이학석사 학위논문

미토콘드리아 기능이상 근육세포에서 RXRα 자극에 의한 IRS1의 양적 회복

Quantitative recovery of IRS1 by RXRα activation in mitochondrial dysfunction-induced myotubes

2013년 2월

서울대학교 대학원

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이 논문을 이학 석사 학위 논문으로 제출함

서울대학교 대학원

분자의학 및 바이오제약학과 분자의학 및 바이오제약학전공

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Abstract

Mitochondrion is a well-known organelle generating energy via metabolic pathway of nutrients, glucose and lipid. Mitochondrial dysfunction leads to impairment of insulin secretion in pancreatic beta cells and insulin resistance in liver and skeletal muscles. Our previous study on the cybrids carrying mtDNA A3243G mutation discovered that the activation of retinoid X receptor α (RXRα), a ligand dependent transcription factor, had restored mitochondrial function, therefore in this study the effect of RXRα activation on insulin signaling pathway impaired by mitochondrial dysfunction was investigated. RXRα as well as insulin receptor substrate 1 (IRS1) were decreased in the myotubes treated with mitochondrial OXPHOS complex inhibitors and in skeletal muscle of high fat/high sucrose-dieted mice. However, RXRα activation by its ligands restored IRS1 expression levels which were down-regulated by mitochondrial OXPHOS complex inhibitors in C2C12 myotubes. In addition, this was accompanied by the recovery of cellular ATP production. RXRα over-expression or activation increased the promoter activity of IRS1 in the transient transfection and luciferase assay system, indicating the possibility that RXRα regulates expression of IRS1 by directly binding to the promoter region. Therefore, these results suggest that RXRα might be a potential therapeutic target to improve insulin signaling in mitochondrial dysfunction-induced insulin resistance.

Key words: Insulin receptor substrate 1, Retinoic X receptor α, RXR specific ligand, mitochondrial dysfunction, insulin resistance, C2C12 myotubes

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Introduction

Diabetes mellitus is a metabolic disease causing high blood glucose either by insulin deficiency or by insulin resistance [1]. Diabetes has become one of the most serious medical concerns as the number of people with diabetes has increased rapidly during last decades all over the world [2]. Over the past two centuries, the enzymes and hormones involved in glucose metabolism [3, 4], the regulatory effects of reversible protein phosphorylation [5], and cyclic AMP on blood glucose levels [6], which contribute to diabetic hyperglycemia, have been identified. Moreover, insulin, released from pancreatic beta cells by the stimulation of nervous system [7] or by the gut hormones [8], plays a key role regulating blood glucose concentration as it stimulates glucose uptake by the target tissues. Type 1 diabetes is caused by autoimmune destruction of pancreatic beta cell which often leads to absolute insulin deficiency [1]. On the other hand, type 2 diabetes has both insulin resistance and insulin deficiency [9, 10].

Insulin resistance is a reduced response to circulating insulin in target tissues such as liver, adipose tissues and muscles [11]. Insulin resistance is a major pathophysiologic abnormality in the development of type 2 diabetes mellitus. It also identifies persons at high risk for cardiovascular disease [12] since it is commonly accompanied by other cardiovascular risk factors such as dyslipidemia,

hypertension and pro-thrombotic factor [13]. Thus insulin resistance has been considered a powerful predictor of diabetes as well as a therapeutic target [11]. Insulin resistance is determined by multiple genetic variations and environmental factors [14]. Recently, the genome-wide association studies (GWAS) have identified more than 80 genes are associated with type 2 diabetes [14]. Among candidate genes for type 2 diabetes, *PPARG* and *IRS1* are well-known genes involved in insulin response [15, 16. 17]. The risk of developing type 2 diabetes goes up to 70% if the individual has both maternal and paternal affected histories [18]. However, the missing heritability can be explained gene-gene interactions [19] or gene-environmental interactions. Although numerous genetic variations are involved in the development of type 2 diabetes, a worldwide epidemic increase of the disease is also associated with the increase of westernized diet, obesity and the decrease of physical activity [20, 21, 22].

