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ORIGINAL ARTICLE

DPP4 deficiency preserves cardiac function via GLP-1 signaling in rats subjected to myocardial ischemia/reperfusion

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Abstract Dipeptidyl peptidase-4 (DPP4) enzyme inhibition has been reported to increase plasma glucagon-like peptide-1 (GLP-1) level for controlling postprandial glucose concentration. Both DPP4 inhibitors and GLP-1 analog have been approved for antihyperglycemic agents. In addition to the insulinotropic effect, GLP-1 signaling was reported to modulate cardiac function. DPP4 inhibition was shown to improve survival rate after myocardial infarction in mice, but the precise mechanism remains unknown. We aimed to compare the cardiovascular responses of ischemia/reperfusion (I/R) between wild-type and DPP4-deficient rats and investigate the underlying mechanism. Rats were subjected to 45 min of coronary artery occlusion, followed by reperfusion for 2 h. Cardiac function was characterized by analyzing pressure–volume loops. As compared to wild-type rats, after I/R, DPP4-deficient rats had better cardiac performance in association with less infarct size and cardiac injury markers (LDH, ANP, and BNP), which could be attenuated by exendin-(9–39), a GLP-1 receptor antagonist. Exendin-(9–39) could diminish the increased phosphorylation levels of myocardial AKT and GSK-3β as well as the higher expression of GLUT4 in post-infarcted DPP4-deficient rats. However, exendin-(9–39) could not completely abrogate the less infarct size in DPP4-deficient rats as compared with that in wild-type rats, implicating the involvement of GLP-1 receptor-independent pathway. In summary, this study demonstrated that the benefit of cardiac protective action against I/R injury was demonstrated in DPP4-deficient rats, which is mediated through both GLP-1 receptor-dependent and receptor-independent mechanisms.

Keywords Glucagon-like peptide-1 · Dipeptidyl peptidase-4 · Ischemia/reperfusion · Cardiac function · Pressure–volume loops · AKT

Abbreviations

DPP4	Dipeptidyl peptidase-4
GLP-1	Glucagon-like peptide-1
ex(9–39)	Exendin-(9–39)
I/R	Ischemia/reperfusion
LDH	Lactate dehydrogenase
PKA	Protein kinase A
PV loop	Pressure–volume loop
GSK	Glycogen synthase kinase
SDF-1	Stromal cell-derived factor-1

Introduction

Dipeptidyl peptidase-4 (DPP4) has three major functions: peptidase activity, adenosine deaminase binding, and extracellular matrix binding (Morimoto and Schlossman 1998). DPP4 known as CD26 is a homodimeric type II transmembrane glycoprotein (Thompson et al. 2007), which is one of the accessory molecules of helper T cells, mediates as a co-stimulatory effect on T cell activation (Morimoto and Schlossman 1998; Thompson et al. 2007; Ohnuma et al. 2008). DPP4 can catalyze the release of dipeptides from the N terminus which contain Pro or Ala in the third amino acid position (Morimoto and Schlossman 1998), such as glucagon-like peptide-1 (GLP-1), chemokines, neuropeptides, and stromal cell-derived factor-1 (SDF-1) (Boonacker and Van

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Noorden 2003; Michel et al. 2008). The action of DPP4 is complicated.

GLP-1 is secreted from the gastrointestinal tract after food intake and stimulates glucose-dependent insulin secretion by activating GLP-1 receptor (Fehmann et al. 1995). GLP-1 receptor is a G protein-coupled receptor, which manipulates intracellular cAMP levels with subsequent PKA activation (Drucker 2006). The major active form of GLP-1 is a GLP-1(7–36) amide (Orskov et al. 1994); after release into the circulation, it undergoes rapid cleavage by DPP4 into a GLP-1(9–36) amide, by the removal of an N-terminal dipeptide (Morimoto and Schlossman 1998). GLP-1(9–36) is characterized by a complete lack of insulinotropic activity (Drucker 2006). GLP-1-based therapies have been approved recently as antihyperglycemic agents, such as GLP-1 analog and DPP4 inhibitor (Doupis and Veves 2008; Michel et al. 2008; Grieve et al. 2009). In addition to the insulinotropic effect, GLP-1 receptor is also expressed in the cardiovascular system (Gros et al. 2003; Ban et al. 2008), which also modulates cardiac function (Barragan et al. 1994; Vila Petroff et al. 2001).

Diabetes is associated with high risk of cardiovascular disease, so the strategy which decreases cardiovascular risk factor in the diabetic subject is an important issue. Myocardial ischemia/reperfusion (I/R) induces a spectrum of events including cardiac dysfunction and cell death, but the management is still limited with high mortality (Hearse and Bolli 1992). Evidence from several studies suggested that GLP-1-based therapies play an important role in protection from myocardial I/R injury (Zhao et al. 2006; Fields et al. 2009; Noyan-Ashraf et al. 2009; Dokken et al. 2010), and the protective effect was diminished by GLP-1 receptor antagonist, cAMP inhibitor, PI3K/AKT inhibitor, and the mitogen-activated protein kinase inhibitor (Bose et al. 2005). In addition, inhibition of DPP4 also improved heart function and survival after myocardial infarction (Zaruba et al. 2009; Sauve et al. 2010). G-CSF administration in combination with DPP4 inhibitor leads to the stabilization of active SDF-1, which attracted stem cells to the heart and improved outcome after myocardial infarction (Zaruba et al. 2009).

We have shown that a prominent GLP-1 production in DPP4-deficient rats was associated with better preservation of cardiovascular function than that in wild-type rats during endotoxemia (Ku et al. 2010), so we hypothesized that GLP-1 signaling exerts a cardioprotective effect in DPP4-deficient rats after I/R. Accordingly, we measured the plasma GLP-1 level after the heart was subjected to I/R and used GLP-1 receptor antagonist to assess whether GLP-1 receptor-dependent pathway plays an important role on DPP4-deficient rats subjected to myocardial infarction.

