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Dipeptidyl peptidase IV inhibition upregulates GLUT4 translocation and expression in heart and skeletal muscle of spontaneously hypertensive rats

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ABSTRACT

The purpose of the current study was to test the hypothesis that the dipeptidyl peptidase IV (DPPIV) inhibitor sitagliptin, which exerts anti-hyperglycemic and anti-hypertensive effects, upregulates GLUT4 translocation, protein levels, and/or mRNA expression in heart and skeletal muscle of spontaneously hypertensive rats (SHRs). Ten days of treatment with sitagliptin (40 mg/kg twice daily) decreased plasma DPPIV activity in both young (Y, 5-week-old) and adult (A, 20-week-old) SHRs to similar extents (~85%). However, DPPIV inhibition only lowered blood pressure in Y-SHRs (119 ± 3 vs. 136 ± 4 mmHg). GLUT4 translocation, total protein levels and mRNA expression were decreased in the heart, soleus and gastrocnemius muscle of SHRs compared to age-matched Wistar Kyoto (WKY) normotensive rats. These differences were much more pronounced between A-SHRs and A-WKY rats than between Y-SHRs and Y-WKY rats. In Y-SHRs, sitagliptin normalized GLUT4 expression in the heart, soleus and gastrocnemius. In A-SHRs, sitagliptin increased GLUT4 expression to levels that were even higher than those of A-WKY rats. Sitagliptin enhanced the circulating levels of the DPPIV substrate glucagon-like peptide-1 (GLP-1) in SHRs. In addition, stimulation of the GLP-1 receptor in cardiomyocytes isolated from SHRs increased the protein level of GLUT4 by 154 ± 13%. Collectively, these results indicate that DPPIV inhibition upregulates GLUT4 in heart and skeletal muscle of SHRs. The underlying mechanism of sitagliptin-induced upregulation of GLUT4 in SHRs may be, at least partially, attributed to GLP-1 .

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1. Introduction

Maintenance of glucose homeostasis relies both on insulin secretion and insulin stimulation of facilitative glucose uptake, which, in mammalian cells, is mediated by a family of glucose transport proteins. In striated muscle (skeletal and cardiac) and adipose tissues, this effect is primarily due to the recruitment of glucose transporter type 4 (GLUT4) from intracellular vesicles to the plasma membrane (James et al., 1988).

Abnormalities in glucose homeostasis have been observed in hypertensive patients and in experimental models of hypertension. Numerous reports have documented that spontaneously hypertensive rats (SHRs) exhibit changes in GLUT4 function, trafficking, and/or expression in insulin-sensitive tissues (Campbell et al., 1995; Chiappe De Cingolani and Caldiz, 2004; Katayama et al., 1997; Lehnen et al., 2011; Paternostro et al., 1995; Reaven et al., 1989). More specifically, most of these studies have found that GLUT4 is downregulated in adipocytes, skeletal and cardiac muscles from hypertensive animals.

Inhibitors of the enzyme dipeptidyl peptidase IV (DPPIV) represent a novel class of anti-hyperglycemic agents that improve the control of blood glucose levels in type 2 diabetic patients primarily by preventing the DPPIV-mediated rapid degradation of the incretin glucagon-like peptide-1 (GLP-1) (Ahren, 2003; Drucker, 2003; Drucker and Nauck, 2006; Holst, 2007). Notably, studies of DPPIV null mice have shown that these animals not only display improved glucose tolerance (Marguet et al., 2000) but also are resistant to the development of obesity induced by a high fat diet (Conarello et al., 2003), which suggests that chronic ablation of DPPIV activity confers metabolic benefits that extend beyond glucose homeostasis per se.

Previous studies have suggested that DPPIV inhibition may have a beneficial effect on blood pressure control as well (Mistry et al., 2008; Okerson et al., 2011; Pacheco et al., 2011;

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Tanaka et al., 2011). The DPPIV inhibitor sitagliptin reduced both systolic and diastolic blood pressure in nondiabetic patients with mild to moderate hypertension (Mistry et al., 2008). Moreover, we have previously shown that long-term inhibition of DPPIV in SHRs beginning at the young, pre-hypertensive stage produces a sustained reduction in blood pressure of approximately 15 mmHg (Pacheco et al., 2011). To date, the mechanisms by which DPPIV inhibition reduces blood pressure have only been partially addressed (Girardi and Di Sole, 2012; Pacheco et al., 2011; Tanaka et al., 2011).

On the basis of these observations, the present study was undertaken to test the hypothesis that DPPIV inhibition may upregulate GLUT4 translocation, protein levels and/or mRNA expression in the skeletal and cardiac muscle of SHRs. The possibility that the effect of DPPIV inhibition was, at least in part, mediated by GLP-1 receptor stimulation and activation of the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signal transduction pathway was also investigated.

2. Material and methods

2.1. Materials

Reagents were purchased from Sigma Chemical Company (Saint Louis, MO) unless stated otherwise. Januvia tablets (Merck & Company, Inc.) containing 100 mg of sitagliptin monophosphate were purchased from a local pharmacy. Rabbit polyclonal antibody against GLUT4 was purchased from Millipore (Temecula, CA), the monoclonal antibody against DPPIV, clone 5E8, and the rabbit polyclonal antibody against the GLP-1 receptor were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and the monoclonal antibody against actin JLA20 was from Calbiochem (San Diego, CA). Anti-pSer/Thr PKA substrate antibody was purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Life Technologies Corporation (Carlsbad, CA).

2.2. Extraction of sitagliptin

Extraction of sitagliptin was performed as previously described by Jackson and Mi (2008) with some modifications. Given the fact that sitagliptin phosphate monohydrate is highly water-soluble, four 100 mg Januvia tablets were added to 10 ml of water and incubated in the refrigerator for 1 h to dissolve the tablets. The suspension was vortexed and centrifuged at 2000 g for 10 min 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).

2.3. Animal procedures

Experimental procedures were performed 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. Studies were conducted in male SHRs at 5 (Y, young, $n=40$) and 20 weeks (A, adult, $n=42$) of age that were randomly divided into two groups and treated twice a day, by oral gavage, with sitagliptin (IDPPIV, 40 mg/day) or vehicle (water) for a ten day period. Age-matched WKY rats served as normotensive controls ($n=40$). The 40 mg/day dosage of sitagliptin was determined based on dose-response experiments previously performed by Pacheco et al. (2011). The animals were housed under constant temperature, humidity and light cycle (12:12 h light:dark) conditions and had *ad libitum* access to standard rat chow and tap water. At the end of the treatment period and after a six hour fast, the animals were anesthetized with an *i.p.* injection of sodium pentobarbital (50 mg/kg BW). Blood samples were collected from the infraorbital plexus, and the animals were subsequently killed by decapitation. The heart and hindlimb skeletal muscles, including the gastrocnemius, soleus, and extensor digitorum longus (EDL), were excised, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent protein and RNA extraction.

2.4. Blood pressure measurements

Systolic blood pressure was recorded in conscious restrained animals by the tail-cuff method (BP-2000 Blood Pressure Analysis System, Visitech Systems, Inc., Apex, NC) one day prior to the administration of either sitagliptin or vehicle (Day 0) and after ten days of treatment (Day 10). Prior to these measurements, the rats were trained for two weeks with the blood pressure device to allow them to become accustomed to the procedure.

2.5. Glucose tolerance test

Glucose tolerance tests were performed on both SHRs and WKY rats on the eighth day of treatment with sitagliptin or the vehicle. Animals were exposed to fasting conditions for six hours (beginning approximately 8:00 AM), weighed, and injected intraperitoneally with a glucose solution (1.0 g/kg body weight). Blood was withdrawn from tail veins of conscious animals at 0, 15, 30, 60, 90, and 120 min after glucose injection. Blood glucose concentration was immediately measured with the ACCU-CHEK ® Performa meter (Roche Diagnostics GmbH, Mannheim, Germany). The area under the curve (AUC) was determined for quantification of the glucose tolerance test.

2.6. Measurements of plasma glucose, insulin, and DPPIV

Plasma samples were obtained by centrifugation of blood samples at 4000 g for 10 min at 4 °C and were stored at −80 °C until they were assayed for glucose and insulin levels and DPPIV activity. Plasma glucose was measured by the hexokinase method using a commercially available kit (Labtest, Lagoa Santa, MG, Brazil). Plasma insulin concentration was measured with an ELISA kit (Millipore) according to the manufacturer's protocol. Plasma DPPIV activity was measured colorimetrically using glycyl-prolyl-para-nitroanilide as a chromogenic substrate as described previously (Pacheco et al., 2011).

2.7. Determination of plasma concentrations of total and active GLP-1

Blood from six rats per experimental group was withdrawn from the retro-orbital plexus, immediately transferred into chilled tubes containing 1 mg/ml EDTA and 10 μM of the DPPIV inhibitor P32/98 (Enzo Life Sciences Inc., Farmingdale, NY) and centrifuged at 4000 g and 4 °C for 10 min. Plasma samples were frozen and stored at −80 °C. Samples were extracted using a solid phase extraction C18-Ecolumn (Strata Phenomenex, Torrance, CA) in accordance with the manufacturer's instructions. The plasma GLP-1 (total) concentration was determined by an enzyme-linked immunosorbent assay (Millipore) according to the manufacturer's instructions. Plasma levels of intact GLP-1 [7–36 amide] were measured with an enzyme-linked immunosorbent assay (Linco Research, St. Charles, MO), which uses an *N*-terminally directed antibody that does not cross-react with the DPPIV cleavage product GLP-1 [9–36 amide].

