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Retinoid X Receptor α Overexpression Alleviates Mitochondrial Dysfunction-induced Insulin Resistance through Transcriptional Regulation of Insulin Receptor Substrate 1

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Mitochondrial dysfunction is associated with insulin resistance and diabetes. We previously showed that retinoid X receptor α (RXRα) played an important role in transcriptional regulation of oxidative phosphorylation (OXPHOS) genes in cells with mitochondrial dysfunction caused by mitochondrial DNA mutation. In this study, we investigated whether mitochondrial dysfunction induced by incubation with OXPHOS inhibitors affects insulin receptor substrate 1 (IRS1) mRNA and protein levels and whether RXRα activation or overexpression can restore IRS1 expression. Both IRS1 and RXRα protein levels were significantly reduced when C2C12 myotubes were treated with the OXPHOS complex inhibitors, rotenone and antimycin A. The addition of RXRα agonists, 9-cis retinoic acid (9cRA) and LG1506, increased IRS1 transcription and protein levels and restored mitochondrial function, which ultimately improved insulin signaling. RXRα overexpression also increased IRS1 transcription and mitochondrial function. Because RXRα overexpression, knock-down, or activation by LG1506 regulated IRS1 transcription mostly independently of mitochondrial function, it is likely that RXRα directly regulates IRS1 transcription. Consistent with the hypothesis, we showed that RXRα bound to the IRS1 promoter as a heterodimer with peroxisome proliferator-activated receptor δ (PPARδ). These results suggest that RXRα overexpression or activation alleviates insulin resistance by increasing IRS1 expression.

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INTRODUCTION

It is well known that insulin resistance precedes the development of diabetes. There are many reports suggesting that impaired mitochondrial function is associated with insulin resistance and diabetes. Previous studies found that the mitochondrial oxidative capacity for glucose and lipids is reduced in the skeletal muscle of type 2 diabetes patients (Kelley and Simoneau, 1994; Lowell and Shulman, 2005; Simoneau and Kelley, 1997). In addition, mitochondrial content also is reduced in the muscle of obese and diabetes patients (Kelley et al., 2002; Ritov et al., 2005). There are several theories to explain the association between mitochondrial dysfunction and insulin resistance. For example, specific lipid metabolites (e.g., ceramide and diacylglycerol) accumulate when mitochondrial function is impaired, and they activate protein kinase C, which inhibits insulin signaling through serine/threonine phosphorylation of insulin receptor substrate (IRS) (Morino et al., 2005). A recent study reported that reactive oxygen species (ROS) production is increased in the mitochondria of obese and insulin-resistant animal models or humans (Anderson et al., 2009). Although the precise mechanism has not been elucidated, higher ROS levels promote serine/threonine stress kinase activity, which inhibits the insulin signaling pathway (Fisher-Wellman and Neufer, 2012). Even though these observations provide evidence for an association between mitochondrial dysfunction and insulin resistance, whether mitochondrial dysfunction is required for the development of insulin resistance is not known (Martin and McGee, 2014).

IRS is an important regulator of insulin signaling, and its activation is inhibited by phosphorylation at serine residues. Inflammatory cytokines such as tumor necrosis factor α (TNFα), free fatty acids, and oxidative stress promote serine phosphorylation of IRS through activating stress-response kinases such as mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK), ultimately disrupting insulin signaling (Gual et al., 2005). In addition, chronic insulin stimulation down-regulates insulin signaling via mammalian target of rapamycin complex (mTORC)-mediated serine/threonine phosphorylation of IRS which promotes the ubiquitination and degradation of IRS (Egawa et al., 2000; Pederson et al., 2001; Sun et al., 1999). We previously showed that the increase in IRS1 protein

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level mediated by ginsenoid Rg3 increases insulin signaling and glucose uptake in L6 myotubes (Kim et al., 2009). Collectively, these results indicate that both IRS protein level and phosphorylation status regulate insulin signaling.

