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α-Lipoic acid ameliorates impaired glucose uptake in LYRM1 overexpressing 3T3-L1 adipocytes through the IRS-1/Akt signaling pathway

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Abstract Overexpression of the *Homo sapiens* LYR motif containing 1 (LYRM1) causes mitochondrial dysfunction and induces insulin resistance in 3T3-L1 adipocytes. α-Lipoic acid (α-LA), a dithiol compound with antioxidant properties, improves glucose transport and utilization in 3T3-L1 adipocytes. The aim of this study was to investigate the direct effects of α-LA on reactive oxygen species (ROS) production and insulin sensitivity in LYRM1 overexpressing 3T3-L1 adipocytes and to explore the underlying mechanism. Pretreatment with α-LA significantly increased both basal and insulin-stimulated glucose uptake and insulin-stimulated GLUT4 translocation, while intracellular ROS levels in LYRM1 overexpressing 3T3-L1 adipocytes were decreased. These changes were accompanied by a marked upregulation in expression of insulin-stimulated tyrosine phosphorylation of IRS-1 and serine phosphorylation of Akt following treatment with α-LA. These results indicated that α-LA protects 3T3-L1 adipocytes from LYRM1-induced insulin resistance partially via its capacity to restore mitochondrial function and/or increase phosphorylation of IRS-1 and Akt.

Keywords α-LA · LYRM1 · Mitochondrial dysfunction · Insulin resistance · Obesity

Introduction

In previous studies, we isolated and characterized *LYRM1*, a novel human gene expressed at high levels in omental adipose tissue of obese patients. LYRM1 protein is localized in the nucleus, with a LYR domain associated with mitochondrial function and energy metabolism (Qiu et al. 2009; Qiu et al. 2007). Further studies demonstrated that overexpression of LYRM1 in 3T3-L1 adipocytes caused abnormal mitochondrial morphology, decreased intracellular ATP synthesis and mitochondrial membrane potential (Cao et al. 2010). In addition, LYRM1 overexpression led to excessive production of intracellular reactive oxygen species (ROS) resulting in reduced insulin-stimulated glucose uptake. Overexpression of LYRM1 in L6 myotubes decreased insulin-stimulated glucose transport through attenuated phosphorylation of insulin receptor substrate (IRS)-1, phosphatidylinositol-3-kinase (PI3K) p85, and Akt (Cao et al. 2010; Kou et al. 2011). These studies indicated that LYRM1 exerts regulatory activities on mitochondrial dysfunction and the development of obesity-related insulin resistance.

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Mitochondria are key organelles involved in glucose and fatty acid metabolism. Growing evidence demonstrates that mitochondrial defects play a critical role in obesity-associated insulin resistance (Kim et al. 2008). In this regard, several reports have suggested notable links between mitochondrial dysfunction and impaired insulin action based on the observation of impaired mitochondrial function in patients and/or mice with obesity and type 2 diabetes (Bournat and Brown 2010; Lowell and Shulman 2005; Pershadsingh 2007; Lu et al. 2010; Turner and Heilbronn 2008), as well as in vitro observations (Lu et al. 2010; Shibata et al. 2010).

Oxidative stress caused by ROS, leads to mitochondrial dysfunction and chronic diseases, such as insulin resistance and cardiovascular disease and associated complications (Shibata et al. 2010; Bashan et al. 2009; Bonnard et al. 2008; Houstis et al. 2006; Forstermann 2008; Wang et al. 2012). α-LA has been identified as a potent antioxidant and free radical scavenger, as well as a cofactor of mitochondrial dehydrogenase complexes which catalyze both carbohydrate and amino acid metabolism (Biewenga et al. 1997). α-LA has been investigated in several studies on prevention or treatment of diabetes and its complications. Although the exact mechanism by which α-LA ameliorates insulin resistance is complicated and unclear, reduced oxidative stress (Pershadsingh 2007; Houstis et al. 2006; Konrad 2005), promotion of mitochondrial synthesis (Shen et al. 2008), improved glucose oxidation (Konrad et al. 1999) and activation of the insulin signaling pathway may be involved (Konrad 2005; Moini et al. 2002; Yaworsky et al. 2000). This study was undertaken to elucidate the effects and mechanism of action of LYRM1 on IR by α-LA treatment.

