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α-Lipoic acid protects 3T3-L1 adipocytes from NYGGF4 (PID1) overexpression-induced insulin resistance through increasing phosphorylation of IRS-1 and Akt

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Abstract NYGGF4 (also called PID1) was demonstrated that it may be related to the development of obesity-related IR. We aimed in the present study to further elucidate the effects of NYGGF4 on IR and the underlying mechanisms through using α-Lipoic acid (LA) treatment, which could facilitate glucose transport and utilization in fully differentiated adipocytes. Our data showed that the LA pretreatment strikingly enhanced insulin-stimulated glucose uptake through increasing GLUT4 translocation to the PM in NYGGF4 overexpression adipocytes. The reactive oxygen species (ROS) levels in NYGGF4 overexpression adipocytes were strikingly enhanced, which could be decreased by the LA pretreatment. NYGGF4 overexpression resulted in significant inhibition of tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt, whereas incubation with LA strongly activated IRS-1 and Akt phosphorylation in NYGGF4 overexpression adipocytes. These results suggest that LA protects 3T3-L1 adipocytes from NYGGF4-induced IR partially through increasing phosphorylation of IRS-1 and Akt and provide evidence that NYGGF4 may be a potential target for the treatment of obesity and obesity-related IR.

Keywords Insulin resistance · NYGGF4 · α-Lipoic acid · IRS-1 · Akt

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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 obesity 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 previous reports, we isolated and characterized a novel human gene NYGGF4 (GenBank accession no. NM_001100818) (Wang et al. 2006), which has been indicated that it might 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 NYGGF4

might play an important role in the development of obesity-related IR although further studies are needed to explore the possible mechanisms. Nowadays, a growing body of evidence suggests that α-Lipoic acid (LA) may have potential therapeutic value in lowering elevated glucose levels in diabetic conditions. Oral or intravenous administration of LA was shown to increase insulin sensitivity in individuals with type 2 diabetes (Jacob et al. 1995, 1996, 1999). Furthermore, recent studies have demonstrated that LA facilitates glucose transport and utilization in fully differentiated adipocytes, as well as in animal models of diabetes (Estrada et al. 1996a; Eason et al. 2002; Moini et al. 2002a). So, we aimed in the present study to further elucidate the effects of NYGGF4 on IR and the underlying mechanisms through using LA treatment.

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/ml G418. From day 2 to day 4, the medium was supplemented with only 100 nmol/l insulin. The cells were then transferred to DMEM containing 10 % FBS and 100 μg/ml G418 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 100 μmol/L or 250 μmol/L α-lipoic acid (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.

Reactive oxygen species (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

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 glucose transporter 4 (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).

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

Consistent with our previous reports (Zhang et al. 2009), NYGGF4 overexpression led to significant inhibition of glucose uptake into adipocytes but basal glucose uptake was similar to that observed in control cells. Treatment of cells with 100 or 250 μM of LA strikingly enhanced insulin-stimulated glucose uptake relative to NYGGF4 overexpression adipocytes without affecting basal glucose uptake (Fig. 1).

GLUT4 translocation

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

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 insulin resistance (Bonnard et al. 2008). As shown in Fig. 3, the ROS levels in NYGGF4 overexpression adipocytes were strikingly enhanced compared with the control cells, while the LA pretreatment significantly decreased the ROS levels in NYGGF4 overexpression adipocytes.

Protein expression and phosphorylation of insulin signaling molecules

LA is known to increase glucose uptake into fully differentiated 3T3-L1 adipocytes by activating the IR/Akt signaling pathway (Moini et al. 2002a; Yaworsky et al. 2000a) and