Retinoid X receptor (RXR) is a member of the nuclear receptor superfamily and regulates the transcription of target genes by forming a heterodimer with various nuclear receptors, including peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs), and thyroid hormone receptors (TRs) (Evans and Mangelsdorf, 2014; Heyman et al., 1992; Kliewer et al., 1992). RXRα expression is reported to be reduced in the high glucose state due to the increase in ROS (Singh et al., 2012). We previously demonstrated that impaired mitochondrial function caused by an mtDNA mutation reduced RXRα by ROS-JNK pathway. Subsequent decrease in the interaction between PGC1α and RXRα resulted in transcriptional repression of oxidative phosphorylation (OXPHOS) enzymes, aggravating mitochondrial dysfunction (Chae et al., 2013). Based on these results, we hypothesized that the reduction of RXRα protein level in the setting of mitochondrial dysfunction is related to insulin signaling impairment. In this study, we investigated whether RXRα protein levels are related to IRS1 expression and determined if RXRα overexpression or activation can restore IRS1 expression and insulin signaling in the setting of mitochondrial dysfunction.

MATERIALS AND METHODS

Cell culture, adenovirus infection, and siRNA transfection

C2C12 myoblasts were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, USA). Differentiation was induced by incubating with DMEM containing 2% horse serum (Invitrogen) for 4 days. COS7 cells were maintained in DMEM supplemented with 10% FBS. Myotubes were treated with 50 multiplicity of infection (MOI) Ad-GFP or Ad-RXRα for 24 h and then incubated with fresh media for 48 h. Myotubes were treated with 9-cis retinoic acid (9cRA, Sigma, USA) or LG101506 (LG1506, Sigma). Myotubes were transfected with nonspecific siRNAs (siNS; Bioneer, Korea) or siRNAs of RXRα (siRXRα; Dharmacon, USA) using RNAiMAX (Invitrogen).

Plasmids, adenovirus, and antibodies

Three DNA fragments from -1845 bp to -875 bp, -155 bp to -875 bp, and -998 bp to -875 bp of the mouse IRS1 gene (+1 indicates translational start site) were inserted upstream of the luciferase gene in the pGL2-Basic (Promega, USA), and named IRS1(-1845)-Luc, IRS1 (-1155)-Luc and IRS1 (-998)-Luc, respectively. Expression vectors were constructed by inserting the cDNAs of RXRα, PPARα, PPARδ and PPARγ into pcDNA 3.1 (Promega). To generate the adenovirus overexpressing human RXRα, RXRα cDNA was inserted into the pAD-Track-CMV vector followed by recombination with a pAdEasy vector (Promega). Ad-RXRα was generated by transfecting the recombinant adenoviral DNA into human embryonic kidney (H293) cells. Antibodies against the proteins were used for western blot analyses and chromatin immunoprecipitation (ChIP): IRS1, pY612IRS1, RXRα PPARδ and RNA Polymerase II (RNA pol II) (Santa Cruz Biotechnology, USA); AKT and pS473AKT (Cell Signaling, USA); and γ-tubulin (Sigma).

ATP measurement

Cellular ATP levels were measured using ATPlite luminescence ATP detection assay system (PerkinElmer, USA). Briefly, C2C12 myotubes were lysed in cell lysis solution and mixed with substrate solution before luminescence was measured.

Western blot analysis

C2C12 myotubes were lysed in 20 mM Tris-HCl (pH 7.4), 5 mM $Na_4P_2O_7$, 100 nM NaF, 2 mM Na_3VO_4, and 1% NP-40 buffer supplemented with protease inhibitors (1 μg/μl aprotinin, 1 μg/μl leupeptin, and 1 mM PMSF). About 20 μg of proteins per lane were separated by SDS-PAGE and then transferred onto nitrocellulose membrane (Whatman, Germany). The bands were visualized by Enhanced Chemi-Luminescence (Pierce).

