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NYGGF4 (*PID1*) effects on insulin resistance are reversed by metformin in 3T3-L1 adipocytes

Jie Qiu · Yu-mei Wang · Chun-mei Shi · Hong-ni Yue · Zhen-Ying Qin · Guan-zhong Zhu · Xin-guo Cao · Chen-bo Ji · Yan Cui · Xi-rong Guo

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Abstract *NYGGF4* (also called *PID1*) is a recently discovered gene that is involved in obesity-related insulin resistance (IR). We aimed in the present study to further elucidate the effects of *NYGGF4* on IR and the underlying mechanisms through using metformin treatment in 3T3-L1 adipocytes. Our data showed that the metformin pretreatment strikingly enhanced insulin-stimulated glucose uptake through increasing GLUT4 translocation to the PM in *NYGGF4* overexpression adipocytes. *NYGGF4* overexpression resulted in significant inhibition of tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt, whereas incubation with metformin strongly activated IRS-1 and Akt phosphorylation in *NYGGF4* overexpression adipocytes. The reactive oxygen species (ROS) levels in *NYGGF4* overexpression adipocytes were strikingly enhanced, which could be decreased by the metformin pretreatment. Our data also showed that metformin increased the expressions of PGC1-α, NRF-1, and TFAM, which were reduced in the *NYGGF4* overexpression adipocytes. These results suggest that *NYGGF4* plays a role in IR and its effects on IR could be reversed by metformin through activating IRS-1/PI3K/Akt and AMPK-PGC1-α pathways.

Keywords Obesity · Insulin resistance · *NYGGF4* · Metformin · Adipocytes

Introduction

Insulin resistance (IR), defined as a condition in which normal insulin concentrations fail to achieve normal glucose metabolism (Kahn 1978), is considered to be an important link between adiposity and the associated risks of type 2 diabetes and cardiovascular disease (Reaven 1988; Qatanani and Lazar 2007; Ferrannini et al. 2007). To date, the mechanisms underlying IR have remained unclear, although numerous studies have indicated that free fatty acids, proinflammatory cytokines, adipocytokines, and mitochondrial function are implicated in the pathogenesis of IR (de Jongh et al. 2004; Kremen et al. 2006; Tilg and Moschen 2006; Kim et al. 2008).

In the previous report, we isolated and characterized a novel human gene *NYGGF4* (GenBank accession no. NM_001100818) (Wang et al. 2006), which has been indicated to be a new interactor of low-density lipoprotein receptor-related protein 1 (*LRP1*) (Caratù et al. 2007; Herz and Strickland 2001). Our further studies revealed that overexpression of *NYGGF4* inhibits insulin-stimulated glucose transport and impaired insulin-stimulated glucose transporter 4 (GLUT4) translocation in mature adipocytes and

Jie Qiu and Yu-mei Wang contributed equally to this work.

J. Qiu
Department of Newborn Infants,
Nanjing Children's Hospital of Nanjing Medical University,
Nanjing 210008, China

Y.-m. Wang · H.-n. Yue
Department of Child Health,
Huai'an Maternity and Child Health Hospital,
Huai'an 223002, China

C.-m. Shi · G.-z. Zhu · X.-g. Cao · C.-b. Ji · X.-r. Guo
Department of Pediatrics, Nanjing Maternity and Child Health Hospital of Nanjing Medical University,
Nanjing 210004, China

C.-m. Shi · Z.-Y. Qin · G.-z. Zhu · X.-g. Cao · C.-b. Ji · X.-r. Guo (✉)
Institute of Pediatrics of Nanjing Medical University,
Nanjing 210029, China
e-mail: xrguo@njmu.edu.cn

Y. Cui (✉)
School of Nursing of Nanjing Medical University,
Nanjing 210029, China
e-mail: cyan@njmu.edu.cn

skeletal myotubes by blocking the IRS-1/PI3K/Akt insulin pathway (Zhang et al. 2009; Wu et al. 2011). This was accompanied by decreased mitochondrial number, abnormal morphology, lower ATP synthesis, increased reactive oxygen species (ROS) levels, and altered mitochondrial gene expression (Zhao et al. 2010). *NYGGF4* knockdown enhances the glucose uptake of adipocytes and reduces intracellular ATP concentration and promotes an increase in mitochondrial transmembrane potential ($\Delta\Psi m$) and ROS levels without affecting mitochondrial morphology or mtDNA (Zhang et al. 2010). Taken together, these results provided evidence that *NYGGF4* might play an important role in the development of obesity-related IR. The IRS1/PI3K/Akt insulin pathway and the mitochondrial dysfunction in adipocytes might be responsible for the development of *NYGGF4*-induced IR. However, the role of *NYGGF4* in IR remains unclear, and further studies are needed to explore the biological function of this gene.