Recently, mitochondrial dysfunction has also been proposed to explain insulin resistance [14]. Mitochondrion is a membrane-enclosed organelle found in most eukaryotic cells and generates adenosine triphosphate (ATP), which is a source of cellular energy [23]. The enzymes of electron transport chain in mitochondrial inner membrane perform redox reactions of oxidative phosphorylation (OXPHOS) to generate ATP in the matrix [23, 24]. These redox reactions, transferring electrons from electron donors to acceptors, are carried out by a series of protein complexes: NADH-coenzyme Q oxidoreductase (complex I), Succinate-Q oxidoreductase (complex II), Electron transfer flavoprotein-Q oxidoreductase, Q-cytochrome c oxidoreductase (complex III) and Cytochrome c

oxidase (complex IV) [23, 25]. This essential organelle is impaired by the genetic mutations [31, 32] or by the environmental factors such as excessive lipid accumulation [26, 27, 28] and immediate OXPHOS complex inhibitors [29, 30]. Recent studies have demonstrated that mitochondrial ATP synthesis and mitochondrial density are decreased in the muscle of insulin-resistant offspring of type 2 diabetic patients [33]. Also, their further studies have shown that mitochondrial substrate oxidation is considerably decreased in insulin-resistant offspring when compared with insulin-sensitive control subjects [34]. These data further support the result that insulin resistance in skeletal muscle is associated with an inherited defect of mitochondrial oxidative phosphorylation activity [34, 35]. Mitochondrial function is also important in insulin secretion. In pancreatic beta cells, mitochondria generates factors that couple nutrient metabolism to the exocytosis of insulin-containing vesicles and the latter process requires an increase in cytosolic Ca^{2+}, which depends on ATP synthesized by the mitochondria [35]. Thus mutations in mitochondrial DNA also cause beta cell dysfunction [36]. Since the majority of diabetic patients have both of insulin deficiency and insulin resistance, mitochondrial dysfunction may play an important role in the pathogenesis of type 2 diabetes. Among mitochondrial DNA (mtDNA) mutations associating with various disease states, the most common mutation found in diabetic patients is the mtDNA A3243G mutation in the mitochondrial DNA-encoded tRNALeu(UUR) gene [32]. Markus et al have reported that subjects with mtDNA A3243G mutation are insulin resistant in skeletal muscle even when beta cell function is not markedly impaired [37]. Since decreased glucose uptake in

skeletal muscle is the major determinant of impaired insulin sensitivity both in diabetic and in non-diabetic subjects [38], studying mitochondrial dysfunction in skeletal muscle seems to be important to understand mitochondrial dysfunction-induced insulin resistance.

Interestingly, we have previously found that retinoid X receptor α (RXRα) is associated with mitochondrial functions and the activation of RXRα by its ligand restores mitochondrial OXPHOS proteins in the cybrids carrying mtDNA A3243G mutation. Retinoid X receptor (RXR) is a ligand-activated transcriptional factor and conserves DNA-binding domain (DBD) for anchoring the protein to specific DNA sequences, ligand-binding domain (LBD) for binding of small lipophilic molecules and transactivation domain for activating the basal transcriptional machinery [39]. Retinoid X receptor (RXR) was firstly found as a partner for retinoic acid receptor (RAR) [39]. However, RXR has been proposed as a unique nuclear receptor that forms diverse heterodimers with PPARs, LXRs as well as RARs, and therefore the activation of RXR by its ligand has pleiotropic effects on numerous biological pathways [39]. The alpha isoform of RXR (RXRα), we have previously studied, belongs to this RXR family [39]. It affects the development of cardiac muscle by up-regulating the expression of both PPAR and LXR target genes [40] and alters cardiac fatty acid oxidation (FAO) capacity when it is reduced by hypoxia [41]. Interestingly, rexinoids, RXR specific ligands which activate RXRs, have been recognized well as insulin-sensitizers like Thiazolidinediones (TZDs) [42, 43, 44]. Some studies have demonstrated that rexinoids administration in type 2 diabetic rodents markedly decreases both hyperglycemia and

hyperinsulinemia [45]. However, rexinoids can activate a variety of RXR-containing heterodimers and RXR homodimers whereas TZDs can only activate the RXR:PPARγ heterodimer [44]. The primary effects of rexinoids involve alterations in gene expression in the liver and skeletal muscle while TZDs have the most profound effects on gene expression in adipose tissue [46]. Recent studies have identified that skeletal muscle is a major target of rexinoid action, where it sensitizes insulin-dependent glucose disposal in diabetic skeletal muscle [47]. Although hypertriglyceridemia is a common side effect with rexinoids treatment to diabetic subjects [48], newly modified rexinoids, LG101506 does not elevate triglycerides [49]. Less has known on their roles in mitochondrial dysfunction-induced states yet. Based on our previous study on the cybrids carrying mtDNA 3234 A>G mutation, the role of RXRα on the regulation of insulin receptor substrate 1 (IRS1) in mitochondrial dysfunction-induced insulin-resistant states was investigated in this study.