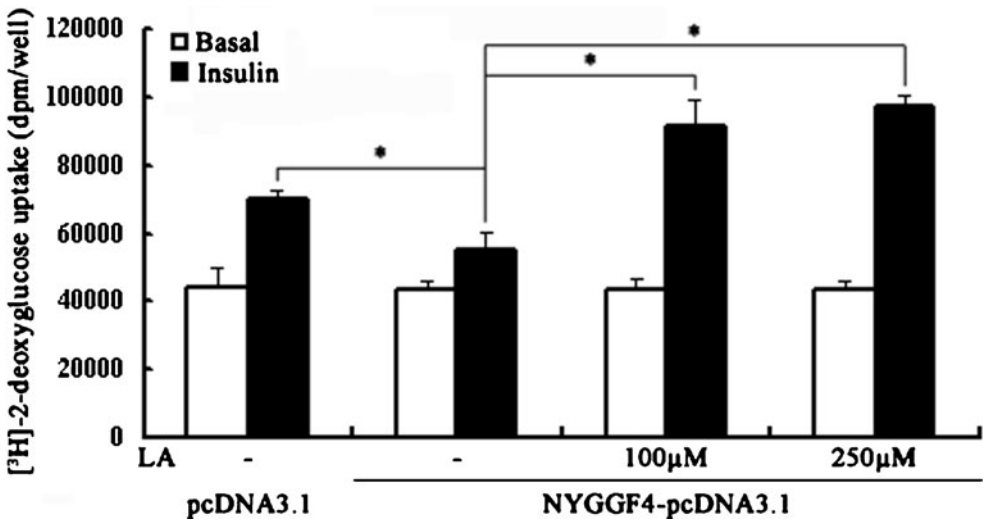

Fig. 1 Effects of LA on glucose uptake into NYGGF4 overexpression adipocytes. 3T3-L1 predipocytes stably transfected with either an empty expression vector (pcDNA3.1) or a NYGGF4 expression vector (NYGGF4-pcDNA3.1) were induced to differentiate. On the 10th day of differentiation, the mature adipocytes were pretreated with or without LA (100 or 250 μM) for 24 h before being incubated with (black columns) or without (white columns) 100 nM insulin for 30 min. 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 (*$P<0.05$)

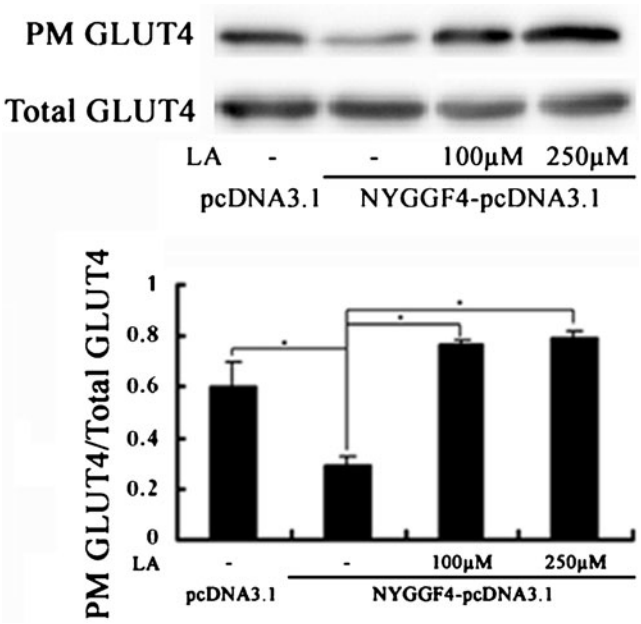

Fig. 2 Effects of LA on the GLUT4 translocation in NYGGF4 overexpression adipocytes. 3T3-L1 preadipocytes transfected with the NYGGF4 expression vector (NYGGF4-pcDNA3.1) or the empty vector (pcDNA3.1) were induced to differentiate. Membrane proteins and total proteins were extracted from the differentiated cells incubated with or without LA (100 or 250 μM) 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)

our previous study have 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. 4, NYGGF4 overexpression resulted in significant inhibition of tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt, whereas incubation with LA 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.

Discussion

NYGGF4 (also called PID1) was isolated and characterized by our group (Wang et al. 2006) and it was demonstrated that it may be related to the development of obesity. Our further studies revealed that overexpression of NYGGF4 inhibits insulin-stimulated glucose transport and impaired insulin-stimulated GLUT4 translocation in mature adipocytes and skeletal myotubes by blocking the IRS1/PI3K/AKT insulin pathway (Zhang et al. 2009; Wu et al. 2011), accompanied by decreased mitochondrial number, abnormal morphology, lower ATP synthesis, increased 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 level without affecting mitochondrial morphology or mtDNA (Zhang et al. 2010). Taken together, these results provide evidence that NYGGF4 might play an important role in the development of obesity-related IR through blocking the IRS1/PI3K/AKT insulin

