Genetic deletion or pharmacological inhibition of dipeptidyl peptidase-4 improves cardiovascular outcomes following myocardial infarction in mice

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Objective: Glucagon-like peptide-1(7-36)amide (GLP-1), is cleaved by dipeptidyl peptidase-4 (DPP-4) to GLP-1(9-36)amide. We examined whether chemical inhibition or genetic elimination of DPP-4 activity impacts cardiovascular function in normoglycemic and diabetic mice following experimental myocardial infarction.

Research Design & Methods: Cardiac structure and function was assessed by hemodynamic monitoring and echocardiography in DPP-4 knockout (Dpp4−/−) mice vs. wild-type (Dpp4+/+) littermate controls and following left anterior descending (LAD) coronary artery ligation-induced myocardial infarction (MI). Effects of sustained DPP-4 inhibition with sitagliptin vs. treatment with metformin were ascertained following experimental MI in a high fat diet-streptozotocin model of murine diabetes. Functional recovery from ischemia-reperfusion (I/R) injury was measured in isolated hearts from Dpp4−/− vs. Dpp4+/+ littermates, and from normoglycemic wild-type (WT) mice treated with sitagliptin or metformin. Cardioprotective signaling in the murine heart was examined by RT-PCR and Western blot analyses.

Results: Dpp4−/− mice exhibited normal indices of cardiac structure and function. Survival post-MI was modestly improved in normoglycemic Dpp4−/− mice. Increased cardiac expression of pAKT, pGSK3β, and ANP was detected in the non-ischemic Dpp4−/− heart and HO-1, ANP and pGSK3β proteins were induced in non-ischemic hearts from diabetic mice treated with sitagliptin or metformin. Sitagliptin and metformin treatment of wild-type diabetic mice reduced mortality following myocardial infarction. Sitagliptin improved functional recovery following I/R injury ex vivo in WT mice with similar protection from I/R injury also manifest in hearts from Dpp4−/− vs. Dpp4+/+ mice.

Conclusions: Genetic disruption or chemical inhibition of DPP-4 does not impair cardiovascular function in the normoglycemic or diabetic mouse heart.
Type 2 diabetes (T2DM) is associated with an increased risk of cardiovascular disease (CVD), hence there is considerable interest in strategies that reduce cardiovascular morbidity and mortality in diabetic subjects. Although aggressive treatment of blood pressure and dyslipidemia reduces cardiovascular events in both non-diabetic and diabetic patients, whether reduction in blood glucose alone reduces cardiac events in subjects with established diabetes remains controversial (1). Moreover, pharmacotherapy of diabetes using agents with unique anti-diabetic mechanisms may be associated with differential and occasionally unexpected adverse effects on cardiovascular outcomes, independent of effects on glucose control (2; 3). Therefore, a detailed understanding of the unique cardiovascular benefits and risks of each anti-diabetic drug used to treat diabetes seems prudent.

The two most recently approved drug categories for the treatment of T2DM, GLP-1 receptor (GLP-1R) agonists and DPP-4 inhibitors exert their anti-diabetic actions largely through potentiation of GLP-1R activation (4). As these agents have only been utilized clinically for several years, there is scant data on cardiovascular outcomes associated with these incretin-based therapies. GLP-1 improves endothelial function in subjects with T2DM (5), and transient GLP-1 administration improves cardiovascular outcomes in subjects with myocardial infarction (MI) or congestive heart failure (CHF) (6; 7). Moreover, preclinical data demonstrates that GLP-1 is cardioprotective when administered prior to the induction of ischemia (8-10). Furthermore, therapy with the GLP-1R agonists exenatide and liraglutide is associated with blood pressure reduction in the majority of treated subjects (11; 12). Hence, although limited, the available data support the hypothesis that anti-diabetic therapy with GLP-1 may be associated with beneficial effects on cardiovascular outcomes. Nevertheless, as GLP-1R agonists may produce weight loss (13), the extent to which the salutary effects of GLP-1R activation on the cardiovascular system in vivo reflect the beneficial consequences of weight loss remains unclear.

In contrast, much less is known about the cardiovascular biology of DPP-4. Unlike therapy with GLP-1R agonists, the use of sitagliptin, saxagliptin or vildagliptin has not been associated with weight loss or sustained improvement in lipid profiles (4). Moreover inhibition of DPP-4 enzyme activity modulates the activity of cardioactive peptides such as brain natriuretic peptide (BNP), neuropeptide Y (NPY), and stromal cell-derived factor-1 (SDF-1) (14) via non-GLP-1 mechanisms of action. More recently, GLP-1(9-36), a peptide metabolite derived from native GLP-1(7-36)amide following cleavage by DPP-4, has been shown to exert cardioprotective actions in rodents (15; 16) and improve cardiovascular function in dogs with CHF (17). Accordingly, to delineate the importance of DPP-4 for cardiovascular biology in vivo, we studied cardiovascular function in Dpp4−/− mice in vivo, in isolated perfused Dpp4+/+ and Dpp4−/− hearts ex vivo, and in wild type diabetic mice subjected to experimental MI and treated with the DPP-4 inhibitor sitagliptin.

