GLP-1 improves palmitate-induced insulin resistance in human skeletal muscle via SIRT1 activity

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Abstract. The present study investigated whether glucagon like peptide-1 (GLP-1) improves glucose uptake through glucose transporter type 4 (GLUT4), mediated by the activation of sirtuin 1 (SIRT1), in skeletal muscle cells with palmitate induced-insulin resistance. The levels of glucose uptake, GLUT4, protein kinase A (PKA), and cyclic adenosine monophosphate (cAMP) were determined in human skeletal muscle myotubes (HSMMs) exposed to palmitate and GLP-1. Then, to determine whether PKA/cAMP were downstream signals of GLP-1, a PKA inhibitor was used. To determine whether SIRT-1 contributes to GLP-1 action in HSMMs with palmitate-induced insulin resistance, the levels of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) deacetylation and SIRT-1 activity were assessed using a SIRT1 inhibitor and small interfering RNA (siRNA). The phosphorylation levels of protein kinase B (Akt) and insulin receptor substrate 1 (IRS-1) as insulin signaling pathways, were assessed in GLP-1-treated HSMMs exposed to palmitate. The influence of SIRT1 on the GLP-1-induced activation of insulin signaling pathway was determined using a SIRT1 inhibitor. GLP-1 restored the palmitate-induced reductions in the levels of glucose uptake, GLUT4 mRNA, GLUT4 promoter activity, and GLUT4 protein in HSMMs. PKA and cAMP, as GLP-1 downstream signals, played a role in this process. GLP-1 increased the deacetylation levels of PGC1α, and stimulated SIRT1 in HSMMs. Moreover, the SIRT1 inhibitor and siRNA of SIRT1 suppressed the effect of GLP-1 on GLUT4 expression in HSMMs exposed to palmitate. The SIRT1 inhibitor also prevented the GLP-1-induced phosphorylation of IRS-1 and Akt in palmitate-treated HSMMs. The present findings suggest that in palmitate-induced insulin-resistant HSMM, GLP-1 activates SIRT1 through the PKA/cAMP pathway, which in turn enhances glucose uptake through GLUT4 and the insulin signaling pathway.

Introduction

Insulin resistance is a major pathogenic factor underlying the development of type 2 diabetes mellitus (T2DM) and obesity (1). Elevated concentrations of nonesterified fatty acids, particularly saturated fatty acids, play a critical role in the induction of insulin resistance (2). This is because saturated fatty acids impair insulin signaling via multiple mechanisms including increases in the activity of stress signaling kinases [(mitogen-activated protein kinase 8 (JNK), inhibitor of nuclear factor-κB kinase (IKK), and protein kinase B (PKC)], enhanced levels of reactive oxygen species, activation of endoplasmic reticulum stress, perturbations in mitochondrial function, and the accumulation of lipid intermediates (3-5). Due to impairments in insulin signaling, C2 myotubes and human skeletal muscle exposed to saturated fatty acids, such as palmitate exhibit a failure of the glucose transporter type 4 (GLUT4) to translocate from the cytosol to the membrane due to impairments in insulin signaling (6,7).

Glucagon-like peptide-1 (GLP-1) is a hormone secreted from intestinal L-cells in response to nutrients and exerts glucose-dependent insulinotropic actions in pancreatic islet cells (8). Additionally, GLP-1 has extra-pancreatic effects that include the generation of increased glucose uptake and the regulation of energy homeostasis in the skeletal muscles of rats and obese humans through the activation of the phosphoinositide 3 kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase pathways (9). Muscle-specific GLP-1-overexpressing transgenic mice exhibit reduced weight gain and improved lipid profiles following a high-fat diet challenge (10), and GLP-1 upregulates the translocation and expression of GLUT4 in the heart and skeletal muscles of spontaneously hypertensive rats (11). Sirtuin 1 (SIRT1), a mammalian ortholog of Sir2, is an NAD-dependent protein deacetylase that is thought to play a critical role in the adaptation of cells to metabolic alterations.
during insulin resistance. The whole-body overexpression of SIRT1 prevents the development of metabolic disorders in mice fed a high-fat diet (12), and the pharmacological activation of SIRT1 increases insulin sensitivity in skeletal muscle in animal models of insulin resistance (13). Forskolin, epinephrine, and oleic acid stimulate the cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) pathway to activate SIRT1 activity, which in turn increases the rate of fatty acid oxidation in skeletal muscle cells (14,15). Exenatide, a GLP-1 agonist, activates the cAMP/PKA pathway and ameliorates hepatic steatosis via SIRT1 activation in obese C57BL/6J mice fed a high-fat diet (16). This result is consistent with investigations of the GLP-1 receptor agonist Exendin-4, which activates the cAMP/PKA pathway, reduces endoplasmic reticulum stress, and leads to attenuation of hepatic steatohepatitis in high-fat diet-induced obese C57BL/6J mice in a SIRT1-dependent manner (17,18). However, GLP-1 also inhibits SIRT1-related deacetylase activity during the mass expansion of pancreatic β-cells (19). Regardless of the direction, it is evident that there is a link between the activities of GLP-1 and SIRT1.