Materials and methods

Experimental animals

The research was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996). Wild-type Fischer 344 served as the genetic background control. The strain DchcHsd-DPP IV− on a Fischer 344 background has lower DPP4 activity (Ku et al. 2010). Fischer 344 rats were purchased from the National Laboratory Animal Center of Taiwan, and DPP4 mutant rats (DchcHsd-DPP IV−) were purchased from Harlan laboratories, Inc. Fourteen- to 16-week-old male rats were used. Animals were maintained under a 12-h light/dark cycle at a controlled temperature (21±2°C) with free access to food and tap water.

Experimental model of myocardial I/R injury in vivo

Rats were anesthetized, and tracheotomy was performed for ventilation by a respirator (model 683, Harvard Rodent Ventilator) with a stroke volume of 15 mL/kg body weight and at a rate of 65 strokes per minute. The electrocardiogram of lead II was monitored. Thoracotomy was performed, and the left anterior descending coronary artery of the heart was ligated by 7-0 silk just proximal to its main branching point. Myocardial ischemia was confirmed by a zone of cyanosis and electrocardiogram S–T segment elevation. Rats were subjected to a completion of 45-min occlusion, followed by reperfusion for 2 h. Sham groups received the same surgery process without ligation. After 2 h of reperfusion, the rats were sacrificed, and the hearts were harvested. The blood plasma samples were collected immediately and stored at −80°C until used.

To investigate the role of GLP-1 receptor on myocardial I/R injury, exendin-(9–39) (ex(9–39); Sigma, St. Louis, MO, USA), a GLP-1 receptor antagonist, was subcutaneously injected at a dose of 45 μg/kg at 30 min before I/R injury operated. We used ex(9–39) to antagonize GLP-1 receptor because the agents have been widely used to estimate the role of receptor-dependent pathway, and the dosage in these experiments would inhibit glucose-induced insulin secretion (Tseng et al. 1999).

Determination of cardiac function in vivo

Cardiac function was estimated in vivo by a pressure–volume catheter (1.9 F; Scisense Instruments, Ontario, Canada) as described previously (Chen et al. 2010; Ku et al. 2010). The catheter was inserted into the carotid

artery and then advanced into the left ventricle for the measurement of pressure and volume. All pressure–volume loop (PV loop) data were analyzed using a cardiac analysis program of PONEMAH Life Sciences Suite from Data Sciences International (DSI Corporate Headquarters, St. Paul, MN, USA). PV loops of basal condition were recorded, and several parameters were chosen for analysis: left ventricular end-systolic (LVESP) and left ventricular end-diastolic pressure (LVEDP), maximal slope of the systolic pressure increment (+dP/dt) and diastolic pressure decrement (−dP/dt), and stroke work (SW). SW is used to assess ventricular function by calculating the area within the PV loop. PV Loops were then determined under condition of preload change, elicited by inferior vena cava occlusion as described previously (Chen et al. 2010; Ku et al. 2010). The end-systolic pressure–volume relation (ESPVR) and end-diastolic pressure–volume relation (EDPVR) were respectively examined by the slope of end-systolic and end-diastolic pressure–volume points, which are load-independent indexes of myocardial contractility and ventricular compliance.

Determination of the myocardial risk size and the infarct size

At the end of reperfusion, the heart was excised and perfused on a Langendorff system with 95% O_2, 5% CO_2 Tyrode's solution (137 mM NaCl, 11.9 mM $NaHCO_3$, 11 mM glucose, 2 mM $CaCl_2$, 1.1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, and 5.4 mM KCl). After the blood was washed out, the coronary artery was ligated again and perfused with 1% evan blue to determine the risk area. The nonischemic myocardium stained blue, and the ischemic myocardium remained red. The risk size was calculated as a percentage of the red area to total ventricular area. Each heart was then sliced horizontally, and the slices were incubated in 1% triphenyltetrazolium chloride (Sigma, St. Louis, MO, USA) prepared in normal saline for 30 min at 37°C, and then placed in 10% formaldehyde for 3 days. The infarct area appeared white, and the infarct size was also calculated as a percentage of the white area to the total risk area.

Determination of plasma GLP-1, glucose, insulin, lactate dehydrogenase, atrial natriuretic peptide, brain natriuretic peptide, and cardiac tumor necrosis factor α concentrations

Plasma GLP-1 levels were measured by ELISA kit (Millipore Corporation, Billerica, MA, USA). An insulin ELISA kit (Mammalian Insulin ELISA; Mercodia AB, Uppsala, Sweden) was used for the determination of plasma insulin concentrations. Plasma glucose and LDH levels were evaluated by a colorimetric analyzer (ARKRAY, SP-4410, Spotchem, Japan). LDH is regarded as the indicator of cardiac injury. Plasma atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) concentrations were detected by ELISA kit (Raybiotech, Inc., CA, USA). Cardiac TNF-α levels were measured by ELISA kit (BD Biosciences, NJ, USA).