RNA preparation and real-time PCR

Total RNAs from the C2C12 myotubes were isolated by using TRIzol (Invitrogen). Real-time polymerase chain reaction (PCR) was performed using SYBR-master mix (Takara, Japan) and an ABI 7500 real-time PCR system (Applied Biosystems, USA). Each sample was analyzed in duplicate. The primers used for amplification were shown: for IRS1, 5′ - GTT GGG CAG AAT AGG CCC TG -3′ (forward) and 5′- AAT GCC TGT CCG CAT GTC AG -3′ (reverse); for RXRα, 5′- ACT GGT AGC CCC CAG CTC AA -3′ (forward) and 5′- GAG CGG TCC CCA CAG ATA GC -3′ (reverse); for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′ - AGG TCG GTG TGA ACG GAT TTG -3′ (forward) and 5′- TGT AGA CCA TGT AGT TGA GGT CA -3′ (reverse).

Transient transfection and luciferase reporter assays

COS7 cells were transfected with 0.3 μg of reporter vectors and expression vectors using Lipofectamine with Plus Reagent (Invitrogen). An expression vector (0.1 μg) for β-galactosidase was also transfected. pcDNA was added to transfect the same amount of DNA in each well. Cells were harvested 24 h after transfection, and luciferase and β -galactosidase activities were measured.

ChIP coupled with quantitative PCR

C2C12 myotubes were infected with Ad-GFP or Ad-RXRα for 2 days and then treated with rotenone for an additional 24 h. After cross-linking and DNA fragmentation, nuclear extracts were subjected to immunoprecipitation with antibodies against RXRα, PPARδ, RNA pol II, and control IgG. The primers used for amplification of the RXRα promoter region between -998 bp and -875 bp were: 5′- AAT GGT AGC GAG CAG GGA G -3′ (forward) and 5′ -CAC TCC AGA GGA GCA AAG CA- 3′ (reverse).

Statistical analysis

Statistical analyses were performed using SPSS version 12.0 (SPSS Inc., USA). Significant differences were assessed using Mann-Whitney U tests. A *P* values below 0.05 were considered statistically significant.

RESULTS

IRS1 and RXRα protein levels are reduced by OXPHOS complex inhibitors

We first determined whether IRS1 and RXRα protein levels were affected by mitochondrial dysfunction. C2C2 myoblasts were differentiated to myotubes, and then treated with the OXPHOS complex inhibitors rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) for 24 h. Both IRS1 and RXRα protein levels were markedly decreased after treatments (Fig. 1A). Real time quantitative PCR was used to determine whether the reduction of IRS1 and RXRα proteins was due to