**MATERIALS & METHODS**

**Animal models & drug treatments:**
Experimental procedures adhered to approved protocols of the University Health Network and Mt. Sinai Hospital Animal Care Committees. Mice were housed under pathogen-free conditions in microisolator cages and maintained on a 12 h light (07:00)/dark (19:00) cycle with access to standard rodent chow and water *ad libitum.*
except where noted. All experiments used age-and sex-matched littermates. Dpp4<sup>−/−</sup> mice were inbred on the C57BL/6 background (18). Experimental animals were derived by crosses between heterozygous Dpp4<sup>+/−</sup> mice to generate Dpp4<sup>−/−</sup> and Dpp4<sup>+/+</sup> littermate mice. All genotypes were confirmed by PCR analyses of tail DNA.

C57BL/6 mice, 4-wk old, were purchased from Taconic (Germantown, NY), and housed as described above, but placed on a high-fat diet (HFD: 45% kcal from fat; Research Diets, New Brunswick, NJ). After 5 wks of HFD, mice were fasted for 5 h, and then injected with a single dose of streptozotocin (STZ; Sigma) (75 mg/kg i.p.) as a freshly prepared solution in 0.1 mM sodium citrate, pH 5.5. Mice were then randomized to treatment with either HFD alone, HFD plus a DPP-4 inhibitor (sitagliptin, 250 mg/kg/day) (19) or HFD plus metformin (450 mg/kg/day). The dose of metformin was chosen based on related studies (20), and following pilot studies demonstrating optimal anti-diabetic actions without significant effects on food intake or body weight. This dose of sitagliptin does not affect food intake yet results in 90% inhibition of DPP-4 (19; 21). Sitagliptin and metformin were supplied by Merck Research Labs (Rahway, NJ).

For RNA and protein analyses by real-time quantitative PCR and Western blot respectively, heart tissue was obtained from separate groups of normoglycemic Dpp4<sup>+/+</sup> or Dpp4<sup>−/−</sup> mice fed regular chow, or wild-type C57BL/6 mice exposed to HFD for 4-wk, given a single dose of STZ (75 mg/kg), and then treated with either HFD alone, or HFD plus (a) metformin, (b) sitagliptin or (c) the GLP-1R agonist liraglutide (Novo Nordisk, Novo Alle, Bagsvaerd, Denmark) (10), 75 µg/kg i.p. twice daily for an additional 7 days. All animals were euthanized by exposure to carbon dioxide.

Isolated heart preparations were from 12 wk-old male Dpp4<sup>−/−</sup> and, Dpp4<sup>+/+</sup> littermates, or separate groups of wild-type C57BL/6 mice. Only isolated mouse hearts exhibiting a heart rate >350 b.p.m. were used. Wild-type C57BL/6 mice were treated with an i.p. injection of either sitagliptin (20 mg/kg) or metformin (125 µg/kg) or saline at 24h and 1h prior to heart excision.

**Metabolic measurements:** Oral glucose tolerance tests (OGTTs) were performed in sitagliptin- or metformin-treated wild-type C57BL/6 mice and untreated controls. Mice were fasted for 16 h, and administered glucose (1.5 mg/g) via oral gavage. HbA1c and blood glucose levels were measured on whole blood using the DCA 2000+ Analyzer and a Glucometer (both Bayer, Toronto, ON). GLP-1(7-36)amide levels were measured by a Meso Scale Discovery (MSD) metabolic sandwich immunoassay (MSD, Gaithersburg, MD) in plasma from mice fasted for 5h.

**Coronary artery ligation:** Experimental MI was induced by LAD artery ligation as described (22). Briefly, 12 wk old male and female Dpp4<sup>−/−</sup> and Dpp4<sup>+/+</sup> mice were anesthetized using 1% isoflurane, intubated, and ventilated with room air using a positive-pressure respirator (model 680, Harvard, South Natick, MA). Left thoracotomy was performed via the fourth intercostal space; the lungs were retracted to expose the heart, and the pericardium was opened. The LAD was ligated with an 8-0 silk suture near its origin between the pulmonary outflow tract and the edge of the left atrium. Acute myocardial ischemia was considered successful when the anterior wall of the left ventricle (LV) turned pale. The lungs were inflated by an increase in positive end-expiratory pressure, and the thoracotomy was closed. Animals were kept on the ventilator until awake. Sham operation differed only in that the 8-0 silk suture was passed under the coronary artery, then removed.

**Ultrasound biomicroscopy in mice after LAD artery ligation:** Male C57BL/6
mice, 10-wk old from Taconic (Germantown, NY), were housed under pathogen-free conditions in microisolator cages and maintained on a 12 h light (07:00)/dark (19:00) cycle. At 11 weeks of age, mice were placed on either a control diet (Research Diets, New Brunswick, NJ), or a diet containing sitagliptin (250 mg/kg/day), or metformin (450 mg/kg/day) for one week prior to LAD ligation. High frequency ultrasound imaging was carried out on day 4 post-MI. Mice were euthanized on day 5 following coronary artery ligation and hearts were collected for RNA and protein analyses.

Isolated heart preparations: Following administration of heparin (1000 IU/kg s.c.) and sodium pentobarbital (200 mg/kg i.p.), hearts were excised, cannulated through the aorta and perfused at 80 mmHg in a Langendorff apparatus with gassed (95% O₂, 5% CO₂) Krebs-Hensleit buffer (mM: 118 NaCl, 4.7 KCl, 11 glucose, 1.2 MgSO₄, 25 NaHCO₃, 1.2 KH₂PO₄, 2.5 CaCl₂) maintained at 37°C (Harvard Apparatus, Holliston, MA) as described (15). For measurement of isovolumetric pressures, a small plastic balloon was inflated in the LV via a small left atrial incision. Both end-systolic (LVESP) and end-diastolic (LVEDP) pressures were monitored, the latter maintained between 4-8 mmHg throughout the experiment. LV developed pressure (LVDP) was calculated as LVESP-LVEDP.