Materials and methods

Antibodies

Primary polyclonal glucose transporter 4 (GLUT4) antibodies and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (1:500; Santa Cruz, CA, USA). Anti-β-actin and anti-IRS-1 antibodies were purchased from Cell Signaling Technology (1:1000, 1:500; Danvers, MA, USA). The phospho-specific polyclonal antibody against IRS-1 (Tyr612) was obtained from Biosource (1:500; Camarillo, CA, USA). Antibodies against Akt and the phosphorylated forms of this protein were obtained from Kangchen (1:1,000; Shanghai, China).

Cell culture and treatment

3T3-L1 cells, which have been extensively used as a model of adipogenic differentiation and insulin action, were cultured, maintained and differentiated as previously described (Student et al. 1980). Briefly, cells were grown in DMEM/high-glucose medium (Gibco, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco) in a 5 % CO_2 environment. Two days after cell growth arrest, differentiation into adipocytes was initiated as follows: medium was supplemented with 0.5 mmol/L 3-isobuty-1-methylxanthine (MIX; Sigma, St. Louis, MO, USA), 1 μmol/L dexamethasone (Sigma) and 10 μg/mL insulin (Sigma) for 2 days. The medium was then changed and supplemented with insulin only for a further 2 days. Thereafter, cells were maintained in DMEM medium containing only 10 % FBS and changed every two days. Ten days after the induction of differentiation, more than 90 % of cells exhibited typical morphology and biochemical properties of adipocytes. After overnight incubation in serum-free DMEM, 3T3-L1 adipocytes were treated with α-LA (100 μmol/L and 250 μmol/L) diluted in DMEM from a 100 mM stock solution dissolved in DMSO. After 12 h incubation in the presence or absence of α-LA, adipocytes were collected for subsequent experiments.

Confocal laser microscopy

The H2-DCFDA probe (Sigma, St. Louis, MO, USA) was used to estimate intracellular ROS levels (Maxwell et al. 1999). Cells were incubated with 5 μmol/L H2-DCFDA for 30 min at 37 °C and then washed three times with pre-warmed phosphate-buffered saline (PBS). Cells were imaged by confocal laser scanning microscopy (Zeiss, Gottingen, Germany).

Flow cytometry

Cells were incubated with 5 μmol/L H2-DCFDA at 37 °C. After 30 min, cultured cells were trypsinized and centrifuged at 1,000 rpm at 4 °C for 5 min, then resuspended in Krebs-Ringer solution buffered with 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and 0.5 % bovine serum albumin (BSA). Cells were analyzed with a FACScan flow cytometer using the CellQuest software (BD Biosciences, San Jose, CA, USA).

Glucose uptake

Glucose uptake assays were performed using a standard protocol with minor modifications (Ceddia et al. 2005). Stably transfected cells were cultured in 12-well plates and induced into mature adipocytes. Cells were serum-starved in DMEM containing 0.5 % FBS for 3 h, washed twice with 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-glucose was added

at a final concentration of 2 μCi/mL. After 10 min at 37 °C, the reaction was terminated by washing three times with ice-cold PBS supplemented with 10 mmol/L D-glucose. Cells were solubilized by adding 200 μL 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 measurement of protein using the bicinchonic acid protein assay kit (Pierce, Rockford, IL, USA). Radioactivity was normalized by protein concentration.