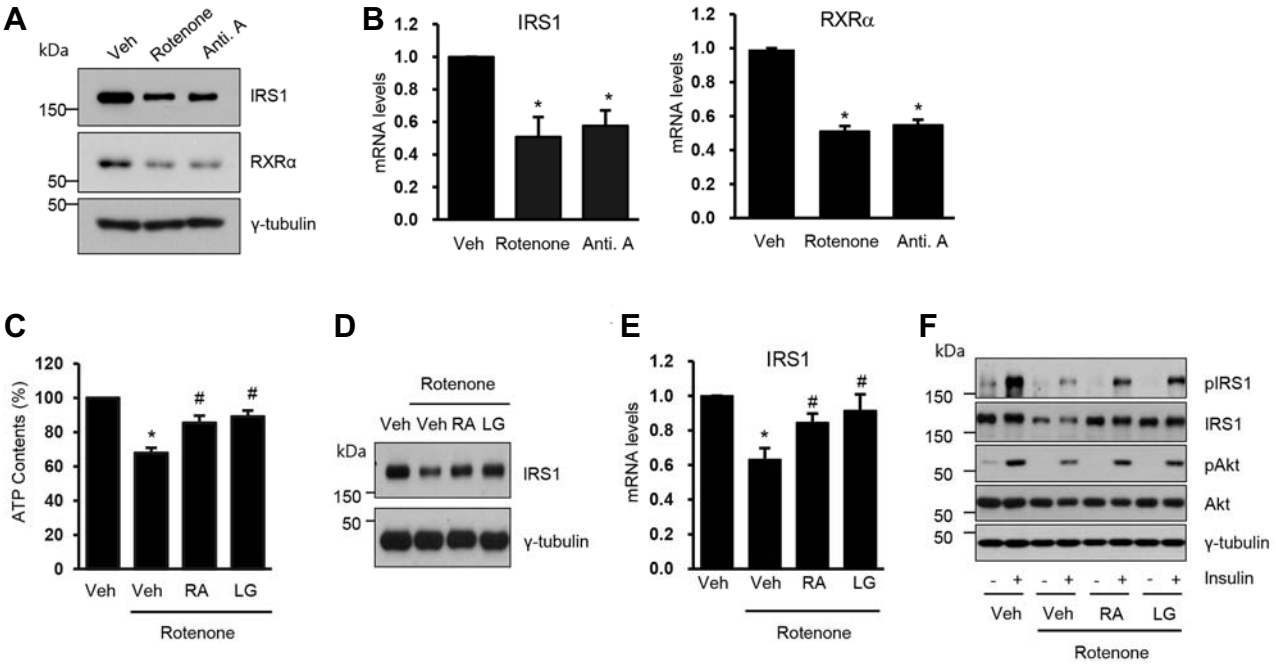

Fig. 1. RXRα agonists restore mitochondrial function and IRS transcription impaired by rotenone. C2C12 myoblasts were differentiated to myotubes and treated with rotenone (3 μM) or antimycin A (10 μM) for 24 h. (A) Cells were lysed and then Western blot analyses were performed with the specific antibodies. (B) Total RNAs were isolated and real time quantitative PCR was performed. The mRNA levels of each gene were normalized by that of GAPDH. The value of Veh was set to 1 and the others were expressed as the relatives to that. The data are the means ± SEM of 3 experiments. *, $P < 0.05$ vs. Veh. (C-F) C2C12 myotubes were treated with rotenone (3 μM) for 24 h and then the media was replaced to the fresh media containing DMSO (Veh), 9cRA (5 μM) or LG1506 (2 μM) for 18 h. (C) Cells were lysed and ATP contents were measured (n = 6). *, $P < 0.05$ vs. Veh; #, $P < 0.05$ vs. rotenone only. (D) Cell lysates were subjected to Western blot analysis. (E) The IRS1 mRNA levels were measured by real time PCR (n = 5). *, $P < 0.05$ vs. Veh; #, $P < 0.05$ vs. rotenone only. (F) Insulin (100 nM) was added 15 min before harvesting. Cell lysates were subjected to the Western blot analysis.

decreased mRNA levels. Both IRS1 and RXRα mRNA levels were significantly reduced following treatment with rotenone or antimycin A (Fig. 1B), suggesting that IRS1 and RXRα transcription are suppressed in the setting of mitochondrial dysfunction.

RXRα agonists reverse the effects of rotenone on IRS1 expression and insulin signaling

Because we previously showed that activation of RXRα by its agonists significantly recovered mitochondrial function in cybrid cells having mutant mitochondrial DNA (Chae et al., 2013), we tested whether RXRα agonist treatment enhances IRS1 expression and restores insulin signaling impaired by mitochondrial dysfunction. C2C12 myotubes were treated with rotenone for 24 h and then treated with two different types of agonists in fresh media for 18 h: 9-cis-retinoic acid (9cRA) and LG101506 (LG1506). ATP levels were increased after the cells were incubated with 9cRA or LG1506, indicating that mitochondria function was partially recovered following treatment with an RXRα agonist (Fig. 1C). IRS1 protein levels, which had been reduced by rotenone treatment, were restored to baseline when 9cRA or LG1506 was added (Fig. 1D). A similar effect was observed with IRS1 mRNA levels (Fig. 1E). In addition to the recovery of IRS1 expression levels, rotenone-induced insulin signaling impairment was significantly alleviated by the addition of either RXRα agonist (Fig. 1F). These results suggest that RXRα activation recovers rotenone-mediated decreases in IRS1 transcription and ATP production and that the quantitative recovery of IRS1 protein is important for normal insulin signaling.