Total and membrane protein extraction of cardiac tissue

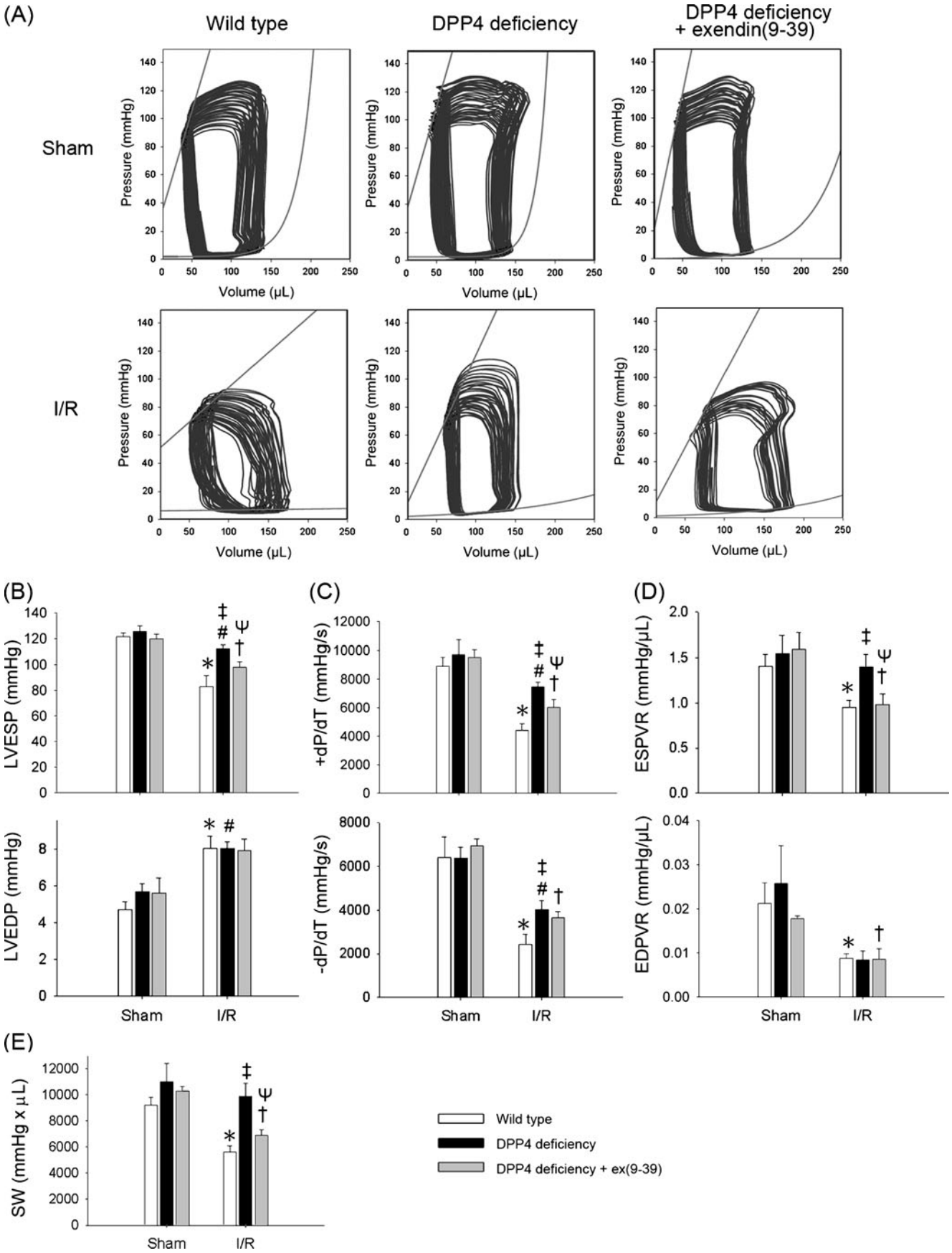
RIPA buffer was used for the total protein extraction. Left ventricles were homogenized in RIPA buffer (Tris–HCl 50 mM, NaCl 150 mM, EGTA 1 mM, EDTA 1 mM, NP-40 1%, sodium deoxycholate 1%) containing cocktail protease and phosphatase inhibitor (Sigma, St. Louis, MO, USA). The supernatant of tissue homogenate was collected after centrifugation (800×*g*, 10 min at 4°C) and defined as total cardiac protein. An ELISA kit (BioVision, Inc., USA) was used to extract cardiac membrane protein for the determination of glucose transporter expression. Protein concentrations were determined by BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Western blotting

Protein samples were denatured for 10 min in boiling sample buffer (31.25 mM Tris–HCl buffer, pH 6.8, containing 10% SDS, 10% 2-mercaptoethanol, 25% glycerol, and 0.00125% bromophenol blue). The myocardial proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences, Boston, MA, USA). The membranes were blocked in 5% nonfat milk dissolved in Tris/phosphate/saline/Tween (TBST) and incubated with primary antibody to GLUT1, GLUT4, AKT, glycogen synthase kinase (GSK)-3β, p-GSK-3β (serine-9) (purchased from Santa Cruz Biotechnology, Inc.), p-AKT (serine-473) (purchased from Cell Signaling Technology, Inc.), and *N*-cadherin (Epitomics, Inc., USA). The membranes were washed by TBST several times, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.). After being washed several times, the signals were detected with the ECL system (Millipore, Bedford, MA, USA). The blots were scanned and quantified by ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

Statistical analysis

All values were presented as means ± SE. The results were analyzed using ANOVA followed by Bonferroni's post hoc tests. $P<0.05$ was considered as significant difference.


(A)
Wild type
DPP4 deficiency
DPP4 deficiency + exendin(9-39)
Sham
I/R
Pressure (mmHg)
Volume (μL)
(B)
LVESP (mmHg)
LVEDP (mmHg)
(C)
+dP/dT (mmHg/s)
-dP/dT (mmHg/s)
(D)
ESPVR (mmHg/μL)
EDPVR (mmHg/μL)
(E)
SW (mmHg x μL)
Sham
I/R
Wild type
DPP4 deficiency
DPP4 deficiency + ex(9-39)

◀ **Fig. 1** Hemodynamic parameters of rats subjected to myocardial I/R. **a** Pressure–volume loops, **b** left ventricular end-systolic (*LVESP*) and left ventricular end-diastolic pressure (*LVEDP*), **c** maximal slope of the systolic pressure increment (+dP/dt) and diastolic pressure decrement (−dP/dt), **d** end-systolic pressure–volume relation (*ESPVR*) and end-diastolic pressure–volume relation (*EDPVR*), and **e** stroke work (*SW*) after the myocardium was subjected to sham surgery or ischemia (45 min) and reperfusion (2 h) in wild-type, DPP4-deficient, and ex(9–39)-treated DPP4-deficient rats. (n=7) *p<0.05 vs. wild-type sham, #p<0.05 vs. DPP4-deficient sham, †p<0.05 vs. DPP4-deficient + ex(9–39) sham, ‡p<0.05 vs. wild-type I/R, Ψp<0.05 vs. DPP4-deficient I/R

