzyme both in plasma and heart.

To determine in which cardiac cell type the upregulation of DPPIV expression occurred, heart sections were analyzed by immunofluorescence using specific cell markers. As demonstrated in Figure 2E, co-staining for von Willebrand factor with DPPIV indicated that upregulated DPPIV expression was confined to the surface of heart endothelial cells. Though the kidney is the principal site of expression of DPPIV, no significant differences were found in renal cortical DPPIV activity and expression between sham and HF rats (Supplementary Figure 2A-B).

**Correlation of plasma DPPIV activity with cardiac dysfunction and congestion**

Similar to what was observed in HF patients, there were significant correlations between plasma DPPIV activity and different parameters of cardiac dysfunction and congestion in the rat model of HF (Figure 3). Plasma DPPIV activity correlated negatively with LV ejection fraction (Pearson $r = -0.70; P< 0.01$) (Figure 3A) and positively with LV end-diastolic pressure (Pearson $r = 0.72; P< 0.001$) (Figure 3B) and with the lung/body weight index (Pearson $r = 0.78; P< 0.001$) (Figure 3C).
Effect of DPPIV inhibition on the cardiac function of experimental HF rats

The rats that were treated with the DPPIV inhibitor exhibited significant attenuation of HF-related dysfunction and remarkably mitigated pulmonary congestion (Table and Figure 4). Figure 4 displays representative P-V loops obtained during transient occlusions of the inferior vena cava in the experimental groups, and the averaged results of cardiac performance are depicted by the bar graphic. The slope of the linear relationship between the end-systolic volumes and pressures (ESPVR) was steeper in the sham rats, suggesting decreased systolic performance in the HF rats. However, the ESPVR of the radiofrequency LV-ablated rats treated with the DPPIV inhibitor sitagliptin was similar to that of the sham rats (Figure 4D, left). In addition, the increased slope of the end-diastolic P-V relationships in the HF group compared with the sham group, which is suggestive of increased chamber stiffness, was significantly mitigated by DPPIV inhibition (Figure 4D, right).

Cardioprotective effects of DPPIV inhibition in experimental HF rats

The cardiac mass and volume are presented in the Table. Although body weight was similar among the groups, the heart weight as a function of body weight was significantly increased in the HF rats. This finding was associated with ventricular dilation (i.e., increased end-diastolic volume), suggesting the occurrence of cardiac remodeling. These parameters were partially preserved by sitagliptin treatment.

Histological analysis of the remodeled myocardium far from the scar demonstrated a significant increase in the average cardiomyocyte nuclear volume in the HF rats compared with the sham rats, which was significantly reduced by DPPIV inhibition (Figure 5A). In addition, the
increased interstitial collagen in reminiscent tissues evidenced in the HF group was significantly attenuated by sitagliptin treatment (Figure 5B) compared with samples from similar regions.

As depicted in Figure 5C, the apoptosis rate was higher in HF rats (0.70 ± 0.03, P<0.05) compared with sham rats (0.18 ± 0.06, P<0.05). The extent of apoptosis was attenuated, but not normalized, by sitagliptin (0.39 ± 0.01, P<0.05 vs. sham and P<0.01 vs. HF).

**Effect of DPPIV inhibition on GLP-1 circulating level, on heart GLP-1 receptor expression and on the stimulation of cardioprotective signaling pathways**

The plasma level of active GLP-1 was 3.2 times greater in the sitagliptin-treated radiofrequency LV-ablated rats than in the HF animals and 2.4 times greater than in the sham rats (Figure 6A). Additionally, there was a significant decrease in plasma GLP-1 in HF rats (~25%) compared with the sham rats (Figure 6A). The level of the GLP-1 receptor in the heart was examined using immunoblotting and normalized to actin. As depicted in Figure 6B, the GLP-1 receptor was significantly more abundant in the HF rats than in the sham rats (118 ± 21%, P<0.05).