RXRα overexpression restores IRS1 transcription in the presence of rotenone

Next, we tested whether RXRα protein level itself affects rotenone-suppressed IRS1 mRNA or protein levels. When RXRα was overexpressed using an adenoviral system, IRS1 protein levels were also increased in the presence of rotenone (Fig. 2A). Consistent with this finding, IRS1 mRNA levels were also increased following RXRα overexpression (Fig. 2B). In addition, RXRα overexpression prevented rotenone-induced insulin signaling impairment (Fig. 2C). We also assessed the effect of RXRα overexpression on mitochondrial function. ATP contents were significantly recovered by RXRα overexpression in the presence of rotenone (Fig. 2D). These results suggest that RXRα overexpression was sufficient to reverse decreased IRS transcription and mitochondrial dysfunction induced by rotenone.

RXRα directly regulates IRS1 transcription

Although our data clearly showed that RXRα activation or overexpression ameliorated mitochondrial dysfunction and increased IRS1 transcription, it was not clear whether RXRα directly regulates IRS1 transcription or if the recovery of IRS1 transcription is a secondary effect of improved mitochondrial function. We therefore tested whether RXRα affected IRS1 transcription in the myotubes that were not treated with rotenone. RXRα overexpression increased IRS1 mRNA and pro-

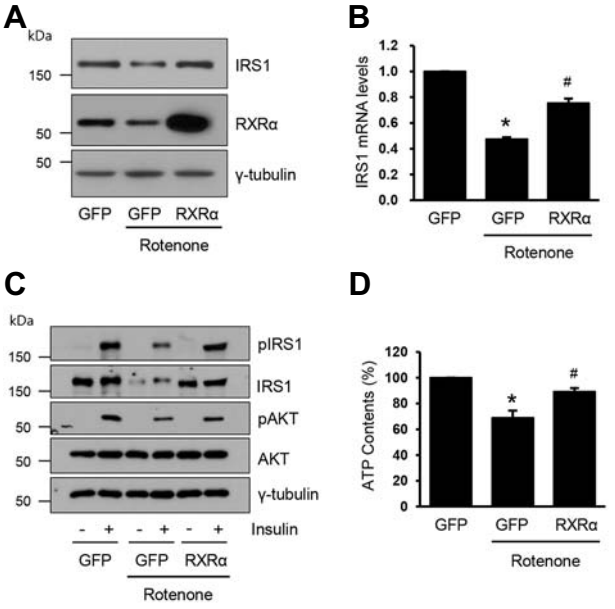

Fig. 2. RXRα overexpression prevents rotenone-induced suppression of IRS1 transcription. C2C12 myotubes were infected with Ad-RXRα (50 MOI) for 48 h and then treated with rotenone for an additional 24 h. (A) Western blot analysis and (B) real-time PCR were performed (n = 6). P < 0.05 vs. Ad-GFP; #, *P* < 0.05 vs. Ad-GFP with rotenone. (C) Insulin was treated for 15 min before harvesting and western blot analysis was performed. (D) Cellular ATP contents were measured (n = 7). *, *P* < 0.05 vs. Ad-GFP; #, *P* < 0.05 vs. Ad-GFP with rotenone.