Results

I/R-induced cardiac dysfunction was recovered in DPP4-deficient rats

The cardiac functions were measured by PV loops in sham and I/R conditions (Fig. 1a). In the sham condition, similar LVESP and LVEDP (Fig. 1b), +dP/dt and −dP/dt (Fig. 1c), ESPVR and EDPVR (Fig. 1d), and SW (Fig. 1e) were found in all groups; however, cardiac dysfunction occurred after I/R. I/R caused a reduction of LVESP (121.3±2.9 mmHg/s in sham and 82.8±8.8 mmHg/s in I/R) and an elevation of LVEDP (4.7 mmHg/s±0.4 in sham and 8.0±0.7 mmHg/s in I/R) in wild-type rats. The contraction and relaxation development rate deteriorated in wild-type rats, being 49.4% and 38.0% of that without I/R treatment, respectively. Stroke work also markedly decreased after I/R (9,190.2±622.9 mmHg×μL in sham and 5,601.5±506.3 mmHg×μL in I/R). Furthermore, load-independent cardiac function indexes attenuated after I/R, both ESPVR and EDPVR values markedly reduced in wild-type rats, being 67.5% and 41.2% of those without I/R treatment, respectively.

DPP4-deficient rats had better cardiac performance after I/R injury (Fig. 1). LVESP attenuated less in DPP4-deficient rats than in wild-type rats (125.3±4.6 mmHg/s in sham and 112.4±2.8 mmHg/s in I/R). LVEDP elevated in DPP4-deficient rats (5.7±0.4 mmHg/s in sham and 8.0±0.4 mmHg/s in I/R), but did not have any significant difference between wild-type and DPP4-deficient rats. The rate of contraction and relaxation development remained at 76.7% and 63.0% of that without I/R treatment, respectively. Stroke work markedly recovered after I/R in DPP4-deficient rats (11,011.2±1,384.6 mmHg×μL in sham and 9,859.6±1,025.5 mmHg×μL in I/R). ESPVR, the load-independent value, remained at 90.5% in DPP4-deficient rats, but not EDPVR. The results showed that there was a less marked suppression of cardiac function after I/R in DPP4-deficient rats.

To understand whether the better resistance of DPP4-deficient rats to I/R-induced cardiac dysfunction is related to the GLP-1 receptor-dependent pathway, ex(9–39), a GLP-1 receptor antagonist, was used. The effect on cardiac function of DPP4-deficient rats after I/R was examined (Fig. 1). In DPP4-deficient rats, ex(9–39) treatment had no significant effects on the cardiac function in the sham condition. However, ex(9–39) treatment attenuated the cardiac function preservation in DPP4-deficient rats after I/R. I/R caused a reduction of LVESP (119.8 mmHg/s±3.6 in sham and 97.9±4.3 mmHg/s in I/R) in ex(9–39)-treated DPP4-deficient rats. The contraction development rate deteriorated in ex(9–39)-treated DPP4-deficient rats, being 63.3% of that without I/R treatment. Stroke work attenuated after I/R (10,285.8±357.5 mmHg×μL in sham and 6,346.0±486.4 mmHg×μL in I/R). ESPVR also reduced in the ex(9–39)-treated DPP4-deficient rats, being 61.9% of that without I/R treatment. The results demonstrated that ex(9–39) diminished the cardiac function preservation in DPP4-deficient rats.

DPP4 deficiency reduced myocardial infarct size after being subjected to I/R

Myocardial infarction plays an important role on cardiac dysfunction following myocardial I/R. The risk size (Fig. 2a) and infarct size (Fig. 2b) were calculated. There

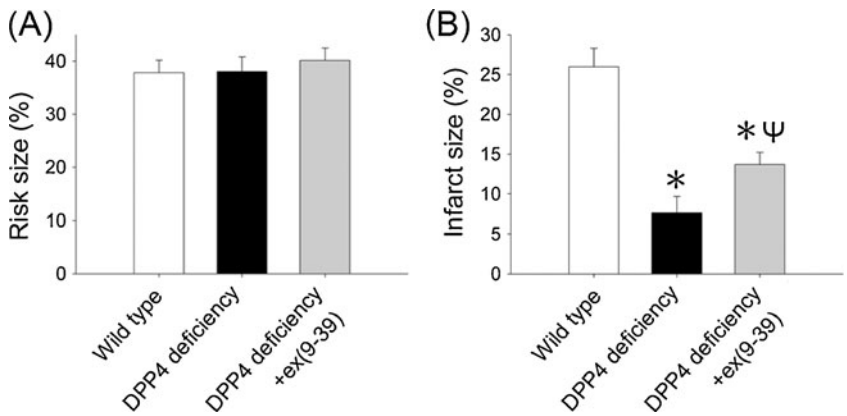


Fig. 2 Wild-type, DPP4-deficient, and ex(9–39)-treated DPP4-deficient rats showed myocardial infarction after being subjected to 45 min of coronary artery occlusion, followed by reperfusion for 2 h. **a** Risk size and **b** infarct size were reported. Ratios of risk area to total ventricular area were defined as risk size, while infarct size was calculated as a percentage of infarct area to risk area. (n=7) *p<0.05 vs. wild-type, Ψp<0.05 vs. DPP4 deficiency

was no significant difference of the risk size among groups, which indicated the position of coronary artery occlusion was similar among groups. I/R-induced infarct size was 26.0±2.1% in wild-type rats, which was markedly reduced to 8.1±2.0% in DPP4-deficient rats. Ex(9–39), an antagonist of GLP-1 receptor, diminished the protective effect in DPP4-deficient rats (13.7±1.6%). However, the infarct size in the ex(9–39)-treated DPP4-deficient rats was still less than that in wild-type rats.

