Original Research

Metformin prevents *LYRM1*-induced insulin resistance in 3T3-L1 adipocytes via a mitochondrial-dependent mechanism

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Abstract

We previously proposed that LYR motif containing 1 (LYRM1)-induced mitochondrial reactive oxygen species (ROS) production contributes to obesity-related insulin resistance. Metformin inhibits ROS production and promotes mitochondrial biogenesis in specific tissues. We assessed the effects of metformin on insulin resistance in LYRM1-over-expressing 3T3-L1 adipocytes. Metformin enhanced basal and insulin-stimulated glucose uptake and GLUT4 translocation, reduced IRS-1 and Akt phosphorylation and ROS levels, and affected the expression of regulators of mitochondrial biogenesis in LYRM1-over-expressing adipocytes. Metformin may ameliorate LYRM1-induced insulin resistance and mitochondrial dysfunction in part via a direct antioxidant effect and in part by activating the adenosine monophosphate-activated protein kinase (AMPK)-PGC1/NRFs pathway.

Keywords: LYRM1, metformin, mitochondrial biogenesis, insulin resistance

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Introduction

Insulin resistance is an early and important defect related to obesity and type-2 diabetes (T2D) which is defined as a diminished ability of the cells or tissues to respond to physiological levels of insulin. To date, the molecular and cellular mechanisms underlying insulin resistance have remained elusive. Mitochondrial dysfunction is increasingly believed to play a crucial role in the pathogenesis of obesity-related diabetes.[1–3] Mitochondrial defects can be induced by increased accumulation of reactive oxygen species (ROS), and additionally, numerous studies have shown that ROS are often involved in the onset and progression of insulin resistance.[2,4–6] It is noteworthy that antioxidant supplements or stimulators of mitochondrial biogenesis may have beneficial effects on insulin resistance by improving mitochondrial function or activating the insulin receptor substrate 1 (IRS-1)/Akt signal pathway.[6–10]

In previous research,[11] we isolated and characterized a novel human gene *LYRM1* (GenBank accession no. NM_020424), which was expressed at a high level in the omental adipose tissue of obese patients. We demonstrated that over-expression of LYRM1-induced insulin resistance, and also led to excessive production of ROS, reduced ATP synthesis, decreased the mitochondrial membrane potential, and induced an abnormal mitochondrial morphology in 3T3-L1 adipocytes.[12] A previous report showed that treatment with α-Lipoic acid (LA), a natural antioxidant, protected against LYRM1-induced insulin resistance, partially via its capacity to restore mitochondrial function and/or increase the phosphorylation of IRS-1 and Akt.[7] Taken together, these results suggest that ROS-induced mitochondrial defects play a relevant role in LYRM1-mediated insulin resistance. However, the mechanism by which LYRM1 induces insulin resistance remains unclear, thus further research to explore the biological function of this gene is essential.

The antidiabetic agent metformin is thought to increase the efficacy of glycemic control, primarily by curtailing hepatic output and increasing insulin-stimulated glucose uptake by muscle and fat.[13,14] A recent investigation showed that metformin prevented mitochondrial-driven oxidative stress by reducing ROS production and improving mitochondrial biogenesis through activating the adenosine monophosphate-activated protein kinase (AMPK)-PGC-1/NRFs axis pathway.[3,9,10,15] We propose that mitochondrial dysfunction is the origin of LYRM1-induced insulin resistance in adipocytes. This study was

undertaken to elucidate the effects and mechanism of action of metformin on LYRM1-induced insulin resistance in adipocytes.

Materials and methods

Cell culture and treatment

The 3T3-L1 preadipocytes were stably transfected with either an empty expression vector (pcDNA3.1Myc/His B) or an LYRM1 expression vector, as described previously.[16] The transfected cells were cultured in DMEM/high-glucose medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 μg/mL neomycin (G418; Roche, Basel, Switzerland) in a 5% CO_2 in air humidified atmosphere at 37°C. After reaching complete confluence (Day 0), the cells were cultured for two days in DMEM supplemented with 10% FBS, 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), and 100 μg/mL G418. The medium was then changed to DMEM supplemented with 10% FBS and 10 μg/mL insulin for a further two days. Thereafter, the cells were maintained in DMEM containing 10% FBS and 100 μg/mL G418, and the media was replenished every two days. Ten days after the induction of differentiation, more than 90% of the cells exhibited the typical morphology and biochemical properties of adipocytes. After overnight incubation in serum-free DMEM, the cells were pretreated with or without metformin (1 mmol/L or 10 mmol/L, Sigma) for 24 h.

Confocal laser microscopy

The H2-DCFDA probe (Sigma) was used to estimate intracellular ROS levels.[17] The cells were treated as described in Section ''Cell culture and treatment,'' incubated with 5 μmol/L H2-DCFDA for 30 min at 37°C, washed three times with prewarmed phosphate-buffered saline (PBS), and then imaged using a confocal laser scanning microscope (Zeiss, Gottingen, Germany).

Flow cytometry

The cells were treated as described in Section ''Cell culture and treatment,'' and then incubated with 5 μmol/L H2-DCFDA at 37°C. After 30 min, the cultured cells were trypsinized, centrifuged at 1000 rpm at 4°C for 5 min, then re-suspended in Krebs–Ringer solution buffered with 4 -(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and 0.5% bovine serum albumin (BSA). The cells were analyzed using a FACScan flow cytometer using CellQuest software (BD Biosciences, San Jose, CA, USA).