White adipose tissue is not only an energy storage site but also a major endocrine and secretory organ actively involved in the regulation of energy balance. Defects in adipose tissue metabolism are one of the links between obesity, IR and diabetes. Metformin is commonly used in the treatment of type 2 diabetes and is used clinically to alleviate IR (Davidson and Peters 1997). It reduces hyperglycaemia by a number of mechanisms, including inhibition of hepatic output and stimulation of glucose uptake in a variety of tissues (Bailey and Turner 1996; Kirpichnikov et al. 2002; Ciaraldi et al. 2002). Metformin does not influence insulin secretion but helps to improve the control of glycaemia by promoting glucose utilization through an AMP-activated protein kinase (AMPK)-mediated stimulation of catabolism in white adipocytes (Bailey and Turner 1996; Kirpichnikov et al. 2002). So, we aimed in the present study to further elucidate the effects of *NYGGF4* on IR and the underlying mechanisms through using metformin treatment in 3T3-L1 adipocytes.

Materials and methods

Cell culture and treatment

3T3-L1 preadipocytes were stably transfected with either an empty expression vector (pcDNA3.1Myc/His B) or a *NYGGF4* expression vector as described previously (Wang et al. 2006). The transfected cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10 % calf serum (Gibco, Carlsbad, CA, USA) and 100 μg/ml neomycin (G418; Roche, Basel, Switzerland). Two days after complete confluence (day 0), the cells were cultured for 48 h in DMEM supplemented with 10 % fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 0.5 mmol/l 1-methyl-3-isobutylxanthine (Sigma, St. Louis, MO, USA), 1 μmol/l dexamethasone (Sigma, St. Louis, MO, USA), 10 μg/ml insulin (Sigma, St. Louis, MO, USA), and 100 μg/mlG418. From day 2 to day 4, the medium was supplemented with only 10 μg/ml insulin. The cells were then transferred to DMEM containing 10 % FBS and 100 μg/mlG418 for the remaining culture period. The cultures were replenished every 2 days. Ten days after the induction of differentiation, more than 90 % of the cells exhibited typical adipocyte morphology.

On the 10th day after induction of differentiation, when more than 90 % of the cells showed the morphological and biochemical properties of adipocytes, the cells were used for the experiments. After overnight incubation in serum free DMEM, the cells were stimulated with 1 mmol/L or 10 mmol/L metformin (Sigma, St. Louis, MO, USA) for 24 h.

Glucose uptake

2-Deoxy-*D*-[^{3}H] glucose (CIC, Beijing, China) uptake was assayed as described previously (Ceddia et al. 2005). The cells were cultured in 6-well plates and were serum starved in DMEM containing 0.5 % FBS for 3 h before the experiments. The cells were then washed twice with phosphate-buffered saline (PBS) and incubated in KRP-HEPES buffer [30 mmol/L HEPES (pH 7.4), 10 mmol/L $NaHCO_3$, 120 mmol/L NaCl, 4 mmol/L KH_2PO_4, 1 mmol/L $MgSO_4$, and 1 mmol/L $CaCl_2$] in the presence or absence of 100 nmol/L insulin for 30 min at 37°C. Labeled 2-deoxy-*D*-[^{3}H]glucose was added to a final concentration of 2 μCi/mL. After 10 min at 37 °C, the reaction was terminated by washing 3 times with ice-cold PBS supplemented with 10 mmol/L *D*-glucose. The cells were solubilized by adding 200 μL of 1 mol/L NaOH to each well, and aliquots of the cell lysate were transferred to scintillation vials for radioactivity counting; the remainder was used for the protein assay.