GLP-1 signaling upregulated in DPP4-deficient rats

In the sham condition, plasma GLP-1 level was 2.9-fold higher in DPP4-deficient rats than that in wild-type rats (Fig. 3a). I/R did not alter GLP-1 level in all groups. Plasma GLP-1 level was still higher after I/R in DPP4-deficient rats than that in wild-type rats. Ex(9–39) did not alter the difference of plasma GLP-1 level in DPP4-deficient rats. Plasma insulin (Fig. 3b) and glucose (Fig. 3c) levels did not show any significant difference among groups in the presence or absence of I/R.

DPP4 deficiency preserved cardiac GLUT1 and GLUT4 levels after I/R

GLP-1 stimulated myocardial glucose uptake in the isolated rat heart (Zhao et al. 2006; Ban et al. 2008). Glucose transporter mediates the transportation of glucose into the cell for energy source. We examined the amount of GLUT1 and GLUT4 translocated to the cell membrane in the heart (Fig. 4). The level of GLUT1 expression in the cardiac membrane was shown (Fig. 4a), and the results were all quantified (Fig. 4b). In the sham condition, similar cardiac GLUT1 expressions were found in all groups (Fig. 4a and b). GLUT1 expression decreased in wild-type rats, being 68.4% of that without I/R treatment, while DPP4-deficient rats remained at 92.9%. Administration of ex(9–39) diminished the preservative effect of GLUT1 expression in DPP4-deficient rats.

In contrast to the wildly expressed GLUT1, GLUT4 is only expressed on striated muscle and adipose tissue. The level of GLUT4 expression in the cardiac membrane was measured (Fig. 4a), and the results were all quantified (Fig. 4c). In the sham condition, the level of cardiac GLUT4 was 1.1-fold higher in DPP4-deficient rats than that in wild-type rats. I/R resulted in a reduction of GLUT4 expression in all groups. However, cardiac GLUT4 remained 1.2-fold higher in DPP4-deficient rats than that in wild-type rats in the treatment of I/R. DPP4-deficient rats treated with ex(9–39) significantly attenuated GLUT4 expression in the presence or absence of I/R.

DPP4 deficiency diminished heart injury after I/R

LDH is a nonspecific marker of cardiac injury. In the sham condition, similar plasma LDH concentrations were found in all groups (Fig. 5a). In response to I/R, plasma LDH level increased in wild-type rats. The elevation was attenuated in DPP4-deficient rats. However, the protective effect was diminished by ex(9–39).

The plethora of mediators, including chemokines, cytokines, and peptides, are upregulated following I/R and associated with tissue injury or repair. The peptide hormones offer substitute markers for cardiac function (Burley et al. 2007). We examined whether plasma ANP and BNP increased during I/R (Fig. 5b and c). In the sham

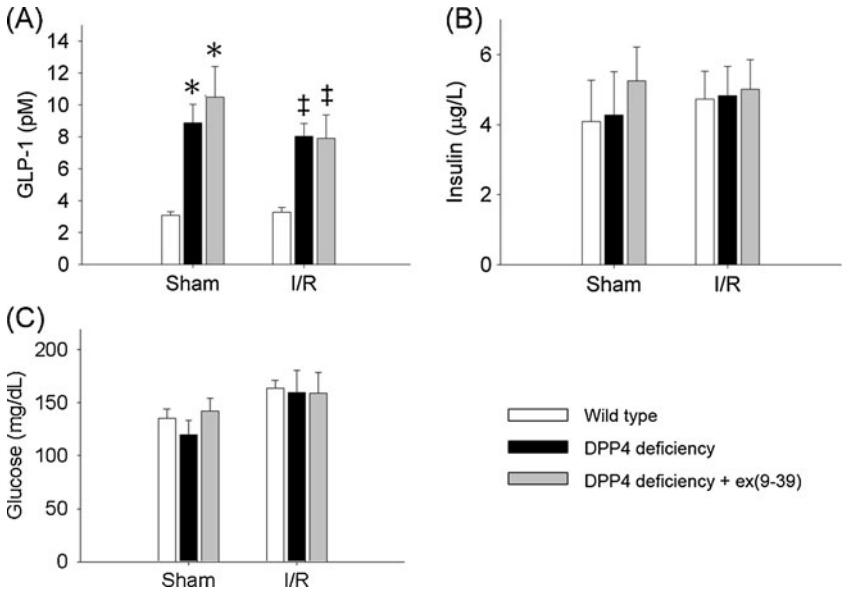


Fig. 3 Plasma levels of **a** GLP-1, **b** insulin, and **c** glucose after the myocardium was subjected to sham surgery or ischemia (45 min) and reperfusion (2 h) in wild-type, DPP4-deficient, and ex(9–39)-treated DPP4-deficient rats (GLP-1 and insulin, $n=8$; glucose, $n=6$ in sham condition and $n=8$ in I/R condition) *$p<0.05$ vs. wild-type sham, ‡$p<0.05$ vs. wild-type I/R