The relationship between GLP-1 and SIRT1 and the effects of GLP-1 on palmitate-induced insulin resistance and GLUT4 regulation in human skeletal muscle remain poorly understood. Thus, the present study investigated whether GLP-1 has a protective effect on human skeletal muscle myotubes (HSMMs) exhibiting palmitate-induced insulin resistance. Moreover, the present study investigated whether the effects of GLP-1 occur through SIRT1 activation.

Materials and methods

Reagents. The present study utilized recombinant human GLP-1 (ProSpec; Ness Ziona), the SIRT1 inhibitor EX527 (cat. no. E-7034; Sigma-Aldrich; Merck KGaA), BSA (cat. no. A9418; Sigma-Aldrich; Merck KGaA), palmitate (cat. no. P5586; Sigma-Aldrich; Merck KGaA), H-89 dihydrochloride (cat. no. 127243-85-0; Merck KGaA) and insulin (cat. no. 19278; Sigma-Aldrich; Merck KGaA), all of which were dissolved in the appropriate medium or PBS prior to use at the required working dilution. Recombinant GLP-1 is an active form of native GLP-1. It consists of 30 amino acids and has a half-life of <2 min, due to its degradation by the DPPIV enzyme in the circulation. The palmitate/BSA conjugates were prepared as described previously (20) and used to treat the cultured cells. Briefly, a 20-mM solution of palmitate in fatty acid soaps were mixed with 5% BSA in PBS at an 8:1 molar ratio.

Cell cultures. The present study used normal HSMMs (Lonza Group, Ltd.). The undifferentiated myoblasts were cultured using a Bulletkit containing basal medium (SkBM; cat. no. CC3161; Lonza Group, Ltd.) at 37°C in an atmosphere of 5% CO₂. The cells were then induced to differentiate using DMEM (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 11500416) supplemented with 2% horse serum (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 10368902), 2 mmol/l glutamine, and 50 U/ml streptomycin and penicillin; the medium was replaced every other day. The normal HSMMs were grown for 6 days prior to harvesting for either the protein or RNA analyses. The differentiated HSMMs were either treated with palmitate (200 µmol/l) or stimulated with recombinant GLP-1 (100 or 200 nmol/l) for 24 h. Cell viability and cytotoxicity under palmitate treatment (100 or 200 µmol/l) were measured using 2-(2-methoxy-4-nitrophenyl)-5-(2,4-disulfophenyl)-2 H tetrazolium monosodium salt (Cell Counting Kit-8; CCK-8) assay (Sigma-Aldrich; Merck KGaA). Briefly, differentiated HSMMs treated with 100 or 200 µM palmitate were cultured in a 96-well tissue culture plate. After 24 h, 10 µl CCK-8 solution was added to each well and the cells were incubated for another 4 h at 37°C. Relative cell viability was determined by scanning with an ELISA reader with a 450 nm filter and calculated based on a CCK-8 assay.