ROS assay

Intracellular ROS generation was determined using 6-carboxy-2, 7-dichlorodihydrofluorescein diacetate (H2-DCFDA) as previously described (Sundaresan et al. 1995). In brief, adipocytes were washed and incubated with H2-DCFDA (Sigma, St. Louis, MO, USA) for 20 min. Cells were then washed several times and gently scraped by a lifter. Following, the cells were suspended in the same media. For detection of intracellular fluorescence, cells were excited using a 488 nm argon ion laser in a flow cytometer (BD Biosciences, San Jose, CA, USA). The dichlorofluorescein emission was recorded at 530 nm. Data were collected from at least 20,000 cells.

Western blot

On the 10th day of differentiation, the cells were serum-starved for 3 h and then incubated with 10 μg/ml insulin for

30 min. Total proteins or phosphorylated proteins were extracted as described previously (Andreozzi et al. 2004). Plasma membrane (PM) proteins were extracted using the Eukaryotic Membrane Protein Extraction Reagent (Pierce, Rockford, IL, USA). Protein levels were quantified using the bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) in accordance with the manufacturer's instructions. After sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE), the proteins (20 μg/lane) were electrophoretically transferred onto a nitrocellulose membrane (Whatman, London, UK). After being blocked with Tris-buffered saline Tween-20 [TBST; 0.14 mol/L NaCl, 0.02 mol/L Tris base (pH 7.6), and 0.1 % Tween] containing 3 % bovine serum albumin (BSA) for 1 h at room temperature, the membrane was hybridized with primary antibodies at an appropriate dilution at 4°C overnight. The membrane was then washed with TBST for 5 min. This step was repeated 5 times. After being washed, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, washed with TBST, and developed with the enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ, USA).

Primary polyclonal GLUT4 antibody and horseradish peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-IRS-1 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). The phospho-specific polyclonal antibody against IRS-1 (Tyr612) was from Biosource (Camarillo, CA, USA). Antibodies against Akt and against the phosphorylated form of this protein were obtained from Kangchen (Shanghai, China). Rabbit polyclonal antibodies against peroxisome proliferator-activated receptor gamma coactivator (PGC) 1-α, mitochondrial DNA transcription factor A (TFAM), and mouse monoclonal antibodies against nuclear respiratory factor-1 (NRF-1) were purchased from Abcam Inc. (Cambridge, CA, USA).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using the paired Student's *t*-test of the SPSS 10.0 statistical software package (SPSS, Chicago, IL, USA). The threshold of significance was defined as $P<0.05$.

Results

Glucose uptake

The transfected 3T3-L1 preadipocytes were induced to differentiate as described in the Materials and methods. Then we measured glucose uptake in 3T3-L1 adipocytes with or without *NYGGF4* overexpression. As shown in Fig. 1, insulin-stimulated glucose uptake was significantly down-regulated in *NYGGF4* overexpression adipocytes but basal glucose uptake was similar compared with control cells. Next, we measured insulin-induced glucose uptake after 1 mmol/L or 10 mmol/L metformin pretreatment. Our data showed that metformin pretreatment strikingly enhanced insulin-stimulated glucose uptake relative to *NYGGF4* overexpression adipocytes without affecting basal glucose uptake.

GLUT4 translocation

In adipocytes, insulin-stimulated glucose uptake is dependent on translocation of the insulin responsive GLUT4 from intracellular storage compartments to the PM (Bryant et al. 2002; Kanzaki 2006). Therefore, we examined the effects of *NYGGF4* on GLUT4 translocation to the PM in response to insulin. The results demonstrated that *NYGGF4* overexpression decreased GLUT4 translocation to the PM but did not alter the total GLUT4 protein content. In addition, the metformin pretreatment increased GLUT4 translocation to the PM compared with the *NYGGF4* overexpression adipocytes without affecting the total GLUT4 protein content (Fig. 2).

Protein expressions and phosphorylations of IRS-1 and Akt

The IRS-1/PI3K/Akt signaling pathway could mediate the effect of metformin to redistribute glucose transporters from an intracellular compartment to the PM (Hundal et al. 1992)