When insulin reaches its target tissues such as liver, muscles and adipose tissue, it interacts with insulin receptor (IR) on the cell surface which has tyrosine kinase activity [50]. Then the activated IR subsequently phosphorylates its substrates such as insulin receptor substrate (IRS) [51]. Insulin receptor substrate 1 (IRS1) belongs to this IRS family and activates Akt/ Phosphatidylinositol 3-Kinase (PI3K) pathway if its tyrosine residue 612 (Tyr^{612}) is phosphorylated [52]. However, this IRS1-mediated insulin signaling pathway is blocked when the serine residue 1101 (Ser^{1101}) is phosphorylated by free fatty acids or TNFα in C2C12 myocytes [53]. Interestingly, a recent genome-wide association study has shown that reduced

protein level of IRS1 is also strongly associated with decreased IRS1-mediated insulin induction in human skeletal muscles [54]. The basal expression level of IRS1 is reduced by the increase of microRNA targeting IRS1 [30] or by the facilitation of ubiquitin-mediated degradation of IRS1 [55]. These have appeared more clearly in mitochondrial dysfunction-induced states [30, 55]. Therefore, it seems important to find the agents which can improve total expression level of IRS1 at least in mitochondrial dysfunction-induced insulin resistant states. Some reports have presented that rexinoids increase insulin-stimulated IRS1 tyrosine phosphorylation or attenuate the level of IRS1 serine phosphorylation in skeletal muscle of db/db mice [47]. There has been no report on its association with IRS1 expression levels in mitochondrial dysfunction-induced states.

Therefore, in this study, the effect of RXRα activation on insulin signaling pathway impaired by mitochondrial dysfunction was investigated. First, the expression level of IRS1 and that of RXRα in insulin-resistant C2C12 myotubes incubated with mitochondrial OXPHOS complex inhibitors as well as in skeletal muscle tissues of mice fed with high fat/ high sucrose diet was examined. Then, how the activation of RXRα restored IRS1 expression impaired by mitochondrial dysfunction and tried to reveal the mechanism involved was investigated.

Materials and Methods

Cell culture and differentiation

C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA, USA). Two days after confluence, differentiation was induced with 1X high glucose Dulbecco's modified Eagle's medium with 2% horse serum (Invitrogen, Carlsbad, CA, USA) for three or four days. Then fresh differentiation inducing media was changed for treatments. Cos7 cells were maintained in DMEM supplemented with 10% fetal bovine serum.

Inducing mitochondria dysfunction and ligand treatment

After three days of differentiation, C2C12 cells were treated with mitochondrial oxidative phosphorylation (OXPHOS) complex inhibitors, 3 μM of rotenone for complex I inhibition and 10 μM of antimycin A for compelx III inhibition, or DMSO for 24 hours in 2% horse serum supplemented DMEM to induce or not to induce mitochondria dysfunction. Afterwards, three kinds of RXR ligands mixed with 2% horse serum DMEM were treated for 18 hours: 5 μM of 9-cis retinoic

acid (Sigma Aldrich, St. Louis, MO, USA), 2 μM of LG101506 (Santa Cruz biotechnology, inc., Santa Cruz, CA, USA) and 2 μM of LG100268 (Sigma Aldrich).

siRNA treatment

Small interfering RNAs (siRNAs) of RXRα (siRXRα) were purchased from Dharmacon (Chicago, IL, USA) and nonspecific siRNAs (siNS, negative control) were purchased from Bioneer (Daejeon, Korea). 50 nM of siRXRα or siNS was mixed with RNAiMAX (Invitrogen) in 100$\mu\ell$ of serum-free DMEM and incubated for 20 minutes at room temperature. The complex was treated to the cells with 400 $\mu\ell$ of serum-free DMEM. After 2.5 hour incubation, 400$\mu\ell$ of DMEM media supplemented with 2% horse serum were added.