2.8. Preparation of microsomal and plasma membrane fractions from heart and skeletal muscles

The skeletal (soleus, EDL and gastrocnemius) and ventricular muscles were excised from rats, minced with opposing razor blades, and homogenized in a *Polytron* PT 2100 homogenizer (Kinmatica, AG, Switzerland) in an ice cold buffer containing 1 mM EDTA, 250 mM sucrose, and 10 mM Tris, pH 7.5. The homogenate was centrifuged at 760 g for 5 min at 4 °C. The supernatant was removed and subjected to 60 min of centrifugation at 31,000 g and 4 °C to pellet the fraction enriched for the plasma membrane. Next, the supernatant was subjected to centrifugation for 60 min at 190,000 g and 4° C to pellet the fraction enriched for microsomes. The protein concentration of both microsomal and plasma membrane fractions was determined by the Lowry method (Lowry et al., 1951).

2.9. Evaluation of GLUT4 total and subcellular distribution

Total cellular GLUT4 protein expression was determined as the sum of both the microsomal and plasma membrane subcellular fractions (TOTAL). The percentage of GLUT4 in the plasma membrane (PM) was calculated as (PM GLUT4/TOTAL GLUT4) × 100. The amounts of TOTAL and plasma membrane GLUT4 are expressed as percentages of the controls (normotensive WKY rats).

2.10. SDS-PAGE and immunoblotting

Equal amounts of protein or volume of plasma were resolved by SDS-PAGE and analyzed by immunoblotting as previously described (Crajoinas et al., 2010). The visualized bands were digitized using an ImageScanner (GE HealthCare) and quantified using the Scion Image Software package (Scion Corporation, Frederick, MD).

2.11. RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from skeletal and cardiac muscles with TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA). The extracted RNA was quantified spectrophotometrically at 260 nm and stored at −80 °C until further analysis. cDNA synthesis was performed with the MML-V Reverse Transcriptase kit (Promega, Madison, WC) according to the manufacturer's recommended protocol. Quantitative real time PCR (RT-PCR) was carried out using the QuantiTech SYBR green PCR kit (Qiagen, Valencia, CA) on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The following oligonucleotide primers were used to detect GLUT4 (BC085757) and cyclophilin (XR 009383): GLUT4 forward: 5′-CATTGCTTCTGGCTATCAC-3′; GLUT4 reverse: 5′-AACATGCTGGTTGAATAGTAG-3′; cyclophilin forward: 5′-GGATTCAT-GTGCCAGGG TGG-3′; and cyclophilin reverse: 5′-CACATGCTTGCCATCCAGCC-3′. The amplification conditions consisted of initial denaturation (95 °C for 10 min) followed by 45 cycles of 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 20 s. The comparative threshold (CT) cycle method was used for data analysis.

2.12. Indirect measurement of PKA activity

Equal amounts (15 μg) of plasma membrane protein isolated from the heart and gastrocnemius muscles of SHR and WKY rats were resolved by SDS-PAGE and analyzed by immunoblotting using an antibody specific for phosphorylated PKA substrates (Gronborg et al., 2002). The visualized bands were digitized using

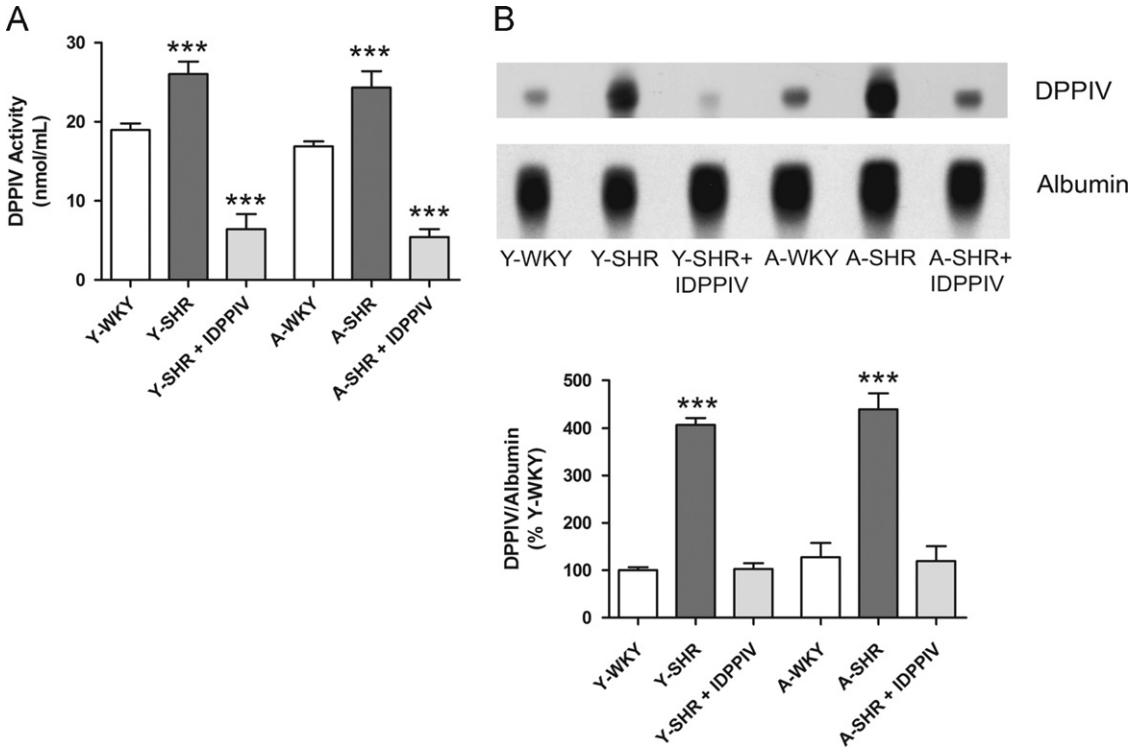

Fig. 1. Plasma dipeptidyl peptidase IV (DPPIV) activity and abundance in sitagliptin-treated and untreated SHRs and age-matched WKY rats. (A) Plasma DPPIV activity was measured by a colorimetric method in young and adult SHRs treated with sitagliptin or vehicle (water) for ten days and in age-matched WKY rats. $n=14$ rats per group, with the exception of the A-SHR and A-SHR+IDPPIV groups in which 15 rats were analyzed per group and (B) The abundance of DPPIV was evaluated in the plasma of young and adult animals by immunoblotting. An equal volume of plasma (0.5 μl) from each animal was subjected to SDS-PAGE, transferred to a PVDF membrane and incubated with a monoclonal antibody against DPPIV. Membranes were stained with Ponceau S prior to antibody incubation and albumin was used as an internal control. Values are the means ± S.E.M. $n=6$ animals/group $^{***}P<0.001$ *vs.* age-matched WKY rats.

an ImageScanner (GE HealthCare) and quantified using the Scion Image Software package (Scion Corporation, Frederick, MD).

2.13. Isolation and culture of rat cardiac myocytes

Cardiac myocytes were prepared by enzymatic disaggregation as previously described (Barreto-Chaves et al., 2000). Briefly, hearts from neonatal (7–10 days old) SHRs were excised, and their ventricles were minced and transferred to a sterile buffer. The tissue was then subjected to multiple enzymatic digestions at 37 °C using a mixture of collagenase and pancreatin. Five subsequent digestions, each lasting for 20 min, were performed. The solution obtained in each digestion was transferred to a tube containing 1 ml of newborn calf serum (NCS) and centrifuged. Each cell pellet was resuspended in NCS. Dissociated cells were pooled. Cardiac myocytes were isolated by layering the cell suspension onto a discontinuous Percoll density gradient. After removing all traces of Percoll, myocytes were cultured in DMEM containing 5% fetal calf serum, 10% horse serum and 100 μM bromodeoxyuridine. Myocytes were serum starved for 48 h prior to drug treatment.

2.14. Measurement of cyclic adenosine monophosphate (cAMP)

Cardiac myocytes were treated for 10 min with 1 mM 3-isobutyl-1-methylxanthine (IBMX) and subsequently incubated for another 30 min with IBMX, IBMX with 50 nM GLP-1, IBMX with 5 μM exendin-9 or IBMX with GLP-1 and exendin-4 . cAMP levels were measured by an enzyme immunoassay (cAMP Direct Biotrak EIA, GE HealthCare) according to the manufacturer's specifications.