Uptake of 2-NBDG by HSMMs. The HSMMs were treated with either palmitate (200 µM) or GLP-1 (200 nmol/l) for 24 h. The cells then were starved for 16 h and pre-incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 2% BSA for 30 min at 37°C. Next, the HSMMs were treated with 500 µmol/l 2-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino-2-deoxyglucose (2-NBDG; cat. no. N13195; Invitrogen; Thermo Fisher Scientific, Inc.) in either the presence or absence of insulin (100 nmol/l) for 3 h at 37°C. The cells were then rinsed three times with ice-cold PBS, lysed with RIPA Lysis and Extraction buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.), and the lysates were centrifuged at 12,000 x g for 30 min at 4°C. The supernatants were measured for fluorescence (excitation: 475 nm; emission: 550 nm) using a SpectraMax Gemini EM microplate reader (Molecular Devices, LLC). The protein concentrations were determined using the Bradford assay (Coomassie Protein Reagent, Pierce; Thermo Fisher Scientific, Inc.).

Semiquantitative reverse transcription-PCR (RT-PCR) analysis. The mRNA expression levels were compared using semi-quantitative RT-PCR performed after RNA extraction using the TRIZol® method (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized by RT-PCR using the Takara RNA PCR kit (AMV) ver 3. supplied by Takara Bio, Inc. The first strand cDNA was synthesized from 1 g total RNA using the AMV reverse transcriptase and the random 9-mer oligo-nucleotide. The RT reaction was performed for 1 cycle under the condition of 30°C for 10 min, 42°C for 30 min, 95°C for 5 min and 5°C for 5 min. Then the PCR reaction was performed using a Takara Shuzo PCR Amplification kit (cat. no. R011; Takara Bio, Inc.) with primer sets specific for different genes. The following PCR primers were used in the present study: GLUT4, 5'-TGG cTG AGc TGA AGG ATG AG-3'; and GAPDH, 5'-CCA ACA ACACGAGACC AG-3' and 5'-CAA ATTGTTGT CATGGATGACC-3'. The thermal conditions for the GLUT4, and GAPDH were denaturation for 30 sec at 95°C, annealing for 30 sec at 56°C, and extension for 30 sec at 72°C. The amplifications of the GLUT4, and GAPDH were performed using 35, and 25-28 cycles, respectively. The amplified PCR products were separated by electrophoresis on a 1.5% agarose gel. Then gel was stained with ethidium bromide for 30 min at room temperature and washed with distilled water for 10 min. The levels of amplified cDNA were quantified by
GLUT4 promoter activity. The HSMMs (5x10^6) were transfected with a reporter plasmid (mouse GLUT4 promoter luciferase reporter) using a pipette-type electroporator (Microporator-Mini; Digital Biotechnology) according to the manufacturer's protocol. The total transfected DNA amount was held constant at 50 ng by the addition of the plasmid vector β-galactosidase (β-gal). For the co-transfection of hG4-luc and β-gal, 5x10^6 HSMMs were mixed with 800 ng mouse GLUT4 promoter luciferase reporter, as previously described (6) and 50 ng β-gal in 100 µl R buffer by microporation at 1,380 V and 30 m width. Following transfection, the HSMMs were induced to differentiate by switching to a differentiation medium for 48 h. Following differentiation, the cells were treated either with or without palmitate and/or GLP-1 for 24 h, and the acetylation status was determined by mixing 100 µl luciferase assay reagent with 20 µl lysate and measuring the luminescence.

ELISA for cAMP. cAMP levels in the cell lysates were evaluated using the Direct cAMP ELISA kit (Enzo Life Sciences, Inc.; cat. no. BML-AK255) according to the manufacturer's protocol. The maximum intensity of the bands was converted to 100% and used to calculate the relative intensity.

Peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) acetylation. To assess the acetylation levels of PGC1α, the HSMMs were transfected using Lipofectamine® 2000 (cat. no. 11668027; Invitrogen; Thermo Fisher Scientific, Inc.) with 3 µg pcDNA3 Flag-PGC1α expression vector. The transfected cells were treated either with or without recombinant GLP-1 for 24 h, and the acetylation status was determined by immunoprecipitating 1 mg protein lysate in a RIPA lysis buffer [20 mmol/l Tris (pH 8.0), 137 mmol/l NaCl, 1 mmol/l MgCl2, 2 mmol/l CaCl2, 1% NP-40, 2 mmol/l vanadate, 1 mmol/l dithiothreitol (DTT) and 2.5 mmol/l phenylmethylsulfonyl fluoride]. The lysates were centrifuged at 12,000 x g for 20 min at 4°C, and aliquots of the supernatant were removed for protein concentration quantification using the Bradford assay (MicroPorator-Mini; Digital Biotechnology) according to the manufacturer's protocol. The total transfected DNA amount was held constant at 50 ng by the addition of the plasmid vector β-galactosidase (β-gal). For the co-transfection of hG4-luc and β-gal, 5x10^6 HSMMs were mixed with 800 ng mouse GLUT4 promoter luciferase reporter, as previously described (6) and 50 ng β-gal in 100 µl R buffer by microporation at 1,380 V and 30 m width. Following transfection, the HSMMs were induced to differentiate by switching to a differentiation medium for 48 h. Following differentiation, the cells were treated either with or without palmitate and/or GLP-1 for 24 h, and the acetylation status was determined by mixing 100 µl luciferase assay reagent with 20 µl lysate and measuring the luminescence.