Animal experiments and preparation of protein and RNA samples

Wild FVB mice, an inbred strain sensitive to Friend leukemia virus B strain, were group-housed in a specific pathogen free facility on 12 hour light/dark cycle at 22-24℃ and fed with normal chow diet (NCD) or high fat/ high sucrose diet (HFHSD) for 12 weeks. After the diets, they were sacrificed. Tissues were collected and carried within -80℃ liquid nitrogen. Randomly picked gastrocnemius muscles were ground in a pre-chilled bowl and divided into two

vials. One vial was lysed in 20mM Tris-HCl, pH 7.4, 5mM $Na_4P_2O_7$, 100nM NaF, 2mM Na_3VO_4, 1% NP-40 buffer supplemented with protease inhibitors (1μg/$\mu\ell$ aprotinin, 1μg/$\mu\ell$ luepeptin and 1mM PMSF) to get protein samples while the other vial was lysed in TRIzol (Invitrogen) to get RNA samples.

Western blot analysis

Cells were lysed in 20mM Tris-HCl, pH 7.4, 5mM $Na_4P_2O_7$, 100nM NaF, 2mM Na_3VO_4, 1% NP-40 buffer supplemented with protease inhibitor (1μg/$\mu\ell$ aprotinin, 1μg/$\mu\ell$ luepeptin and 1mM PMSF). The whole-cell lysates were sonicated 15 seconds for two times, and cell debris was removed by centrifugation (13,000 rpm) for 30 minutes at 4℃. About 20~30μg of proteins were separated on the SDS-polyacrylamide gels. Separated proteins were transferred onto nitrocellulose membrane (Whatman, Dassel, Germany). The membrane was blocked with 5% skim milk in Tween-20-Tris-buffered saline (TBS-T) for 1 hour at room temperature, and it was incubated with the specific primary antibody for overnight at 4℃. Membranes were probed with specific antibodies and bands were visualized by enhanced chemi-luminescence (Pierce, IL, USA).

RNA preparation and Real-time PCR

Total RNAs of differentiated C2C12 cells were isolated using TRIzol

(Invitrogen) according to the manufacturer's instructions. To prepare cDNA, 10ul of reaction buffer (Invitrogen), 5$\mu\ell$ of 100mM DTT, 2.5$\mu\ell$ of 10mM dNTP, 1$\mu\ell$ of Oligo dT, 0.5$\mu\ell$ of RNase inhibitor, 2$\mu\ell$ of RTase and 1μg of RNA were mixed and RNase free water was added up to 50$\mu\ell$. The mixture was incubated at 37℃ for 1 hour and at 72℃ for 10minutes using PCR system. Expression levels of genes were determined by using SYBR-master mix (Takara, Otsu, Shiga, Japan) and AB 7500 Real-time PCR system (Applied Biosystems, CA, USA). The primers for PCR of the genes were as follows: *irs1* (5'-CGA TGG CTT CTC AGA CGT-3' and 5'-CAG CCC GCT TGT TGA TGT TG-3') and *rxrα* (5'-ACT GGT AGC CCC CAG CTC AA-3' and 5'-GAG CGG TCC CCA CAG ATA GC-3'). Samples were prepared repetitively and each sample was analyzed in duplicates.

Plasmids and antibodies

The mouse IRS1 promoter fragments from -1845bp to -875bp, from -1155bp to -875bp and from -998bp to -875bp were inserted into the region upstream of the luciferase gene of the pGL2-Basic vector (Promega, Madison, WI, USA) while the mouse IRS1 promoter fragment from -4674bp to +375bp, inserted in pGL3-Basic vector, was provided by Dr. Wonho Kim in NIH. Antibodies against RXRα and IRS1 were purchased from Santa Cruz Biotechnology while antibody against pY (pY612) IRS1 was purchased from Invitrogen.

ATP bioluminescent somatic cell assay

Cells were lysed in somatic cell ATP releasing reagent (Sigma Aldrich, St. Louis, MO, USA), to increase membrane permeability and to release cellular ATP. Then these cellular ATPs were reacted with ATP assay mix (Sigma Aldrich, St. Louis, MO, USA), containing luciferase, luciferin, $MgSO_4$, DTT, EDTA, bovine serum albumin (BSA) and tricine buffer salts. The relative amount of ATP released was determined by the light emitted from above reaction.