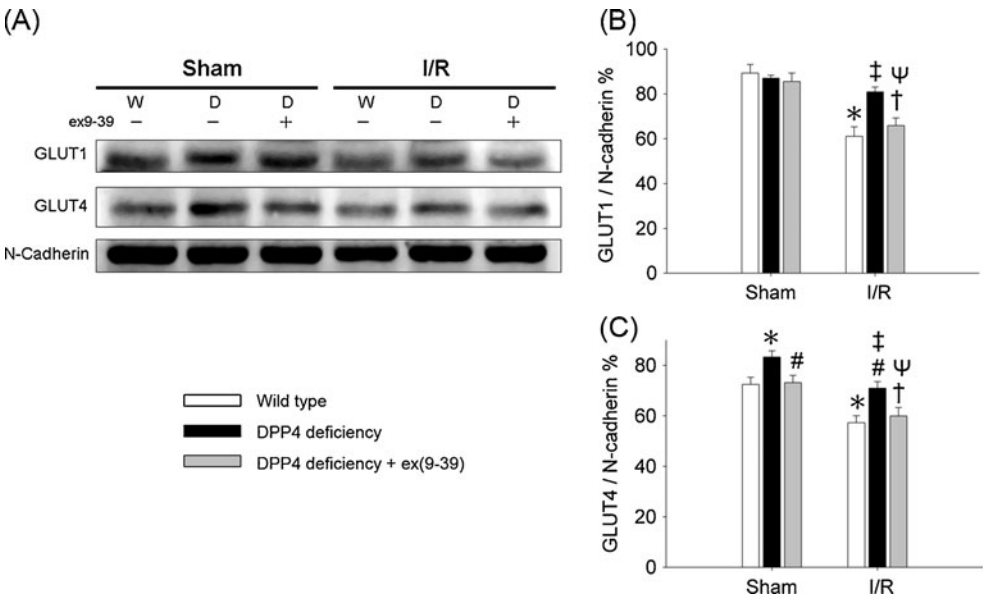


Fig. 4 Effects of I/R on cardiac membrane GLUT1 and GLUT4 levels in wild-type, DPP4-deficient, and ex(9–39)-treated DPP4-deficient rats. Proteins were detected after the myocardium was subjected to sham surgery or ischemia (45 min) and reperfusion (2 h). **a** Original Western blots were reported. Results of densitometry of **b** GLUT1 and **c** GLUT4. *W* indicated wild type, and *D* indicated DPP4 deficiency (n=4). *N*-cadherin is an internal control of membrane protein. *p<0.05 vs. wild-type sham, #p<0.05 vs. DPP4-deficient sham, †p<0.05 vs. DPP4-deficient + ex(9–39) sham, ‡p<0.05 vs. wild-type I/R, Ψp<0.05 vs. DPP4-deficient I/R

condition, similar plasma ANP and BNP concentrations were found in all groups. In response to I/R, plasma ANP and BNP levels increased in wild-type rats. The elevations were less in DPP4-deficient rats; however, ex(9–39) increased plasma BNP level in response to I/R.

Cardiac TNF-α level was also measured (Fig. 5d). Similar cardiac TNF-α concentrations were found in the sham condition. I/R slightly increased TNF-α release in wild-type rats, which was less in DPP4-deficient rats. However, ex(9–39) did not show any significant increase of cardiac TNF-α level in DPP4-deficient rats.

DPP4 deficiency improved cardiac AKT and GSK-3β phosphorylation levels after I/R

Activation of the AKT-dependent signaling has been shown to activate the survival pathway, prevent apoptosis, and protect the myocardium after I/R (Fujio et al.

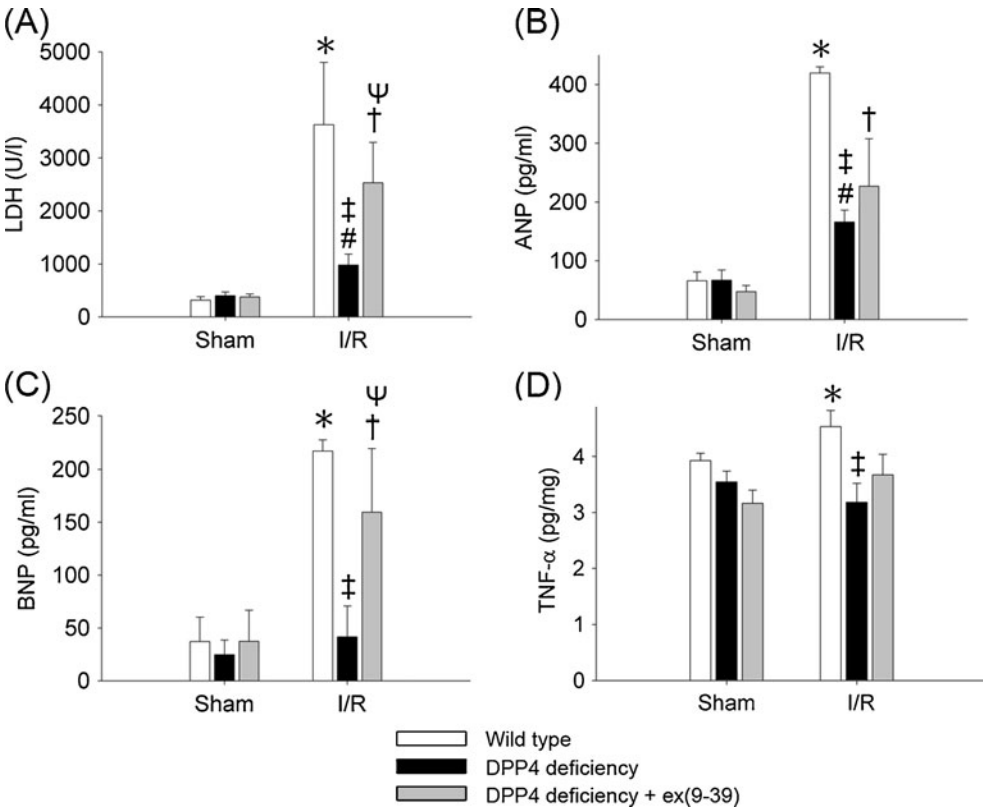


Fig. 5 Plasma levels of **a** LDH, **b** ANP, **c** BNP, and **d** cardiac levels of TNF-α after the myocardium was subjected to sham surgery or ischemia (45 min) and reperfusion (2 h) in wild-type, DPP4-deficient, and ex(9–39)-treated DPP4-deficient rats. (LDH, n=6 in sham condition and n=8 in I/R condition. ANP, BNP, and TNF-α, n=8) *p<0.05 vs. wild-type sham, #p<0.05 vs. DPP4-deficient sham, †p<0.05 vs. DPP4-deficient + ex(9–39) sham, ‡p<0.05 vs. wild-type I/R, Ψp<0.05 vs. DPP4-deficient I/R

2000; Mullonkal and Toledo-Pereyra 2007; Ravingerova et al. 2007). We examined the levels of AKT phosphorylation in the myocardium of wild-type and DPP4-deficient rats in the presence or absence of I/R (Fig. 6a), and the results were all quantified (Fig. 6b). In the sham condition, the level of myocardial AKT phosphorylation was 2.1-fold higher in DPP4-deficient rats than that in wild-type rats. I/R increased cardiac AKT phosphorylation level in wild-type rats. The level of myocardial AKT phosphorylation was equivalent in DPP4-deficient rats in the presence and absence of I/R. However, the level of cardiac AKT phosphorylation in DPP4-deficient rats subjected to I/R is 1.3-fold higher than that in wild-type rats. Administration of ex(9–39) in DPP4-deficient rats markedly diminished the incremental level of myocardial AKT phosphorylation in the presence or absence of I/R.