Sitagliptin remarkably increased cardiac GLP-1 receptor expression compared with the sham rats (180 ± 28%, P< 0.01) and with the HF rats (63 ± 5%, P< 0.05). The signaling pathways transduced downstream of the cardiac GLP-1 receptor were examined using ELISA and immunoblotting. Sitagliptin treatment increased cardiac PKA activity compared to HF and sham rats, suggesting the activation of the cAMP-PKA pathway (Figure 6C). Similarly, the ratio of phosphorylated to total Akt (114 ± 27%, P< 0.01) increased in the hearts of the sitagliptin-treated radiofrequency LV-ablated rats compared with the HF rats and (104 ± 30%, P< 0.01) compared with the sham rats (Figure 6D). The expression of Bcl-2 and Bax, apoptosis-related proteins downstream of Akt, were also examined (Figure 6E). Cardiac Bcl-2 expression was decreased in
HF rats relative to sham (70 ± 4%, P<0.001) and to sitagliptin-treated radiofrequency LV-ablated rats (42 ± 2%, P<0.05). Conversely, Bax expression was increased in the heart of HF rats relative to sham (126 ± 13%, P<0.01) and to sitagliptin-treated radiofrequency LV-ablated rats (36 ± 3, P<0.05). Consistent with the data shown in Figure 5C, the Bcl-2 to Bax ratio was decreased in HF rats compared to sham, and this reduction was significantly mitigated by treatment with sitagliptin.

**Effect of DPPIV inhibition on total BNP circulating level, on kidney function and on the stimulation of renoprotective signaling pathway**

Plasma total BNP was greater in the HF rats than in the sham rats and the radiofrequency LV-ablated rats treated with sitagliptin (Figure 7A). Despite the higher circulating total BNP, renal PKG activity was lower in HF rats compared with sham rats (28 ± 3%, P<0.05) and with sitagliptin-treated radiofrequency LV-ablated rats (36 ± 3%, P<0.05) (Figure 7B). As previously shown, urinary output (Figure 7C), urinary sodium (Figure 7D), and glomerular filtration rate (GFR) (Figure 7E) were not significantly different between HF and sham rats. Treatment with sitagliptin did not alter urinary flow or fractional sodium excretion compared to sham and HF rats. However, as shown in Figure 7E, GFR was modestly but significantly increased by sitagliptin compared to HF rats.

**Discussion**

The present work demonstrated that circulating DPPIV activity correlates with poorer cardiovascular outcomes in human and experimental HF. The upregulation of DPPIV activity and expression are increased on the surface of heart cells in HF rats, particularly endothelial cells, suggesting that this peptidase may be directly involved in cardiac dysfunction.
Furthermore, we determined that treating radiofrequency LV-ablated rats with the DPPIV inhibitor sitagliptin significantly attenuates HF-related cardiac remodeling and dysfunction. The cardioprotective effects of DPPIV inhibition seem to be partially attributed to increased GLP-1 bioavailability and stimulation of the cardiac GLP-1 receptor. These results confirm and extend accumulating evidence that the inhibition of DPPIV ameliorates cardiac function in experimental models of cardiovascular disease.

Enhanced serum or plasma DPPIV activity has been noted in patients with cardiovascular and/or metabolic diseases such as type 1 and 2 diabetes,17,18 obesity19 and pulmonary hypertension.20 This work demonstrated that circulating DPPIV activity is increased in HF patients and in radiofrequency LV ablation-induced HF rats. The higher DPPIV activity observed in HF suggests that this condition may involve greater degradation of a wide range of DPPIV substrates that possess cardioactive, vasoactive and/or renal effects. The reduced bioavailability of these molecules after myocardial injury may lead to HF aggravation and decompensation. Accordingly, in addition to the fact that DPPIV-knockout mice exhibit increased survival after experimental myocardial infarction,21 our observation that DPPIV correlates with poorer cardiovascular outcomes in patients and in a rat model further substantiates the potential therapeutic benefits of DPPIV inhibition in HF.