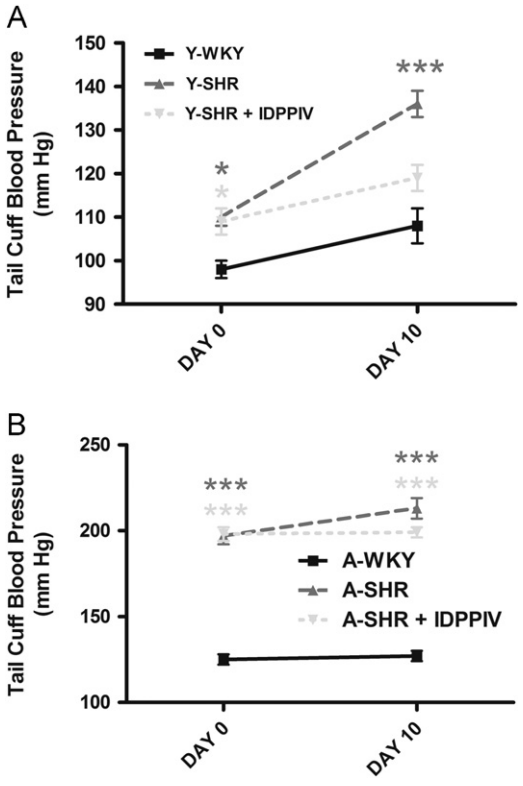

Fig. 2. Systolic blood pressure of young (Y-SHRs) and adult SHRs (A-SHRs) before and after treatment with sitagliptin and of age-matched WKY rats. Systolic blood pressure (SBP) was measured by the tail cuff method in young (A) and adult (B) rats before and after sitagliptin (IDPPIV) or vehicle treatment. WKY rats received no treatment. Values are the means ± S.E.M. $n=14$ rats per group, with the exception of the A-SHR and A-SHR+IDPPIV groups in which 15 rats were analyzed per group. *$P<0.05$ and ***$P<0.001$ *vs.* age-matched WKY.

2.15. Statistical analysis

Data are expressed as the means ± S.E.M. Comparisons among groups were made by two-way analysis of variance (ANOVA), except for assessing changes on blood pressure, in which repeated measures analysis of variance (rANOVA) was applied. The Bonferroni method was used to adjust the P value for multiple comparisons. The results were considered significant when $P<0.05$.

3. Results

3.1. Effect of sitagliptin on plasma DPPIV activity

Fig. 1 shows that DPPIV activity was significantly increased in the plasma of SHRs compared to WKY rats (Fig. 1A). Sitagliptin treatment inhibited the activity of DPPIV in the plasma of both young and adult SHRs to similar extents (85 ± 7%, $P<0.001$ and 88 ± 8%, $P<0.001$, respectively). As shown in Fig. 1B, increased DPPIV activity in SHRs compared to WKY rats was accompanied by a greater abundance of the peptidase in the plasma of hypertensive animals. Surprisingly, sitagliptin not only inhibited plasma DPPIV catalytic activity but also decreased the circulating levels of the enzyme (Fig. 1B).

3.2. Effect of sitagliptin treatment on blood pressure

Fig. 2A shows the effect of sitagliptin treatment on the blood pressure of young SHRs. Baseline systolic blood pressures were similar between the two groups of young SHRs (Y-SHR and Y-SHR+IDPPIV) and were slightly but significantly higher than in the Y-WKY rat group. Treatment with sitagliptin for ten days

Table 1
Body weight (BW), food and water intake, plasma glucose and insulin concentrations of young SHR treated with the DPPIV inhibitor sitagliptin (Y-SHR+IDPPIV) or water (Y-SHR) and age-matched WKY (Y-WKY).

	Y-WKY ($n=14$)	Y-SHR ($n=14$)	Y-SHR+IDPPIV ($n=14$)
BW at day 0 (g)	167 ± 15	123 ± 8[a]	125 ± 7[a]
BW at day 10 (g)	205 ± 15	151 ± 7[a]	154 ± 7[a]
% BW gain	22.7 ± 1.8	22.7 ± 1.3	23.2 ± 1.7
Food intake (g/kg)	209 ± 17	206 ± 15	196 ± 11
Water intake (ml/kg)	151 ± 6	145 ± 6	147 ± 4
Glucose (mM)	5.98 ± 0.14	5.81 ± 0.24	5.60 ± 0.29
Insulin (ng/ml)	1.31 ± 0.26	1.43 ± 0.13	1.23 ± 0.17

All parameters are expressed as means ± S.E.M.

[a] $P<0.001$ *vs.* Y-WKY. The number of animals in each experimental group is shown in parenthesis.

Table 2
Body weight (BW), food and water intake, plasma glucose and insulin concentrations of adult SHR treated with the DPPIV inhibitor sitagliptin (A-SHR+IDPPIV) or water (A-SHR) and age-matched WKY (A-WKY).

	A-WKY ($n=14$)	A-SHR ($n=15$)	A-SHR+IDPPIV ($n=15$)
BW at day 0	417 ± 8	319 ± 7[b]	323 ± 8[b]
BW at day 10	425 ± 8	325 ± 10[b]	328 ± 8[b]
% BW gain	2.1 ± 0.2	1.7 ± 0.4	1.6 ± 0.3
Food intake (g/kg)	131 ± 11	130 ± 9	126 ± 7
Water intake (ml/kg)	85 ± 4	89 ± 4	87 ± 3
Glucose (mM)	6.06 ± 0.20	6.12 ± 0.32	6.01 ± 0.34
Insulin (ng/ml)	1.47 ± 0.12	2.58 ± 0.27[a]	1.36 ± 0.20

All parameters are expressed as means ± S.E.M.

[a] $P<0.01$.

[b] $P<0.001$ *vs.* A-WKY. The number of animals in each experimental group is shown in parenthesis.

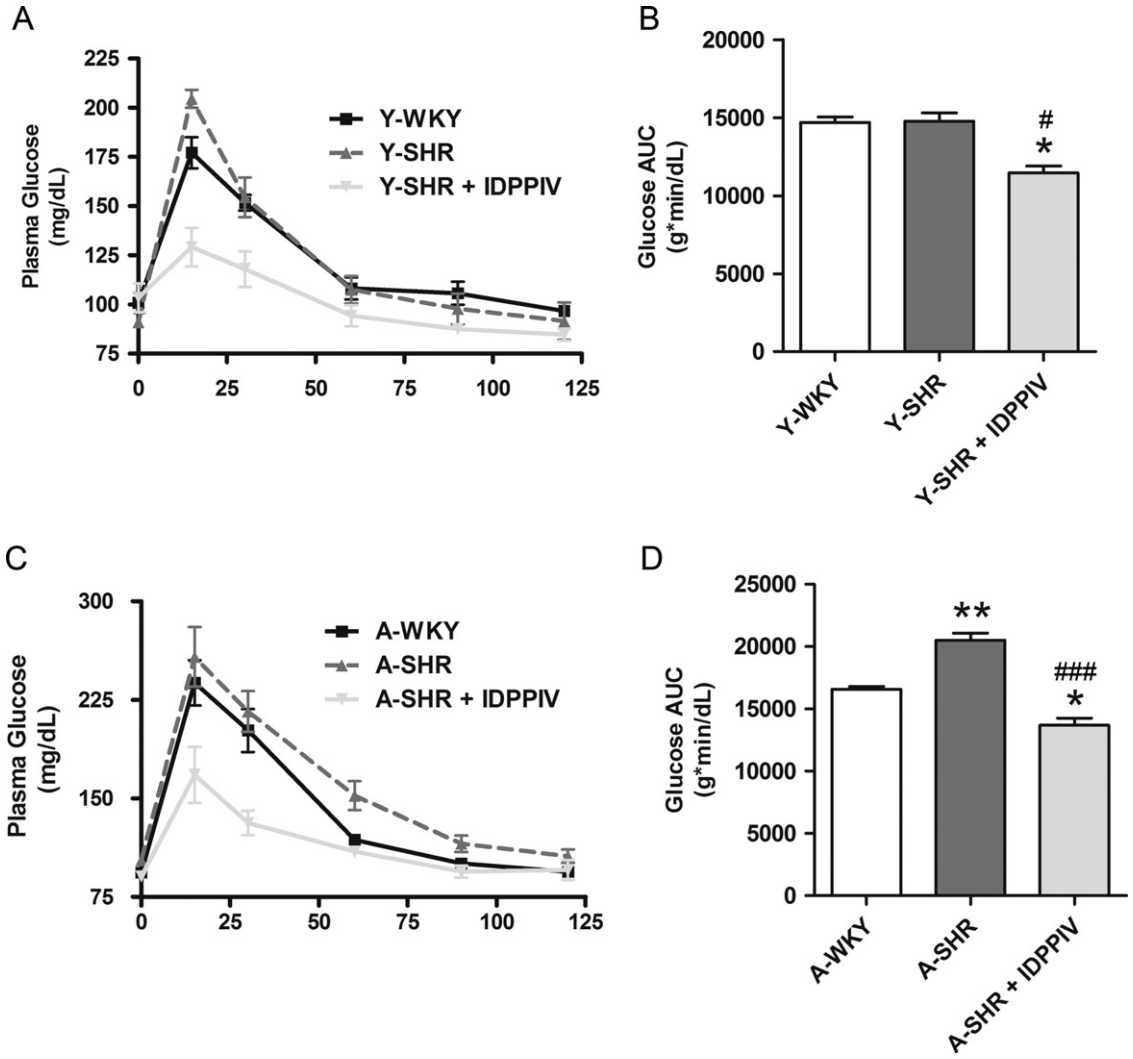

Fig. 3. Glucose Tolerance Test in sitagliptin-treated and untreated SHRs and age-matched WKY rats. Intraperitoneal glucose tolerance tests were performed following a six-hour fast on young (A) and adult (B) SHRs treated with or without sitagliptin (IDPPIV) and age-matched WKY rats after an *i.p.* injection of D-glucose (1 g/kg of body weight). Glucose was measured in blood samples from conscious rat tail veins at the indicated time intervals. (C) and (D) Glucose area under the curve (AUC) values are presented as the means ± S.E.M. $n=8$ animals/group $^{*}P<0.05$ and $^{**}P<0.01$ *vs.* age-matched WKY rats. $^{\#}P<0.05$ and $^{\#\#\#}P<0.001$ *vs.* age-matched SHR.

suppressed systolic blood pressure increases in the Y-SHR+IDPPIV group (119 ± 3 *vs.*136 ± 4 mmHg in the Y-SHR group; $P<0.05$). The mean value of systolic blood pressure in the Y-SHR+IDPPIV group was not significantly different from age-matched Y-WKY rats (119 ± 3 *vs.* 107 ± 4 mmHg, respectively; n.s.), whereas blood pressure continued to increase in vehicle-treated Y-SHRs (Fig. 2A).