Measurement of histone deacetylase (HDAC) activity. HDAC activity in the cell nucleus was measured using an HDAC colorimetric assay kit (cat. no. N9331-100; BioVision, Inc.) according to the manufacturer's protocol. Briefly, HSMMs treated with GLP-1 at various times. Nuclei were isolated in hypotonic buffer solution (20 mM Tris-HCl, pH 7.4, 10 mM NaCl and 3 mM MgCl2) with 0.5% of NP40, followed by centrifugation at 1,700 x g for 10 min at 4°C. The reaction was started by mixing 50 µl nuclear extract and 50 µl siruimt assay buffer (fluorescence-conjugated human p53 peptides and NAD+). The reaction was incubated for 30 min at room temperature without light, after which 10 µl developer was added. The absorbance was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Quantitative RT-PCR. Total RNA was extracted from the HSMMs using TRIZol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized by RT-PCR using the Takara RNA PCR kit (AMV) ver 3. (cat. no. RR019) supplied by Takara Bio, Inc. The first strand cDNA was synthesized by RT-PCR using the Ex Taq (Takara Bio, Inc.) and the following primers: GLUT4 forward, 5'-TTTCTCTGCGTTGCTTTGCCTC-3'; reverse 5'-TCCCCAGGCCAGCTTCAATTG-3'; GAPDH forward, 5'-GAGTCAACGGATTTGAGCCT-3' and reverse 5'-GAC AAGCTTTGGTCTCAC-3'; and SIRT1 forward, 5'-TTG GACTCGTGATGTCCA-3' and reverse 5'-CATTTCCTC TGCCCGTAAGG-3'. The PCR cycle included a holding stage for 30 sec at 95°C and cycling stage for 5 sec at 95°C and
30 sec at 60°C. Changes in GLUT4 or SIRT1 gene expression were normalized to the housekeeping gene GAPDH and were quantified using the 2−ΔΔCt method (21).

Small interfering RNAs. The 21-nucleotide small interfering RNA (siRNA) duplexes for green fluorescent protein (GFP; 5′-GUUCAGCCGUGCCCAGATT-3′), SIRT1 (5′-GGA UAGAGCCUCACAUGCAAU-3′), and GLUT4 (5′-GAUAACAGGUCAAU-3′) were designed and created at Genolution Pharmaceuticals. The cells were transfected with the siRNA oligonucleotides and cDNA (pcDNA3 plasmid) using a pipette-type electroporator (Digital Biotechnology) according to the manufacturer’s protocol (pulse voltage, 1,005 V; pulse width, 35 msec; pulse number 2). Briefly, the cells were transfected with 100 pg of each siRNA, cDNA, or 3 µg pcDNA3 in 100 µl R bu-er. After transfection, the cells were seeded in six-well plates, allowed to differentiate for 3 days and treated with or without GLP-1.