Ischemia-reperfusion protocol: Hearts underwent 20- and 40-min equilibrium- and perfusion-phases respectively during which hemodynamic parameters were recorded. Global ischemia- and reperfusion-phases were produced by clamping and restoring inflow for 30- and 40-min respectively. For direct infusion of sitagliptin (5µM) (vs. PBS), the agent was added to the perfusion buffer for the final 20 min of the perfusion phase. Recovery of LVDP was measured at the end of reperfusion and expressed as % of LVDP at the end of perfusion (i.e. prior to ischemia) (15).

Histology: Animals were anesthetized using 3% isoflurane/97% air. The chest was opened to expose the heart, where an apical injection of KCl (1M) was used to arrest the heart in diastole. The heart was then perfusion-fixed with 4% buffered formalin at physiological pressure. Hearts were post-fixed in formalin, embedded in paraffin, sectioned at 6 µm, and stained with hematoxylin and eosin (H&E) or Masson’s Trichrome. Cardiac morphometry was performed with LEICA QWin V3 software (2003) using digital planimetry of images obtained from mid-ventricular cross-sections. Infarcted LV area was calculated as a % of total LV area.

Quantitative RT-PCR: Total RNA was prepared from mouse heart using Tri-Reagent (Sigma-Aldrich). First-strand cDNA synthesis employed random hexamers and the Superscript II (Invitrogen) system. Real-time PCR analysis was carried out using Taqman gene expression assays and Universal PCR master mix (Applied Biosystems), using the ABI prism 7900 Sequence Detection System. Primers employed included mouse Akt1 (Mm00437443_m1), Mmp9 (Mm00442991_m1), Ho1 (Mm00516005_m1), Ppara (Mm00440939_m1), Gsk3β (Mm00444911_m1), PI3K (Mm01282781_m1), β-actin (Mm00607939_s1) (Applied Biosystems), and Gapdh (Mm99999915-g1). Relative mRNA transcript levels were quantified with the 2^ΔΔCT method, using β-actin as an internal control.

Western blots: Extracts from whole hearts were prepared as described (23). Following SDS-PAGE, proteins were electrotransferred onto a Hybond-C nitrocellulose membrane (Amersham, Piscataway, NJ). Blots were incubated O/N at 4°C with 1⁰ Ab. Horseradish peroxidase-
conjugated 2° Ab and enhanced chemiluminescence (Amersham, Piscataway, NJ) were used to detect proteins. Primary Abs include p-GSK3β (Ser9) 1:2000 (Cell Signaling, Danvers, MA), ANP 1:500 (Santa Cruz, Santa Cruz, CA), HO-1 1:5000 (Stressgen, Ann Arbour, MA), p-AKT (Ser 473) and total AKT 1:1000 (Cell Signaling), and HSP90 1:2000 (BD Biosciences, San Jose, CA).

Statistics: Data are presented as mean ± S.E.M except where noted. Analyses were performed using Prism software (Version 4.02; GraphPad Software, San Diego, CA). Differences in the number of surviving animals were analyzed using Kaplan-Meier Survival analyses. The remaining results were analyzed using Analysis of Variance (ANOVA) followed by Bonferroni post hoc tests. P<0.05 was considered statistically significant.

RESULTS

Cardiac structure and function in Dpp4^-/- mice. We first verified that Dpp4^-/- mice used in our studies retained the phenotype of improved glucose tolerance and reduced DPP-4 activity as originally described (18). Consistent with previous findings, oral glucose tolerance was improved and plasma DPP-4 activity was markedly reduced in Dpp4^-/- mice (Supplementary Figure 1A-C which can be found in an online appendix at http://diabetes.diabetesjournals.org ). Heart weights did not differ between 12 wk-old sex-matched Dpp4^-/- and Dpp4^+/+ mice (Fig. 1B, P=NS). High-resolution echocardiography did not detect any differences in LV-wall thicknesses, LV-end systolic and -end diastolic dimensions, mitral and aortic flow velocities, LV systolic and diastolic areas, LV outflow tract diameter, aortic ejection time, left atrial size or fractional shortening between Dpp4^-/- and Dpp4^+/+ mice (Table 1, P=NS for all comparisons). Hence, genetic disruption of the Dpp4 gene in mice is not associated with baseline abnormalities in cardiac structure or function.

Myocardial infarction outcomes in normoglycemic Dpp4^-/- mice. To determine whether disruption of the Dpp4 gene modifies the response to cardiac injury, we induced MI in non-diabetic 12-wk old male and female Dpp4^-/- and Dpp4^+/+ mice via permanent surgical LAD ligation (Fig. 1B). At the pre-defined endpoint of 4 wk post-MI, Dpp4^-/- mice exhibited a ~20% absolute increase in survival as compared to Dpp4^+/+ littermate controls (Fig. 1C). Post-MI, the hearts of Dpp4^-/- and Dpp4^+/+ mice underwent similar compensatory hypertrophy (Fig. 1D). Although infarct size was reduced in Dpp4^-/- mice, this difference was not statistically significant (Fig. 1E).