As shown in Fig. 2B, both groups of adult SHRs (A-SHR and A-SHR+IDPPIV) had markedly higher systolic blood pressures than the normotensive control A-WKY rats both before and after sitagliptin administration. Inhibition of plasma DPPIV in the adult SHRs with established hypertension produced only a non-statistically significant trend towards reduced systolic blood pressure compared to the vehicle-treated SHR control (198 ± 6 *vs*. 205 ± 7 mmHg, respectively; n.s.).

3.3. *Effect of sitagliptin treatment on body weight and glucose homeostasis*

Body weight was greater in both young and adult WKY rats than in age-matched SHRs before and after the ten-day experimental period (Tables 1 and 2). Weight gain and food and water consumption were unaltered by treatment with sitagliptin (Tables 1 and 2). Plasma glucose and insulin were measured after a six-hour fast (8:00–14:00 h). There were no significant differences in blood glucose between SHRs and WKY rats at all ages studied (Tables 1 and 2). Plasma insulin levels were not significantly different between the strains at young ages. Adult SHRs displayed an enhanced plasma insulin concentration under fasting normoglycemia. Inhibition of DPPIV by sitagliptin completely restored plasma insulin levels in the A-SHR+IDPPIV group.

Fig. 3 illustrates the plasma glucose response of Y-SHRs (Fig. 3A and B) and A-SHRs (Fig. 3C and D) treated with and without sitagliptin and of age-matched WKY rats at various times after an *i.p.* injection of 1 mg/kg glucose. There were no significant differences in glucose response between Y-SHRs and Y-WKY rats (Fig. 3A and B). In contrast, A-SHRs exhibited an increased glucose response compared to A-WKY rats (Fig. 3C and D). Glucose AUC responses after glucose load were significantly reduced by sitagliptin treatment in Y-SHRs and A-SHRs compared to the age-matched vehicle-treated SHRs and normotensive controls (Fig. 3).

3.4. *Effect of sitagliptin on GLUT4 protein expression and subcellular localization in heart and skeletal muscle*

The effects of sitagliptin on GLUT4 protein abundance and subcellular localization were evaluated in various striated muscle

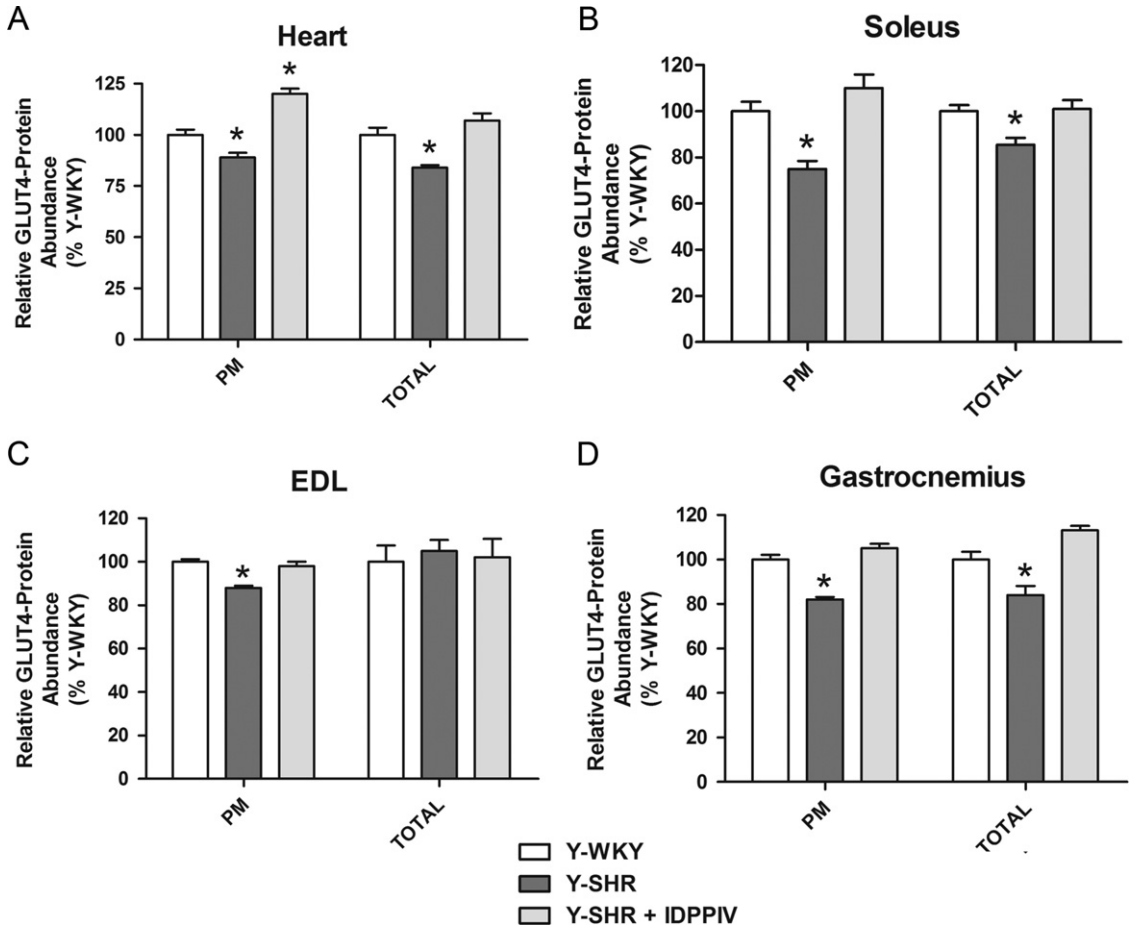

Fig. 4. GLUT4 protein expression in heart and skeletal muscle of sitagliptin-treated and untreated young SHRs and age-matched WKY rats. Graphical representation of the total protein levels and plasma membrane expression of GLUT4 in (A) heart, (B) gastrocnemius, (C) soleus, and (D) extensor digitorum longus (EDL) muscle of young SHRs treated with water (Y-SHR) or sitagliptin (Y-SHR+IDPPIV) for ten days and age-matched WKY (Y-WKY) rats. Values are presented as the means ± S.E.M. $n=6$ animals/group $^{*}P<0.05$ *vs.* Y-WKY.

types of the young (Fig. 4) and adult (Fig. 5) SHRs. As depicted in Fig. 4, the amount of GLUT4 in the plasma membrane fraction was reduced in the heart cells of Y-SHRs compared to those of Y-WKY rats ($-11.0\pm0.3\%$) (Fig. 4A) and in each of the skeletal muscles examined, which included the slow-twitch oxidative soleus ($-25.0\pm1.0\%$) (Fig. 4B), the fast-twitch glycolytic EDL ($-12.0\pm0.2\%$) (Fig. 4C), and the fast-twitch glycolytic and oxidative gastrocnemius ($-18.0\pm0.9\%$) (Fig. 4D). Reductions in the GLUT4 plasma membrane levels in the heart, soleus and gastrocnemius were accompanied by a slight but significant decrease in total GLUT4 protein expression. The total GLUT4 protein levels in these three types of muscle cells were normalized to the Y-SHRs treated with sitagliptin for ten days (Y-SHR+IDPPIV). There were no statistically significant differences between GLUT4 expression in the plasma membrane of skeletal muscle cells from the Y-SHR+IDPPIV group and Y-WKY rats (Fig. 4B–D). Interestingly, DPPIV inhibition resulted in a significant increase ($+20.0\pm0.5\%$) in GLUT4 expression in the plasma membranes of heart muscle cells from sitagliptin-treated Y-SHRs compared to normotensive Y-WKY rats (Fig. 4A).

As shown in Fig. 5, reduction of GLUT4 expression in the plasma membranes of heart and skeletal muscle cells was much more pronounced in A-SHR *vs.* A-WKY rats than in Y-SHR *vs.* Y-WKY rats. A-SHRs displayed decreased amounts of GLUT4 in the plasma membrane fractions from the heart ($-32.0\pm1.0\%$) (Fig. 5A), soleus ($-35.0\pm3.4\%$) (Fig. 5B), EDL ($-15.0\pm0.6\%$) (Fig. 5C), and gastrocnemius ($-30.0\pm1.8\%$) (Fig. 5D) muscles compared to age-matched WKY rats. Decreased plasma membrane GLUT4 expression was associated with lower GLUT4 levels in the heart and in red muscles enriched with oxidative type I and IIa myofibers (soleus and gastrocnemius, respectively) (Fig.5). Inhibition of DPPIV by sitagliptin increased GLUT4 plasma membrane levels and total protein expression in the heart, soleus, and gastrocnemius of in the A-SHR+IDPPIV group to levels greater than those found in the normotensive adult animals. Total GLUT-4 expression in the EDL was not altered in SHRs compared to WKY rats and remained unchanged in sitagliptin-treated rats.