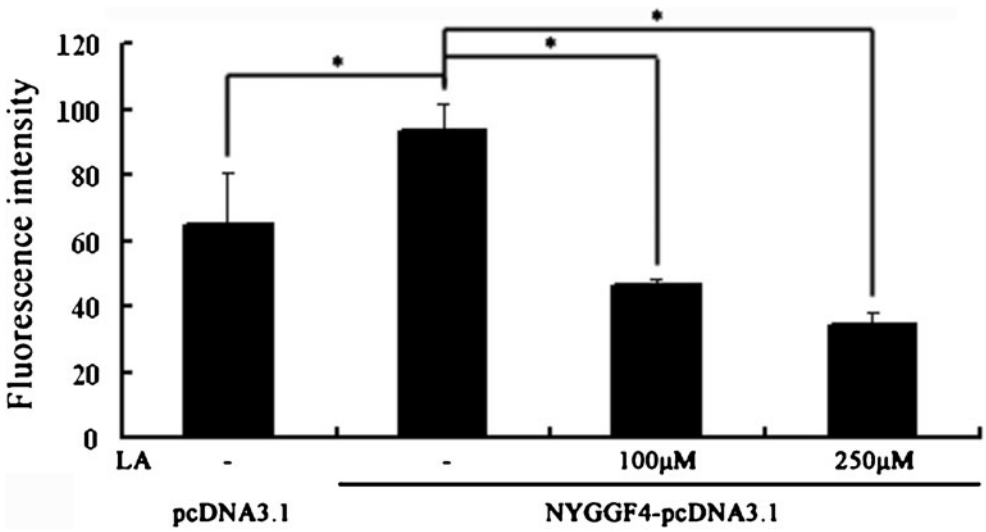

Fig. 3 Effects of LA on the ROS levels in NYGGF4 overexpression adipocytes. 3T3-L1 preadipocytes transfected with the NYGGF4 expression vector (NYGGF4-pcDNA3.1) or the empty vector (pcDNA3.1) were induced to differentiate as described in the Materials and Methods. On the 10th day of differentiation, the mature adipocytes were pretreated with or without LA (100 or 250 μM) 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)

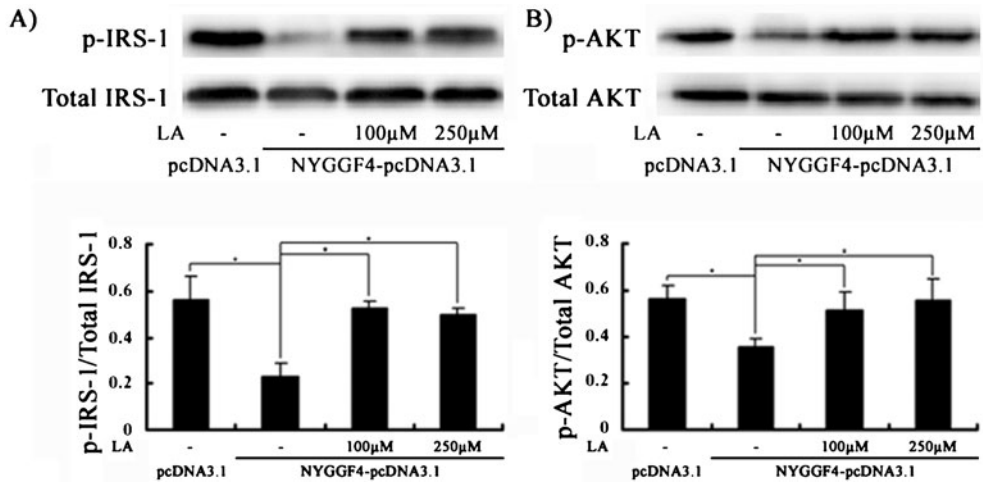

Fig. 4 Effects of LA on IRS-1 and Akt phosphorylation in NYGGF4 overexpression adipocytes. 3T3-L1 predipocytes stably transfected with either an empty expression vector (pcDNA3.1) or a NYGGF4 expression vector (NYGGF4-pcDNA3.1) were induced to differentiate. The mature adipocytes were incubated with or without LA (100 or 250 μM) for 24 h and the phosphorylation states of the IRS-1 (**a**) and Akt (**b**) were determined by Western blot analysis. Each bar represents mean ± SD of three independent experiments (*$P<0.05$)

pathway and mitochondrial dysfunction. Therefore, we hypothesized that NYGGF4 may be a potential target for the treatment of obesity-related IR and further studies were needed to reveal the underlying mechanisms.