Long-term treatment of radiofrequency LV-ablated rats with sitagliptin increased circulating active GLP-1 by approximately 3-fold. Surprisingly, the hearts of radiofrequency LV-ablated rats treated with sitagliptin expressed significantly higher levels of the GLP-1 receptor compared with the sham and HF rats. Taken together, these findings suggest that enhanced coupling of GLP-1 to its cardiac receptor may occur, and this enhanced coupling may represent one possible mechanism underlying the observed sitagliptin-induced cardioprotection after...
radiofrequency myocardial injury. Accordingly, we observed that the cardioprotective signaling pathways transduced downstream of the GLP-1 receptor,\(^7\) including protein kinase A, Akt, and the anti-apoptotic protein Bcl-2, were activated by sitagliptin treatment.

Several independent lines of evidence from both animal and human studies indicate that GLP-1,\(^8,22,23\) GLP-1 analogs\(^{24}\) and GLP-1 receptor agonists\(^{23,25}\) exert cardioprotective benefits in HF. However, far fewer studies have examined the role of DPPIV inhibition in preventing or reversing HF. The administration of sitagliptin (30 mg/kg/day) to pigs with pacing-induced HF improved their LV hemodynamics, reduced their heart rate and preserved their renal function.\(^{26}\) Conversely, the administration of the DPPIV inhibitor vildagliptin (15 mg/kg/day) to rats either before or after myocardial infarction did not prevent or reverse HF-related cardiac dysfunction.\(^{27}\) In line with those findings in pigs, we discovered that the administration of sitagliptin (40 mg/kg, twice daily) to radiofrequency LV-ablated rats has a protective effect on LV hemodynamic function. Of note, those two previous studies and ours differ in terms of the dose of the DPPIV inhibitor used, the frequency of administration and how the inhibitors were orally administered (drinking water vs. gavage). Thus, as mentioned,\(^{27}\) the lack of vildagliptin-induced cardioprotection in rats with post-myocardial infarct-induced HF may be due at least in part to the low dosage employed. The use of a low dose produced a lower level of circulating DPPIV inhibition compared with our study (~70% vs. 90%) and possibly a lower level of enzyme catalytic inhibition on target tissues, such as the heart, blood vessels and kidneys, where the DPPIV substrates exert their protective actions.

The findings that serum DPPIV activity correlates with serum sodium level in HF patients and with lung congestion in HF rats imply that higher DPPIV activity results in worse cardiovascular outcomes in human and experimental HF. Of note, we previously discovered that
DPPIV physically\textsuperscript{28} and functionally\textsuperscript{29, 30} interacts with the Na\textsuperscript{+}/H\textsuperscript{+} exchanger isoform 3 (NHE3), the major apical transcellular pathway for sodium reabsorption in the renal proximal tubule. Our group also determined that NHE3-mediated sodium reabsorption is increased in the proximal tubule of rats with HF,\textsuperscript{15} which may contribute to lower sodium and water excretion, extracellular volume expansion and peripheral and pulmonary edema. Enhanced NHE3 transport activity may also diminish the sodium delivery to the distal nephron, thus conferring resistance to the natriuretic actions of BNP in patients and experimental models of HF.

Inhibitors of DPPIV,\textsuperscript{29, 30} native GLP-1,\textsuperscript{31} and GLP-1 receptor agonists\textsuperscript{32} inhibit NHE3 activity. Therefore, from a therapeutic point of view, DPPIV inhibition may improve the renal handing of sodium and water by increasing the GLP-1 bioactivity and inhibiting NHE3-mediated sodium reabsorption in the renal proximal tubule. This, in turn, may create a higher responsiveness to the renal actions of BNP in the distal nephron of HF patients and experimental models. This hypothesis is corroborated by our findings that (i) sitagliptin-treated radiofrequency LV-ablated rats display a remarkable reduction in congestive HF parameters compared with LV-ablated rats not given sitagliptin; (ii) the sitagliptin-treated rats exhibit lower circulating total BNP but higher renal cortical PKG activity than in the HF rats; and (iii) renal cortical NHE3 protein expression is decreased by (46 ± 4%, P<0.05) in sitagliptin-treated rats compared to HF rats (Supplementary Figure 2C). Furthermore, DPPIV inhibition may directly increase the bioactivity of BNP(1-32) and potentiate its vascular and renal actions. In this regard, Gomez and colleagues discovered that acute i.v. administration of BNP(1-32) to sitagliptin-treated HF pigs improved cardiac performance and contractility, whereas no beneficial effect was observed when BNP(1-32) was administered to HF pigs treated with placebo.\textsuperscript{26} Moreover, we found that sitagliptin-treated rats display higher GFR than HF rats, which may be mediated through an
increase in the active form of BNP. The assay we used to measure BNP does not distinguish among the several circulating molecular forms of BNP. Thus, further investigation will be necessary to address the question of whether DPPIV inhibition is capable of increasing the ratio of BNP(1-32) to BNP(3-32) in HF.