Western blot analysis. The levels of GLUT4 (1:1,000; cat. no. 2213; overnight at 4°C), GAPDH (14C10; 1:2,000; cat. no. 2118; 2 h at room temperature), Akt (1:2,000; cat. no. 9272; overnight at 4°C), and PKA (PKA-c-α; 1:2,000; cat. no. 4782; overnight at 4°C) were determined using antibodies from Cell Signaling Technology, Inc. SIRT1 (1:10,000; cat. no. SC-15404; overnight at 4°C), and IRS-1 (1:2,000; cat. no. SC-559; overnight at 4°C) antibodies were obtained from Santa Cruz Biotechnology, Inc. Cytoskeletal actin antibodies (1:10,000; cat. no. A300-491A; 1 h at room temperature) were obtained from Bethyl Laboratories, Inc. The levels of IRS-1, Akt, and PKA were determined using antibodies specific for phospho-IRS-1 (p-IRS-1; PY612; 1:500; cat. no. MBS624304; BioSource, Inc.), phospho-Akt (p-Akt; Ser473; 1:1,000; cat. no. 9275; Cell Signaling Technology, Inc.) and phospho-PKA C (p-PKA C; Thr197; 1:1,000; cat. no. 4781; Cell Signaling Technology, Inc.) overnight at 4°C. The HSMMs were homogenized at 4°C in lysis buffer [20 mmol/l Tris (pH 8.0), 137 mmol/l NaCl, 1 mmol/l MgCl2, 2 mmol/l CaCl2, 1% NP-40, 2 mmol/l vanadate, 1 mmol/l DTT, 2.5 mmol/l phenylmethylsulfonyl fluoride], 0.12 g/ml nicotinamide, 1 mol/l trichostatin A, and the resulting lysate was centrifuged for 20 min at 12,000 x g and 4°C. Aliquots of the supernatant were removed for protein quantification using the Bradford assay (Coomassie Protein Reagent; Pierce; Thermo Fisher Scientific, Inc.). The supernatant was incubated in SDS sample buffer (100 mmol/l DTT and 100 µg sample) for 5 min at 95°C and resolved by SDS-PAGE on a 10% gel, and the separated proteins were transferred to a polyvinylidene fluoride membrane (EMD Millipore). After blocking with 3% skimmed milk for 30 min at room temperature, the membranes were incubated with primary antibodies as mentioned above and then a peroxidase-conjugated anti-rabbit IgG secondary antibody (1:3,000; cat. no. SC2357; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. All immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Amersham ECL Prime Western Blotting Detection Reagent; cat. no. RPN2232; GE Healthcare), and the band intensities were determined using a Quantity One-4.6.8 (basic) analysis software (Bio-Rad Laboratories, Inc.), which is a one-dimensional image analysis program. The levels of each protein were normalized to the total protein values.

Statistical analysis. All results are presented as the mean ± standard deviation consisting of at least three independent experiments, and all statistical analyses were performed using SPSS (version 19.0; IBM Corp.). A Student's t-test was used for comparison of two dependent groups and ANOVA with post-hoc tests (Bonferroni test) was used for comparison of multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

GLP-1 reverses palmitate-induced insulin resistance in HSMMs. When treated with palmitate (100 or 200 µmol/l) in HSMMs, no significant viability reduction was noted (Fig. S1). The expression of the GLP-1 receptor in human skeletal muscle tissue was confirmed (Fig. S2) and then analysed further; there was an increase following exposure to recombinant GLP-1 (200 nmol/l). To demonstrate the effects of GLP-1 in HSMMs exposed to palmitate, the levels of 2-NBDG uptake, GLUT4 mRNA, and GLUT4 promoter activity were measured in palmitate-treated (200 µmol/l) or palmitate-(200 µmol/l) and GLP-1-treated (100 or 200 nmol/l) cells. Compared with basal conditions, there was a significant increase in glucose uptake in HSMMs exposed to GLP-1 (P<0.05; Fig. 1A) and a significant decrease in glucose uptake in HSMMs exposed to palmitate (P<0.05; Fig. 1A). The co-administration of palmitate and GLP-1 (200 nmol/l) significantly restored the palmitate-induced reduction in glucose uptake compared with treatment with palmitate (200 µmol/l) alone (P<0.05) regardless of whether insulin was present (Fig. 1A).