Glucose uptake

Glucose uptake assays were performed using a standard protocol with minor modifications.[18] Stably transfected cells were cultured in 12-well plates and induced to differentiate into mature adipocytes. The 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. The cells were solubilized by adding 200 μL 1 mol/L of NaOH to each well and aliquots of the cell lysate were transferred to scintillation vials for radioactivity counting. The remainder of the lysate was used for the protein assay with the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Radioactivity counts were normalized to the protein concentration of each sample.

Western blotting

Transfected cells were induced to mature into adipocytes as described in Section ''Cell culture and treatment.'' After stimulation with metformin for 24 h, the cells were starved for 3 h and then incubated with 100 nmol/L insulin for 30 min. Total and phosphorylated proteins were extracted using the nuclear protein and cytoplasm protein extraction kit (Beyotime, Hangzhou, China) according to the instructions provided by the manufacturer. Plasma membrane (PM) proteins were extracted using the eukaryotic membrane protein extraction reagent (Pierce). Protein concentrations were quantified using the BCA protein assay kit (Pierce) in accordance with the manufacturer's instructions. Proteins (30 μg/lane) were separated by 10% sodium dodecy1 sulfate-polyacrylamide electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with Tris-buffered saline Tween-20 (TBST) buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween-20) containing 5% BSA for 1 h at room temperature and then incubated at 4°C overnight in TBST buffer containing the primary antibodies. The membranes were then washed three times with TBST for 15 min, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, washed four times with TBST buffer for 10 min, and developed using an enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ, USA).

The primary polyclonal glucose transporter 4 (GLUT4) antibody and horseradish peroxidase-conjugated secondary antibody 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 and 1:500, respectively; 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 phosphorylated AKT were obtained from Kangchen (1:1000; Shanghai, China). Rabbit polyclonal antibodies against peroxisome proliferator-activated receptor gamma coactivator 1 -α (PGC 1 -α), PGC 1 -β, mitochondrial DNA transcription factor A (TFAM), and mouse monoclonal antibodies against nuclear respiratory factor-1 (NRF-1) and NRF-2 were purchased from Abcam Inc. (Cambridge, CA, USA).

Statistical analysis

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

Results

LYRM1 over-expression-induced reductions in insulin-stimulated glucose uptake and GLUT4 translocation are reversed by metformin

Studies have demonstrated that insulin stimulates glucose transport into adipocytes, by inducing translocation of the insulin-responsive glucose transport protein GLUT4 from intracellular storage to the PM.[19] LYRM1 and empty vector stably transfected 3T3-L1 preadipocytes were induced to differentiate, then treated with or without metformin (1 mmol/L and 10 mmol/L). Over-expression of LYRM1 led to insulin resistance, as indicated by decreased insulin-stimulated glucose uptake (Figure 1), and downregulated GLUT4 translocation to the PM in response to insulin (Figure 2). However, basal glucose uptake (Figure 1) and the total GLUT4 protein content (Figure 2) did not significantly alter in LYRM1-over-expressing adipocytes relative to empty vector control cells. Pretreatment of LYRM1-over-expressing adipocytes with 1 mmol/L or 10 mmol/L metformin strikingly enhanced baseline and insulin-stimulated glucose uptake (Figure 1), and 10 mmol/L metformin significantly increased GLUT4 translocation to the PM compared to untreated LYRM1-over-expressing adipocytes (Figure 2). It is noteworthy that metformin protected against the impairments in glucose uptake and GLUT4 translocation to the PM induced by LYRM1.

Effects of metformin on insulin-stimulated phosphorylation of insulin-signaling molecules in LYRM1-over-expressing adipocytes

Our previous study showed that over-expression of LYRM1 inhibits glucose transport in rat skeletal muscles via attenuating the phosphorylation of PI3K and Akt.[20] Metformin is known to increase glucose uptake into human ovarian granulosa cells by activating the PI3K/Akt pathway[8]; however, metformin increases glucose uptake in human adipocytes by activating the AMPKα pathway.[9] Next, we investigated the protein expression levels and phosphorylation of IRS-1 and Akt in differentiated LYRM1 and empty vector stably transfected adipocytes, either when untreated or treated with metformin. As shown in Figure 3, over-expression of LYRM1 considerably downregulated the insulin-stimulated phosphorylation of IRS-1 and Akt, whereas pretreatment with 10 mM metformin strongly activated IRS-1 and Akt phosphorylation in LYRM1-over-expressing adipocytes. Metformin had no significant effects on the total protein contents of these signaling molecules.

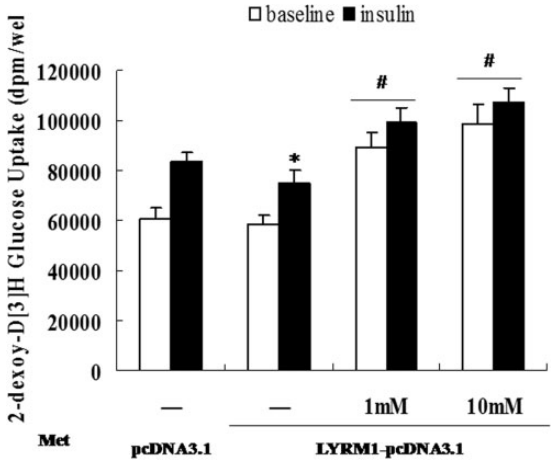

Figure 1 Effect of metformin (Met) on glucose uptake in LYRM1-over-expressing adipocytes. 3T3-L1 preadipocytes stably transfected with an empty expression vector (pcDNA3.1) or LYRM1 expression vector (LYRM1-pcDNA3.1) were induced to differentiate as described in Section ''Cell culture and treatment.'' Ten days after differentiation, the mature adipocytes were incubated in serum-free DMEM overnight and then stimulated with or without metformin (1 or 10 mM) for 24 h. Uptake of 2-deoxy-*D*-[3H] glucose was measured as described in Section ''Glucose uptake.'' Values shown are the mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.01$ vs. pcDNA3.1 adipocytes; $^{\#}P < 0.01$ vs. insulin-stimulated LYRM1-pcDNA3.1 adipocytes