The stimulus that increases the circulating level and activity of DPPIV in HF remains to be determined. Unfortunately, few data are available regarding the origin of the soluble form of DPPIV. This phenomenon has been attributed to the proteolytic cleavage of DPPIV from the surface of peripheral lymphocytes, especially T-lymphocytes, through an as yet unidentified sheddase. Indeed, some reports have demonstrated that lymphocyte DPPIV expression is decreased in pathological conditions in which the serum DPPIV activity is elevated. Currenty, one can only speculate that the sheddase that releases DPPIV from the cell surface to the blood stream is upregulated in HF as well as in other cardiovascular and metabolic diseases in which the serum DPPIV activity is higher than in healthy subjects.

Sources of Funding

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Disclosures

None.
References


**Table.** Biometric and hemodynamic parameters of sham and experimental heart failure rats treated with the DPPIV inhibitor sitagliptin (HF + IDPPIV) or untreated (HF)

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<th>Sham (n = 9-12)</th>
<th>HF (n = 12-16)</th>
<th>HF + IDPPIV (n = 11-16)</th>
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<td>406 ± 12</td>
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Values are means ± SEM. BW, body weight; HR, heart rate; MAP, mean arterial pressure; LVSP, left ventricular (LV) systolic pressure; LVEDP, LV end-diastolic pressure; CO, cardiac output; EF, ejection fraction; SW, stroke work; +dP/dt_max and −dP/dt_max, maximal rate of LV pressure increment and decrement, respectively; Tau, time constant of LV pressure decay; TPR, total peripheral resistance; SV, stroke volume; EDV, end-diastolic volume. *P<0.05 and **P<0.01 vs. sham; /P<0.05 and /P<0.01 vs. HF.
Figure Legends

**Figure 1. Circulating DPPIV activity in heart failure (HF) patients and normal subjects.**

(A) Frequency distribution of serum DPPIV activity from 190 patients with HF and 42 control subjects without cardiovascular disease. In both groups, serum DPPIV activity exhibited a Gaussian distribution. (B) Average serum DPPIV activity in HF patients and control subjects. The values are the means ± SD. ***P<0.001 vs. CTRL.

**Figure 2. DPPIV activity and expression in the plasma and heart of a rat model of chronic heart failure.** DPPIV activity in plasma (A) and (B) heart membrane proteins from radiofrequency LV ablation-induced HF rats treated (HF+ IDPPIV) or not (HF) with the DPPIV inhibitor sitagliptin and sham rats. (C) An equal volume of plasma (0.5 μL) from each animal was subjected to SDS-PAGE, transferred to PVDF membranes and incubated with an antibody against DPPIV. The membranes were stained with Ponceau S prior to antibody incubation, and albumin was used as an internal control. (D) Equal amounts of protein (50 μg for DPPIV and 2.5 μg for actin) from the heart membrane fractions isolated from sham or HF rats were subjected to immunoblotting for DPPIV and actin; the latter was used as an internal control. The values are the means ± SEM. The number of animals analyzed in each group is indicated within the bar. **P<0.01 and ***P<0.001 vs. sham; #P<0.05 and ###P<0.001 vs. HF. (E) Representative images of the heart sections from radiofrequency LV ablation-induced HF rats (HF) and sham rats co-stained with antibodies against DPPIV (red) and the endothelial cell marker von Willebrand factor (green). The scale bar is 20 μm.
Figure 3. Plasma DPPIV activity correlates with the severity of cardiac dysfunction and congestion in the rat model of heart failure. Correlations between plasma DPPIV activity and (A) ejection fraction, (B) end-diastolic pressure and (C) lung weight indexed by body weight in radiofrequency LV ablation-induced HF rats. The filled symbols represent the mean ± SEM of the sham group. The correlation coefficients and P values were obtained using Pearson’s correlation test and the lines represent linear regression plotting.