To explore mechanisms mediating the increased survival of Dpp4^-/- mice post MI, we analyzed cardiac mRNA and protein levels of known cardioprotective genes. Normoglycemic non-ischemic Dpp4^-/- mice exhibited small but non-significant increases in Akt1, Gsk3β, Ppara, PI3K, and Ho1 transcripts (Fig. 2B-F). Moreover hearts from Dpp4^-/- mice contained higher levels of phosphorylated AKT (pAKT), pGSK3β (pGSK3β), and atrial natriuretic peptide (ANP) (Fig. 2G-I), proteins known to be regulated by GLP-1R agonists (10) and associated with cardioprotection in vivo (24-27).

Treatment of diabetic mice with metformin or sitagliptin pre and post-MI. As Dpp4^-/- mice are resistant to the development of STZ-induced diabetes (28), we assessed whether reduction of DPP-4 activity is cardioprotective in diabetic Dpp4^+/+ mice. Wild-type mice were placed on a HFD for 4 wks, rendered diabetic with STZ, and maintained for an additional 12 wks on HFD alone or on HFD plus either sitagliptin or metformin (Fig. 3A). After 8 wks on drug treatment or HFD alone, mice were subjected
to LAD ligation and observed for an additional 4 wks (Fig. 3A). Random blood glucose (Fig. 3B), levels of HbA1c (Fig. 3C), and oral glucose tolerance (Fig. 3D-E) were improved to a similar extent with body weights remaining comparable (Fig. 3F) in mice treated with sitagliptin or metformin. Moreover, plasma levels of active GLP-1(7-36)amide were increased to a similar extent in sitagliptin- vs. metformin-treated mice (Fig. 3G).

Cumulative survival assessed up to 4 wks following LAD ligation was improved in mice treated with either sitagliptin or metformin, compared to mice on HFD/STZ alone (Fig. 4A). A significant increase in heart:body weight ratios post-MI was observed only in diabetic mice treated with metformin (Fig. 4B). No differences in infarct size were observed between the 3 groups (Fig. 4C).

To identify potential mechanism(s) underlying improved survival post-MI in sitagliptin- and metformin-treated diabetic mice, we assessed cardiac mRNA and protein levels of candidate pro-survival genes in separate groups of diabetic animals treated for 1 wk with either sitagliptin, metformin, or the GLP-1R agonist, liraglutide. No significant changes were detected in mRNA levels of PI3k, Akt, Ho1, Mmp9, and Pparα after treatment with these anti-diabetic agents (Fig. 5B-F). In contrast, sitagliptin, metformin, and liraglutide increased expression of ANP (Fig. 5G). Sitagliptin and liraglutide, but not metformin, activated the prosurvival kinase AKT (Fig. 5H), whereas all 3 drugs increased levels of HO-1 (Fig. 5I). A modest but non-significant increase in levels of phospho-GSK3β was also observed with all 3 anti-diabetic agents (Fig. 5J).

We next examined whether metformin or sitagliptin treatment of HFD-fed mice produced changes in gene and protein expression in the mouse heart after LAD ligation (Fig. 6A). Levels of Ho1 and Gsk3β RNA transcripts were modestly increased (Fig. 6D,E), whereas Akt1, PI3k, and Pparα RNA transcripts were significantly increased in the post-ischemic heart after metformin treatment (Fig. 6B,C,F). In contrast, sitagliptin treatment was not associated with significant changes in levels of cardioprotective mRNA transcripts post MI (Fig. 6B-F). Neither metformin nor sitagliptin treatment produced detectable changes in levels of AKT, ANP, GSK3β, or HO-1 proteins assessed at day 5 post-MI (Fig 6G-J). Similarly, although heart rate was increased in metformin-treated mice, we did not detect other significant differences in parameters of cardiac function in sitagliptin vs. metformin-treated mice after myocardial infarction (Table 2).

Ischemia-reperfusion injury and metformin vs. DPP-4 inhibition in normoglycemic mouse hearts. To determine whether cardioprotective actions of sitagliptin are observed in the normoglycemic murine heart, we acutely administered sitagliptin, metformin or saline (PBS) to non-diabetic wild-type mice in vivo before assessing recovery of LVDP following I/R injury to their hearts ex vivo (15). Parallel experiments included I/R injury in hearts from normoglycemic Dpp4-/- and Dpp4+/- animals, as well as testing cardioprotective actions of an acute ex vivo sitagliptin infusion (20 min) vs. placebo (PBS) immediately prior to wild-type hearts undergoing I/R injury (Fig. 7A).

Acute administration of metformin or sitagliptin in vivo improved recovery from subsequent I/R injury in normoglycemic mice (Fig. 7B). Recovery of LVDP was also greater in Dpp4-/- hearts vs. Dpp4+/- littermate controls (Fig. 7B). By contrast, sitagliptin administered to the coronary circulation ex vivo (and immediately before I/R injury) exerted no direct cardioprotective actions in isolated mouse hearts (Fig. 7C). Taken together, these data show that the cardioprotective effects of genetic or
pharmacological inhibition of DPP-4 activity are not strictly glucose-dependent and depend on one or more DPP-4-dependent actions in vivo.