There was an elevation in GLUT4 mRNA in HSMMs treated with GLP-1 compared with basal conditions (P<0.05; Fig. 1B). When palmitate and GLP-1 were co-administered, GLP-1 significantly restored the palmitate-induced reductions in GLUT4 mRNA, compared with palmitate (200 µmol/l) alone (P<0.05; Fig. 1B). GLUT4 promoter activity was measured using a luciferase assay. Similar to the glucose uptake and GLUT4 mRNA results, GLP-1 increased GLUT4 promoter activity (P<0.05 for both 100 and 200 nmol/l GLP-1; Fig. 1C) and palmitate decreased GLUT4 promoter activity (P<0.05; Fig. 1C) compared with basal conditions. When GLP-1 and palmitate were co-administered, GLP-1 (100 and 200 nmol/l) significantly restored GLUT4 promoter activity levels, compared with palmitate (200 µmol/l) alone (P<0.05 for both 100 and 200 nmol/l GLP-1 with palmitate; Fig. 1C). There were also consistent results in GLUT4 protein expression in GLP-1-only, palmitate-only, and GLP-1 and palmitate treatment conditions (Fig. 1D). To determine whether the enhanced glucose uptake of GLP-1 by HSMMs was mediated by GLUT4, glucose uptake in cells treated with siRNA targeting GLUT4 was measured. The knockdown of GLUT4 significantly decreased glucose uptake (P<0.05; Fig. 1E and F). These findings suggest GLP-1 restored palmitate-induced decreases in glucose uptake via restoration of GLUT4 expression and GLUT4 promoter stimulation in HSMMs. In other words, GLP-1 influences glucose uptake directly in human skeletal muscles under conditions of insulin resistance as well as via insulinotropic actions.

cAMP and PKA signaling cascades acts as downstream signals of GLP-1 in HSMMs. The classical downstream
GLP-1 leads to the deacetylation of PGC1α and increases SIRT1 activity in HSMMs. To determine the linkage between GLP-1 and SIRT1 in HSMMs, the deacetylation levels of PGC1α were assessed after GLP-1 (200 nmol/l) was added to HSMMs overexpressing Flag-tagged PGC1α. SIRT1 has been shown to induce PGC1α deacetylation (22). Immunoblot assays using the indicated antibodies were performed after HSMMs expressing Flag-tagged PGC1α were immunoprecipitated by antibodies targeting the Flag tag. GLP-1 decreased the levels of acetylated PGC1α (Fig. 3A), indicating the presence of GLP-1-activated deacetylases, such as SIRT1. Thus, in HSMMs treated with GLP-1, SIRT1 activity was proportional to the GLP-1 concentration (Fig. 3B). PKA is an enhancer of SIRT1 activity (23) and a downstream signal of GLP-1, as also demonstrated above. The PKA inhibitor, H89, suppressed SIRT1 activity which was stimulated by GLP-1 (200 nmol/l; Fig. 3C). In a further experiment, involvement of HDAC-mediated deacetylation, in addition to SIRT1 activity in GLP-1-treated cells was ruled out (Fig. 3D).

Inhibition of SIRT1 activity suppresses GLP-1-induced GLUT4 expression in HSMMs. To confirm the role of SIRT1 during GLP-1 activity in HSMMs, the SIRT1 inhibitor EX527 was applied to the cells, and GLUT4 mRNA levels were assessed using reverse transcription PCR. EX527 (10 µmol/l) significantly repressed GLP-1-induced GLUT4 mRNA expression in HSMMs exposed to GLP-1 alone (200 nmol/l; P<0.05; Fig. 4A) and with co-administered palmitate (200 µmol/l) and GLP-1 (200 nmol/l; P<0.05; Fig. 4B). Simultaneously, SIRT1 activity was measured in cells treated with GLP-1 and/or palmitate and/or EX527. GLUT4 mRNA expression levels were correlated with the levels of SIRT1 activity (Fig. 4C). To confirm the SIRT1 dependence of GLP-1 activity in HSMMs, the cells were treated with SIRT1 siRNA. The transfection
efficiency on the expression of SIRT1 was demonstrated with the si-SIRT1 plasmid (Fig. S3). Consistent with the results obtained using the SIRT1 inhibitor, SIRT1 siRNA significantly suppressed the GLP1-induced enhancement of GLUT4 expression in HSMMs with/without palmitate (P<0.05; Fig. 4D; palmitate, 200 µmol/l, P<0.05; Fig. 4E). These findings

Figure 2. Changes in cAMP and PKA signals according to the GLP-1 concentration and exposure time, and the effect of the PKA inhibitor on GLP-1-induced GLUT4 expression. The cAMP and PKA signaling cascades act as downstream signals of GLP-1 in human skeletal muscle myotubes. (A) cAMP levels are significantly elevated for 24 h in GLP-1-treated cells. (B) Increase in p-PKA C levels as a function of the GLP-1 concentration. (C) PKA inhibitor blocks the enhancement of GLUT4 protein expression by GLP-1. *P<0.05 vs. basal condition; †P<0.05. cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; GLP-1, glucagon like peptide-1; GLUT4, glucose transporter type 4; p, phosphorylated.