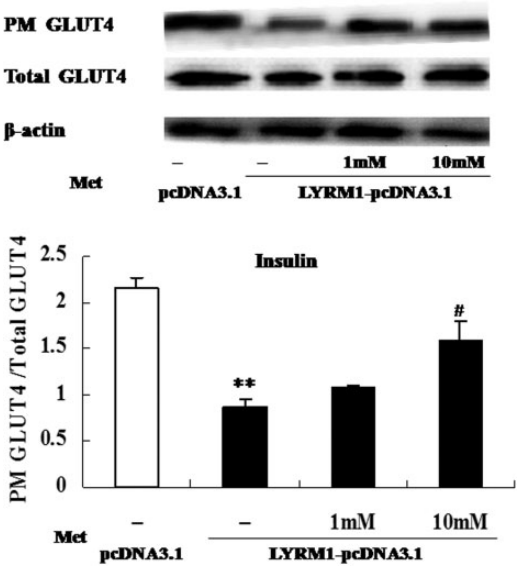

Figure 2 Effect of metformin (Met) on GLUT4 translocation in LYRM1-over-expressing adipocytes. 3T3-L1 preadipocytes stably transfected with an empty expression vector (pcDNA3.1) or a LYRM1 expression vector (LYRM1-pcDNA3.1) were induced to differentiate as described in Section ''Cell culture and treatment.'' Ten days after the induction of differentiation, the mature adipocytes were incubated in serum-free DMEM overnight and then stimulated with or without metformin (1 or 10 mM) for 24 h. Membrane and total proteins were extracted from the differentiated cells after incubation with 100 nM insulin for 30 min. Western blotting was performed using antibodies against GLUT4; quantification of protein expression was based on grayscale analysis. Values shown are the mean ± SD of three independent experiments performed in triplicate. $^{**}P < 0.01$ vs. pcDNA3.1 adipocytes; $^{\#}P < 0.01$ vs. insulin-stimulated LYRM1-pcDNA3.1 adipocytes

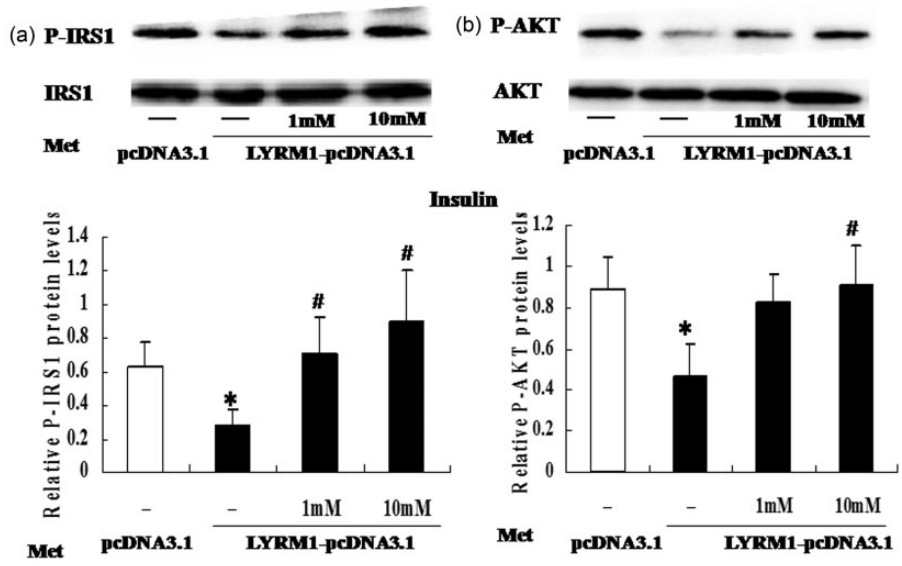

Figure 3 Effect of metformin (Met) on IRS-1 and Akt phosphorylation in LYRM1-over-expressing adipocytes. 3T3-L1 preadipocytes stably transfected with an empty expression vector (pcDNA3.1) or a LYRM1 expression vector (LYRM1-pcDNA3.1) were induced to differentiate as described in Section ''Cell culture and treatment.'' Ten days after the induction of differentiation, the mature adipocytes were incubated in serum-free DMEM overnight and then stimulated with or without metformin (1 or 10 mM) for 24 h. Total and phosphorylated proteins were extracted from differentiated cells after incubation with 100 nmol/L insulin for 30 min, and Western blotting was performed; quantification of protein expression was based on grayscale analysis. Values shown are the mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.01$ vs. pcDNA3.1 adipocytes; $^{\#}P < 0.01$ vs. insulin-stimulated LYRM1-pcDNA3.1 adipocytes

Increased ROS levels due to LYRM1 over-expression are prevented by metformin

Endogenous ROS are mainly generated in the mitochondria. Importantly, a causative role for ROS in mitochondrial dysfunction and obesity-related insulin resistance has been suggested.[4,5,21] Consistent with our previous study,[12] the levels of ROS in LYRM1-over-expressing adipocytes were significantly increased relative to the empty vector control cells; however, treatment with metformin (1 nmol/L or 10 nmol/L) for 24 h significantly decreased the levels of ROS in LYRM1-over-expressing adipocytes (Figure 4). These results indicate that metformin reduces the oxidative stress induced by over-expression of LYRM1 by inhibiting the generation of ROS.