**DISCUSSION**

Analysis of the cardiovascular profile of anti-diabetic agents involves ascertainment of the effects of each drug on the myocardium and endothelium, and on secondary risk factors such as control of blood pressure and cholesterol. Although preclinical studies may be useful in generating hypotheses about the putative cardiovascular actions of different drug classes, the results of subsequent clinical studies have not always been concordant with predictions made from preclinical analyses. For example, although both thiazolidinediones, pioglitazone, and rosiglitazone exert beneficial effects on inflammation and endothelial function (29), pioglitazone, but not rosiglitazone, is associated with reduced cardiovascular events in human studies (3; 30). Similarly, although data from both preclinical (31) and clinical studies (32) suggests that metformin therapy may be cardioprotective, the mechanisms through which metformin therapy is associated with cardioprotection remain poorly understood. Moreover, sulfonylureas have been associated with increased rates of death from CVD in some (33) but not all studies (32).

We have now investigated the cardiovascular consequences arising from genetic elimination or pharmacological inhibition of DPP-4 activity. DPP-4 has 3 major functions: adenosine deaminase binding, peptidase activity, and extracellular matrix binding, all of which potentially influence the activity of the immune and/or endocrine systems (34). Although DPP-4 cleaves and inactivates several cardioactive peptides, including neuropeptide Y, BNP, SDF-1 and GLP-1, there is little information on the cardiovascular consequences of reduced or absent DPP-4 activity. Normoglycemic Dpp4-/- mice exhibit normal cardiac structure and function in the basal state, yet increased survival following experimental MI. Whether the increased survival following LAD ligation is directly due to loss of DPP-4 activity per se in cardiomyocytes or blood vessels, or indirectly due to the subsequent upregulation of cardioprotective molecules such as GLP-1 (18), or SDF-1 (35) cannot be inferred from the present study. Zaruba and colleagues also observed modest improvements in survival following experimental MI in Dpp4-/- mice or in WT mice treated with a DPP-4 inhibitor and more robust improvements in survival were observed following administration of G-CSF, findings attributed to SDF-1-dependent mobilization of cardiac stem cells (35). Our studies extend their observations through examination of the cardiovascular effects of DPP-4 inhibition in diabetic mice, and by demonstrating that direct sitagliptin administration into the circulation of the ischemic mouse heart is not directly cardioprotective ex vivo, suggesting that acute reduction of cardiac DPP-4 activity is not sufficient to produce cardioprotection.

Our findings demonstrating that both sitagliptin and the GLP-1R agonist liraglutide upregulated levels of cardioprotective proteins in the non-ischemic myocardium suggest a possible role for GLP-1 in the context of enhanced survival following DPP-4 inhibition and experimental MI. Nevertheless, we did not observe a sustained induction of cardioprotective proteins in sitagliptin-treated murine hearts when the same proteins were examined after MI. Moreover, we did not detect significant changes in infarct size or cardiac function after MI that might directly account for the improved survival seen with genetic or chemical reduction of DPP-4 activity. Hence, the precise mechanisms mediating the improvements in survival observed following pharmacological
treatment with sitagliptin in diabetic mice or genetic reduction of DPP-4 activity in normoglycemic Dpp4-/- mice require further investigation, ideally through examination of whether DPP-4 inhibitors exert cardioprotective actions in Glp1r-/- mice.

Western blot analysis of proteins in non-ischemic hearts demonstrated that both sitagliptin and metformin therapy induced an overlapping set of cardioprotective proteins. Metformin is thought to exert its cardioprotective actions through distinct mechanisms requiring activation of AMP kinase and endothelial nitric oxide (31). Intriguingly, administration of metformin has also been associated with reduction of DPP-4 activity (36) and increased circulating levels of GLP-1 in both rodent (37) and clinical studies (38) and we detected increased levels of GLP-1 in both metformin- and sitagliptin-treated mice. Accordingly, the extent to which therapy with sitagliptin and metformin produces an overlapping spectrum of actions reflecting similarities in their mechanism(s) of action through enhanced levels of GLP-1 requires further clarification.

Both metformin and sitagliptin significantly increased survival in diabetic mice, possibly due to a comparable reduction in blood glucose achieved with either agent. Hyperglycemia is a risk factor for a poor outcome after MI in humans (39) and there is considerable interest in determining whether intensive glucose control safely and consistently improves outcomes post-MI (40). Similarly, hyperglycemia is known to be associated with reduced survival and impaired LV function in mice following MI (41-43), and it seems likely that reduction in the severity of hyperglycemia contributes to improved survival perhaps independent of the anti-diabetic mechanisms unique to each agent under study.

In contrast, the increased survival observed in normoglycemic Dpp4-/- mice after MI supports the concept that reduction of DPP-4 activity may be cardioprotective in the absence of hyperglycemia (35). Similarly, our observations demonstrating that genetic or chemical inhibition of DPP-4 is associated with enhanced recovery of LVDP in the normoglycemic ischemic murine heart _ex vivo_, suggests that DPP-4 modifies cardiovascular outcomes independent of glucoregulation, and provides a useful model for future studies. Given the increasing interest in employing strategies based on DPP-4 inhibition for the treatment of diabetes, a more detailed understanding of the role of DPP-4 in the normal and diabetic cardiovascular system is clearly warranted.