Western blotting

Transfected cells were induced to mature into adipocytes. After incubation with α-LA for 12 h, cells were starved for 3 h and then incubated with 100 nmol/L insulin for 30 min. Total and phosphorylated proteins were extracted according to the instructions provided by the manufacturer(Beyotime, Hangzhou,China). 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 protein assay kit in accordance with the instructions provided by the manufacturer (Pierce, Rockford, IL, USA). Proteins (30 μg/lane) were separated by 10 % sodium dodecy1 sulfate-polyacrylamide electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Membranes were blocked with TBST buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01%Tween-20) containing 5 % BSA for 1 h at room temperature. Membranes were incubated at 4 °C overnight in TBST buffer containing each of the primary antibodies described, washed three times with TBST for 15 min and then hybridized with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Membranes were washed four times with TBST buffer for 10 min and developed with an enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ, USA).

Statistical analysis

Each experiment was performed at least three times. All data are expressed as means ± SD. 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 $P<0.05$.

Results

α-LA decreased intracellular ROS levels in LYRM1-overexpressing 3T3-L1 adipocytes

A causative role of ROS has been indicated in obesity and type 2 diabetes (Bashan et al. 2009; Bonnard et al. 2008; Wang et al. 2012). Intracellular ROS are mainly produced in the mitochondria. In order to confirm the role of the antioxidant α-LA, ROS production was investigated in LYRM1-overexpressing adipocytes. As shown in Fig. 1, ROS levels were dramatically increased in LYRM1-overexpressing adipocytes, and this effect was blocked by incubation with α-LA (100 μmol/L and 250 μmol/L) for 12 h. These results suggest that α-LA pretreatment protected cells against an LYRM1-induced increase in ROS content.

Effects of α-LA on basal and insulin-stimulated glucose uptake in LYRM1-overexpressing 3T3-L1 adipocytes

α-LA was used to investigate the role of ROS generation in LYRM1-induced insulin resistance. Nine days after initiation of differentiation, LYRM1-overexpressing adipocytes were exposed to α-LA (100 μmol/L and 250 μmol/L) for 12 h. As shown in Fig. 2, LYRM1 overexpression led to significant inhibition of glucose uptake into adipocytes although basal glucose uptake was similar to that observed in control cells. These observations are consistent with our previous reports (Cao et al. 2010). In parallel with the reduction in ROS production, pretreatment with α-LA (100 μmol/L and 250 μmol/L) led to marked increases in baseline and insulin-stimulated glucose uptake. These data suggest that α-LA protects against the impairment in glucose uptake induced by LYRM1.

Effects of α-LA on insulin-stimulated GLUT4 translocation in LYRM1-overexpressing adipocytes

GLUT4 is the dominant glucose transporter responsible for insulin-stimulated glucose transport in 3T3-L1 adipocytes. Therefore, PM GLUT4 content was assessed. Consistent with previous studies (Moini et al. 2002; Rudich et al. 1999), treatment with α-LA (50–250 μmol/L) increased glucose transport in a dose-dependent manner, reaching a maximum at 250 μmol/L. As shown in Fig. 3, overexpression of LYRM1 decreased insulin-stimulated GLUT4 translocation to the PM but had no effect on total GLUT4 protein. However, α-LA pretreatment significantly increased GLUT4 translocation to the PM compared with that of LYRM1 overexpressing adipocytes. These results show that α-LA protected against the impaired insulin-stimulated GLUT4 translocation induced by LYRM1.

Effects of α-LA on insulin-stimulated phosphorylation of insulin signaling molecules in LYRM1-overexpressing adipocytes

α-LA stimulates IRS-1-associated PI3K and Akt activity in 3T3-L1 adipocytes (Moini et al. 2002; Rudich et al. 1999). Overexpression of LYRM1 has been shown previously to