LYRM1-induced defects in mitochondrial biogenesis are reversed by metformin

Our previous studies revealed that over-expression of LYRM1 induced mitochondrial impairment in mature adipocytes and skeletal myotubes.[12,20] PGC1-α, PGC1-β, NRF1, NRF2, and TFAM can be measured as major sensors and regulators of mitochondrial oxidative phosphorylation (OXPHOS) function and/or mitochondrial biogenesis.[22–24] Furthermore, there is considerable circumstantial evidence to suggest that metformin directly normalizes intracellular ROS generation,[10,25] and promotes mitochondrial biogenesis, at least in part by activating the AMPK-PGC1/NRF pathway.[10,26,27] Figure 5 demonstrates the effects of metformin on the expression of mitochondrial biogenesis regulators in LYRM1-over-expressing cells. PGC1-α and PGC1-β protein expressions were markedly upregulated in LYRM1-over-expressing adipocytes compared to empty vector control cells. Conversely, NRF1 and NRF2 protein expressions were downregulated in LYRM1-over-expressing adipocytes compared to empty vector control cells, whereas the levels of TFAM protein expression decreased slightly. Treatment with metformin decreased the protein expressions of PGC1-α, PGC1-β, and increased the protein expressions of NRF1, NRF2, and TFAM in LYRM1-over-expressing adipocytes.

Discussion

We previously reported that over-expression of LYRM1 induced mitochondrial defects in adipocytes, which may be responsible for the development of LYRM1-mediated insulin resistance.[7,12] Metformin is a widely prescribed drug for the treatment of T2D which improves glycemic control, mainly through the inhibition of hepatic glucose output and stimulation of glucose disposal in the target tissues.[8,14,28] Despite the fact that it is routinely prescribed, metformin has also been reported to reduce oxidative stress via exerting antioxidant activity and promote mitochondrial biogenesis via activation of the AMPK-PGC-1/NRFs pathway.[10,25,26,29] On the basis of these observations, we investigated the effects of metformin on indicators of oxidative stress and mitochondrial biogenesis (intracellular ROS, PGC1-α, PGC1-β, NRF-1, NRF-2 and TFAM) in LYRM1-over-expressing adipocytes.

Uncontrolled elevated levels of ROS can potentially result in oxidative damage within the mitochondria and compromise mitochondrial function. ROS and mitochondrial dysfunction have been implicated in the etiology of insulin resistance.[4,5,21] In addition, pharmacological agents that can reduce ROS generation and/or promote mitochondrial biogenesis may have valid beneficial effects against insulin resistance and its complications.[6,7,9,22] PGC-1 is a major regulator of energy metabolism and mitochondrial

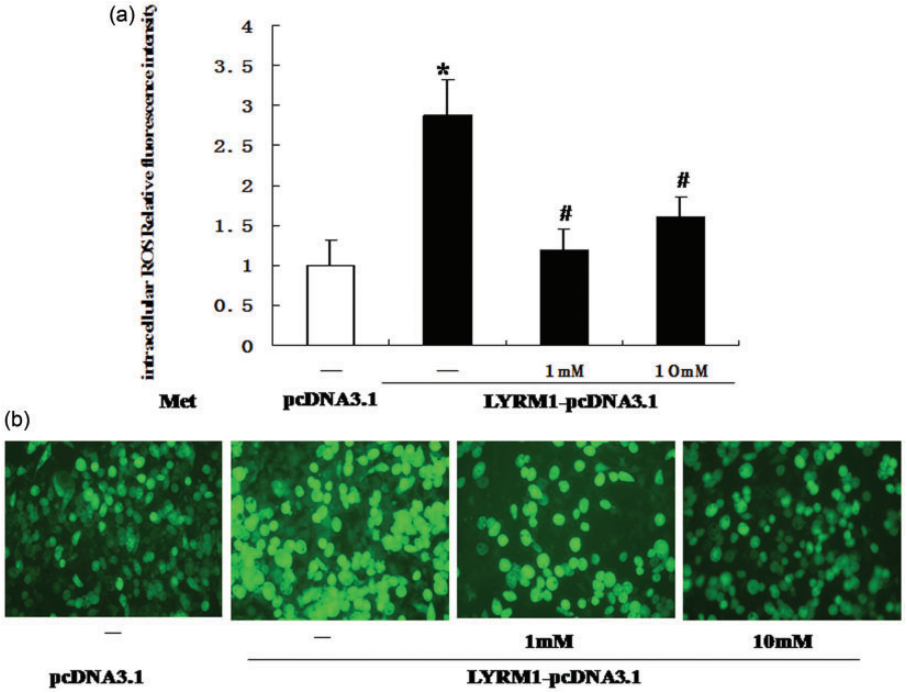

Figure 4 Effect of metformin (Met) on the ROS levels in LYRM1-over-expressing adipocytes. 3T3-L1 preadipocytes stably transfected with an empty expression vector (pcDNA3.1) or a LYRM1 expression vector (LYRM1-pcDNA3.1) were induced to differentiate as described in Section ''Cell culture and treatment.'' Ten days after the induction of differentiation, the mature adipocytes were incubated in serum-free DMEM overnight and then stimulated with or without metformin (1 or 10 mM) for 24 h. ROS levels were determined by detection of the probe H2-DCFDA using a FACScan flow cytometer (a), or a confocal laser scanning microscope at ×400 magnification (b). Values shown are the mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.01$ vs. pcDNA3.1 adipocytes; $^{\#}P < 0.01$ vs. insulin-stimulated LYRM1-pcDNA3.1 adipocytes. (A color version of this figure is available in the online journal.)