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Table 1. Echocardiography-defined dimensional and functional parameters in *Dpp4<sup>+/+</sup>* and *Dpp4<sup>-/-</sup>* mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dpp4&lt;sup&gt;+/+&lt;/sup&gt; (n=5)</th>
<th>Dpp4&lt;sup&gt;-/-&lt;/sup&gt; (n=5)</th>
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<tbody>
<tr>
<td><strong>Aortic Flow</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic outflow velocity (cm/s)</td>
<td>72.8 ± 4.4</td>
<td>63.6 ± 2.9</td>
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<tr>
<td>Aortic valve ejection time (ms)</td>
<td>68.5 ± 3.9</td>
<td>69.6 ± 3.6</td>
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<tr>
<td><strong>Mitral Flow</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak E velocity (cm/s)</td>
<td>51.7 ± 2.3</td>
<td>47.7 ± 5.0</td>
</tr>
<tr>
<td>Deceleration time (ms)</td>
<td>42.5 ± 3.5</td>
<td>47.1 ± 1.3</td>
</tr>
<tr>
<td><strong>Left ventricular chamber dimensions by M mode</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left Atrium Atrium size (mm)</td>
<td>1.74 ± 0.06</td>
<td>1.66 ± 0.03</td>
</tr>
<tr>
<td>LV end diastolic dimension (mm)</td>
<td>3.85 ± 0.22</td>
<td>3.87 ± 0.09</td>
</tr>
<tr>
<td>LV end systolic diameter (mm)</td>
<td>2.45 ± 0.18</td>
<td>2.62 ± 0.08</td>
</tr>
<tr>
<td>LV outflow tract diameter (mm)</td>
<td>1.17 ± 0.03</td>
<td>1.14 ± 0.02</td>
</tr>
<tr>
<td>LV systolic wall thickness (mm)</td>
<td>0.74 ± 0.30</td>
<td>0.76 ± 0.01</td>
</tr>
<tr>
<td>Posterior wall thickness (mm)</td>
<td>0.77 ± 0.04</td>
<td>0.74 ± 0.02</td>
</tr>
<tr>
<td>Fractional Shortening (%)</td>
<td>36.42 ± 2.17</td>
<td>32.85 ± 1.04</td>
</tr>
</tbody>
</table>

Table 1. Genetic elimination of DPP-4 activity is not associated with abnormalities in parameters of CV function. Transthoracic echocardiographic studies were carried out in male 12-wk old *Dpp4<sup>+/+</sup>* and *Dpp4<sup>-/-</sup>* mice. Mice were lightly anesthetized using 3% isoflurane/97% oxygen. Two-dimensional and M-mode echocardiography, as well as pulsed Doppler analyses, were performed by a blinded observer using a Hewlett-Packard 5500 ultrasound device (Hewlett-Packard Co., Palo Alto, CA) and a 12-MHz phased array and 15 MHz Doppler probes. Three M-mode recordings of end-systolic and end-diastolic LV internal diameters, and end-diastolic LV posterior wall thickness and LV size were made. A single mean measurement was then calculated for each mouse. cm/s: centimeters/second; m/s: meters/second; mm: millimeter; ms: millisecond. Data are expressed as mean ± S.E. Acquisition of images and analysis of data were carried out as previously described (44; 45).
Table 2. Ultrasound biomicroscopy-defined cardiac hemodynamic, functional and dimensional parameters in mice treated with either control, sitagliptin, or metformin on day 4 post-LAD ligation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=6)</th>
<th>Sitagliptin (n=6)</th>
<th>Metformin (n=5)</th>
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<tbody>
<tr>
<td><strong>Aortic flow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>441 ± 17</td>
<td>423 ± 19</td>
<td>526 ± 23*</td>
</tr>
<tr>
<td>VTImax (cm)</td>
<td>2.64 ± 0.14</td>
<td>2.40 ± 0.08</td>
<td>2.39 ± 0.30</td>
</tr>
<tr>
<td>AO diameter (mm)</td>
<td>1.04 ± 0.04</td>
<td>1.06 ± 0.03</td>
<td>1.04 ± 0.03</td>
</tr>
<tr>
<td>LV SV (ul)</td>
<td>22.38 ± 1.24</td>
<td>21.10 ± 1.33</td>
<td>20.34 ± 3.07</td>
</tr>
<tr>
<td>CO (ml/min)</td>
<td>9.82 ± .046</td>
<td>8.95 ± 0.71</td>
<td>10.70 ± 1.66</td>
</tr>
<tr>
<td><strong>Mitral Flow</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>447 ± 13</td>
<td>433 ± 16</td>
<td>792 ± 33</td>
</tr>
<tr>
<td>Peak E velocity (cm/s)</td>
<td>67.6 ± 3.0</td>
<td>62.6 ± 2.4</td>
<td>53.4 ± 6.2</td>
</tr>
<tr>
<td>Peak A velocity (cm/s)</td>
<td>48.0 ± 49</td>
<td>36.0 ± 40</td>
<td>54.6 ± 73</td>
</tr>
<tr>
<td>Peak E/A ratio</td>
<td>1.47 ± 0.15</td>
<td>1.89 ± 0.29</td>
<td>0.99 ± 0.08</td>
</tr>
<tr>
<td><strong>Left Ventricular Chamber dimensions by mmode</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>493 ± 35</td>
<td>458 ± 15</td>
<td>541 ± 18</td>
</tr>
<tr>
<td>LV EDD (mm)</td>
<td>4.26 ± 0.15</td>
<td>4.28 ± 0.19</td>
<td>4.43 ± 0.16</td>
</tr>
<tr>
<td>LV ESD (mm)</td>
<td>3.54 ± 0.27</td>
<td>3.68 ± 0.21</td>
<td>3.89 ± 0.22</td>
</tr>
<tr>
<td>FS (%)</td>
<td>17.3 ± 3.5</td>
<td>14.2 ± 1.2</td>
<td>12.4 ± 2.4</td>
</tr>
</tbody>
</table>

