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Knockdown of NYGGF4 increases glucose transport in C2C12 mice skeletal myocytes by activation IRS-1/PI3K/AKT insulin pathway

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Abstract NYGGF4, an obesity-related gene, is proposed to be involved in the development of insulin resistance. Skeletal muscle is a primary target organ for insulin and NYGGF4 showed a relatively high expression level in skeletal muscle. Therefore, this study aimed to explore the effect of NYGGF4 on insulin sensitivity of skeletal muscle cells. RNA interference (RNAi) was adopted to silence NYGGF4 expression in mice C2C12 skeletal myocytes. A remarkably increased insulin-stimulated glucose uptake and GLUT4 translocation was observed in NYGGF4 silencing C2C12 cells. Importantly, the enhanced glucose uptake induced by NYGGF4 silencing could be abrogated by the PI3K inhibitor LY294002. In addition, the crucial molecules involved in PI3K insulin signaling pathway were detected by western blotting. The results showed that NYGGF4 knockdown dramatically activate the insulin-stimulated phosphorylation of IRS-1 and AKT. Taken together, these data demonstrate that NYGGF4 knockdown increases glucose transport in myocytes by activation of the IRS-1/PI3K/AKT insulin pathway.

Keywords NYGGF4 · Glucose uptake · Insulin signaling · Skeletal myocytes

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Introduction

In recent decades, the incidence of obesity has increased drastically worldwide (Jeffery and Sherwood 2008). Obesity constitutes a serious public health problem that is causally linked to the pathogenesis of insulin resistance, metabolic syndrome and type-2 diabetes (T2D) (Kopelman 2000; Spiegelman and Flier 2001; Visscher and Seidell 2001). Individual genetic make-up is considered to be one of the important factors that lead to obesity, and multiple genes, such as renin and resistin, are considered to have a role in this area (Araki et al. 2008; Fowler et al. 2009). NYGGF4, a novel identified human gene, was found to be expressed higher in obese subjects (Wang et al. 2006). Our previous study revealed that overexpression of NYGGF4 attenuated insulin sensitivity in adipocytes by inhibition insulin-stimulated glucose uptake (Zhang et al. 2009), which indicated that NYGGF4 might be involved in obesity-associated insulin resistance. It is known that skeletal muscle is a primary target organ for insulin and skeletal muscle insulin resistance is a common metabolic disorder in the obese (Lillioja et al. 1993). Since NYGGF4 is relatively highly expressed in skeletal muscle (Wang et al. 2006), the effect of NYGGF4 on insulin sensitivity in skeletal muscle drew our attention. Therefore, in this study, we knocked down the NYGGF4 gene expression in C2C12 mice skeletal myocytes by RNA

interference to evaluate the effect of NYGGF4 on insulin sensitivity in skeletal muscle cells.

Materials and methods

Antibodies

Primary polyclonal glucose transporter 4 (GLUT4) antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (1:1000; Santa Cruz, CA, USA). Anti-phospho-insulin receptor (IR) beta (Tyr1146), anti-IR beta, and anti-IRS-1 were purchased from Cell Signaling Technology (1:800; Danvers, MA, USA). The phospho-specific polyclonal antibody against IRS-1 (Tyr612) was obtained from Biosource (1:500; Camarillo, CA, USA). Antibody against Akt was obtained from Kangchen (1:800; Shanghai, China).

Cell culture and differentiation

C2C12 mice myoblasts were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and maintained in 5 % CO2 in an air humidified atmosphere at 37 °C. The short hairpin (sh)-RNA (pGPU6-NYGGF4-shRNA) used for NYGGF4 knockdown and the negative control vector (pGPU6-NC-shRNA) was designed and constructed previously by Dr Zhang (Zhang et al. 2010), a member of our research team. These vectors were transfected independently into C2C12 myoblasts using the Lipofectamine gene transfection reagent Hilymax. After 48 h, the cells were cultured in a selective medium that contained 800 μg/ml gentamycin(G418; Sigma, St. Louis, MO, USA) to select positive cell clones. A stable single clone was isolated and identified after 2 weeks in culture. Then the cells were grown on 6-well plates and when the cells reached 80 % confluence, differentiation was induced by switching the growth medium to DMEM supplemented with 2 % fetal bovine serum, 800 μg/ml gentamycin for 7 days.

RNA extraction and quantitative real-time PCR

Total RNAs from the C2C12 cells transfected with pGPU6-NYGGF4-shRNA or pGPU6-NC-shRNA were extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed using an AMV Reverse Transcriptase kit (Promega, Madison, WI, USA) using a random hexamer primer. Real-time PCR was performed using an Applied Biosystems 7300 Sequence Detection System (ABI 7300 SDS; Foster City, CA, USA) by following the manufacturer's protocols. The sequences of the primers and TaqMan probes (Shenggong, Shanghai, China) were described previously (Zhang et al. 2010).