Figure 3. Effects of GLP-1 on SIRT1 activity in human skeletal muscle myotubes. GLP-1 elevates both (A) the deacetylation of PGC1α and (B) SIRT1 activity. *P<0.05. (C) PKA inhibitor (H89) reduces SIRT1 activity. *P<0.05 vs. basal condition. (D) GLP-1 has no effect on HDAC activity. IP, immunoprecipitation; Ac-Lys, acetylated lysine residues; HDAC, histone deacetylase; SIRT1, sirtuin 1; GLP-1, glucagon like peptide-1; GLUT4, glucose transporter type 4; PGC1α, peroxisome proliferator-activated receptor γ 1α.
suggest the restoration of GLP-1-induced GLUT4 expression was mediated by SIRT1 in HSMMs exposed to palmitate.

The SIRT1 inhibitor EX527 suppresses the GLP-1-induced phosphorylation of IRS-1 and Akt in HSMMs exposed to palmitate. The present study investigated whether GLP-1 influenced the activation of insulin signaling pathways and whether SIRT1 was involved in the reversal of insulin resistance by GLP-1 in HSMMs exposed to palmitate. The differentiated HSMMs were treated with palmitate, recombinant GLP-1, and/or EX527 for 24 h and then stimulated with insulin for 30 min. The phosphorylation levels of IRS-1 and Akt (proteins related to insulin signaling pathways) in HSMMs exposed to palmitate (200 µmol/l) and GLP-1 (200 nmol/l) were simultaneously determined by adding EX527 (10 µmol/l) to the cells. GLP-1 increased, while palmitate decreased the phosphorylation of IRS-1 in HSMMs exposed to insulin. Palmitate decreased the phosphorylation of proteins in the insulin signalling cascade, such as IRS-1 and Akt (Fig. 5). When GLP-1 and palmitate were co-administered, GLP-1 significantly reversed the palmitate-induced reduction in insulin signaling in HSMMs exposed to insulin compared with palmitate (200 µmol/l) and insulin (100 nmol/l; P<0.05; Fig. 5). Moreover, in HSMMs exposed to both palmitate and GLP-1 in conjunction with insulin, EX527 limited the GLP-1-induced enhancement of phosphorylated IRS-1 and Akt compared with palmitate (200 µmol/l), GLP-1 (200 nmol/l), and insulin (100 nmol/l; P<0.05; Fig. 5). These findings suggest that GLP-1 functioned through SIRT1 and improved the palmitate-induced repression of insulin signaling pathways in HSMMs. A model of the present study is presented in Fig. 6.

Discussion

The present study demonstrated that GLP-1 restores palmitate-induced reductions in glucose uptake and GLUT4 expression, and improves the palmitate-induced repression of insulin signaling pathways in HSMMs. These actions of GLP-1 were shown to be mediated by SIRT1. The glucose-lowering effect of GLP-1 involves several mechanisms, the most important of which is enhancement of glucose-dependent insulin secretion from pancreatic β cells. GLP-1 also has extra-pancreatic actions, including in adipose tissue and skeletal muscle, both of which are major peripheral target organs for glucose control (24,25). Independent of the incretin effect, GLP-1 plays a role in the stimulation of glucose disposal in insulin-sensitive tissues (26-28) and increases glucose uptake and GLUT1/GLUT4 expression in adipose tissue (24). In human skeletal muscle, GLP-1 stimulates glycogen synthesis and glucose uptake (25,29). In the present study, the increased glucose uptake of GLP-1 in HSMMs was shown to be mediated by GLUT4. GLP-1 seems to influence glucose uptake by...
increasing both GLUT4 levels at the plasma membrane and total GLUT4 levels, although this has yet to be confirmed in other studies (29-31). For example, a previous study reported that GLP-1 has no direct effect on glucose uptake in human skeletal muscle (32). However, short-term treatment (1-2 h) with GLP-1 in individuals with normal blood glucose may have little effect on GLUT4 translocation and total GLUT4 expression; this may explain the differences between studies regarding the effects of GLP-1 on the glucose concentration (31).