In adipocytes, one of insulin's important effects is the ability to increase the rate of cellular glucose transport (Ducluzeau et al. 2002) which is mediated by the translocation of insulin-sensitive GLUT4 from intracellular vesicles to the PM. Consistent with our previous work, we found that NYGGF4 overexpression could significantly reduce insulin-stimulated glucose transport by decreasing GLUT4 translocation to the PM in mature adipocytes and had no effect on basal glucose uptake. As previously reported (Yaworsky et al. 2000b; Konrad et al. 2001; Moini et al. 2002b), LA increased uptake of glucose into 3T3-L1 adipocytes. In the present study, we demonstrated that the LA pretreatment strikingly enhanced insulin-stimulated glucose uptake through increasing GLUT4 translocation to the PM in NYGGF4 overexpression adipocytes. These findings showed that LA could improve NYGGF4-induced IR.

Nowadays, there is growing evidence that the development of mitochondrial dysfunction in adipocytes is an early step in the pathogenesis of obesity-associated IR (Guilherme et al. 2008). Our previous studies using NYGGF4 overexpression and knockdown adipocytes showed that mitochondrial dysfunction might be responsible for the development of NYGGF4-induced IR. Mitochondria are the major source of ROS owing to the continuous generation of superoxide (Barja 1999; Turrens 2003). Exposure to high level of ROS can induce mitochondrial alteration (Bonnard et al. 2008) and possibly IR (Anderson et al. 2009; Houstis et al. 2006) because intracellular redox status plays an important role in the modulation of insulin action. Furthermore, it has been reported that LA stimulates glucose uptake into 3T3-L1 adipocytes by changing the intracellular redox status (Moini et al. 2002b). In this present study, our data showed that the ROS levels in NYGGF4 overexpression adipocytes were strikingly enhanced, which could be decreased by the LA pretreatment. On the basis of these data, we hypothesized that elevated ROS production in NYGGF4 overexpressing adipocytes might lead to the loss of insulin sensitivity, and that the antioxidant activity of LA contributes to its beneficial effects in improving the NYGGF4-induced IR.

Defects in the insulin signaling pathway are central to the development of obesity and related diseases, such as IR (Jackson 2006). Activation of PI 3-kinase was shown to be necessary for insulin-stimulated translocation of GLUT4 from a sequestered intracellular pool to the PM (Yonezawa et al. 1992; Czech and Corvera 1999). Nowadays, insulin signaling has been established to regulate mitochondrial function (Cheng et al. 2009; Cheng and White 2010; Liu et al. 2009; O'Neill et al. 2007). In mammalian cells, AKT activity is closely correlated with mitochondrial function (Tapodi et al. 2005) and the proposed molecular mechanism for IR caused by mitochondrial dysfunction is an increase in ROS that activates serine/threonine kinase. This leads to an increase in the serine phosphorylation of IRS-1 (Kim et al. 2008; Schrauwen and Hesselink 2004). Furthermore, mechanistic studies conducted in insulin-responsive cells in culture demonstrated that LA rapidly stimulates glucose uptake by activating the insulin-signaling pathway (Yaworsky et al. 2000a). It has been demonstrated that LA stimulates glucose uptake into 3T3-L1 adipocytes by increasing phosphorylation of IR and IRS-1, to enhance PI 3-kinase and Akt1 activities, which elevates GLUT4 content in the PM (Yaworsky et al.

2000a; Konrad et al. 2001; Ramath et al. 1999; Estrada et al. 1996b). Combined with our previous results that NYGGF4 induced IR in adipocytes through attenuated tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt (Zhang et al. 2009), we examined the protein expression and phosphorylation levels of IRS-1 and AKT with or without LA. Our data in this present study which showed that overexpression of NYGGF4 inhibits insulin-stimulated glucose uptake through an attenuated phosphorylation levels of IRS-1 and AKT is consistent with our previous results (Zhang et al. 2009). Incubation with LA strongly activated IRS-1 and Akt phosphorylation in NYGGF4 overexpression adipocytes. The results certified again the hypothesis that overexpression of NYGGF4 inhibited insulin-stimulated glucose uptake in adipocytes mainly through blocking the IRS1/PI3K/AKT insulin pathway and mitochondrial dysfunction might be involved in the pathogenesis of NYGGF4-induced IR, which can be changed by LA.

Collectively, these results suggest that LA protects 3T3-L1 adipocytes from NYGGF4-induced IR partially through increasing phosphorylation of IRS-1 and Akt and provide evidence that NYGGF4, a new obesity candidate gene, may be a potential target for the treatment of obesity and obesity-related IR.

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