Glucose uptake

A 2-Deoxy-D-[^{3}H] glucose uptake was assayed as described previously (Ceddia et al. 2005). Briefly, cells were starved in serum-free DMEM for 4 h, and then washed twice with phosphate buffered saline (PBS) and incubated in KRP-HEPES buffer[30 mmol/L HEPES (pH 7.4), 10 mmol/L NaHCO3, 120 mmol/L NaCl, 4 mmol/L KH2PO3, 1 mmol/L MgSO4, and 1 mmol/L CaCl2] in the presence or absence of 30 μmol/L LY294002 for 30 min, and then the cells were treated with vehicle or 100 nM insulin for 30 min. Glucose uptake was measured by adding 2 μCi of 2-deoxy-D-[3H] glucose (CIC, Beijing, China) for 10 min. The assay was terminated with two quick washes of ice-cold PBS supplemented with 10 mmol/L D-glucose. The cells were solubilized by adding 200 μL 1 mol/L NaOH to each well. Aliquots of the resultant cell lysate were transferred to scintillation vials for radioactivity counting, whereas the remainder was used for the protein assay.

Western blotting

After 7 days of differentiation, the cells were starved for 3 h and then incubated with or without 100 nmol/L insulin. Next, the cells were washed with ice-cold PBS and lysed in protein lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1 % Triton X-100, 200 mM sodium fluoride, and 4 mM sodium orthovanadate that contained protease inhibitors, pH 7.5) for 1 h on ice. Total proteins and phosphorylated proteins were extracted. Protein levels were quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) in accordance with the manufacturer's instructions. Proteins (40 μg/lane) were separated by 10 % sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were blocked with 5 % bovine serum albumin (BSA) in TBST buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05 % Tween-20). The membrane was incubated at 4 °C overnight in TBST plus 5 % BSA that contained each of the primary antibodies described. The membrane was then washed with TBST for 15 min. This step was repeated three times. After being washed, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membrane was then washed with TBST for 10 min. This step was repeated four times, and the membrane was then developed with the enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ, USA).

Statistical analysis

All data are expressed as means±standard error of the means (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) with the SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL, USA). The threshold of significance was defined as a P-value <0.05.

Results

Suppression of NYGGF4 expression by small interfering RNA

The knock down efficiency of NYGGF4-specific shRNA in C2C12 cells was evaluated using real-time PCR. Relative NYGGF4 mRNA levels in the transfectants were normalized against mRNA levels of an endogenous control gene, β-actin. As shown in Fig. 1, cells transfected with pGPU6-NYGGF4 shRNA showed a significantly reduced transcription of NYGGF4 mRNA when compared with that of non-transfected C2C12 cells and the negative control vector pGPU6-NC-shRNA- transfected C2C12 cells, respectively ($P<0.05$). There was no significant difference in mRNA transcription between pGPU6-NC-shRNA-transfected and non-transfected C2C12 cells ($P>0.05$).

Effects of NYGGF4 knockdown on basal and insulin-stimulated glucose uptake in C2C12 myocytes

To test whether NYGGF4 affects insulin sensitivity, we assessed glucose uptake in C2C12 myocytes with or without NYGGF4 silencing. As shown in Fig. 2, the insulin-stimulated glucose uptake was significantly enhanced in

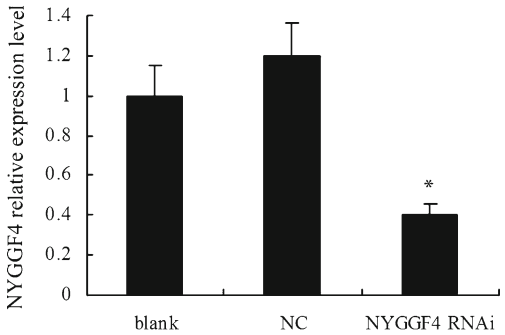

Fig. 1 Knockdown of NYGGF4 mRNA expression by siRNA. The level of the NYGGF4 mRNA relative to β-actin in pGPU6-NYGGF4 shRNA-transfected C2C12 skeletal myocytes was examined by real-time PCR and by comparison with pGPU6-negative control (NC) shRNA-transfected and -untransfected C2C12 skeletal myocytes . *$P<0.05$ vs negative control