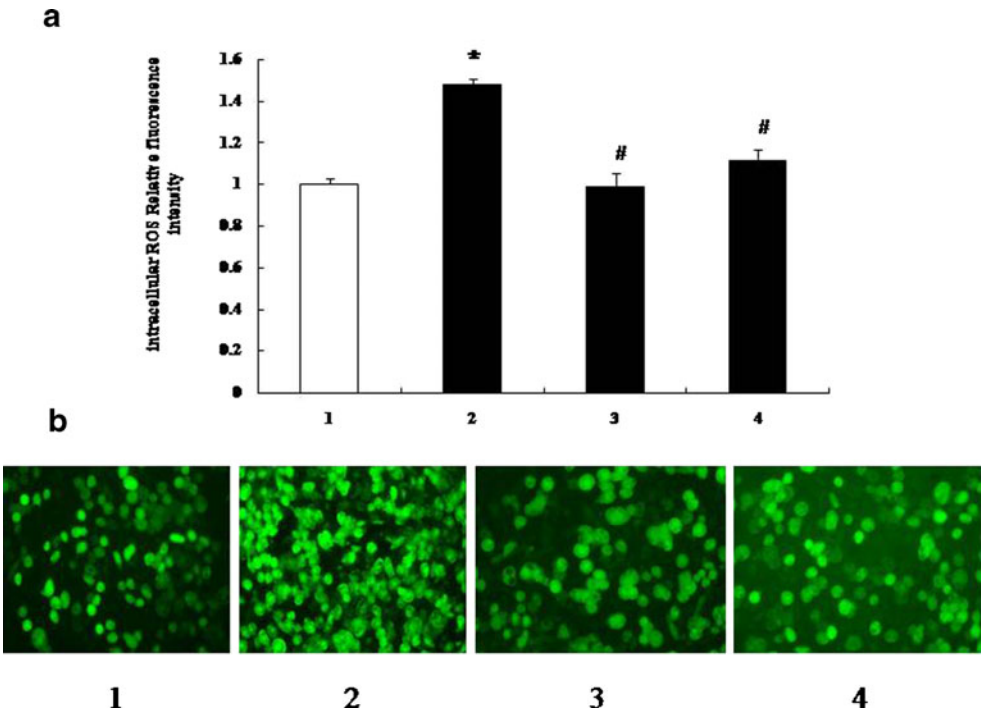

Fig. 1 α-LA decreases cellular levels of reactive oxygen species (ROS) in LYRM1-overexpressing adipocytes. Mature adipocytes were incubated with α-LA for 12 h before ROS levels were determined by detection of a H2-DCFDA probe using a FACScan flow cytometer (N=6) (**a**) and a confocal laser scanning microscope (×400 magnification) (**b**). 1, pcDNA3.1; 2, LYRM1-pcDNA3.1; 3, LYRM1-pcDNA3.1+α-LA100 μmol/L; 4, LYRM1-pcDNA3.1+α-LA250 μmol/L. $^{*}P$<0.001 vs. pcDNA3.1; $^{\#}P$<0.01 vs. LYRM1- pcDNA3.1

inhibit glucose transport in L6 cells via attenuated phosphorylation of IRS-1 and PI3K/Akt. To assess whether the protective effect of α-LA is related to a preserved ability of insulin to activate IRS-1 and Akt, expression and insulin-stimulated phosphorylation of these proteins were investigated. As shown in Fig. 4, LYRM1 considerably reduced insulin-stimulated phosphorylation these proteins. α-LA pretreatment at 250 μmol/L considerably increased insulin-induced phosphorylation of IRS-1 and Akt. In contrast, the increase observed at 100 μmol/L was marginal and no significant difference was detected in the total protein content of these signaling molecules. These data indicate that α-LA prevents the impairment of insulin-stimulated IRS-1 and Akt activity in LYRM1-overexpressing adipocytes.

Discussion

Recent data indicate that the prevalence of overweight and obese children and adolescents continues to rise around the world. Furthermore, being overweight or obese can result in many negative health consequences such as diabetes, cardiovascular disease, osteoarthritis and some forms of cancer, with associated increases in the healthcare requirements and