Transient transfection and Luciferase reporter assay

Cos7 cells were seeded at 12-well plates the day before transfection. The 0.3μg IRS1 promoter-luciferase reporter vector, 0.1μg expression vector for RXRα, and 0.05μg CMV-β-galactosidase were mixed with 3$\mu \ell$ of Plus reagent (Invitrogen) in 50$\mu \ell$ of serum-free DMEM and incubated for 15 minutes at room temperature. Then 50$\mu \ell$ of serum-free DMEM containing 2$\mu \ell$ of Lipofectamine reagent (Invitrogen) was added and incubated for additional 15 minutes at room temperature. The complex was treated to cells with 400$\mu \ell$ of serum-free DMEM. After 3 hours, medium was changed to DMEM supplemented with 10% fetal bovine serum. Twenty four hours after transfection, cells were harvested using reporter lysis buffer and luciferase activities were determined using the luciferase assay system (Promega) and Lumet LB9507 (Berthold, Bad Wildbad, Germany). For normalization of transfection efficiency, β-galactosidase activity was used. For

β-galactosidase activity assay, 10$\mu\ell$ of cell lysate was mixed with 3$\mu\ell$ 100X $MgCl_2$ (0.1M $MgCl_2$, 4.5M β-mercaptoethanol), and 66$\mu\ell$ 1X *O*-nitrophenyl-β-D-galactosidose. β-galactosidase activity was measured by spectrophotometry at 420nm (VersaMax; Molecular devices, Sunnyvale, CA, USA).

Statistical analysis

SPSS, version 20.0 (SPSS inc., Chicago, IL), was used for statistics analysis of data. By using student's t-test and one-way analysis of variance (ANOVA), the differences between means were measured. Additional Spearman's rank correlation test was performed to evaluate the association between genes and proteins. Data were expressed as mean ± standard error, and a p-value less than 0.05, 0.01, 0.001 denoted the presence of statistically significant difference.

Results

I. The association between the expression levels of RXRα and IRS1

RXRα and IRS1 are decreased by mitochondrial OXPHOS complex inhibition in C2C12 myotubes.

To evaluate the association between RXRα and IRS1 protein levels in the myotubes having mitochondrial dysfunction, differentiated C2C12 myotubes were incubated with mitochondrial oxidative phosphorylation (OXPHOS) complex inhibitors, rotenone and antimycin A, for 24 hours. The protein level of IRS1 was significantly decreased when the cells were incubated with mitochondrial OXPHOS complex inhibitors while the protein level of RXRα were significantly decreased only in the cells incubated with OXPHOS complex I inhibitor, rotenone (**Fig. 1**). Likewise, IRS1 mRNA levels were decreased in the mitochondrial OXPHOS complex-inhibited conditions while the RXRα mRNA levels were decrease only in the myotubes incubated with rotenone (**Fig. 2**). These data may suggest that mitochondrial dysfunction, especially the damage of mitochondrial OXPHOS complex I, affects the expression level of RXRα and that of IRS1.

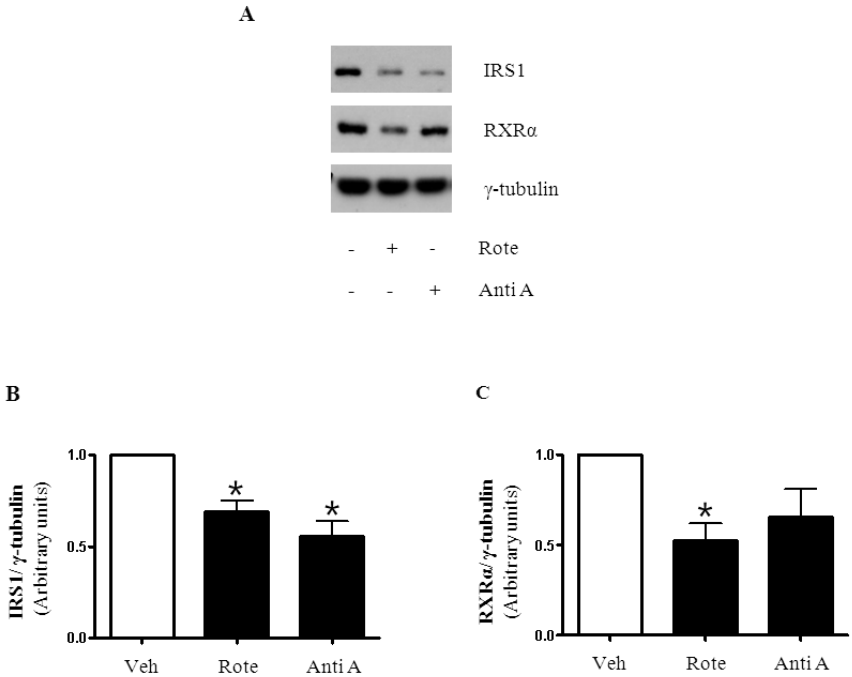


Figure 1. Protein levels of RXRα and IRS1 in mitochondrial dysfunction-induced C2C12 myotubes.