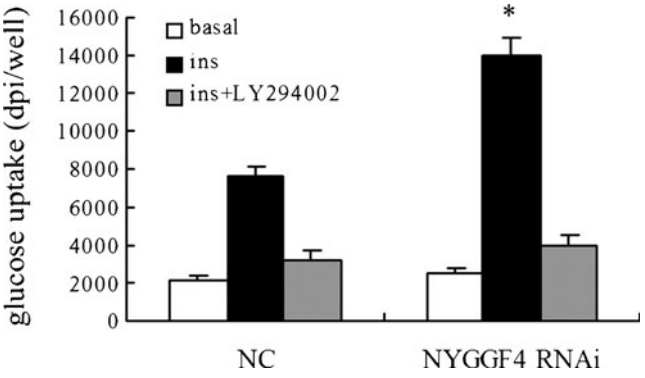

Fig. 2 Effects of NYGGF4 knockdown on glucose uptake in C2C12 myocytes. C2C12 skeletal myocytes transfected with NYGGF4 knockdown vector or pGPU6-negative control (NC) were induced to differentiate into C2C12 skeletal myocytes . On the 10th day of differentiation, the mature myocytes were pretreated with or without LY294002 for 30 min before being treated with 100 nM insulin. Glucose uptake was then measured for 10 min, as described in the Materials and Methods. Values shown are the means±SE of three independent experiments performed in triplicate. *$P<0.05$ vs insulin-stimulated negative control

NYGGF4 silencing myocytes compared with the negative control cells ($P<0.05$). This occurred concurrently with a slight increase in basal 2-deoxy [^{3}H] glucose uptake ($P>0.05$). Moreover, NYGGF4 silencing-induced glucose uptake could be completely blocked by the coincubation with the PI3K inhibitor (LY294002).

Effects of NYGGF4 knockdown on basal and insulin-stimulated GLUT4 translocation in C2C12 myocytes

We examined the effects of NYGGF4 on the protein expression and translocation of GLUT4 in response to insulin in C2C12 myocytes. The results demonstrated that NYGGF4 knockdown did not affect the total intracellular GLUT4 abundance, but dramatically stimulated the translocation of GLUT4 from intracellular storage compartments to the cell surface (Fig. 3).

Effects of NYGGF4 on protein expression and insulin-stimulated phosphorylation of insulin-signaling molecules

We investigated the effect of NYGGF4 on the molecules involved in insulin signaling for glucose uptake to explore the underlying mechanisms. NYGGF4 knockdown resulted in significant enhancement of insulin-induced phosphorylation of IRS-1 and AKT, the upstream and downstream molecules of PI3K. NYGGF4 knockdown did not affect tyrosine phosphorylation of IR. As shown in Fig. 4, there is no significant difference in the total protein content of these signaling molecules.

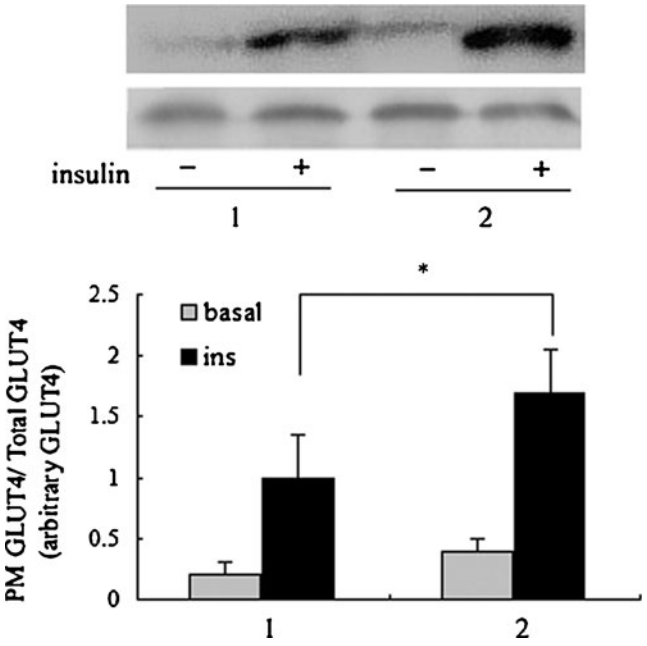

Fig. 3 Effect of NYGGF4 knockdown on GLUT4 translocation.C2C12 skeletal myocytes transfected with the NYGGF4 knockdown vector (2) or the pGPU6-negative control vetor (1) were induced to differentiate into C2C12 skeletal myocytes . Membrane proteins and total proteins were extracted from C2C12 skeletal myocytes which were incubated with (+) or without (−) 100 nmol/L insulin for 30 min. Western blotting was performed using an antibody against GLUT4. Values shown are the means±SE of three independent experiments performed in triplicate. *P<0.05 vs insulin-stimulated control (cells transfected with empty vector)

Discussion

Obesity has become a major public health problem in the world and is causally linked to the pathogenesis of insulin resistance. Skeletal muscle is an important determinant of systemic insulin sensitivity as it contributes 80 % of insulin-stimulated glucose uptake (Ceddia et al. 2005). NYGGF4 is a newly discovered obesity-related gene and is high expressed in skeletal muscle. Our previous study showed that overexpression of NYGGF4 inhibited insulin-induced glucose uptake in skeletal myotubes (Wu et al. 2011). In the present study, we found that inhibition NYGGF4 expression by RNAi significantly augmented insulin-stimulated glucose uptake in C2C12 skeletal muscle cells. This result reconfirmed that NYGGF4 is involved in regulation of insulin sensitivity in skeletal muscle cells.