Table 2. Ultrasound biomicroscopy-defined cardiac hemodynamic, functional and dimensional parameters in mice treated with either control, sitagliptin, or metformin on day 4 post-LAD ligation. Twelve-week old C57Bl/6 mice treated with control, sitagliptin, or metformin for one week prior to experimental cardiac ischemic injury were subject to high-frequency ultrasound imaging. AO: aortic orifice; CO: cardiac output; FS: left ventricular fractional shortening; HR: heart rate; LV SV: left ventricular stroke volume; LV EDD: left ventricular end-diastolic diameter; LV ESD: left ventricular end-systolic diameter; VTImax: velocity-time integral of Doppler flow waveform by tracing the maximal velocity; bpm: beats per minute; cm: centimeters; cm/s: centimeters/second; ml/min: milliliters/minute; mm: millimeters; µl: microlitres. In mitral inflow, the peak E velocity represents the maximal velocity of the early diastolic wave caused by active left ventricular relaxation. The peak A velocity represents the maximal velocity caused by atrial contraction in late diastole. Data are expressed as mean ± SE. *p<0.05 compared to control. Acquisition of images and analysis of data were carried out as previously described (44; 45). *= p< 0.05, vs. control
FIGURE LEGENDS:

Figure 1. Dpp4−/− mice exhibit increased survival following myocardial ischemic injury. (A) Heart weight (HW):body weight (BW) ratios in 12 week old Dpp4+/- vs. Dpp4−/− mice not exposed to LAD ligation (B) Experimental scheme for analysis of normoglycemic Dpp4−/− and Dpp4+/+ mice on a regular chow diet. (C) MI was induced by permanent coronary artery ligation (LAD) and survival was assessed in male and female 12 week-old Dpp4+/+ and Dpp4−/+ mice over subsequent 4 wk. (D) Hypertrophy is observed in Dpp4+/+ and Dpp4−/− mice following MI. (E) Infarct size was determined through quantitative histological analysis of hearts from male and female Dpp4+/+ and Dpp4−/− mice. *p<0.05; numbers in brackets correspond to number of male and female mice (combined) per treatment

Figure 2. Dpp4−/− hearts express increased levels of proteins associated with cardiomyocyte survival. Experimental outline for analysis of basal RNA and protein expression in Dpp4+/+ and Dpp4−/− mice (A). Relative levels of mRNA transcripts for Akt1 (B), Gsk3β (C), Ppara (D), PI3k (E), and Hox (F), in non-ischemic hearts from 12 week-old Dpp4+/+ vs. Dpp4−/− mice assessed by quantitative real-time PCR and normalized to levels of β-actin transcripts in the same samples. n=6 per group. Relative levels of pGSK3β (G), ANP (H), and pAKT1 (I) determined by Western blot analysis of protein extracts from hearts of 12 week-old Dpp4+/+ and Dpp4−/− mice. *p<0.05, n=3 for each genotype.

Figure 3. Sitagliptin and metformin reduce blood glucose levels and increase plasma GLP-1(7-36)amide levels in diabetic mice. Male C57BL/6 mice were placed on HFD (45% fat) for 4 wks (A). At the start of wk 5, mice were injected with a single dose of STZ (75 mg/kg), and then randomized into three treatment groups: (1) HFD/STZ alone, (2) HFD/STZ + sitagliptin (250 mg/kg), or (3) HFD/STZ + metformin (450 mg/kg) for an additional 8 wks. At wk 12, mice underwent LAD ligation or control sham surgery. At wk 16, surviving mice were euthanized and infarct size was measured. Levels of random fed blood glucose (B) and HbA1c (C) were significantly reduced in mice treated with sitagliptin or metformin. Oral glucose tolerance (D,E) was significantly improved in sitagliptin (n=29) or metformin (n=23)-treated mice as compared to HFD alone (n=23). (F) Body weight (BW) in mice treated with HFD/STZ alone, sitagliptin or metformin (n=22-23 per treatment). Plasma active GLP-1 is increased in mice treated with sitagliptin or metformin (n=14 per treatment) (G). *p<0.05, *** p<0.001 vs. untreated HFD/STZ group.

Figure 4. Outcomes following LAD ligation in diabetic mice treated with sitagliptin or metformin. (A) MI was induced by coronary artery ligation (see Fig. 3A) and significant increases in survival were observed in diabetic C57BL/6 mice following treatment with either sitagliptin or metformin, compared to HFD/STZ mice alone. (B) Cardiac hypertrophy was observed in metformin-treated mice following MI. (C) Histological analysis reveals similar infarct size between treatment groups. *p<0.05, ***p<0.001, numbers in brackets correspond to number of mice per treatment.