C2C12 skeletal myocytes were induced to differentiate and the differentiated myotubes were treated with DMSO (vehicle) or mitochondrial oxidative phosphorylation (OXPHOS) complex inhibitors, 3μM of rotenone to inhibit OXPHOS complex I or 10 μM of Antimycin A to inhibit OXPHOS complex III, for 24 hours. Afterwards, cells were harvested and lysed to get proteins for analysis. Proteins were then subjected to SDS-PAGE and immunoblot analysis. **A.** IRS1 and RXRα proteins detected by Western blot under above mitochondrial dysfunction-induced states. The band intensity of IRS1 or RXRa was normalized by that of γ-tubulin; **B.** IRS1 protein level and **C.** RXRα protein level in each group. The data were represented as means ± S.E. of three independent experiments *, $p < 0.05$ vs. the value of vehicle.

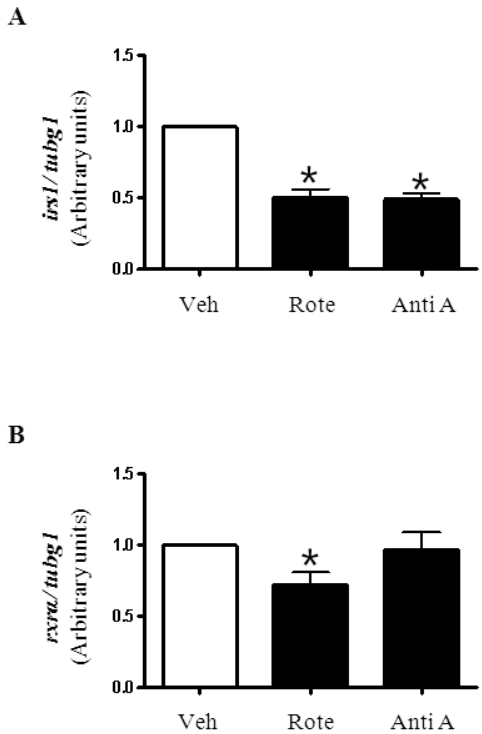


Figure 2. mRNA levels of RXRα and IRS1 in mitochondrial dysfunction-induced C2C12 myotubes.

RNAs were isolated from the same condition presented in Figure 1. RNAs were subjected by real-time PCR with specific primers for IRS1 and RXRα. The mRNA expression level of each gene was normalized by that of *tubg1*; **A.** IRS1 mRNA levels and **B.** RXRα mRNA levels in each group. The data were represented as means ± S.E of three independent experiments *, $p < 0.05$ vs. the value of vehicle.

Small interfering RNA of RXRα down-regulates IRS1 expression.

To confirm the causal relationship between RXRα and IRS1, the differentiated myotubes were transfected with small interfering RNA of RXRα (siRXRα) and the mRNA expression levels of IRS1 were investigated. Indeed, knock-down of RXRα down-regulated IRS1 mRNA levels in C2C12 myotubes (**Fig. 3**). In contrast, the mRNA expression levels of IRS2 were not down-regulated by the decrease of RXRα (**Fig. 3A**). Thus this data verified that the decrease of RXRα expression is at least partly involved in the decrease of IRS1 expression in the myotubes, and which may contribute to the insulin-resistant states of the cells whose mitochondrial OXPHOS functions were impaired.

RXRα and IRS1 are decreased in skeletal muscle tissues of high fat/ high sucrose diet-fed mice.

To compare the expression levels of RXRα and IRS1 in response to diet *in vivo*, twelve FVB mice were fed with normal chow diet (NCD) or high fat/ high sucrose diet (HFHSD) for 12 weeks and they were sacrificed to get proteins. Samples were grouped by their diets and the protein levels of RXRα and IRS1 were detected by Western blot. Interestingly, the protein levels of RXRα and IRS1 were also decreased in the gastrocnemius skeletal muscles of the mice fed with high fat/ high sucrose diet (HFHSD) (**Fig. 4**). Likewise, RNA samples were prepared in the same parts of tissues, and subjected to real-time PCR with specific