3.5. *Effect of sitagliptin on GLUT4mRNA expression in heart and skeletal muscle*

The relative amounts of GLUT4mRNA in heart and skeletal muscle cells derived from sitagliptin-treated and untreated SHRs and WKY rats were determined by real time reverse transcription-PCR (Figs. 6 and 7). As shown in Fig. 6, the GLUT4mRNA levels were significantly reduced in the heart ($-18\pm1\%$), soleus ($-17\pm1\%$), and gastrocnemius ($-9\pm1\%$) muscles of Y-SHRs compared to young normotensive rats (Y-WKY). There were no statistically significant differences in GLUT4 gene expression between young SHRs treated with sitagliptin for ten days (Y-SHR+IDPPIV) and Y-WKY rats in all muscle tissues analyzed.

As depicted in Fig. 7, the expression of GLUT4 mRNA was significantly reduced in the heart ($-38\pm1\%$), soleus ($-22\pm1\%$) and gastrocnemius ($-22\pm1\%$) of A-SHRs compared to A-WKY rats.

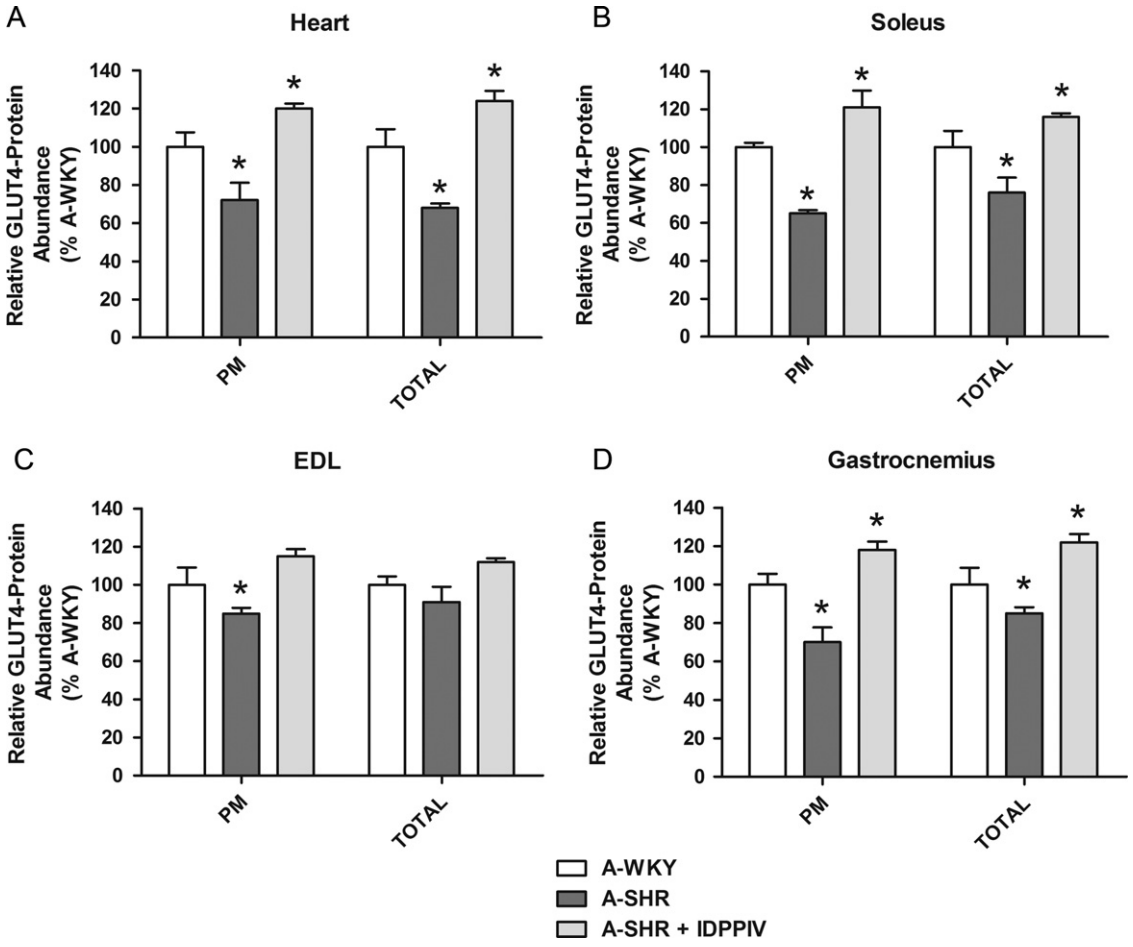

Fig. 5. GLUT4 protein expression in heart and skeletal muscle of sitagliptin-treated and untreated adult SHRs and age-matched WKY rats. Graphical representation of the total protein levels and plasma membrane expression of GLUT4 in (A) heart, (B) soleus, (C) extensor digitorum longus (EDL), and (D) gastrocnemius muscle of adult-SHRs treated with water (A-SHR) or sitagliptin (A-SHR+IDPPIV) for ten days and age matched WKY (A-WKY) rats. Values are presented as the means ± S.E.M. $n=6$ animals/group $^{*}P<0.05$ *vs.* A-WKY.

Following a similar pattern of changes in total protein expression, treatment with sitagliptin increased GLUT4 mRNA expression in the heart, soleus, and gastrocnemius of the A-SHR+IDPPIV group to levels greater than those of normotensive adult animals (A-WKY).

3.6. Effect of sitagliptin treatment on the circulating levels of GLP-1

The effect of DPPIV inhibition on circulating levels of the incretin hormone GLP-1 in young and adult SHRs is reported in Fig. 8. Plasma levels of total GLP-1 (tGLP-1) did not vary significantly among young animals (Fig. 8A). On the other hand, the levels of intact GLP-1, GLP-1[7–36], were 2.3 times greater in sitagliptin-treated Y-SHRs than in Y-SHRs treated with water and 2.2 times greater than in Y-WKY rats (Fig. 8B). Additionally, there was a non-significant trend towards a decrease in plasma tGLP-1 in both the A-SHR and A-SHR+IDPPIV groups compared to A-WKY rats (Fig. 8A). Similar to the findings in young animals, treatment with sitagliptin remarkably increased the plasma levels of GLP-1[7–36] in the adult SHRs compared to both A-SHRs given water and to A-WKY rats (Fig. 8B).

3.7. Effect of sitagliptin treatment on the levels of PKA-mediated phosphorylation in the heart and gastrocnemius

The effect of DPPIV inhibition on PKA activity was estimated by immunoblotting. As shown in Fig. 9A, sitagliptin treatment increased the levels of phosphorylated PKA substrates in the plasma membrane of heart cells from both young and adult SHRs compared to age-matched vehicle-treated SHR and WKY rats. Conversely, DPPIV inhibition did not alter the levels of proteins phosphorylated by PKA in the plasma membrane of gastrocnemius cells (Fig. 9B).

3.8. Direct effects of GLP-1 on SHR cardiomyocytes

As illustrated in Fig. 10A, the treatment of neonatal SHR cardiac myocytes with 50 nM GLP-1 for 30 min increased the intracellular levels of cAMP by 114 ± 7%. Additionally, as demonstrated in Fig. 10B, incubation of SHR cardiomyocytes with GLP-1 for 24 h induced an increase in GLUT4 protein expression by 154 ± 13%. Both responses were blocked in the presence of the GLP-1 receptor antagonist exendin-9 .

4. Discussion

The present study demonstrates that inhibition of DPPIV by sitagliptin upregulates GLUT4 translocation and expression at both the protein and mRNA level in cardiac and oxidative red muscles of young and adult spontaneously hypertensive rats (SHRs). Interestingly, in adult SHRs, DPPIV inhibition upregulates striated muscle GLUT4 expression to levels to a greater extent than those found in age-matched normotensive animals.