Figure 4. Effect of DPPIV inhibition on the end-systolic P-V relationship (ESPVR) and end-diastolic P-V relationship (EDPVR) in the rat model of HF. The original recordings of the representative P-V loops at different preloads during vena cava occlusion demonstrated differences in the ESPVR and EDPVR between (A) sham and (B) HF as well as (C) between HF and HF+DPPIV inhibitor. (D) The overall slope values of the ESPVR (left) as an index of systolic performance and EDPVR (right) as an index of diastolic stiffness. **P<0.01 and ***P<0.001 vs. sham; ##P<0.01 and ###P<0.001 vs. HF.

Figure 5. Effect of DPPIV inhibition on cardiac remodeling and apoptotic rates in the rat model of heart failure. (A) Myocardial hypertrophy was assessed by measuring the cardiomyocyte nuclear volumes in hematoxylin-eosin-stained heart samples. (B) Interstitial collagen was evaluated in equivalent regions of the remote myocardium from each group. (C) The number of apoptotic nuclei was evaluated at ×40 magnification in six random fields per section and expressed as TUNEL-positive nuclei per total nuclei and the number of TUNEL-positive nuclei over the total number of nuclei per field (For more details, see Supplemental Table 2). *P<0.05, **P<0.01 and ***P<0.001 vs. sham; ##P<0.01 and ###P<0.001 vs. HF.
Figure 6. Effects of DPPIV inhibition on GLP-1 circulating level, GLP-1 receptor expression in the heart and the activation of cardioprotective signaling pathways. (A) Circulating active GLP-1(7-36) was measured using ELISA in sham, radiofrequency LV ablation-induced HF rats (HF) and LV-ablated rats treated with sitagliptin for six weeks (HF + IDPPIV). (B) Representative immunoblots and graphical representation of heart membrane proteins isolated from sham rats, HF rats or HF + IDPPIV rats probed with an antibody against the GLP-1 receptor. (C) PKA activity was measured by ELISA. (D-E) Representative immunoblots and graphical representation of heart membrane proteins from the three groups of rats probed with (D) antibodies against phosphorylated and total Akt and (E) antibodies against Bcl-2 and Bax. Anti-actin was used as an internal control. The values are the means ± SEM. n = 6 rats/group. *P< 0.05, **P< 0.01 and ***P< 0.001 vs. sham. #P< 0.05 and ###P< 0.001 vs. HF.

Figure 7. Effect of DPPIV inhibition on total BNP circulating level, PKG activity and renal function. (A) Circulating total BNP. (B) Activity of PKG in renal cortex of sham, radiofrequency LV ablation-induced HF rats (HF) and LV-ablated rats treated with sitagliptin for six weeks (HF + IDPPIV) were measured by ELISA. (C) Urine output was measured gravimetrically. (D) Urinary sodium. (E) The inulin clearance was used to measure glomerular filtration rate (GFR). The values are the means ± SEM. The number of animals analyzed in each group is indicated within the bar. **P< 0.01 and ***P< 0.001 vs. sham. *P< 0.05, **P<0.01 and ###P< 0.001 vs. HF.
Circulating Dipeptidyl Peptidase IV Activity Correlates with Cardiac Dysfunction in Human and Experimental Heart Failure


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Circulating Dipeptidyl Peptidase IV Activity Correlates with Cardiac Dysfunction in Human and Experimental Heart Failure