GSK-3β is one of the downstream targets of AKT. Phosphorylation of GSK-3β by AKT results in GSK-3β inactivation and exerts cardioprotective effect (Juhaszova et al. 2009). To understand whether the incremental level of AKT phosphorylation is related to the alterations in GSK-3β phosphorylation, we investigated the effect of I/R on GSK-3β phosphorylation in wild-type and DPP4-deficient rats (Fig. 6a), and the results were all quantified (Fig. 6c). In the sham condition, the level of myocardial GSK-3β phosphorylation was 1.5-fold higher in DPP4-deficient rats than that in wild-type rats. I/R did not significantly alter myocardial levels of GSK-3β phosphorylation in both wild-type and DPP4-deficient rats, but there was still 1.5-fold higher GSK-3β phosphorylation in DPP4-deficient rats than in wild-type rats. DPP4-deficient rats treated with ex(9–39) significantly attenuated the GSK-3β phosphorylation in the presence or absence of I/R.

Discussion

The present study demonstrated that the cardiac performance after I/R injury in DPP4-deficient rats was better than that in wild-type rats, which was mainly resulted from GLP-1 receptor-mediated cardioprotective effect via increasing the phosphorylation of AKT and GSK-3β and preserving glucose transporter GLUT4 expression to maintain glucose utilization. A GLP-1 receptor-independent mechanism was also identified in alleviating infarct size in DPP4-deficient rats for the incomplete blockade by exendin-(9–39), a GLP-1 receptor antagonist. Furthermore, GLP-1(9–36) did not potentiate the cardioprotective effect in DPP4-deficient rats, indicating the ceiling effect of GLP-1 signaling in DPP4-deficient rats.

Cardiac energy metabolism is drastically altered during I/R. One strategy is to optimize cardiac energy metabolism and oxygen consumption, by decreasing the use of fatty acids as a fuel and by increasing the use of glucose as a fuel (Ussher and Lopaschuk 2008). The strategy is beneficial to allow the heart to produce energy more efficiently and reduce the degree of acidosis associated with I/R (Ussher and Lopaschuk 2008). In our study, there was no alteration in plasma glucose and insulin levels in the presence or absence of I/R; however, GLUT1 and GLUT4 expression in the cardiac membrane is preserved in DPP4-deficient rats after being subjected to I/R. Indeed, GLP-1 increased GLUT-1 expression in dilated cardiomyopathy dogs, which was independent of plasma insulin level elevation, but was associated with p38 MAP kinase activation (Bhashyam et al. 2010). In addition, GLP-1 facilitated AKT and AMPK signaling and consequent with GLUT4 translocation in murine model of dilated cardiomyopathy (Vyas et al. 2011).

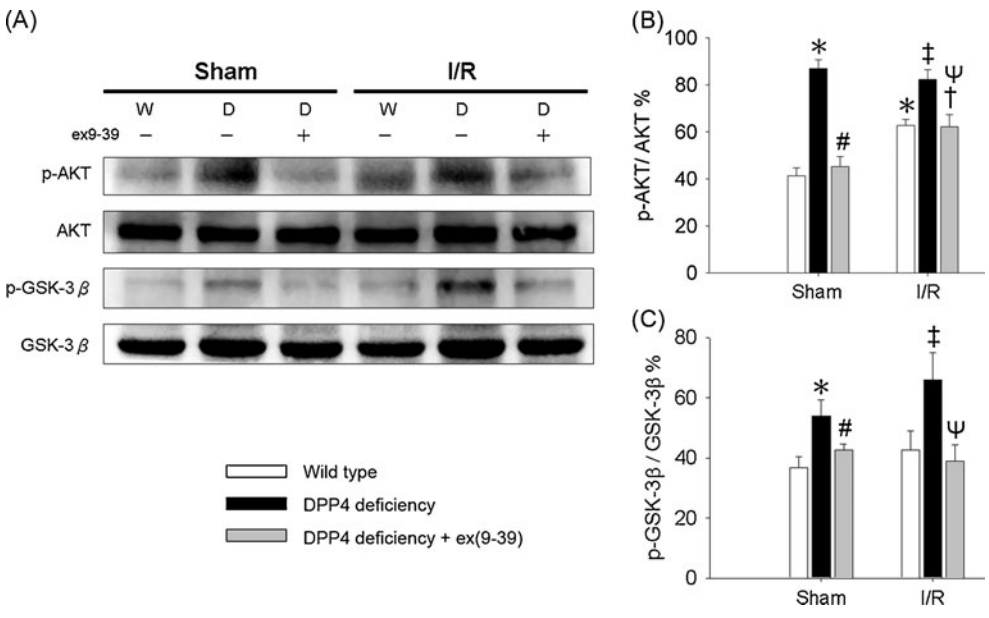


Fig. 6 Effects of I/R on cardiac AKT and GSK-3β phosphorylation levels in wild-type, DPP4-deficient, and ex(9–39)-treated DPP4-deficient rats. Proteins were detected after the myocardium was subjected to sham surgery or ischemia (45 min) and reperfusion (2 h). **a** Original Western blots were reported. Results of densitometry of **b** AKT and **c** GSK-3β phosphorylation. *W* indicated wild type, and *D* indicated DPP4 deficiency (n=4). *p<0.05 vs. wild-type sham, #p<0.05 vs. DPP4-deficient sham, †p<0.05 vs. DPP4-deficient + ex(9–39) sham, ‡p<0.05 vs. wild-type I/R, Ψp<0.05 vs. DPP4-deficient I/R

Improving glucose utilization in DPP4-deficient rats may be one of the beneficial effects in maintaining cardiac function through GLP-1 receptor signaling.