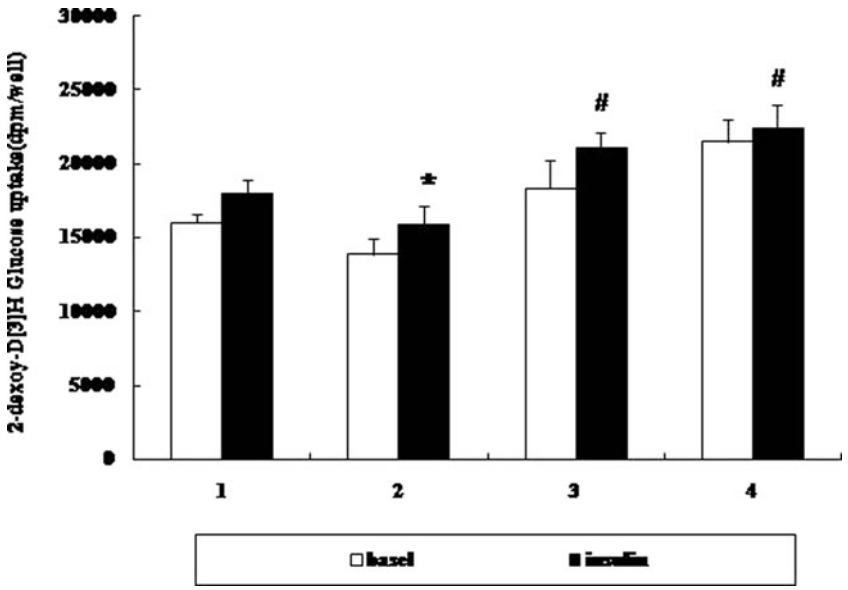

Fig. 2 Effect of α-LA on glucose uptake in LYRM1-overexpressing adipocytes. Fully differentiated adipocytes were incubated in serum-free DMEM overnight and then treated with α-LA for 12 h. Uptake of 2-deoxy-D-[3H]glucose was measured as described in the Materials and methods section. (N=6). 1, pcDNA3.1; 2, LYRM1- pcDNA3.1; 3, LYRM1-pcDNA3.1+α-LA 100 μmol/L; 4, LYRM1-pcDNA3.1+α-LA 250 μmol/L. $^{*}P$<0.05 vs. pcDNA3.1; $^{\#}P$<0.01 vs. LYRM1-pcDNA3.1