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Supplemental Methods

Reagents and antibodies – Januvia tablets (Merck & Company, Inc.) containing 100 mg of sitagliptin monophosphate were purchased from a local pharmacy. Sitagliptin was extracted as previously described by Jackson & Mi^1 with some modifications. Briefly, four 100 mg Januvia tablets were added to 10 ml of water and incubated in the refrigerator for 1 hour to dissolve the tablets. The suspension was vortexed and centrifuged at 2,000 g for 10 minutes to remove the majority of the excipients. The supernatant was then diluted in water to a final concentration of 20 mg/ml sitagliptin (determined based on the labeled amount of sitagliptin in the tablet). The monoclonal antibody against DPPIV, clone 5E8, and the polyclonal antibodies against GLP-1 receptor, BCl2, and Bax were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The monoclonal antibody against actin JLA20 was from Calbiochem (San Diego, CA). Antibodies against total and phospho-Akt (Ser473) were from Cell Signaling Technology (Beverly, MA). Secondary antibodies were from Life
Technologies Corporation (Carlsbad, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Selection of patients with HF – One hundred ninety HF patients from an ongoing inception cohort from the General Outpatient Clinic of the Heart Institute of USP were included in this study. The ascertainment period was from 2005 to 2010. After enrollment, the serum samples were frozen at -80°C until analysis. The HF diagnosis was made according to previously published criteria, and the classification of HF etiology followed previous recommendations. Patients with symptomatic HF of varying etiology and a LVEF ≤ 45% on two-dimensional transthoracic Doppler echocardiography were eligible for enrollment into the cohort. We excluded patients with cardiomyopathy due to valvular heart disease who would be candidates for conventional surgical treatment, such as valve repair or replacement, including patients with hypertrophic cardiomyopathy, chronic obstructive pulmonary disease, recent myocardial infarction and/or unstable angina. Patients with severe renal or hepatic dysfunction, severe peripheral artery disease, cerebrovascular disease, active infection, coexisting neoplasm or active peptic ulcer disease were excluded. In addition, 42 healthy subjects with no prior history of HF or cardiovascular disease were selected as controls.

Animal protocols, surgical procedures and drug treatment – Experimental HF was induced in male Wistar rats via left ventricular (LV) myocardial injury using radiofrequency catheter ablation, as previously described. Sham-operated rats underwent left thoracotomy and were mock-ablated.

Blood collection and plasma preparation to measure active GLP-1 and total BNP - Blood was withdrawn from the retro-orbital plexus, immediately transferred into chilled tubes containing EDTA (1 mg/ml) and 10 µM of the DPPIV inhibitor P32/98.
(Enzo Life Sciences Inc., Farmingdale, NY) and centrifuged at 4000×g at 4 °C for 10 min. Samples were extracted using a solid-phase extraction C18-E column (Strata Phenomenex, Torrance, CA) in accordance with the manufacturer’s instructions.

**Renal function** – Rats were anesthetized with a mixture of ketamine, xylazine, and acepromazine (64.9, 3.20, and 0.78 mg/kg subcutaneously, respectively) and placed on a heated surgical table to maintain body temperature. After a tracheostomy, polyethylene catheters were inserted into the jugular vein and the urinary bladder for inulin infusion and urine collection, respectively. To control mean arterial pressure and allow blood sampling, a PE-60 catheter was inserted into the right carotid artery. Glomerular filtration rate was determined by measuring the clearance of inulin as follows. First, a loading dose of inulin (100 mg/kg in 0.9% saline) was administered. Subsequently, continuous infusions of inulin (10 mg/kg in 0.9% saline) were given at 0.04 ml/min. Three consecutive 30-min periods of urine collection were performed. Blood samples were obtained at the beginning and the end of the experiment. Plasma and urine sodium concentrations were measured by flame photometry (Micronal B262, São Paulo, SP, Brazil), and inulin was determined using the anthrone method 6.