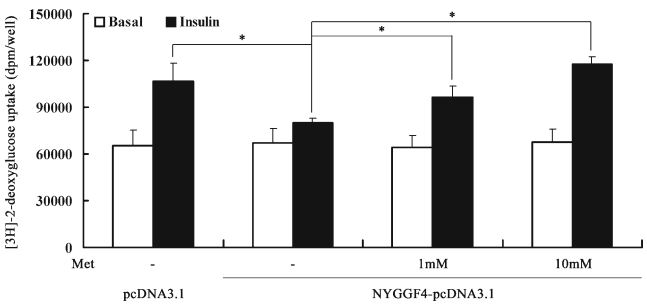

Fig. 1 Effects of metformin (Met) on glucose uptake into *NYGGF4* overexpression adipocytes. 3T3-L1 predipocytes stably transfected with either an empty expression vector (pcDNA3.1) or a *NYGGF4* expression vector (*NYGGF4*-pcDNA3.1) were induced to differentiate. On the 10th day of differentiation, the mature adipocytes were pretreated with or without metformin (1 or 10 mM) for 24 h before being incubated with (*black* columns) or without (*white* columns) 100 nM insulin for 30 min, followed by measurement of the 2-Deoxy-*D*-[^{3}H] glucose uptake. Glucose uptake was then measured as described in the Materials and Methods. Values shown are the means ± SD of three independent experiments performed in triplicate (*$P<0.05$)

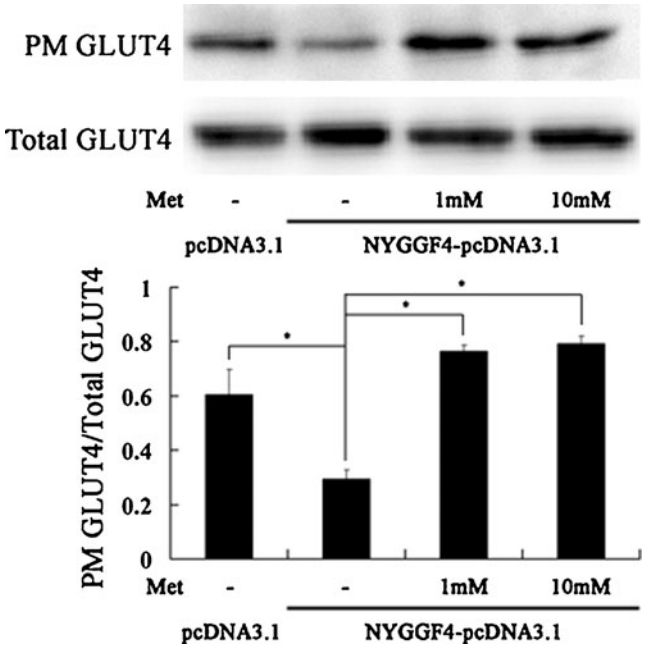

Fig. 2 Effects of metformin (Met) on the GLUT4 translocation in *NYGGF4* overexpression adipocytes. 3T3-L1 preadipocytes transfected with the *NYGGF4* expression vector (*NYGGF4*-pcDNA3.1) or the empty vector (pcDNA3.1) were induced to differentiate. Membrane proteins and total proteins were extracted from the differentiated cells incubated with or without metformin (1 or 10 mM) as described in the Materials and Methods. Immunoblotting was performed using antibodies against GLUT4. The results are representative of those obtained from three independent experiments (*$P<0.05$)

and our previous study has shown that overexpression of *NYGGF4* inhibits glucose transport in 3T3-L1 adipocytes via attenuated phosphorylation of IRS-1 and Akt. Therefore, we next investigated the protein expression and phosphorylation of IRS-1 and Akt. As shown in Fig. 3, *NYGGF4* overexpression resulted in significant inhibition of tyrosine phosphorylation of IRS-1 (A) and serine phosphorylation of Akt (B), whereas incubation with metformin strongly activated IRS-1 and Akt phosphorylation in *NYGGF4* overexpression adipocytes. There is no significant difference in the total protein contents of these signal molecules.

ROS assay

Mitochondria are the primary source of endogenous ROS, which are generated as a by-product of metabolic reactions within this organelle (Andreyev et al. 2005). It has been proposed that there may be a causative link between elevated mitochondrial ROS generation and mitochondrial dysfunction and IR (Bonnard et al. 2008). The ROS levels in *NYGGF4* overexpression adipocytes were strikingly enhanced compared with the control cells, while pretreatment of cells with metformin significantly decreased the ROS levels in *NYGGF4* overexpression adipocytes (Fig. 4).