biogenesis, which binds to and co-activates the transcriptional activity of the NRFs on the promoter for the mitochondrial transcription factor A (*TFAM*).[10,22,27,30,31] TFAM is a direct regulator of mitochondrial DNA (mtDNA) transcription and replication.[31] Contrary to what others have shown in this field, in that metformin normalized the levels of mtROS through activation of the PGC-1/NRFs axis pathway,[10,27,30] we found that the intracellular levels of ROS, PGC-1α, and PGC-1β expressions were markedly increased in LYRM1-over-expressing adipocytes, and were all decreased by metformin. PGC-1α over-expression caused hepatic insulin resistance, manifested by higher glucose production and diminished insulin suppression of gluconeogenesis; meanwhile, PGC-1α over-expression improved muscle insulin sensitivity, as evidenced by elevated insulin-stimulated Akt phosphorylation and peripheral glucose disposal.[32] Importantly, high levels of PGC-1 expression may actually reduce glucose oxidation and lead to the accumulation incomplete oxidation products, which may further decrease insulin sensitivity.[24,33,34] Mitochondrial function is impaired in LYRM1-over-expressing adipocytes[12]; therefore, we speculate that PGC-1 expression is probably upregulated as a transient compensatory response to increased ROS levels, and potentially decreased by metformin due to the ability of metformin to modulate ROS levels. The PGC-1-related co-activators, the NRFs, bind specifically to the *TFAM* promoter and are critical for the regulation of mitochondrial oxidative activity and biogenesis.[22,31] In addition, metformin is reported to reduce mtROS by induction of manganese superoxide dismutase (MnSOD) and promote mitochondrial biogenesis via activating the AMPK-PGC-1/NRFs pathway.[10,26] Another important finding of this study is that LYRM1 significantly reduced the expression of the NRFs and moderately reduced TFAM expression in adipocytes; however, metformin increased expression of the NRFs and TFAM in adipocytes. This result confirms that LYRM1 induces mitochondrial defects, and that increased levels of ROS might be a unifying mechanism that promotes mitochondrial alterations, which can be reversed by metformin. Although these considerable observations cannot be readily explained, one may conceive that the protective effect of metformin could be mediated, at least in part, via the recently reported antioxidant features of this insulin-sensitive drug,[6,25,27,30] as well as its ability to activate mitochondrial biogenesis.[10,26,27,30] Based on our previous findings that LYRM1 plays an important role in the generation of mitochondrial defects and the pathogenesis of obesity-related insulin resistance by blocking the IRS1/PI3K/Akt insulin axis pathway,[7,20] we investigated whether therapeutic concentrations of metformin (1 and 10 mmol/L)[25,26] could reduce insulin resistance by improving mitochondrial function.

In 2007–2008, a large population-based study among adults 20 years of age or older revealed that the prevalence of diabetes and prediabetes in China was 9.7% and 15.5%, respectively.[35] Although the precise factors which lead to insulin resistance have not yet been elucidated, there is