Figure 5. Treatment of diabetic C57BL/6 mice with sitagliptin, metformin, or liraglutide leads to increased expression of cardioprotective proteins. Diabetes was induced in HFD-fed STZ-treated WT C57Bl/6 mice (A). Levels of mRNA transcripts in hearts from mice treated with HFD/STZ
alone, or HFD/STZ plus sitagliptin, metformin, or liraglutide for 1 wk. Relative levels of PI3k (B), Akt1 (C), Ho1 (D), Mmp9 (E) and Ppara (F), were assessed by quantitative real-time PCR and normalized to levels of β-actin transcripts in the same samples. n=6 per group. Western blot analysis for ANP (G), AKT (H), HO-1(I) and p-GSK3β(J) using heart extracts from wild-type mice on HFD/STZ alone, or HFD/STZ plus either sitagliptin, metformin, or liraglutide. *p<0.05, ***p<0.001, n=3 per treatment.

**Figure 6.** Gene and protein expression after LAD occlusion in hearts from wild type mice treated with metformin or sitagliptin. Non-diabetic 10 wk old mice were treated with sitagliptin or metformin for 1 wk (A), and cardiac levels of mRNA transcripts for Akt1 (B), PI3k (C), Ho1 (D), Gsk3β (E) and Ppara (F) were determined by real time PCR, normalized to the values of Gapdh mRNA transcripts in the same sample. Western blot analysis was used to ascertain levels of pAKT (G), ANP (H), pGSK3β (I) and HO-1 (J) 5 days after LAD ligation. HSP90 was used as an internal control protein. ***p<0.001, n= 5 mice per group.

**Figure 7.** Functional recovery after I/R injury in the murine heart. (A) Experimental protocol for ischemia-reperfusion (I/R) injury in isolated mouse hearts. (B) Intraperitoneal (IP) injections of sitagliptin and metformin in 12 wk old wild-type mice in vivo reduced I/R injury of their hearts ex vivo. 12 wk-old Dpp4−/− mice display significantly greater improvement in functional recovery following I/R as compared to Dpp4+/+ littermate controls. (C) Direct 20 min infusion of sitagliptin started (arrow) prior to ischemia did not improve functional recovery following I/R injury in the isolated heart from wild-type (WT) mice. *p<0.05, numbers in brackets correspond to number of mice per treatment.
Figure 1

A

B

Dpp4<sup>+/+</sup> and Dpp4<sup>−/−</sup> mice

Normal chow diet (7% fat)

Age (wks)

0

12

16

LAD ligation

Endpoint: Infarct size

C

Percent survival

Days post myocardial infarction

D

E

Percent Infarct (%)

Dpp4 genotype
Figure 2

A. Dpp4/+ and Dpp4−/− mice

Normal chow diet (7% fat)

Heart tissue collection for RNA and protein analyses

B. Akt

C. Gsk3β

D. Ppara

E. Pdk4

F. Ho1

G. p-GSK3β

H. ANP

I. p-AKT

HSP90

HSP90

Relative units

Relative units

Relative units
Figure 3

A. Wild-type C57BL/6 mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age (wks)</th>
<th>STZ 75 mg/kg</th>
<th>OGTT</th>
<th>LAD</th>
<th>Endpoint</th>
<th>Infarct size</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Fat Diet (45%)</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. High Fat Diet (45%)</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. High Fat Diet + Sitagliptin (250 mg/kg)</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3. High Fat Diet + Metformin (450 mg/kg)</td>
<td>20</td>
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</tr>
</tbody>
</table>

B. Blood glucose (mmol/L) over time

C. HbA1c (%)

D. OGTT

E. AUC (glucose)

F. BW (g) over weeks

G. GLP-1(7-36) amide
Figure 4

A

B

C

Days post myocardial infarction

Survival (%)

0 5 10 15 20 25 30

HFD/STZ Sham (7)
Sitagliptin LAD (17)
Metformin LAD (16)

HFD/STZ Sham (7)
Sitagliptin LAD (17)
Metformin LAD (16)

HW (mg) BW (g)

0 1 2 3 4 5

(5) (9) (17) (16)

HFD/STZ Sham
HFD/STZ LAD
Sitagliptin Sham
Sitagliptin LAD
Metformin Sham
Metformin LAD

Infarct size

Percent Infarct (%)

0 5 10 15 20 25 30

HFD/STZ (5)
Sitagliptin (15)
Metformin (15)
Figure 5

A. Wild-type C57BL/6 mice

<table>
<thead>
<tr>
<th>Age (wks)</th>
<th>STZ 75 mg/kg</th>
<th>Heart tissue collection for RNA and protein analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. Phk

C. Akt

D. Hnf

E. Mnp9

F. Ppara

G. ANP

H. p-AKT

I. HO-1

J. p-GSK3β
Figure 6

Wild-type C57BL/6 mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Sitagliptin (250 mg/kg)</th>
<th>Metformin (450 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (wks)</td>
<td>11</td>
<td>12</td>
<td>12 wks + 4 days</td>
</tr>
<tr>
<td>LAD Ligation</td>
<td>Ultrasound Biomicroscopy</td>
<td>Heart tissue collection for Real-time PCR and Western analyses</td>
<td></td>
</tr>
</tbody>
</table>

B. Akt1

C. P13k

D. Ho1

E. Gsk3β

F. Pparaα

*** indicates statistical significance.
Figure 7

A

<table>
<thead>
<tr>
<th>20 min</th>
<th>40 min</th>
<th>30 min</th>
<th>40 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrate</td>
<td>Perfusion</td>
<td>Ischemia</td>
<td>Reperfusion</td>
</tr>
</tbody>
</table>

B

![Graph showing LVEDP (mmHg) over time for different treatments](image)

C

![Graph showing LVEDP (mmHg) over time for different infusions](image)