Protein expressions of PGC1-α, NRF-1, and TFAM

PGC1-α, which could bind to and coactivate the transcriptional function of NRF-1 on the promoter for TFAM, is an important regulator of intracellular ROS levels by mitochondria (Kukidome et al. 2006; St-Pierre et al. 2003; Vallea et al. 2005). Furthermore, metformin inhibited the intracellular ROS production, by promotion of mitochondrial biogenesis by activating the AMPK-PGC1-α pathway (Kukidome et al. 2006). Therefore, we examined the PGC1-α, NRF-1, and TFAM protein levels which were significantly decreased in

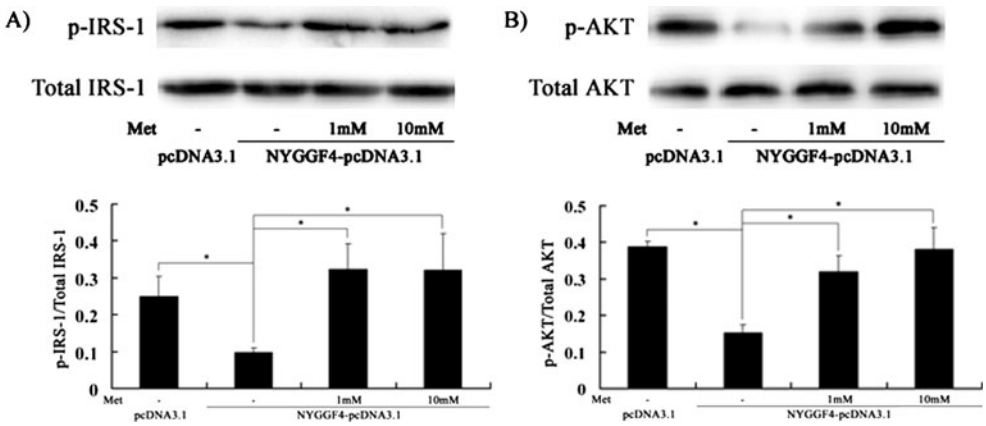

Fig. 3 Effects of metformin (Met) on IRS-1 and Akt phosphorylations in *NYGGF4* overexpression adipocytes. 3T3-L1 predipocytes stably transfected with either an empty expression vector (pcDNA3.1) or a *NYGGF4* expression vector (*NYGGF4*-pcDNA3.1) were induced to differentiate. On the 10th day of differentiation, the cells were serum-starved for 3 h and then incubated with 100 nmol/L insulin for 30 min. The mature adipocytes were incubated with or without metformin (1 or 10 mM) for 24 h and the phosphorylation states of the IRS-1 (**a**) and Akt (**b**) were determined by Western blot analysis. Values represent densitometric analysis of those obtained from three independent experiments (*$P<0.05$)

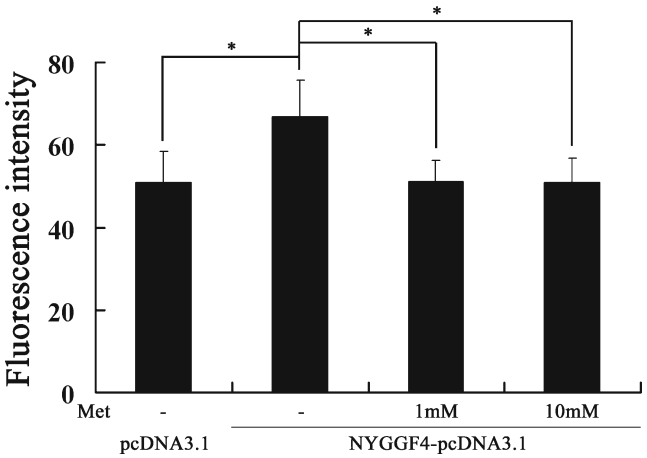

Fig. 4 Effects of metformin (Met) on the ROS levels in *NYGGF4* overexpression adipocytes. 3T3-L1 preadipocytes transfected with the *NYGGF4* expression vector (*NYGGF4*-pcDNA3.1) or the empty vector (pcDNA3.1) were induced to differentiate as described in the Materials and Methods. On the 10th day of differentiation, the mature adipocytes were pretreated with or without metformin (1 or 10 mM) for 24 h and the ROS levels in the adipocytes were determined by using the H2-DCFDA probe with a flow cytometer. Values shown are the means ± SD of three independent experiments performed in triplicate (*P<0.05)

NYGGF4 overexpression adipocytes compared with control cells. Addition of metformin, however, increased the protein expressions of PGC1-α, NRF-1, and TFAM in 3T3-L1 adipocytes (Fig. 5).