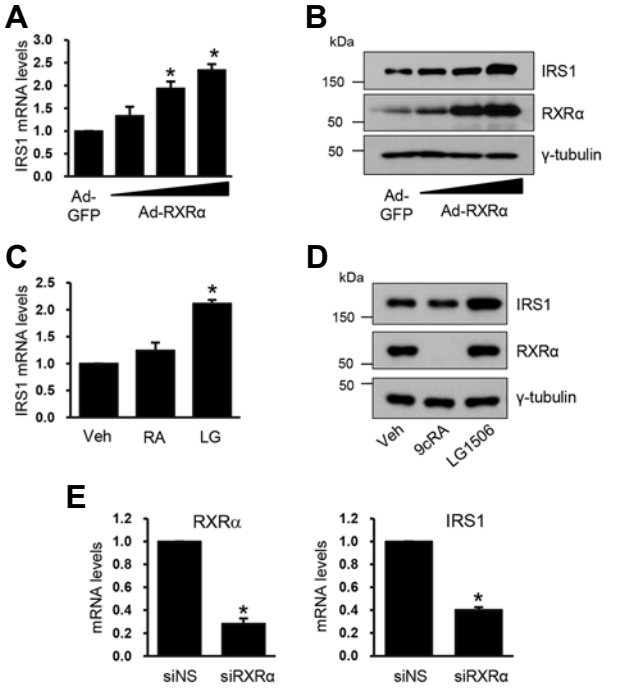

Fig. 3. RXRα directly regulates IRS1 transcription. (A, B) C2C12 myotubes were infected with Ad-RXRα in different dosages (10, 30 and 50 MOI) for 48 h. Cell lysates were subjected to real time PCR (A) and western blot analysis (B). The mRNA levels of control cells (Ad-GFP infected) was set to 1 and the other values were expressed as the relatives to that (n = 4). *, *P* < 0.05 vs. Ad-GFP infected cells. (C, D) C2C12 myotubes were treated with 9cRA (5 μM) or LG1506 (2 μM) for 24 h, and then real time PCR (C) and western blot analysis (D) were performed (n = 5). *, *P* < 0.05 vs. Veh. (E) C2C12 myotubes were transfected with siNS or siRXR (50 nM). Total RNAs were prepared 3 days after siRNA transfection and then real time PCR was performed (n = 3). *, *P* < 0.05 vs. siNS.

tein levels in a dose-dependent manner (Figs. 3A and 3B). Similarly, activation of RXRα by LG1506 treatment also increased IRS1 mRNA and protein levels. However, the RXRα agonist 9cRA did not increase IRS1 mRNA or protein levels (Figs. 3C and 3D). We also examined the effect of RXRα knockdown on IRS1 mRNA levels and found that they were significantly reduced when siRNAs against RXRα were transfected into C2C12 myotubes (Fig. 3E). These results suggest that RXRα is directly involved in IRS1 transcription independently of mitochondrial function, even though 9cRA did not increase IRS1 mRNA levels.

RXRα binds to the IRS1 promoter and augments its activity

To confirm that RXRα directly binds to the *IRS1* promoter and activates the transcription of IRS1, DNA fragments containing three different lengths of 5′-flanking region of the mouse IRS1 gene (from -1845, -1155, or -998 bp to -875 bp) were linked to the luciferase reporter gene and then transfected into COS7 cells with an RXRα expression vector (Fig. 4A). RXRα increased and LG1506 further increased the luciferase activities of the (-1845)-Luc and (-1155)-Luc constructs but not the (-998)-Luc construct (Fig. 4B). This finding suggests that a *cis*-acting element(s) for RXRα is located between -1155 bp and -998 bp in the IRS1 promoter. In contrast, 9cRA did not increase the luciferase activity further (data not shown). Next, we identified the partner of RXRα for binding to the IRS1 promoter region. Based on a report that LG1506 is a specific activator for the RXRα/PPAR heterodimer, we tested whether PPARα, PPARδ, and PPARγ, could increase the luciferase activity of the IRS1 (-1155)-Luc construct. Co-transfection of PPARδ expression vectors further increased luciferase activity, but PPARα and PPARγ vectors did not (Fig. 4C), indicating that the RXRα/PPARδ heterodimer binds to the promoter region of the IRS1 gene. To confirm that RXRα and PPARδ directly bind to the region between -1155 bp and -998 bp, ChIP was performed with RXRα, PPARδ, or RNA Pol II antibodies in C2C12 myotubes, and real-time PCR was performed using primers flanking the region (from -1155 bp to -998 bp). While bindings of RXRα, PPARδ, and RNA Pol II were decreased following rotenone treatment, bindings of these factors to the region were significantly increased when RXRα was overexpressed. These results indicated that IRS1 transcription was augmented by RXRα overexpression (Fig. 4D). Collectively, RXRα forms a heterodimer with PPARδ that directly binds to the *IRS1* promoter region between -1155 bp and -998 bp and regulates IRS1 transcription level.