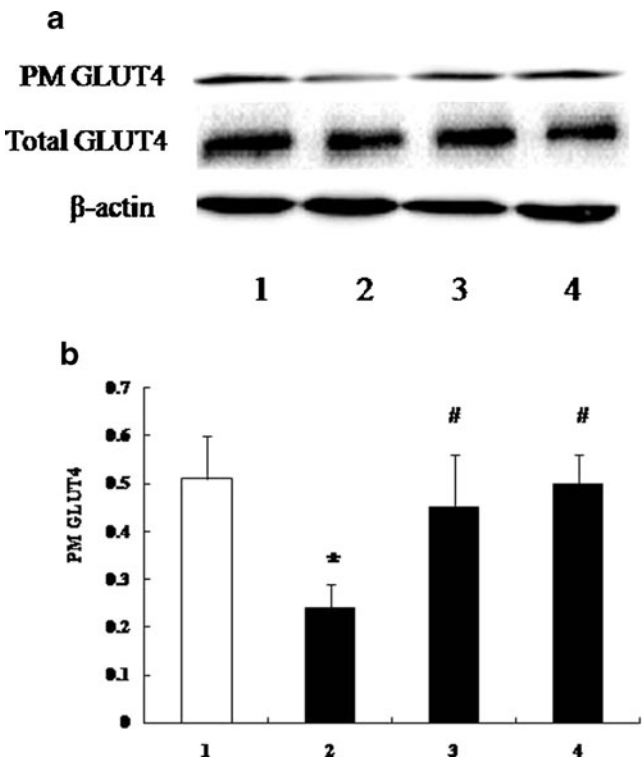

Fig. 3 Effect of α-LA on GLUT4 translocation. 3T3-L1 preadipocytes transfected with LYRM1 or the empty vector were induced to differentiate. **a**, Cells were incubated for 12 h with α-LA . Membrane and total proteins were extracted from differentiated 3T3-L1 adipocytes incubated with 100 nmol/L insulin for 30 min. Western blotting was performed using an antibody against GLUT4. 1, pcDNA3.1; 2, LYRM1- pcDNA3.1; 3, LYRM1-pcDNA3.1+α-LA 100 μmol/L; 4, LYRM1-pcDNA3.1+α-LA 250 μmol/L. **b**, Quantification of protein expression described in 3A based on grayscale analysis. Values shown are the means ± SE of three independent experiments performed in triplicate. $^{*}P<0.01$ vs. pcDNA3.1; $^{\#}P<0.01$ vs. insulin-stimulated LYRM1-pcDNA3.1

economic burden (2002). Although the impact of lifestyle and environmental factors are likely to be significant, it is clear that obesity has high heritability (Walley et al. 2009). *LYRM1* is a novel gene that is involved in obesity-associated insulin resistance and mitochondrial dysfunction in adipocytes (Qiu et al. 2009; Cao et al. 2010; Zhang et al. 2012). Therefore, we hypothesized that LYRM1 is a potential target for the treatment of obesity-related IR and that increased ROS levels are an important trigger for LYRM1-induced IR.

The mitochondrial respiratory chain is the major source of ROS. However, increasing evidence indicates that increased ROS levels induce mitochondrial dysfunction (Choksi et al. 2004) and lead to insulin resistance (Kim et al. 2008; Bashan et al. 2009; Houstis et al. 2006). More importantly, resolution of oxidative stress can reverse insulin resistance (Shibata et al. 2010; Bashan et al. 2009; Bonnard et al. 2008; Houstis et al. 2006; Patti and Corvera 2010). Furthermore, ROS levels have been clearly demonstrated using the indicator of intracellular oxidant, H2DCF-DA (Nishikawa et al. 2000). Prior studies have demonstrated that insulin-stimulated glucose transport is mediated by the GLUT4 transporter, as is the case for adipocytes (Giovannone et al. 2000; Kanzaki 2006). In this study, we demonstrated that LYRM1 dramatically increased ROS levels and reduced insulin-stimulated glucose uptake mediated by the GLUT4 transporter. However, pretreatment with α-LA(100 μmol/L and 250 μmol/L) for 12 h decreased the ROS levels and prevented this loss of insulin activity in accordance with previous reports (Moini et al. 2002; Estrada et al. 1996; Maddux et al. 2001). This suggests a possible association between elevated ROS production and LYRM1-induced insulin resistance and furthermore, that the α-LA-mediated protection of insulin sensitivity is the result of an antioxidant effect.

The actions of insulin maintain glucose homeostasis and other metabolic effects. Insulin resistance is an early and important defect associated with obesity and type 2 diabetes mellitus, which is defined as decreased sensitivity to the effects of physiological levels of insulin on glucose and lipid metabolism in target tissues. Previous studies have shown that multiple defects in insulin signal transduction lead to insulin resistance (Shibata et al. 2010; Bashan et al. 2009; Houstis et al. 2006; Saltiel and Kahn 2001). Insulin signaling involves a complex network initiated by insulin binding to its receptor resulting in increased insulin receptor autophosphorylation and phosphorylation of insulin receptor substrates, such as IRS-1, on tyrosine residues. IRS protein phosphorylation leads to activation of two main signaling pathways: the PI3K-Akt pathway, which is essential for insulin-dependent translocation of GLUT4 from intracellular vesicles to the PM and nutrient metabolism and the Ras-mitogen-activated (MAPK) pathway, which is largely responsible for the effects of insulin on cell growth, differentiation and mitogenesis (Giovannone et al. 2000; Estrada et al. 1996). Our earlier study showed that LYRM1 inhibits glucose transport in rat skeletal muscles via attenuation of IRS-1, PI3K (p85) and Akt phosphorylation although no effects on ERK, p38, and JNK phosphorylation were detected (Kou et al. 2011). In this study, we focused on the IRS-Akt signaling pathway and further examined the effects of α-LA on the protein content and phosphorylation levels of molecules involved in insulin signaling. Beneficial effects of α-LA treatment of type 2 diabetes (T2DM) and associated complications have been reported (Poh and Goh 2009). Furthermore, investigation of the mechanism of the effects of α-LA on insulin responsive cells demonstrated increased glucose uptake and utilization by modulation of the insulin signal transduction pathway (Moini et al. 2002; Yaworsky et al. 2000; Estrada et al. 1996; Poh and Goh 2009) and antagonism of oxidative stresses (Houstis et al. 2006; Rudich et al. 1999; Poh and Goh 2009). In 3T3-L1 adipocytes and/or in L6 myotubes, α-LA at concentrations exceeding 500 μmol/L