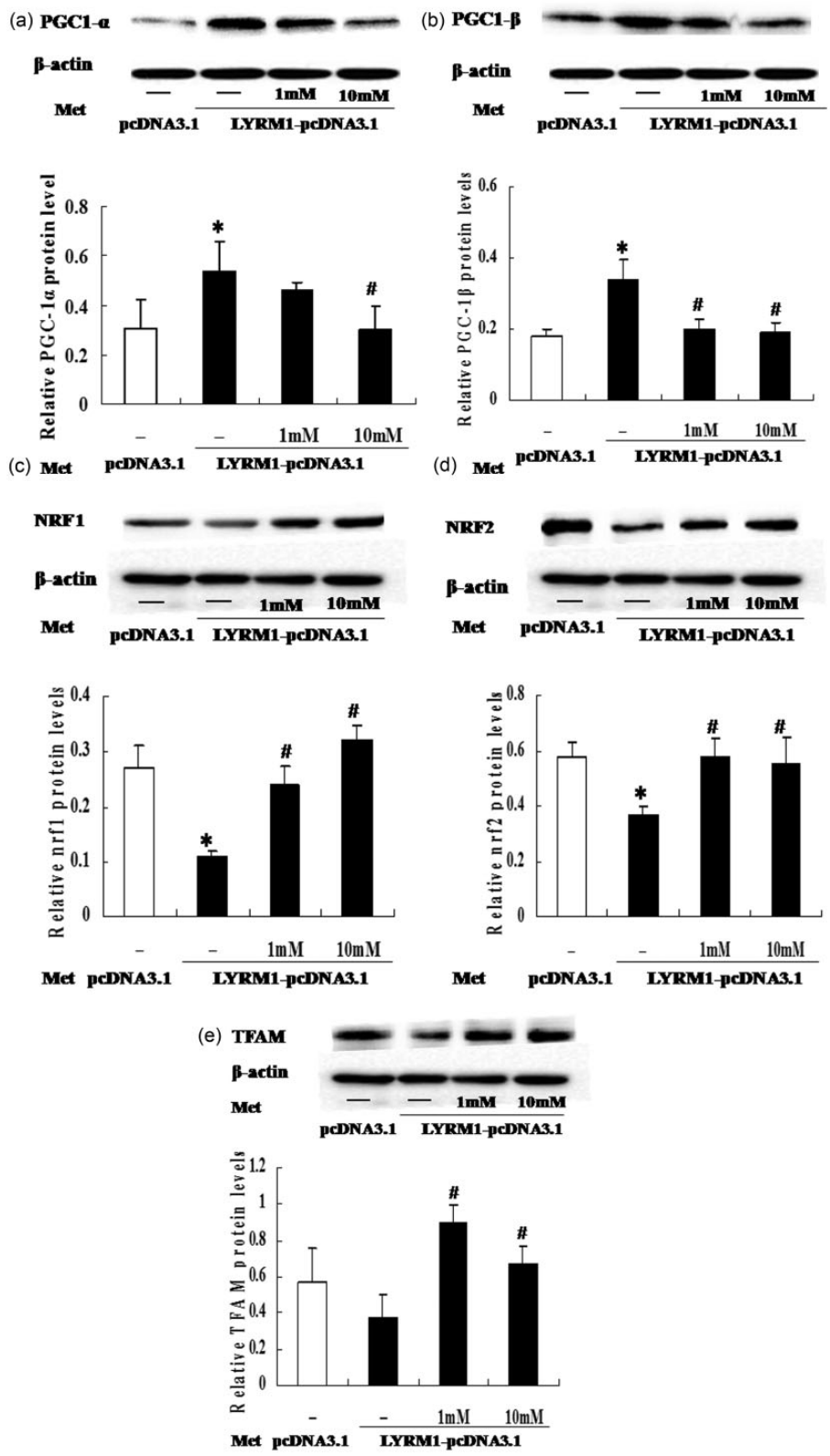

Figure 5 Effects of metformin (Met) on the protein expression levels of PGC1-α, PGC1-β, NRF1, NRF2, and TFAM in LYRM1-over-expressing adipocytes. The mature adipocytes were incubated in serum-free DMEM overnight and then stimulated with or without metformin (1 or 10 mM) for 24 h. The protein expression levels of PGC1-α (a), PGC1-β (b), NRF1 (c), NRF2 (d), and TFAM (e) were analyzed by Western blotting and normalized to the levels of β-actin; quantification of protein expression was based on grayscale analysis. Values shown are the mean ± SD of three independent experiments performed in triplicate. $^{*}P < 0.01$ vs. pcDNA3.1 adipocytes; $^{\#}P < 0.01$ vs. insulin-stimulated LYRM1-pcDNA3.1 adipocytes

growing evidence to indicate a causative link between excessive ROS generation and mitochondrial dysfunction in obesity-related insulin resistance.[4,5,21] Insulin-stimulated glucose uptake is mediated by the translocation of GLUT4 from intracellular vesicles to the PM of adipocytes.[19] The insulin-signaling axis pathway is a complex network, and the PI3K/Akt pathway is essential for insulin-dependent translocation of GLUT4 from the intracellular pool to the PM.[20,36] Hence, multiple defects at many potential levels of insulin signal transduction have been indicated as a mechanism underlying insulin resistance.[5,37] In parallel with our previous results,[7,20] over-expression of LYRM1 reduced insulin-stimulated glucose uptake by decreasing GLUT4 translocation to the PM, which could be reversed by metformin (10 mM) pretreatment. Another potentially interesting finding of this study was that 10 mM metformin considerably increased the insulin-stimulated tyrosine phosphorylation of IRS1 and serine phosphorylation of Akt, which were markedly decreased in LYRM1-over-expressing 3T3-L1 adipocytes. This observation suggests that metformin may directly or indirectly interact with multiple levels of the insulin-mediated signaling cascade in various cells.[8,38,39] In line with these previous studies, we observed that pharmacological concentrations of metformin could reverse both the reduced activation of insulin-signaling molecules and GLUT4 translocation observed in LYRM1-over-expressing adipocytes. However, other studies have shown that metformin had no effect on the insulin-mediated signaling cascaded in human adipocytes of type 2 diabetic and in fish **s**keletal muscle cells.[9,28,29] In the present study, it was difficult to determine if metformin exerted direct actions on the IRS1/PI3K/Akt-signaling transduction pathway in LYRM1-over-expressing 3T3-L1 adipocytes; further study is required to elucidate if metformin directly affects the IRS1/PI3K/Akt-signaling transduction pathway in LYRM1-over-expressing 3T3-L1 adipocytes. However, it should be noted that the changes in the phosphorylation of IRS1 and Akt associated with metformin pretreatment were accompanied by alterations in the levels of ROS and mitochondrial biogenesis indicators, all of which are affected by LYRM1.

Consequentially, increased levels of ROS impair mitochondrial function; yet mitochondrial dysfunction in turn produced excessive levels of ROS and lipid byproducts, producing a vicious positive feed-back loop.[5,21] Additionally, ROS directly impair insulin-signaling cascades via activating various serine kinases that phosphorylate IRS or enhance the degradation of IRS and Akt, which ultimately contributes to insulin resistance.[4–6,40] In addition, antioxidant supplements (metformin, alpha-lipoic acid, N-acetyl-*L*-cysteine) or mitochondrial biogenesis stimulators (metformin, thiazolidinediones) may enhance insulin sensitivity, at least in part, by improving mitochondrial function.[4,7,15,21,26,28] In this study, the primary markers of mitochondrial biogenesis were improved by metformin. Our findings suggest that LYRM1-induced insulin resistance is partially due to the harmful effects of excessive ROS to the mitochondria in adipocytes.

Overall, this study indicates that LYRM1-induced mitochondrial dysfunction may be involved in the development of insulin resistance in adipocytes. Additionally, metformin may reverse LYRM1-induced insulin resistance by directly activating the AMPK-PGC1/NRF axis pathway or indirectly ameliorating the IRS1/Akt-signaling cascade.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. X-GC and X-RG designed the study and wrote the paper; Z-YQ, MZ, Y-mD, Y-MW, G-zZ performed the research; Y-PZ, C-BJ, JQ analyzed the data. Z-YQ and MZ contributed equally to the work.