**Evaluation of cardiac function** - Surgical procedures for hemodynamic assessment were performed as previously described.7 In brief, anesthetized rats (ketamine 50 mg/kg and xylazine 10 mg/kg, i.p.) were placed on a heated rodent operating table (37°C). Thereafter, a microtip Pressure-Volume catheter (Mikro-Tip® 1.4 F SPR 839, Millar Instruments Inc., Houston, TX) was positioned into the LV cavity by means of right carotid artery catheterization. After 10-15 minutes of measurement under steady-state conditions, LV performance was evaluated by determination of P-V relationships during gradual changes in preload obtained by gently compressing the inferior cava vein with a swab. To assess cardiac performance, the end-systolic P-V
relation (ESPVR) and the relationships of LV volume with EDP (EDPVR), dP/dt_{max}, and stroke work were used. LV stiffness was assessed by the slope of the linear regression line fitted with end-diastolic P-V relationships. At the end of each experiment, 50 µl of hypertonic saline was injected intravenously, and from the shift of P-V signals, parallel conductance volume (V_P) was calculated and used for correction of cardiac mass volume. Data were acquired for computer analysis (PVA Software, Millar Instruments Inc., Houston, TX) using the LabChart 7 Software System (PowerLab, ADInstruments, Bella Vista, NSW, Australia). At the end of the hemodynamic measurements, rats were killed by decapitation, and their hearts and lungs were immediately removed.

*Heart membrane protein isolation* – Excised hearts from rats were minced with opposing razor blades and homogenized in a Polytron PT 2100 homogenizer (Kinematica, AG, Switzerland) in an ice-cold buffer containing 1 mM EDTA; 250 mM sucrose; 10 mM Tris, pH 7.5; and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL). The homogenate was centrifuged at 4,000 rpm for 10 min at 4°C. The supernatant was removed and subjected to 90 min of centrifugation at 30,000 rpm and 4°C to pellet the fraction enriched for the plasma membrane. The protein concentration was determined by the Lowry method. 

*SDS-PAGE and immunoblotting* – Plasma or heart membrane protein samples were solubilized in Laemmli sample buffer and separated by SDS-PAGE using 7.5% polyacrylamide gels. For immunoblotting, proteins were transferred to PVDF membranes (Millipore Immobilon-P, Millipore, Bedford, MA) at 350 mA for 8-10 hours at 4°C with a TE 62 transfer electrophoresis unit (GE HealthCare). Membranes were incubated first in Blotto (5% non-fat dry milk or 5% bovine serum albumin and 0.1% Tween 20 in PBS, pH 7.4) for 1 hour to block nonspecific antibody binding, followed by overnight
incubation in primary antibodies diluted in Blotto (1:200-1:50,000). The sheets were then washed in Blotto and incubated for 1 hour with an appropriate HRP-conjugated secondary antibody diluted 1:2,000 in Blotto. After washing five times in Blotto and twice in PBS (pH 7.4), an enhanced chemiluminescence detection (ECL) system (GE HealthCare) was used for visualization of the bound antibodies. The visualized bands were digitized using an ImageScanner (GE HealthCare) and quantified using the Scion Image Software package (Scion Corporation, Frederick, MD).

**Immunofluorescence** – Hearts were fixed with formaldehyde, paraffin-embedded and sectioned. An antigen retrieval step was used in all experiments, by heating samples in a citrate buffer (Spring-Bioscience) to 95°C for 30 min. Dual-fluorescence immunostaining was performed by pre-incubating heart sections with 5% bovine serum albumin and then with anti-DPPIV antibody (1:50) and human anti-rabbit von Willebrand factor antibody (1:50) overnight at 4°C. Then, sections were washed and incubated with Alexa Fluor 555 (1:400) and Alexa Fluor 488 secondary antibodies (1:400) for 90 min. Immunofluorescence staining was detected using a Carl Zeiss 510 LMS confocal system connected to an Axiovert microscope.