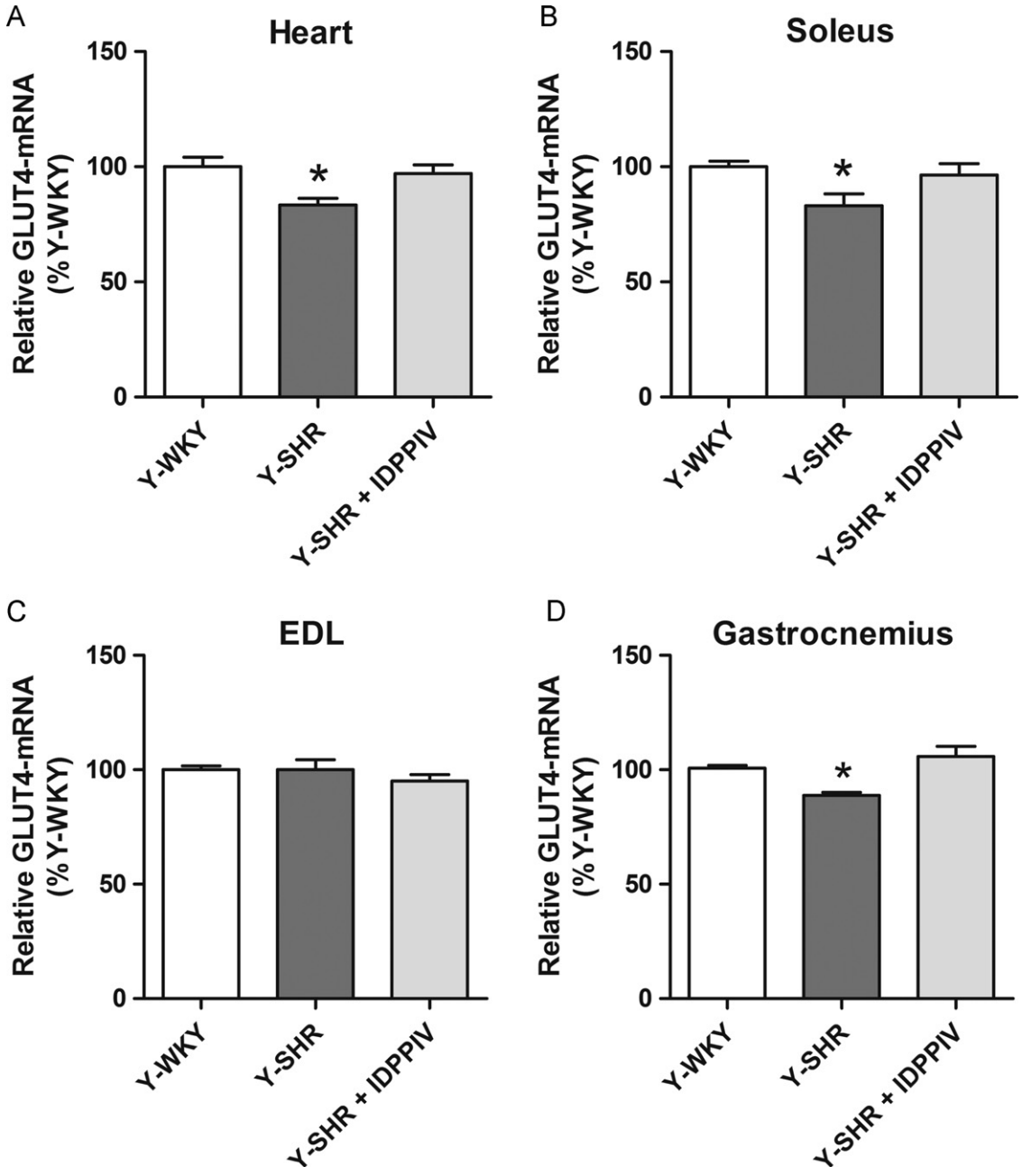

Fig. 6. GLUT4-mRNA expression in heart and skeletal muscle of sitagliptin-treated and untreated young SHRs and age-matched WKY rats. Graphical representation of the relative mRNA-GLUT4 expression levels in (A) heart, (B) soleus, (C) extensor digitorum longus (EDL), and (D) gastrocnemius muscle of young SHRs treated with water (Y-SHR) or sitagliptin (Y-SHR + IDPPIV) for ten days and age-matched WKY (Y-WKY) rats. GLUT4 mRNA expression was quantified by real time RT-PCR. Expression of the GLUT4 transcript was normalized to cyclophilin mRNA. Values are presented as the means ± S.E.M. $n=6$ rats/group, $^{*}P<0.05$ *vs.* Y-WKY.

This observation is in accordance with our finding that adult sitagliptin-treated SHRs exhibit an increased rate of plasma glucose decay in following an *i.p.* injection of 1 mg/kg glucose compared to age-matched WKY rats, suggesting that inhibition of plasma DPPIV facilitates glucose uptake in the striated muscles of SHRs.

Facilitated glucose uptake in muscle fibers is mediated by increases in the plasma membrane level of GLUT4 via insulin- and/or muscle contraction-mediated GLUT4 translocation (Lauritzen and Schertzer, 2010; Thorens et al., 1990). The present study showed that at an age of 5–6 weeks, SHRs display lower levels of GLUT4 in the plasma membrane of all types of striated muscle cells analyzed (cardiac, oxidative and glycolytic myofibers) compared with age-matched WKY rats. Reduced plasma membrane GLUT4 protein levels in heart and skeletal oxidative muscles are accompanied by a decrease in total GLUT4 protein and mRNA levels. Conversely, the expression level of GLUT4 was not significantly different between SHRs and WKY rats in EDL muscles. Additionally, no change in GLUT4 expression was observed in the EDL muscles of sitagliptin-treated SHRs. This result may be related to the fact that stimulation of glucose transport by insulin is greater in red muscles enriched with oxidative type I and IIa myofibers (soleus and gastrocnemius muscles) than white muscles enriched with glycolytic type IIb myofibers (EDL) (James et al., 1985).

Glucose intolerance and/or hyperinsulinemia have been reported to be present in many patients with essential hypertension. In accordance with previous studies (Buchanan et al., 1992;

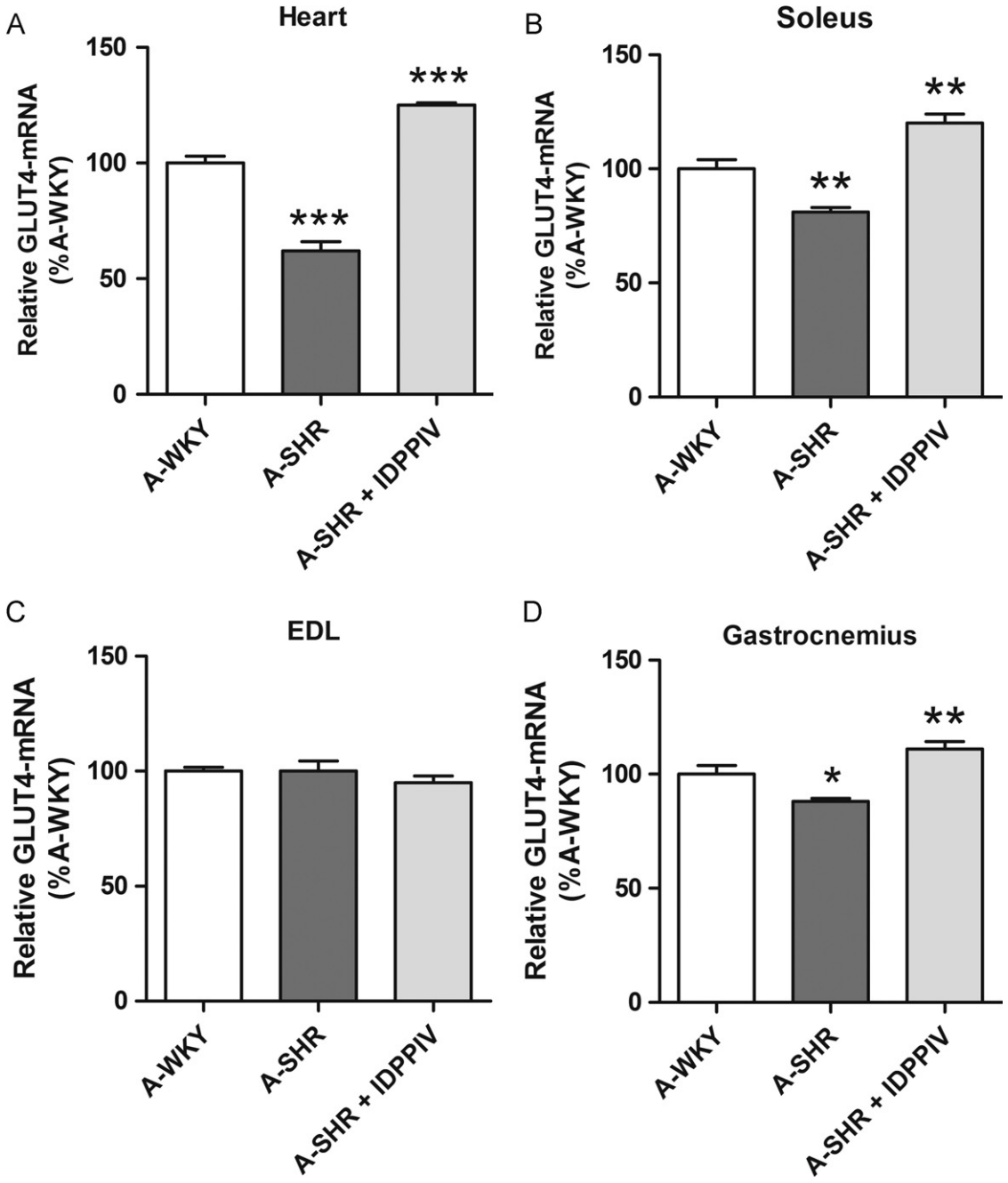

Fig. 7. GLUT4 mRNA expression in heart and skeletal muscle of sitagliptin-treated and untreated adult SHRs and age-matched WKY rats. Graphical representation of the relative GLUT4 mRNA expression levels in (A) heart, (B) soleus, (C) extensor digitorum longus (EDL), and (D) gastrocnemius muscle of adult-SHRs treated with water (A-SHR) or sitagliptin (A-SHR+IDPPIV) for ten days and age matched WKY (A-WKY) rats. GLUT4 mRNA expression was quantified by real time RT-PCR. Levels of GLUT4 mRNA were normalized to those of the internal control, cyclophilin mRNA. Values are presented as the means ± S.E.M. $n=6$ rats/group, $^{*}P<0.05$; $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* A-WKY.