DISCUSSION

We found that IRS1 and RXRα protein levels were dramatically reduced when mitochondrial dysfunction was induced by OXPHOS complex inhibitors in myotubes. Based on this finding,

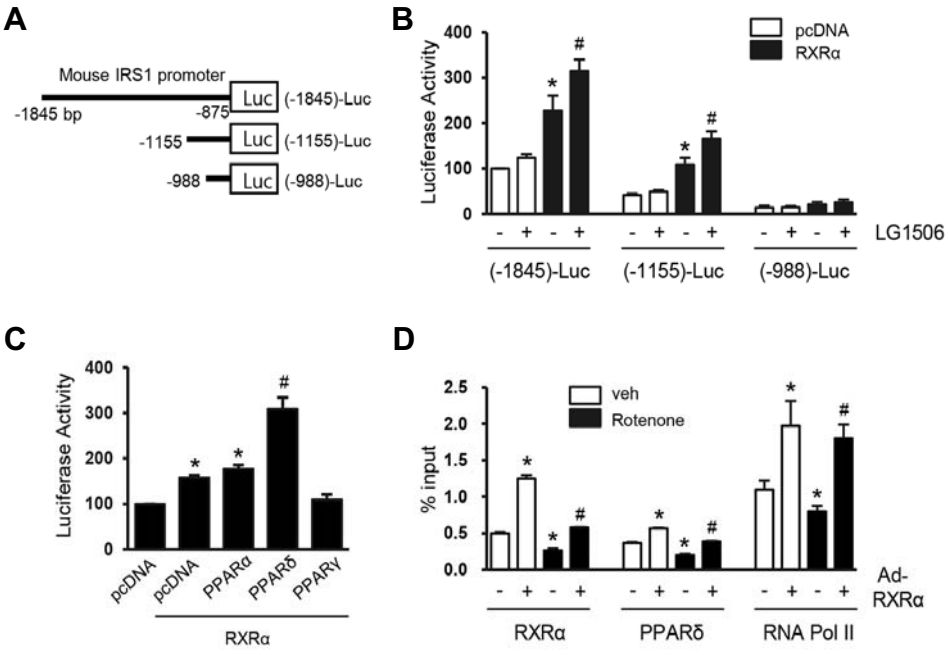

Fig. 4. RXRα directly binds to the IRS1 promoter. (A) A diagram of three different lengths of IRS1 promoter-luciferase constructs, IRS1 (-1845)-Luc, IRS1 (-1155)-Luc and IRS1 (-998)-Luc. (B) COS7 cells were transfected with one of the IRS1 promoter-Luc constructs and RXRα expression vector (pcDNA-RXRα). Three hours after the transfection, cells were treated with LG1506 (2 μM) for an additional 18 h. Luciferase activity of the cells transfected with IRS1 (-1845)-Luc was set to 100 and the other values were expressed as the relatives to that (n = 5). *, $P < 0.05$ vs pcDNA without LG1506 for each construct. #, $P < 0.05$ vs. pcDNA-RXRα without LG1506. (C) COS7 cells were transfected with IRS1 (-1155) Luc, expression vectors for RXRα, PPARα, PPARδ or PPARγ. Luci ferase activity of the cells transfected with IRS1 (-1155)-Luc without RXRα or PPAR expression vectors was set to 100 and the others were expressed as the relatives to that (n = 4). *, $P < 0.05$ vs. cells transfected with IRS1 (-1155)-Luc only. #, $P < 0.05$ vs. cells transfected with IRS1 (-1155)-Luc and pcDNA-RXRα (D) C2C12 myotubes were infected with Ad-RXRα (50 MOI) for 48 h, and then treated with rotenone for an additional 24 h. Cells were subjected to ChIP. Data are the means ± SEM (n = 3). *, $P < 0.05$ vs. Ad-GFP infected cells not treated with rotenone. #, $P < 0.05$ vs. Ad-GFP infected cells treated with rotenone.