Discussion

In adipose cells, GLUT4 is the primary glucose-transporter isoform mediating insulin-stimulated glucose transport (Cushman and Wardzala 1980; Suzuki and Kono 1980). In the basal state, the majority of the GLUT4 is sequestered into intracellular vesicle compartments which translocate to the PM following insulin stimulation, thereby allowing glucose entry. Metformin, however, is able to provoke increases in insulin-stimulated glucose transport by substantially enhancing the insulin-stimulated translocation of GLUT4 transporters from the cytosol to the PM. In this study, our results revealed that overexpression of *NYGGF4* decreased the insulin-stimulated glucose uptake of 3T3-L1 adipocytes by decreasing GLUT4 translocation to the PM, which can be reversed by metformin treatment.

Glucose transport into the adipocytes is acutely regulated by insulin through the activation of a series of intracellular proteins (Taniguchi et al. 2006), which ultimately results in the translocation of GLUT4 from an intracellular pool to the PM of adipocytes to enhance glucose transport (Klip and Ishiki 2005). Consistent with our previous work (Zhang et al. 2009), the result of this study showed that *NYGGF4* decreased the insulin-stimulated tyrosine phosphorylation of IRS1 and serine phosphorylation of Akt. It is generally accepted that IRS-1 functions upstream of PI3K pools required for activation of Akt which is required for insulin stimulated GLUT4 translocation and/or glucose transport in

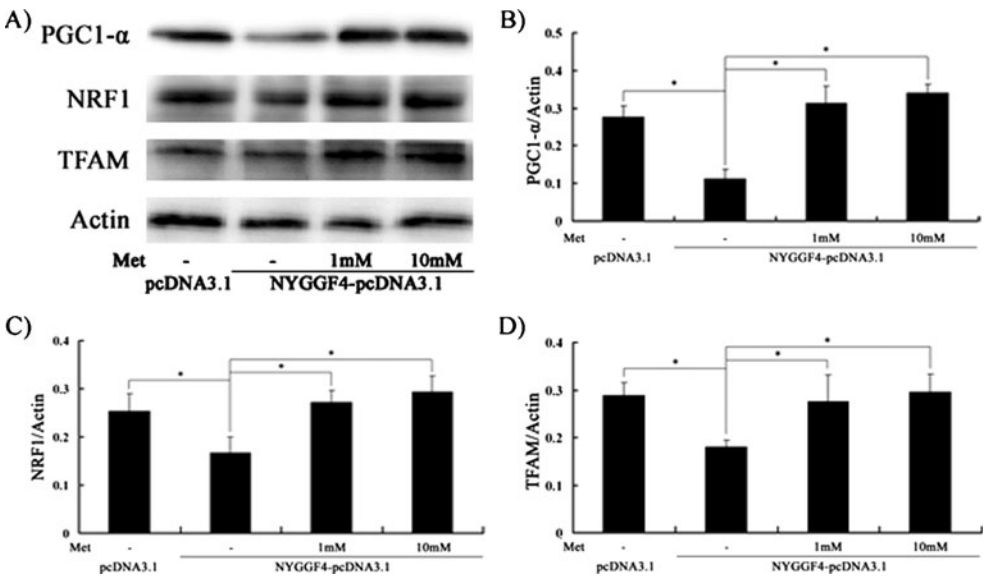

Fig. 5 Effects of metformin (Met) on the PGC1-α, NRF-1, and TFAM protein expression levels in *NYGGF4* overexpression adipocytes. The transfected 3T3-L1 preadipocytes were induced to differentiate as described in the Materials and methods. On the 10th day of differentiation, the cells were serum-starved for 3 h and then incubated with 100 nmol/L insulin for 30 min. Cells were preincubated with metformin at indicated concentrations for 24 h. The protein levels of PGC1-α (A and B), NRF-1 (A and C), and TFAM (A and D) in adipocytes were analyzed using Western blot analysis and normalized to the actin levels. Values represent densitometric analysis of those obtained from three independent experiments (*P<0.05)