Chen et al., 1994; Lehnen et al., 2011), we found that adult SHRs have normal blood glucose levels but an increased blood insulin concentration. Additionally, we have found that a 10-day treatment with sitagliptin normalizes plasma insulin levels in adult hypertensive animals. Previously reported results demonstrated that adult SHRs are hyperinsulinemic and normoglycemic because of an enhanced sensitivity of their pancreatic beta cells to glucose and that the SHR beta-cell hyperfunction is due to an increase in the catalytic activity of glucokinase (Chen et al., 1994). Interestingly, a correlation between DPPIV treatment, beta-cell function and glucokinase activity has recently been reported by Ding et al. (2001). Their study shows that the DPPIV substrate, GLP-1, stimulates post-translational activation of glucokinase and that this might be one of the possible mechanisms by which GLP-1 exerts its insulinotropic effects on beta cells.

Several possible mechanisms may account for the events triggered by DPPIV inhibition because DPPIV degrades a variety of peptides known to modulate cardiovascular and/or metabolic function (Brandt et al., 2006; Lambeir et al., 2003; Mentlein et al., 1993a, 1993b; Palmieri and Ward, 1983; Shioda et al., 1998). The results from this report demonstrate that sitagliptin-treated SHRs have increased plasma concentrations of the active form of GLP-1 compared to SHRs treated with vehicle and WKY rats. The activity of GLP-1 is transduced by a classical seven transmembrane

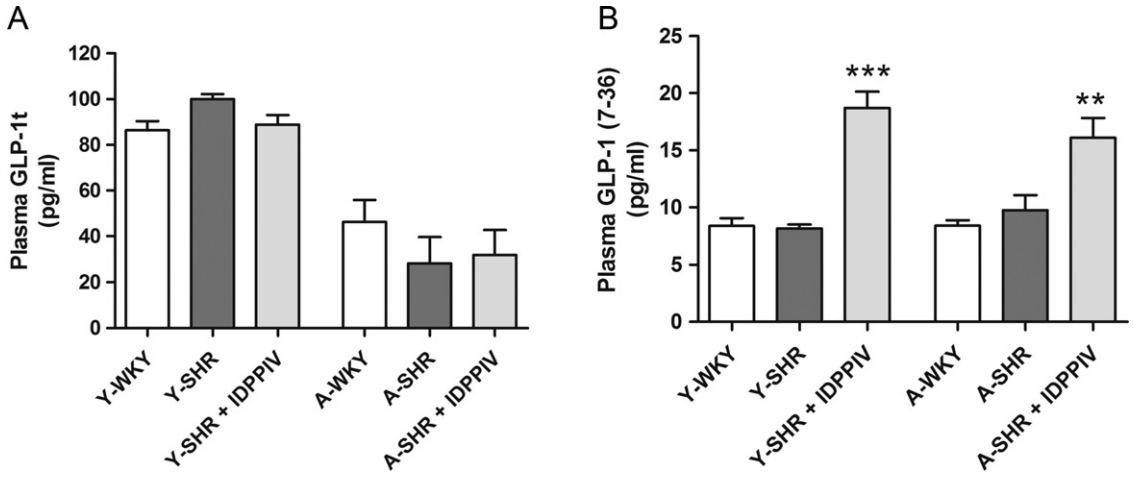

Fig. 8. Plasma GLP-1 levels of sitagliptin-treated and untreated young and adult SHR and age-matched WKY rats. Circulating levels of (A) total GLP-1 (GLP-1t) and (B) active GLP-1(7–36) were measured by ELISA in young (Y) and adult (A) SHRs treated with sitagliptin (IDPPIV) or vehicle (water) for ten days and in age-matched WKY rats. Values are presented as the means ± S.E.M. $n=6$ rats/group. $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* age-matched WKY.

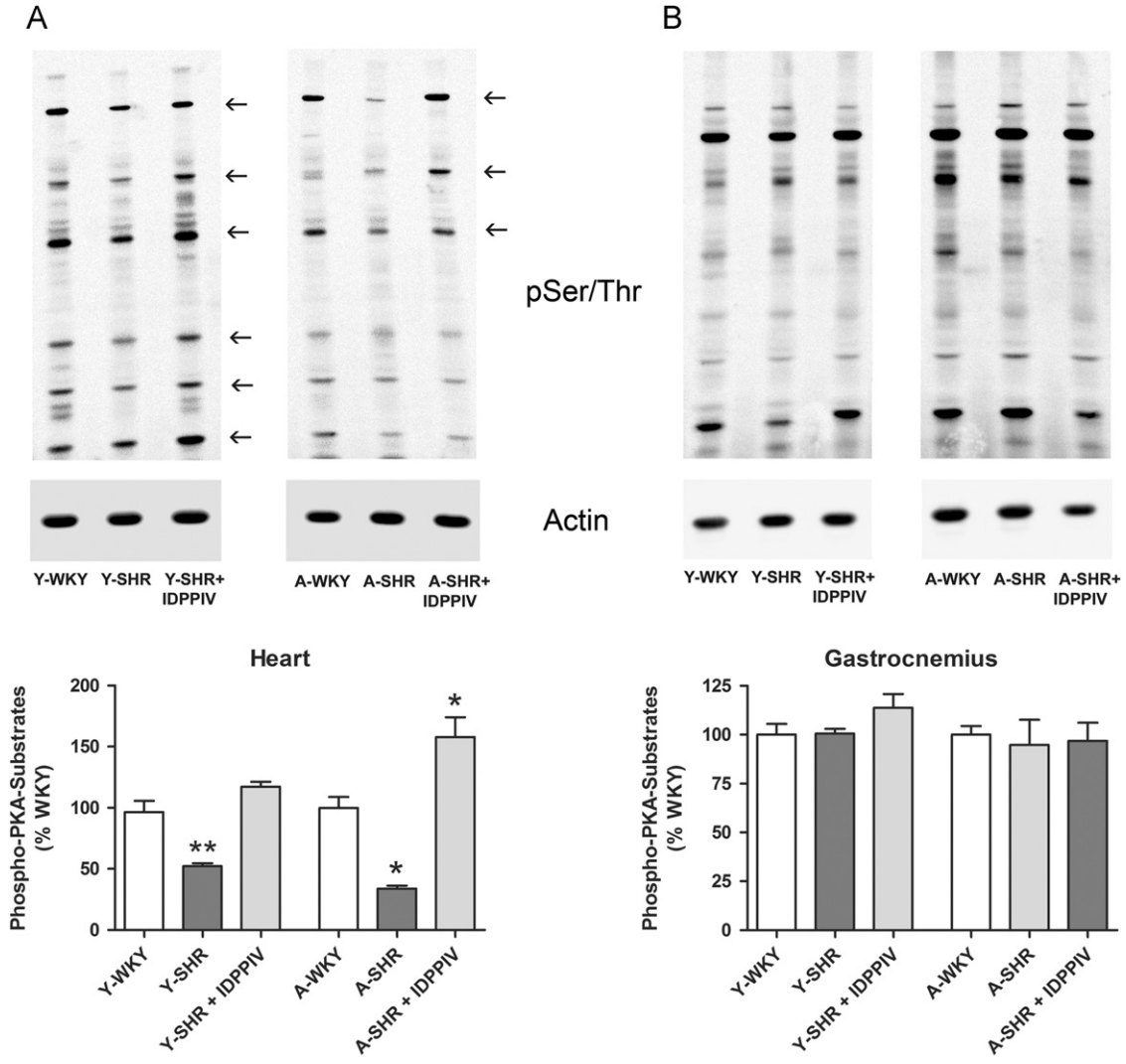

Fig. 9. Levels of PKA-phosphorylated substrates in the plasma membranes of heart and gastrocnemius muscle cells isolated from sitagliptin-treated and untreated young and adult SHRs and age-matched WKY rats. Phosphorylation levels of PKA substrates in the plasma membranes of (A) heart and (B) gastrocnemius muscle cells from young (Y) and adult (A) SHRs treated with sitagliptin (IDPPIV) or vehicle (water) for ten and age-matched WKY rats were evaluated by immunoblotting using an antibody that recognizes proteins containing a phospho-Ser/Thr residue with arginine residues at the −3 and −2 positions (i.e., the PKA consensus site). Top: Equal amounts of protein (15 μg) from each animal were subjected to SDS-PAGE, transferred to a PVDF membrane and incubated with the anti-pSer/Thr antibody. Actin was used as an internal control. Bottom: The sum total of all phospho-PKA proteins per lane was estimated by densitometry and normalized to actin. The arrows indicate the proteins whose phosphorylation was increased by sitagliptin treatment. The combined data from 3 experiments are represented as columns in a bar graph. Values are presented as the means ± S.E.M. $^{*}P<0.05$ and $^{**}P<0.01$ *vs.* age-matched WKY.