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REFERENCES

1. Bournat JC, Brown CW. Mitochondrial dysfunction in obesity. *Curr Opin Endocrinol Diabetes Obes* 2010;**17**:446–52
2. Lu H, Koshkin V, Allister EM, Gyulkhandanyan AV, Wheeler MB. Molecular and metabolic evidence for mitochondrial defects associated with beta-cell dysfunction in a mouse model of type 2 diabetes. *Diabetes* 2010;**59**:448–59
3. Kim JA, Wei Y, Sowers JR. Role of mitochondrial dysfunction in insulin resistance. *Circ Res* 2008;**102**:401–14
4. Wang X, Gu C, He W, Ye X, Chen H, Zhang X, Hai C. Glucose oxidase induces insulin resistance via influencing multiple targets in vitro and in vivo: the central role of oxidative stress. *Biochimie* 2012;**94**:1705–17
5. Bashan N, Kovsan J, Kachko I, Ovadia H, Rudich A. Positive and negative regulation of insulin signaling by reactive oxygen and nitrogen species. *Physiol Rev* 2009;**89**:27–71
6. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 2006;**440**:944–8
7. Qin ZY, Zhang M, Guo XR, Wang YM, Zhu GZ, Ni YH, Zhao YP, Qiu J, Kou CZ, Qin R, Cao XG. α-Lipoic acid ameliorates impaired glucose uptake in LYRM1 overexpressing 3T3-L1 adipocytes through the IRS-1/Akt signaling pathway. *J Bioenerg Biomembr* 2012;**44**:579–86
8. Rice S, Pellatt LJ, Bryan SJ, Whitehead SA, Mason HD. Action of metformin on the insulin-signaling pathway and on glucose transport in human granulosa cells. *J Clin Endocrinol Metab* 2011;**96**:E427–35
9. Grisouard J, Timper K, Radimerski TM, Frey DM, Peterli R, Kola B, Korbonits M, Herrmann P, Krahenbuhl S, Zulewski H, Keller U, Muller B, Christ-Crain M. Mechanisms of metformin action on glucose transport and metabolism in human adipocytes. *Biochem Pharmacol* 2010;**80**: 1736–45
10. Kukidome D, Nishikawa T, Sonoda K, Imoto K, Fujisawa K, Yano M, Motoshima H, Taguchi T, Matsumura T, Araki E. Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes* 2006;**55**:120–7
11. Qiu J, Ni YH, Gong HX, Fei L, Pan XQ, Guo M, Chen RH, Guo XR. Identification of differentially expressed genes in omental adipose tissues of obese patients by suppression subtractive hybridization. *Biochem Biophys Res Commun* 2007;**352**:469–78