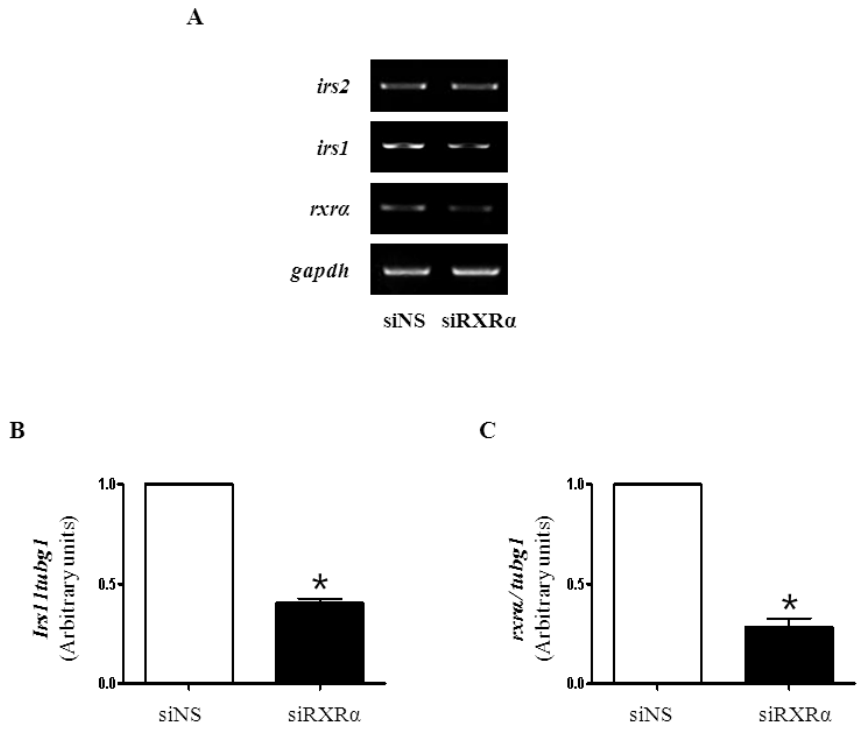


Figure 3. Effects of RXRα knock-down on mRNA expression level of IRS1 in C2C12 myotubes.

C2C12 skeletal myocytes were induced to differentiate and the differentiated myotubes were transfected with non-specific siRNAs (siNS, negative control) or siRNAs against RXRα (siRXRα). Three days after the transfection, cells were harvested and prepared for quantitative analysis. Isolated RNAs were quantitated by real-time PCR using specific primers for IRS1 and RXRα. The mRNA expression level of each gene was normalized by that of *tubg1*. **A.** A representative RT-PCR products of IRS1, IRS2 and RXRα after 29 cycles. The mRNA expression levels of **B.** IRS1 or **C.** RXRα were represented as means ± S.E. of three independent experiments *, $p < 0.05$ vs. the value of siNS**.**

primers for IRS1, IRS2, RXRα, RARα and RARβ. All the mRNA levels, investigated in this study, were not statistically decreased in the HFHSD group (**Fig. 5**) yet the p values of the decrease of IRS1 (**Fig. 5A**), IRS2 (**Fig. 5B**) and RXRα (**Fig. 5C**) mRNA levels in HFHSD were close to the statistically efficient value, 0.05.

The expression levels of RXRα are significantly correlated with the expression levels of IRS1 in skeletal muscle tissues.

In addition, to evaluate further the association between the expression levels of RXRα and IRS1 in skeletal muscle tissues, Spearman's rank correlation test was performed with the quantitated results above (**Fig. 5**). Regardless of the expression level difference between individuals and diets, the protein levels of RXRα and IRS1 showed statistically meaningful association (**Fig. 6**). It was also interesting that the mRNA expression levels of IRS1 and IRS2 were significantly correlated with the mRNA expression levels of RXRα (**Fig. 7A, D**) as well as with the mRNA expression levels of RARα, which is one of partners of RXRα activation (**Fig. 7B, E**) in skeletal muscles. Another partners for RXRα, RARβ, however, was not significantly correlated with the mRNA expression levels of IRS1 or with the levels of IRS2 (**Fig. 7C, F**).