**Morphometric analysis** - Histological analyses were performed to evaluate tissue remodeling. The observers were blinded to the experimental group. Five-micrometer sections of paraffin-embedded tissue were mounted onto slides and stained with hematoxylin and eosin for myocyte analysis. Picrosirius red was used to evaluate fibrosis and quantify the injury scar. A computerized image acquisition system (Leica Imaging Systems, Bannockburn, IL) was used for the analyses. As an estimate of myocyte hypertrophy, the average nuclear volume was determined in 50–70 cardiomyocytes cut longitudinally (acquired in 5 randomized 400× magnification fields per animal) and calculated according to the following equation: nuclear volume = \( \pi \times D^2 \)
$\times \frac{d^2}{6}$ (d = shorter nuclear diameter; D = longer diameter), as previously described 9. 10. Interstitial fibrosis in the remodeled LV was evaluated as the area occupied by collagen fibers, excluding stained ablation scar and perivascular fibers. After digitalization, the red-stained areas were quantified as the average percentage of the total area from each of 5 randomized 200× magnification fields per animal. Myocardial lesion was quantified as the percentage of the LV perimeter containing scar tissue. In addition, the thickness of the scarred myocardial wall was determined in the mid-portion of the injury in 4 transverse LV histological sections.

*Terminal transferase-mediated dUTP nick end labeling (TUNEL) assay* – DNA fragmentation was detected using an in situ cell death detection kit, AP (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer’s instructions. Briefly, tissue sections were deparaffinized in citrisolv (Fisher Scientific Company, Pittsburgh, PA), rehydrated in serial alcohol dilutions and permeabilized with 0.5% triton X-100 in 0.1% sodium citrate. The reaction with terminal deoxynucleotidyl transferase and alkaline phosphatase conversion was performed and the cross-sections were examined by light microscopy. The percentage of TUNEL-positive nuclei was quantified by an observer blinded to the three conditions using Leica Qwin 2.2 Q500IW software. We counted TUNEL-positive for cardiomyocytes and infiltrating cells. Supplemental table 2 shows the average cell count for each group.
Supplemental Tables

Table 1: Clinical characteristics of the studied HF population

<table>
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<td>Mean age (SD)</td>
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<tr>
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<tr>
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<td>Black</td>
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<td>Left ventricular diameter (SD)</td>
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<tr>
<td>Etiology (%)</td>
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Table 2: Tunel-assay for apoptotic nuclei

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<td>Total of nuclei analyzed (cells per field)</td>
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<td>143 ± 5</td>
<td>116 ± 4#</td>
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<td>Total of nuclei analyzed (cells per µm²)</td>
<td>1645 ± 85</td>
<td>1902 ± 70</td>
<td>1544 ± 48#</td>
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<tr>
<td>Tunel-positive nuclei (cells per field)</td>
<td>21 ± 7</td>
<td>105 ± 4***</td>
<td>46 ± 2****</td>
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<tr>
<td>Tunel-positive nuclei (cells per µm²)</td>
<td>284 ± 88</td>
<td>1404 ± 55***</td>
<td>608 ± 21****</td>
</tr>
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</table>

Values are means ± SE; n = 5–7 animals per group. *P < 0.05 and ***P<0.001 vs. control; ###P<0.001 vs. HF.
Serum DPPIV activity in 190 HF patients plotted against different parameters from the respective patient. DPPIV activity exhibited significant correlations with (A) LVEF (B) age, (C) plasma Na$^+$ concentration and (D) hemoglobin.
Figure 2 - Activity and expression of dipeptidyl peptidase IV and expression of Na⁺/H⁺ exchanger isoform 3 (NHE3) in the rat kidney. (A) DPPIV activity in renal cortical membrane proteins from radiofrequency LV ablation-induced HF rats (HF) and sham rats. (B) Equal amounts of protein (20 µg for DPPIV and 5.0 µg for actin) from the renal cortex of sham or HF rats were subjected to immunoblotting for DPPIV and actin. (C) Equal amounts of protein (20 µg for NHE3 and 5.0 µg for actin) from the renal cortex of sham, HF rats, or LV-ablated rats treated with sitagliptin for six weeks (HF + IDPPPIV) were subjected to immunoblotting for NHE3 and actin. The number of animals analyzed in each group is indicated within the bar. The values are the means ± SEM. *P< 0.05 vs. Sham and #P< 0.05 vs. HF.
Supplemental References