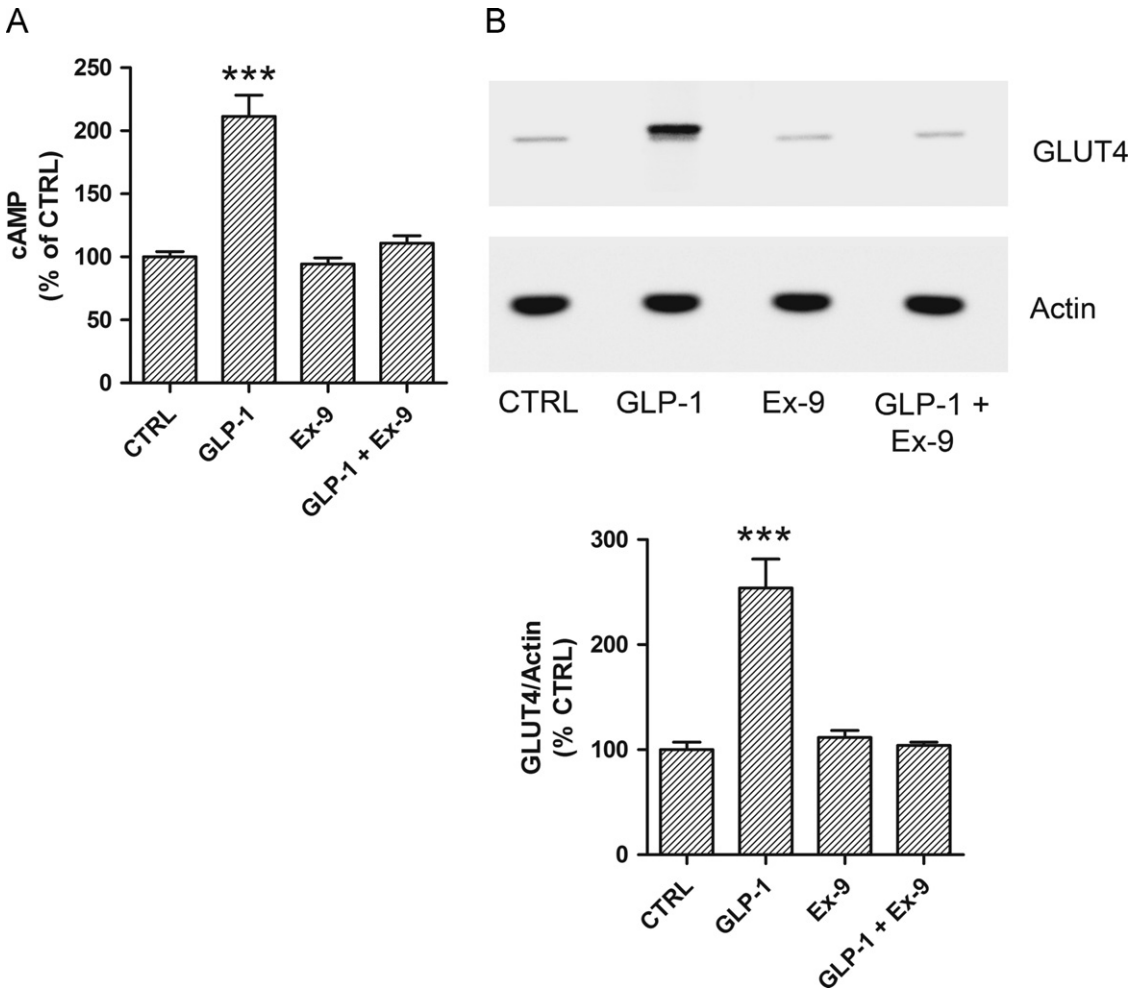

Fig. 10. Effect of GLP-1 receptor stimulation on intracellular cAMP and GLUT4 levels in cardiomyocytes isolated from neonatal SHRs. (A) Cells were incubated at 37 °C with culture medium containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) (CTRL), IBMX and GLP-1 (50 nM), IBMX and exendin-9 (Ex-9) (5 μM), or a combination of IBMX, GLP-1 and exendin-9 for 30 min prior to lysis. The generation of intracellular cAMP was measured by an enzyme immunoassay (cAMP Direct Biotrak EIA, GE Healthcare) according to the manufacturer's instructions. Each assay was performed in triplicate, and the mean value of four assays was calculated. (B) Top: Cardiomyocytes were treated for 24 h with vehicle, 50 nM GLP-1, 5 μM Ex-9 or GLP-1 and Ex-9. Immunoblot analyses were performed using a polyclonal antibody against GLUT4. Expression of GLUT4 was normalized to the actin control. Bottom: The levels of GLUT4 and actin were quantified by densitometry, and the combined data from 4 experiments are represented as columns in a bar graph $^{***}P < 0.001$ *vs.* CTRL.

G protein-coupled receptor that is expressed in pancreatic islet cells, as well as in the heart, kidneys, and vasculature, among other tissues. It is worth mentioning that GLP-1 receptor mRNA appears to be absent in rat skeletal muscles (Bullock BP, 1996). Based on these observations, it can be postulated that DPPIV inhibition-induced upregulation of GLUT4 may be due to a direct effect on GLP-1 levels in the heart, may be due to an indirect effect derived from the normalization of insulin levels and/or may involve other peptidergic systems.

Ligand activation of the GLP-1 receptor stimulates adenylate cyclase via Gαs, which leads to an increase in intracellular cAMP and activation of PKA. In the present study, we found that the levels of PKA-phosphorylated proteins are increased in heart muscle cell plasma membranes isolated from young and adult sitagliptin-treated SHRs compared to SHRs treated with vehicle. Conversely, no differences were found in PKA-mediated phosphorylation of plasma membrane proteins isolated from gastrocnemius muscle cells. These results suggest that increased GLP-1 bioactivity may directly stimulate the GLP-1 receptor in the hearts of SHRs, triggering a cascade of signaling events that culminates in increased GLUT4 expression. Consistent with this hypothesis, our results demonstrate that GLP-1 increases cAMP and GLUT4 expression in isolated cardiac myocytes via GLP-1 receptor stimulation.

The observation that GLP-1 and GLP-1 receptor antagonists modulate GLUT4 is not unprecedented. The GLP receptor agonist exenatide improves glucose homeostasis and increases GLUT4 protein expression and glucose uptake in cardiac muscle from an animal model of dilated cardiomyopathy (Vyas et al., 2011). Moreover, Villanueva-Penacarrillo et al. (2001) have previously shown that GLP-1 may upregulate GLUT4 expression at both the transcriptional and translational levels in normal and diabetic animals. Taken together, the results of our study and others indicate that drugs that mimic GLP-1 or prolong its endogenous half-life have the potential to upregulate GLUT4 function, trafficking to the plasma membrane and total expression.

In the current study, it appears that the beneficial effects of DPPIV inhibition on metabolic control in SHRs are not likely to be associated with its effect on blood pressure because (1) DPPIV inhibition significantly lowers blood pressure only in young SHRs, (2) despite normalizing blood insulin levels in adult SHRs, sitagliptin does not reduce their blood pressure, and (3) DPPIV inhibition increases GLUT4 expression in heart and skeletal muscles of both young and adult SHRs. Indeed, the upregulation of striated muscle GLUT4 expression in adult SHRs *vs.* age-matched WKY rats is greater than the differences in expression found among the younger groups. Of note, Katayama et al. (1994)

have suggested that hyperinsulinemia does not seem to be involved in the pathogenesis of hypertension in SHRs. This report was supported by the observation that the anti-diabetic agent troglitazone (CS-045) improved insulin resistance and decreased systolic blood pressure in other models of hypertension, such as obese Zucker rats (Yoshioka et al., 1993) and fructose-fed rats (Lee et al., 1994); however, despite inducing significant improvements in glucose tolerance in SHRs, troglitazone failed to reduce blood pressure in these animals (Katayama et al., 1994).

An intriguing novel finding of this study is that treatment with sitagliptin not only decreases plasma DPPIV activity but also dramatically reduces the circulating levels of the peptidase in SHRs. Additionally, both young and adult hypertensive animals display higher plasma activity and abundance of the peptidase compared to age-matched WKY rats. The origin of plasma DPPIV has been attributed to its proteolytic cleavage from the surface of peripheral lymphocytes, especially T-lymphocytes, through an as yet unidentified sheddase (i.e., an enzyme that sheds the extracellular portion of transmembrane proteins) (Lambeir et al., 2003). Nonetheless, it is tempting to speculate that this unknown sheddase might be upregulated in essential hypertension and that inhibitors of DPPIV may downregulate the activity of this protease. Important areas for future work will be to identify the sheddase that cleaves DPPIV from the cell surface and to understand how this protease is regulated under physiological and pathophysiological conditions.

In summary, the results of the present study demonstrate that the DPPIV inhibitor sitagliptin upregulates GLUT4 translocation and expression in skeletal and cardiac muscles of SHRs. The underlying mechanism for sitagliptin-induced upregulation of GLUT4 may be, at least partially, attributed to a corresponding increase in plasma GLP-1[7–36] levels. Furthermore, our study sheds light upon new mechanisms by which DPPIV inhibitors improve glycemic control.

Acknowledgments

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