SIRT1 is a promising treatment target for T2DM due to its roles in the regulation of glucose and lipid metabolism, insulin sensitization, and stimulation of insulin secretion (2,33-35). In pancreatic β cells, GLP-1 inhibits SIRT1 activity which stimulates the mass expansion of β cells, while SIRT1 acts as a negative regulator of β cell proliferation and prevents the action of GLP-1 (19). In the mouse liver, a GLP-1 analogue activated SIRT1 and improved fat accumulation (16,17). However, the relationship between GLP-1 and SIRT1 in skeletal muscle cells, is unclear. Thus, in the present study whether SIRT1 influenced the action of GLP-1 during glucose uptake was examined. The results of the present study showed that GLP-1 activated SIRT1 and restored GLUT4 expression in HSMMs treated with palmitate.

The present study was also able to demonstrate that GLP-1 ameliorates insulin resistance via the activation of insulin signaling pathways in insulin-resistant HSMMs. The inhibition of IRS and Akt phosphorylation by a GLP-1 agonist enhanced GLUT4 translocation in palmitate-treated muscle cells (36). Moreover, in the present study, SIRT1 was shown to be involved in the GLP-1-induced activation of insulin signaling.
pathways, leading to enhancement of IRS and Akt phosphorylation. In contrast to pancreatic \( \beta \) cells, the cell signaling cascades associated with GLP-1 in skeletal muscles have yet to be fully characterized. GLP-1 has been shown to activate the P3K/Akt, p70S6K, p42, and p44 mitogen-activated protein kinase pathways in rat and human skeletal muscles (9,37), but GLP-1 also influences glucose uptake via P3K-dependent and Akt-independent mechanisms (9,30,38). GLP-1 improves insulin sensitivity via insulin-mediated mechanisms including enhanced glucose disposal and suppression of endogenous glucose production in patients with type 2 diabetes, obesity, and normal glucose tolerance (38-40). Additionally, apart from its incretin effects, GLP-1 potentiates insulin action in depancreatized dogs (41). The effects of SIRT1 on insulin sensitivity remain unclear. SIRT1 may be the mediator that links caloric restriction to enhanced levels of insulin sensitivity within skeletal muscles (41).

Although GLP-1 receptors are present in skeletal muscles, they appear to be functionally different from those located in pancreatic \( \beta \) cells, because muscle-specific GLP-1 receptors do not increase cAMP levels (23,42). The present study reconfirmed that GLP-1 receptors are expressed in human skeletal muscles, which is consistent with the first report of GLP-1 receptor expression in human skeletal muscles (29). In contrast to previous studies of rat myotubes (43,44), the present study showed that GLP-1 increased glucose uptake and GLUT4 expression through the cAMP/PKA pathway in HSMMs. cAMP levels rose 15 min after GLP-1 treatment and peaked at a six-fold increase over basal levels. A PKA inhibitor also suppressed the GLP-1 effect on GLUT4 expression. Moreover, downstream involvement of SIRT1 in the signaling pathway of GLP-1 was also determined. The activity of SIRT1 is controlled by various mechanisms, including expression changes through transcription factors, post-translational modifications, the formation of complexes with other proteins, and altered NAD\(^+\) levels (43). SIRT1 activity is modulated by phosphorylation via the cAMP/PKA pathway (14,15). In another study, SIRT1-mediated resveratrol was shown to be associated with an improvement of GLUT4 expression in muscles from T2DM mice (44). In conjunction with previous results, the findings of the present study suggest that SIRT1 mediates the GLP-1 induced enhancement of glucose uptake and GLUT4 expression through the cAMP/PKA pathway in HSMMs. The results of the present study provide evidence of the involvement of SIRT1 in the GLP-1-induced enhancement of glucose disposal and insulin signaling in insulin-resistant HSMMs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

JJ, TK, SH, HK, DK, YK and KL contributed to the study design. JJ, SC, HL and EH performed the experiments for data acquisition. JJ, SC and EH performed the statistical analyses. TK, SH, HK, DK, HL and YK interpreted the experimental results. JJ and SC contributed to the drafting of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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