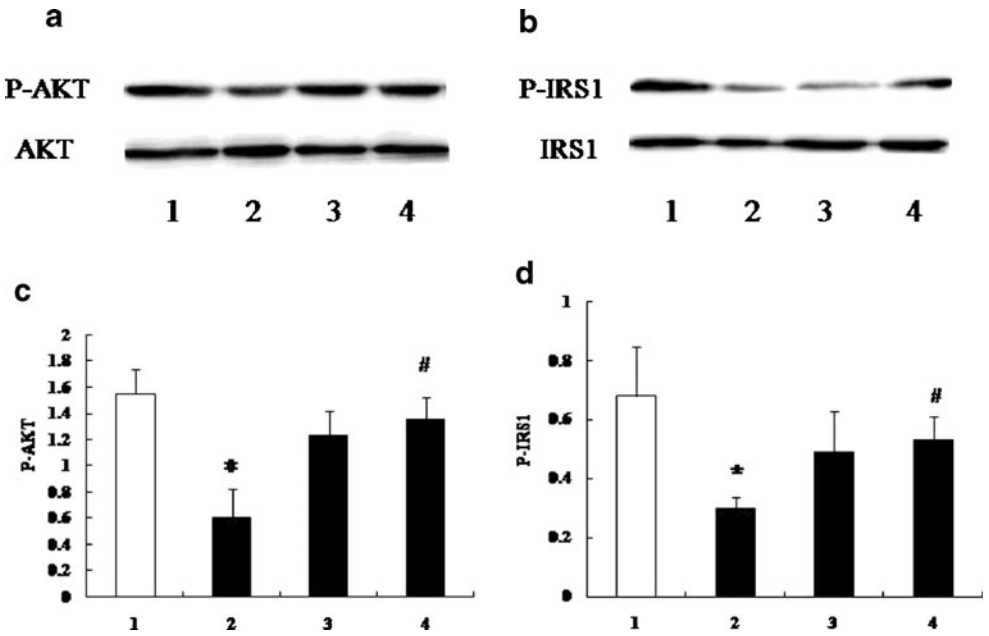

Fig. 4 Effect of α-LA on insulin signal transduction. 3T3-L1 preadipocytes transfected with LYRM1 or the empty vector were induced to differentiate. **a** and **b**, Cells were incubated for 12 h with α-LA. Total and phosphorylated proteins were extracted from differentiated 3T3-L1 adipocytes incubated with 100 nmol/L insulin for 30 min. Western blotting was performed using corresponding antibodies. 1, pcDNA3.1; 2, LYRM1-pcDNA3.1; 3, LYRM1-pcDNA3.1+α-LA 100 μmol/L; 4, LYRM1-pcDNA3.1+α-LA 250 μmol/L. **c** and **d**, Quantification of protein expression described in 4A and 4B based on grayscale analysis. Values shown are the means ± SE of three independent experiments performed in triplicate. $^{*}P<0.01$ vs. pcDNA3.1; $^{\#}P<0.01$ vs. insulin-stimulated LYRM1-pcDNA3.1