we investigated the relationship between IRS1 and RXRα expression. We showed that RXRα overexpression or activation by its agonists accelerated the recovery of mitochondrial function and also increased IRS1 transcription, which in turn activated the insulin signaling pathway. Our data also indicate that RXRα binds to the IRS1 promoter and thus directly affects its transcription. LG1506 is reported as a heterodimer-selective modulator of RXRα: it activates RXRα: PPAR but not RXRα: LXRα or RXRα: FXRα heterodimers (Leibowitz et al., 2006). Consistently, our data showed that PPARδ bound to the IRS1 promoter, suggesting that RXRα: PPARδ heterodimers are involved in IRS1 transcriptional regulation.

Unlike LG1506, treatment of 9cRA increased IRS1 transcription when mitochondria were damaged but did not affect IRS1 transcription under control conditions. At this time, we cannot justify why 9cRA did not increase IRS1 transcription levels. We observed that RXRα protein levels were dramatically reduced by the addition of 9cRA but not LG1506 (Fig. 3D). Ligand-dependent ubiquitination of RXRα has been reported by several researchers: conformational changes induced by binding of specific ligands such as RA or LGD1268 are recognized by ubiquitination enzymes, which leads to proteasome-mediated degradation of RXRα (Gianni et al., 2003; Osburn et al., 2001). In contrast, binding of other types of ligand such as LG1506 may not induce the conformational change preferential for ubiquitination. The difference may explain the differential regulation of IRS1 transcription by 9cRA and LG1506. Based on our study, we postulate that RXRα increases IRS1 transcription by two different mechanisms: directly binding to the IRS1 promoter and indirectly recovering mitochondrial dysfunction. However, further studies are needed to determine how mitochondrial function is related to IRS1 transcription.

Although we confirmed that RXRα bound to the promoter region between -1155 bp and 998 bp of the IRS1 gene, the precise binding site remains to be identified. It is well known that RXR:PPAR heterodimers bind to the direct repeat motif-1 (DR-1); however, a well conserved DR1 site was not found in the region, indicating that they may bind to the region with quite low affinity. This could explain the findings that RXRα abundance was important for the binding and that 9cRA did not further increase IRS1 promoter activity (data not shown).

Our results showed that IRS1 mRNA and protein levels were dramatically reduced following treatment with OXPHOS inhibitors. A previous report showed that the induction of specific miRNAs is involved in suppressing IRS1 expression in the setting of mitochondrial dysfunction (Ryu et al., 2011). Regarding the involvement of RXRα in the regulation of IRS1 transcription in our study, reduced RXRα levels are likely partially involved in the suppression of IRS1 transcription in cells with impaired mitochondria. In addition to mitochondrial dysfunction, reduced IRS1 protein levels are often observed in the muscles of insulin-resistant animal models and myotubes (Macotela et al., 2011; Yuzefovych et al., 2012). These findings suggest that the reduction of IRS1 protein levels is a typical phenomenon of insulin resistance, however, little is known about IRS1 transcriptional regulation. Our data provide evidence that IRS1 transcription is regulated by RXRα. There are several reports showing *in vivo* study of RXRα agonists: LG100268 improves glycemic control and decreases cardiovascular risk in diabetic mice, and LG100754, a selective RXR:PPARγ agonist, decreases glucose levels of *db/db* mice (Cesario et al., 2001; Lenhard et al., 1999). Because, according to our study, LG1506 has beneficial effects on insulin resistance in myotubes, it will be worth to do *in vivo* study for clinical application.

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