The phosphorylation of AKT can activate the survival pathway to protect the myocardium from apoptosis after I/R injury (Fujio et al. 2000; Mullonkal and Toledo-Pereyra 2007; Ravingerova et al. 2007). Inhibition of AKT signaling by PI3K/AKT inhibitor or genetic deletion abrogated the protective effect (Mullonkal and Toledo-Pereyra 2007). Conversely, overexpression of AKT in the heart limited infarct size after I/R (Hua et al. 2007). GSK-3β is a constitutively active enzyme and can be inactivated when phosphorylated by AKT, which results in elevating the threshold for mPTP opening thus increasing the tolerance to I/R (Nishihara et al. 2007; Juhaszova et al. 2009). We observed an increased level of AKT and GSK-3β phosphorylation correlated with infarct size attenuation in DPP4-deficient rats. Treatment with GLP-1 receptor antagonist decreased the levels of AKT and GSK-3β phosphorylation, which was associated with loss of cardioprotection. Liraglutide, a GLP-1 receptor agonist, was reported to increase cardiac AKT and GSK-3β phosphorylation levels in mice which were associated with survival rate improvement and infarct size reduction after myocardial infarction (Noyan-Ashraf et al. 2009). We speculated that higher plasma GLP-1 level increased myocardial AKT phosphorylation in DPP4-deficient rats may lead to increased phosphorylation and inactivation of GSK-3β, which exerted cardioprotective effect after I/R.

GLP-1 is released from the distal small intestine in response to nutrient ingestion (Fehmann et al. 1995). Lack of DPP4 activity resulted in accumulation of plasma GLP-1 in DPP4-deficient rats. Evidence from several studies suggested that GLP-1 receptor agonist improved cardiac function in animal models of experimental cardiac dysfunction, such as dilated cardiomyopathy (Nikolaidis et al. 2004a), myocardial infarction (Noyan-Ashraf et al. 2009), and endotoxemia (Ku et al. 2010). Long-term infusion of GLP-1 also showed beneficial effect and improved cardiac function in patients of myocardial infarction and heart failure (Nikolaidis et al. 2004b; Sokos et al. 2006). Our data supported the importance of GLP-1 receptor signaling pathway in the preservation of cardiac function as well as in reducing infarct size. However, the infarct size in ex(9–39)-treated DPP4-deficient rats after being subjected to I/R was less than that in wild-type rats, implicating that the protective effect in DPP4-deficient rats was more than the GLP-1 receptor-dependent pathway, or the dose of ex(9–39) cannot block GLP-1 receptor completely, but the dosage was reported to block glucose-induced insulin secretion (Tseng et al. 1999). Indeed, it has been shown that GLP-1 improved cardiac function after I/R in GLP-1 receptor knock-out Langendorff-perfused heart (Ban et al. 2008). Growing reports supported the biological actions of GLP-1 (9–36), the metabolite of GLP-1, in the cardiovascular system. Administration of GLP-1(9–36) after the heart was subjected to global ischemia improved cardiac function recovery (Sonne et al. 2008; Ban et al. 2010). GLP-1(9–36) increased the level of cAMP and improved cell viability during hypoxia/reoxygenation in neonatal cardiomyocytes, which were blocked by ex(9–39) (Ban et al. 2010). In addition, GLP-1(9–36) also protected cardiomyocytes from ischemic and oxidative stress-induced injury in the GLP-1 receptor knock-out model (Ban et al. 2010). We also administrated GLP-1(9–36) in DPP4-deficient rats but did not find further decrease in infarct size (data not shown). The results indicated that GLP-1 and its metabolite may share the same pathway, which is involved in both GLP-1 receptor-dependent and receptor-independent signaling, and the effect may reach the maximal response in DPP4-deficient rats. Therefore, we cannot detect the potentiation effect of GLP-1(9–36) in decreasing infarct size. Further investigation is needed to clarify the underlying mechanism of GLP-1 receptor-independent pathway.

The inflammation system plays a prominent role in I/R injury, the cytokines being elevated after myocardial infarction (Zhang and Chen 2008). TNF-α is crucially involved in the pathogenesis and progression of myocardial I/R injury, while anti-TNF-α treatment improves the recovery of cardiac function (Kleinbongard et al. 2011). Lower cardiac TNF-α concentration was found after I/R in DPP4-deficient rats than in wild-type rats. This finding conforms to the previous study that GLP-1 exerted anti-inflammatory effect. GLP-1 inhibits IFN-γ-induced NO production by suppression of TNF-α production in MIN6N8a mouse β cells (Hahm et al. 2008). Exendin-4 suppresses LPS-induced mRNA expression of TNF-α, MCP1, and NFκB translocation to the nucleus in macrophage (Arakawa et al. 2010). The anti-inflammatory effect of GLP-1 may contribute to the protective effect in DPP4-deficient rats. Furthermore, DPP4 inhibitor was able to suppress the proliferation of human lymphocytes stimulated with mitogens or allogeneic cells, and it had been evaluated as a treatment of multiple sclerosis and rheumatoid arthritis in animal models (Williams et al. 2003; Reinhold et al. 2007). Whether DPP4 deficiency-induced functional change contributes to the cardioprotective effect in DPP4-deficient rats cannot be excluded in this study.

In conclusion, the present study identified the cardioprotective mechanisms in DPP4-deficient rats. One is through GLP-1 receptor to ameliorate the cardiac dysfunction and reduce infarct size via decreasing TNF-α expression and increasing the phosphorylation of AKT and GSK-3β in association with maintaining GLUT4 expression. The other is mediated through GLP-1 receptor-independent pathway to

reduce cardiac injury, which needs further study to clarify the detailed signaling.

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