exerted insulinomimetic action, resulting in increased glucose transport via direct activation of PI3K/Akt activity (Yaworsky et al. 2000; Estrada et al. 1996). However, lower concentrations of α-LA (50–300 μmol/L) protected insulin-stimulated glucose transport mainly by modulating the cellular redox state (Moini et al. 2002; Rudich et al. 1999; Maddux et al. 2001). In this study, overexpression of LYRM1 inhibited insulin-induced glucose uptake via attenuation phosphorylation levels of IRS-1 and Akt, which is accordance with our previous work (Kou et al. 2011). However, 250 μmol/L α-LA strongly upregulated IRS-1 and Akt phosphorylation in LYRM1 overexpressing cells, although only marginal changes were detected at 100 μmol/L. Thus, the effects of α-LA on glucose transport can be attributed either to its effect on insulin signaling or to antioxidant properties or possibly, to a combination of the two mechanisms.

Our previous work indicated that LYRM1 induced mitochondrial impairment in 3T3-L1 adipocytes, which was characterized by abnormal mitochondrial morphology, lower ATP synthesis and mitochondrial membrane potential and increased ROS levels (Cao et al. 2010). A growing body of evidence indicates a causative link between mitochondrial ROS generation, mitochondrial dysfunction and insulin resistance (Kim et al. 2008; Bashan et al. 2009; Houstis et al. 2006; Wang et al. 2012; Moini et al. 2002). Indeed, ROS is detrimental to mitochondrial function and the subsequent impairment in the ability to oxidize fatty acids may induce lipid (fatty acyl-CoA, diacylglycerol) accumulation resulting in exacerbation of ROS production. These effects are directly correlated with insulin resistance and the mechanism underlying IR is proposed to involve activation of serine/threonine kinases and serine phosphorylation of IRS-1 (Kim et al. 2008; Bashan et al. 2009; Bloch-Damti et al. 2006; Boden 2011). It has been reported that fat or muscle cells exposed to oxidants exhibit enhanced degradation of IRS/Akt molecules, which could be attributed to increased phosphorylation of IRS1 on Ser307 and decreased phosphorylation of Akt on Ser473 (Bloch-Damti et al. 2006; Shay and Hagen 2009; Demozay et al. 2008; Schrauwen and Hesselink 2004).

Our data showed that increased ROS production was associated with attenuated phosphorylation of IRS-1 and Akt in LYRM1 overexpressing cells. Therefore, we hypothesized that insulin signal transmission from IR to Akt may constitute an oxidation-sensitive step in the insulin signaling cascade. It was demonstrated that enhanced antioxidant capacity of these cells restored mitochondrial integrity and protected against this signaling (Pershadsingh 2007; Bonnard et al. 2008; Houstis et al. 2006; Moini et al. 2002; Yaworsky et al. 2000; Rudich et al. 1999; Estrada et al. 1996). To confirm the role of ROS in LYRM1 overexpressing adipocyte insulin sensitivity, we demonstrated that antioxidant treatment with α-LA protected against both LYRM1-induced IR and ROS production. Activation of the IRS-1/Akt signaling pathway is

essential for GLUT4 translocation (Giovannone et al. 2000). Furthermore, α-LA has been shown to exert antioxidant properties at low doses (Moini et al. 2002; Rudich et al. 1999; Maddux et al. 2001). In accordance with these observations, the present study demonstrated that α-LA pretreatment resulted in marked augmentation of IRS-1 and Akt phosphorylation in LYRM1 overexpressing adipocytes and that LYRM1-induced IR can be ameliorated by blocking the increase in ROS levels. However, the exact oxidation-sensitive step which is protected by α-LA treatment remains to be elucidated.

In summary, our results demonstrate that overexpression of LYRM1 inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes via attenuated phosphorylation of IRS-1 and Akt. Furthermore, α-LA provided partial protection against LYRM1-induced IR by restoration of mitochondrial function. These observations provide evidence that LYRM1 is a potential target for the treatment of obesity and obesity-related IR and that antioxidant therapy may be a useful strategy in insulin resistant states.

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Conflict of interests Relevant to this paper no potential conflict of interests is declared by the authors.

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