3T3-L1 adipocytes (Kohn et al. 1996). This pathway could mediate the effect of metformin to redistribute glucose transporters from an intracellular compartment to the PM (Hundal et al. 1992). Recently, metformin was shown to ameliorate defects in insulin signaling in vitro, involving activation of IRS-1 and associated PI3K activity (Yuan et al. 2003; Kumar and Dey 2002), although these findings were not replicated in muscle from diabetic human subjects (Kim et al. 2002). Furthermore, metformin was able to increase phosphorylation of Akt in the presence of insulin in different cell types (Yuan et al. 2003; Kumar and Dey 2002; Pryor et al. 2000; Sonntag et al. 2005). In the current study, we found that metformin can reverse both signalling and the related GLUT4-trafficking defects associated with *NYGGF4* overexpression. Based on these results, we speculated that the downregulated phosphorylation level of Akt resulting from attenuated tyrosine phosphorylation of IRS-1 might impair insulin-stimulated GLUT4 translocation and glucose uptake in *NYGGF4* overexpression adipocytes. Metformin reverses the decrease in tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt induced by *NYGGF4* overexpression.

Currently, accumulating evidence strongly suggests that the development of mitochondrial dysfunction in adipocytes is an early step in the pathogenesis of obesity-associated IR (Guilherme et al. 2008). The proposed molecular mechanism for IR caused by mitochondrial dysfunction is an increase in ROS. Apart from producing energy, mitochondria are the major source of ROS owing to the continuous generation of superoxide, a byproduct of the electron transport chain. Uncontrolled increase of these oxidants is detrimental to mitochondria, which damages mitochondrial proteins, DNA, and lipids in membrane components, resulting in mitochondrial dysfunction. Consequentially, ROS impairs insulin signaling and causes IR (Fridlyand and Philipson 2006). Our data showed that overexpression of *NYGGF4* in 3T3-L1 adipocytes elevated intracellular ROS level, which indicated that mitochondrial dysfunction might be responsible for the development of *NYGGF4*-induced IR. We also investigated the effect of metformin on the *NYGGF4* overexpression adipocytes and observed a marked decrease in ROS levels. On the basis of these data, we hypothesized that elevated ROS production in *NYGGF4* overexpression adipocytes might contribute to the loss of both mitochondrial function and insulin sensitivity; while pretreatment of cells with metformin might improve the effects of *NYGGF4* on IR, at least in part, by reduced accumulation of ROS.

Several lines of evidence suggested that PGC1-α, identified as an upstream regulator of mitochondrial number and function (Vallea et al. 2005), is an important regulator of intracellular ROS levels by mitochondria (Kukidome et al. 2006; St-Pierre et al. 2003; Vallea et al. 2005). So, we next investigated whether metformin inhibits ROS by activating PGC1-α in *NYGGF4* overexpression adipocytes. Western Blot findings from the present study indicated that the PGC1-α protein level was significantly decreased in *NYGGF4* overexpression adipocytes which was significantly increased by metformin. This suggested that PGC1-α may serve as an adaptation mechanism for protection against ROS in *NYGGF4* overexpression adipocytes. Genetic and biochemical studies strongly suggested that PGC1-α could bind to and coactivate the transcriptional function of NRF-1 on the promoter for TFAM, a direct regulator of mitochondrial DNA replication/transcription and a stimuli for the regulation of mitochondrial biogenesis and function (Wu et al. 1999). Hence, it is conceivable that PGC1-α regulates mitochondrial function partly through modulating the amounts and/or function of these two key sets of regulators, which act together to bring about mitochondrial biogenesis (Poyton and McEwen 1996). In addition, it has been reported also that metformin inhibits the intracellular ROS production by promotion of mitochondrial biogenesis through activating the AMPK-PGC1-α pathway (Kukidome et al. 2006). Our data also showed that metformin increased the expressions of NRF-1 and TFAM, which decreased in the *NYGGF4* overexpression adipocytes. These results revealed that mitochondrial dysfunction might be involved in the pathogenesis of *NYGGF4*-induced IR and metformin normalizes *NYGGF4*-induced ROS production by promotion of mitochondrial biogenesis through the activation of AMPK-PGC1-α pathway. Our findings suggested that a blockade of *NYGGF4*-induced ROS production though the AMPK-PGC1-α pathway could therefore be useful in the design of new pharmacological approaches to prevent IR.

In conclusion, *NYGGF4* plays a role in IR and its effects on IR could be reversed by metformin through activating IRS-1/PI3K/Akt and AMPK-PGC1-α pathways.

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