Numerous studies have identified that insulin-stimulated glucose uptake in skeletal muscle cells is dependent on the translocation of the insulin-responsive glucose transporter 4 (GLUT4) from intracellular storage compartments to the plasmalemma (Jørgensen et al. 2009; Guma et al. 1995). So we detected the protein expression and translocation of GLUT4 in NYGGF4-knockdown C2C12 cells. The results demonstrated that NYGGF4 silencing did not affect the total amount of intracellular GLUT4 expression, but dramatically stimulated the translocation of GLUT4 from intracellular storage compartments to the cell surface, which thereby produced greater augmentation of glucose uptake in response to insulin.

It has been established that two pathways are necessary for insulin-stimulated glucose transport; a phosphatidylinositol 3-kinase (PI3-K)-dependent pathway and a PI3-K-independent pathway (Saltiel and Pessin 2002). In this study, we found that the enhanced insulin-stimulated glucose uptake in NYGGF4 silenced C2C12 cells could be abrogated by the PI3K inhibitor LY294002. This suggest that silencing of NYGGF4 enhanced insulin-stimulated glucose uptake is via a PI3-kinase dependent signaling

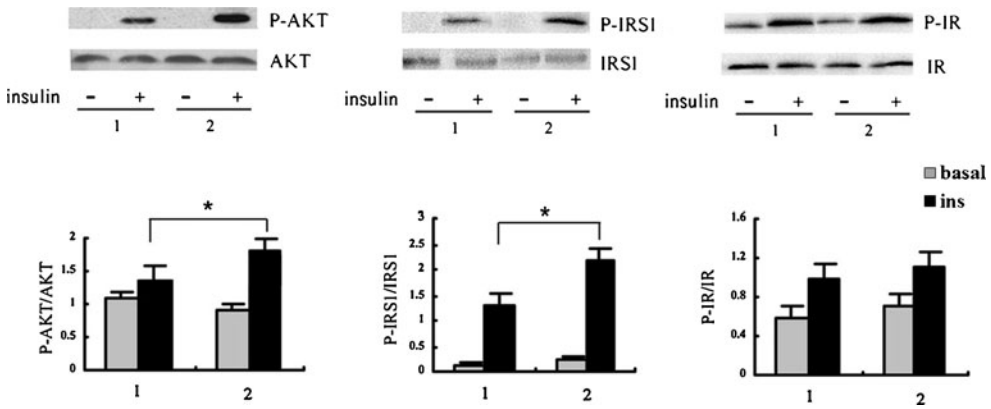

Fig. 4 Effect of NYGGF4 on insulin-signaling transduction. C2C12 skeletal myocytes transfected with NYGGF4 vetor (2) or the pGPU6-negative control vetor (1) were induced to differentiate into C2C12 skeletal myocytes. The cells were incubated with (+) or without (−) 100 nmol/L insulin for 30 min. Then the cell lysates were analyzed by SDS-PAGE, blotted onto a membrane, and then probed with antibodies against a selection of molecules that are known to be involved in the PI3K insulin signal pathway. The results are representative of three independent experiments. The total protein concentrations and phosphorylation levels for IR, IRS-1 and AKT, respectively, are represented. Values shown are the means±SE of three independent experiments performed in triplicate. *P<0.05 vs insulin-stimulated control (cells transfected with empty vector)

pathway. The PI3K signaling cascade is activated by insulin binding to its receptor (IR), recruiting IR substrates and PI3K. Activated PI3K targets downstream activation of Akt which subsequently modulates GLUT4 translocation and glucose uptake (Taniguchi et al. 2006; Kim et al. 1999). We further examined the critical nodes in the PI3K insulin-signaling network: IR, IRS and AKT. The results showed that silencing of NYGGF4 increased the phosphorylation level of IRS-1 and AKT, but did not affect phosphorylation of IR and the total protein content of these molecules. Therefore, we concluded that NYGGF4 silencing augmented insulin-stimulated glucose uptake in skeletal muscle cells by activation of the IRS1/PI3K/AKT insulin pathway. In accordance, our data also demonstrated an insulin-dependent increase of GLUT4 in the plasma membrane fraction after NYGGF4 silencing. Based on these results, we proposed that NYGGF4 might be a potential target for the treatment of obesity-related insulin resistance.

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