12. Cao XG, Kou CZ, Zhao YP, Gao CL, Zhu C, Zhang CM, Ji CB, Qin DN, Zhang M, Guo XR. Overexpression of LYRM1 induces mitochondrial impairment in 3T3-L1 adipocytes. *Mol Genet Metab* 2010;**101**:395–9
13. Bailey CJ, Turner RC. Metformin. *N Engl J Med* 1996;**334**:574–9
14. Hundal RS, Krssak M, Dufour S, Laurent D, Lebon V, Chandramouli V, Inzucchi SE, Schumann WC, Petersen KF, Landau BR, Shulman GI. Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* 2000;**49**:2063–9
15. Fujisawa K, Nishikawa T, Kukidome D, Imoto K, Yamashiro T, Motoshima H, Matsumura T, Araki E. TZDs reduce mitochondrial ROS production and enhance mitochondrial biogenesis. *Biochem Biophys Res Commun* 2009;**379**: 43–8
16. Qiu J, Gao CL, Zhang M, Chen RH, Chi X, Liu F, Zhang CM, Ji CB, Chen XH, Zhao YP, Li XN, Tong ML, Ni YH, Guo XR. LYRM1, a novel gene promotes proliferation and inhibits apoptosis of preadipocytes. *Eur J Endocrinol* 2009;**160**:177–84
17. Maxwell DP, Wang Y, McIntosh L. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *Proc Natl Acad Sci U S A* 1999;**96**:8271–6
18. Ceddia RB, Somwar R, Maida A, Fang X, Bikopoulos G, Sweeney G. Globular adiponectin increases GLUT4 translocation and glucose uptake but reduces glycogen synthesis in rat skeletal muscle cells. *Diabetologia* 2005;**48**:132–9
19. Kanzaki M. Insulin receptor signals regulating GLUT4 translocation and actin dynamics. *Endocr J* 2006;**53**:267–93
20. Kou C, Cao X, Qin D, Ji C, Zhu J, Zhang C, Zhu C, Gao C, Chen R, Guo X, Zhang M. Over-expression of LYRM1 inhibits glucose transport in rat skeletal muscles via attenuated phosphorylation of PI3K (p85) and Akt. *Mol Cell Biochem* 2011;**348**:149–54
21. Bonnard C, Durand A, Peyrol S, Chanseaume E, Chauvin MA, Morio B, Vidal H, Rieusset J. Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J Clin Invest* 2008;**118**:789–800
22. Scarpulla RC. Metabolic control of mitochondrial biogenesis through the PGC-1 family regulatory network. *Biochim Biophys Acta* 2011;**1813**:1269–78
23. Arduini A, Serviddio G, Escobar J, Tormos AM, Bellanti F, Vina J, Monsalve M, Sastre J. Mitochondrial biogenesis fails in secondary biliary cirrhosis in rats leading to mitochondrial DNA depletion and deletions. *Am J Physiol Gastrointest Liver Physiol* 2011;**301**:G119–27
24. Patti ME, Corvera S. The role of mitochondria in the pathogenesis of type 2 diabetes. *Endocr Rev* 2010;**31**:364–95
25. Kane DA, Anderson EJ, Price JW III, Woodlief TL, Lin CT, Bikman BT, Cortright RN, Neufer PD. Metformin selectively attenuates mitochondrial H2O2 emission without affecting respiratory capacity in skeletal muscle of obese rats. *Free Radic Biol Med* 2010;**49**:1082–7
26. Morales AI, Detaille D, Prieto M, Puente A, Briones E, Arevalo M, Leverve X, Lopez-Novoa JM, El-Mir MY. Metformin prevents experimental gentamicin-induced nephropathy by a mitochondria-dependent pathway. *Kidney Int* 2010;**77**:861–9
27. Suwa M, Egashira T, Nakano H, Sasaki H, Kumagai S. Metformin increases the PGC-1alpha protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo. *J Appl Physiol* 2006;**101**:1685–92
28. Ciaraldi TP, Kong AP, Chu NV, Kim DD, Baxi S, Loviscach M, Plodkowski R, Reitz R, Caulfield M, Mudaliar S, Henry RR. Regulation of glucose transport and insulin signaling by troglitazone or metformin in adipose tissue of type 2 diabetic subjects. *Diabetes* 2002;**51**:30–6
29. Magnoni LJ, Vraskou Y, Palstra AP, Planas JV. AMP-activated protein kinase plays an important evolutionary conserved role in the regulation of glucose metabolism in fish skeletal muscle cells. *PLoS One* 2012;**7**:e31219
30. Benton CR, Nickerson JG, Lally J, Han XX, Holloway GP, Glatz JF, Luiken JJ, Graham TE, Heikkila JJ, Bonen A. Modest PGC-1alpha overexpression in muscle in vivo is sufficient to increase insulin sensitivity and palmitate oxidation in subsarcolemmal, not intermyofibrillar, mitochondria. *J Biol Chem* 2008;**283**:4228–40
31. Ikeuchi M, Matsusaka H, Kang D, Matsushima S, Ide T, Kubota T, Fujiwara T, Hamasaki N, Takeshita A, Sunagawa K, Tsutsui H. Overexpression of mitochondrial transcription factor a ameliorates mitochondrial deficiencies and cardiac failure after myocardial infarction. *Circulation* 2005;**112**:683–90
32. Liang H, Balas B, Tantiwong P, Dube J, Goodpaster BH, O'Doherty RM, DeFronzo RA, Richardson A, Musi N, Ward WF. Whole body overexpression of PGC-1alpha has opposite effects on hepatic and muscle insulin sensitivity. *Am J Physiol Endocrinol Metab* 2009;**296**:E945–54
33. Choi CS, Befroy DE, Codella R, Kim S, Reznick RM, Hwang YJ, Liu ZX, Lee HY, Distefano A, Samuel VT, Zhang D, Cline GW, Handschin C, Lin J, Petersen KF, Spiegelman BM, Shulman GI. Paradoxical effects of increased expression of PGC-1alpha on muscle mitochondrial function and insulin-stimulated muscle glucose metabolism. *Proc Natl Acad Sci U S A* 2008;**105**:19926–31
34. Miura S, Kai Y, Ono M, Ezaki O. Overexpression of peroxisome proliferator-activated receptor gamma coactivator-1alpha down-regulates GLUT4 mRNA in skeletal muscles. *J Biol Chem* 2003;**278**:31385–90
35. Yang W, Lu J, Weng J, Jia W, Ji L, Xiao J, Shan Z, Liu J, Tian H, Ji Q, Zhu D, Ge J, Lin L, Chen L, Guo X, Zhao Z, Li Q, Zhou Z, Shan G, He J, China National D, Metabolic Disorders Study G. Prevalence of diabetes among men and women in China. *N Engl J Med* 2010;**362**:1090–101
36. Giovannone B, Scaldaferri ML, Federici M, Porzio O, Lauro D, Fusco A, Sbraccia P, Borboni P, Lauro R, Sesti G. Insulin receptor substrate (IRS) transduction system: distinct and overlapping signaling potential. *Diabetes Metab Res Rev* 2000;**16**:434–41
37. Shibata M, Hakuno F, Yamanaka D, Okajima H, Fukushima T, Hasegawa T, Ogata T, Toyoshima Y, Chida K, Kimura K, Sakoda H, Takenaka A, Asano T, Takahashi S. Paraquat-induced oxidative stress represses phosphatidylinositol 3-kinase activities leading to impaired glucose uptake in 3T3-L1 adipocytes. *J Biol Chem* 2010;**285**:20915–25
38. Kumar N, Dey CS. Metformin enhances insulin signalling in insulin-dependent and-independent pathways in insulin resistant muscle cells. *Br J Pharmacol* 2002;**137**:329–36
39. Yuan L, Ziegler R, Hamann A. Metformin modulates insulin postreceptor signaling transduction in chronically insulin-treated Hep G2 cells. *Acta Pharmacol Sin* 2003;**24**:55–60
40. Bloch-Damti A, Potashnik R, Gual P, Le Marchand-Brustel Y, Tanti JF, Rudich A, Bashan N. Differential effects of IRS1 phosphorylated on Ser307 or Ser632 in the induction of insulin resistance by oxidative stress. *Diabetologia* 2006;**49**:2463–73

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