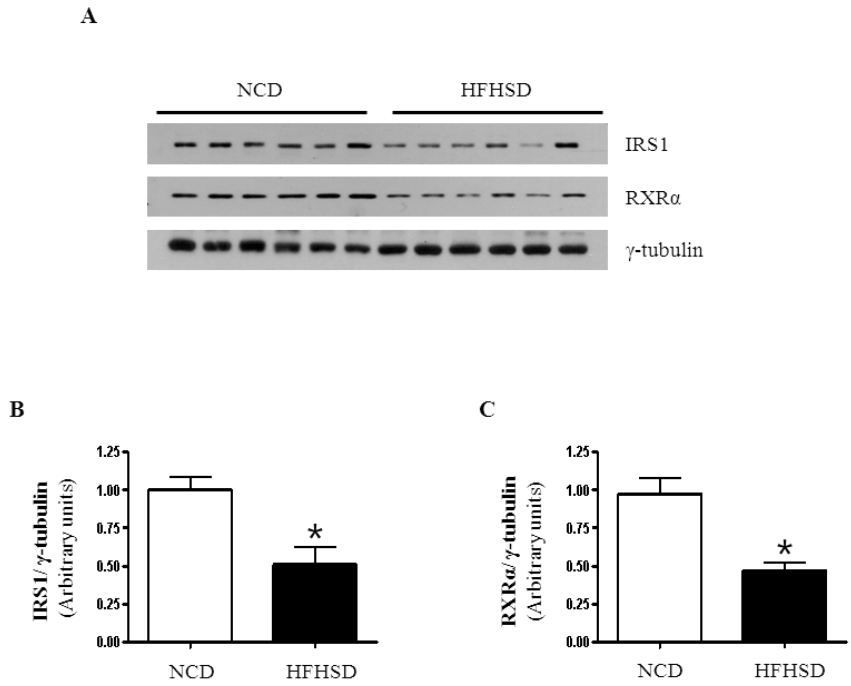


Figure 4. Protein levels of RXRα and IRS1 in the skeletal muscle of mice fed with high fat/high sucrose diet.

Male FVB mice were fed with normal chow diet (NCD, n = 6) or with high fat/ high sucrose diet (HFHSD, n = 6) for 12 weeks, and then sacrificed. Gastrocnemius muscle tissues were collected, and proteins were prepared for immune-blotting. **A.** IRS1 and RXRα proteins in NCD or in HFD detected by western blot. The band intensity of each lane was normalized by that of γ-tubulin; **B.** IRS1 protein level and **C.** RXRα protein level *, $p < 0.05$ vs. the value of NCD.

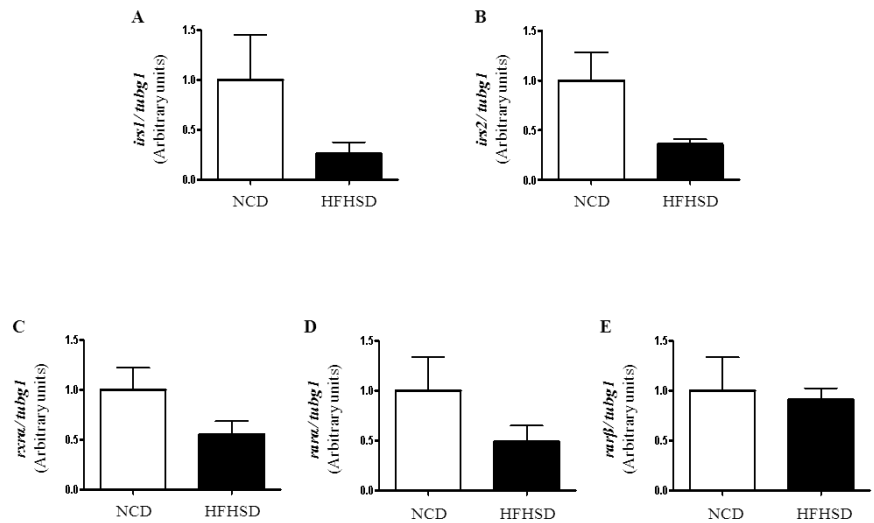


Figure 5. mRNA expression levels of IRS1 and RXRα in the skeletal muscle of mice fed with high fat/ high sucrose diet.

RNAs were isolated from the same individuals presented in the Figure 4 (NCD, n=6 and HFHSD, n=6). RNAs were subjected to real-time PCR with specific primers for IRS1, IRS2, RXRα, RARα and RARβ. Afterwards, the mRNA expression level of each gene was normalized by that of tubg1; the mRNA expression level of **A.** IRS1, **B.** IRS2, **C.** RXRα, **D.** RARα, and **E.** RARβ.

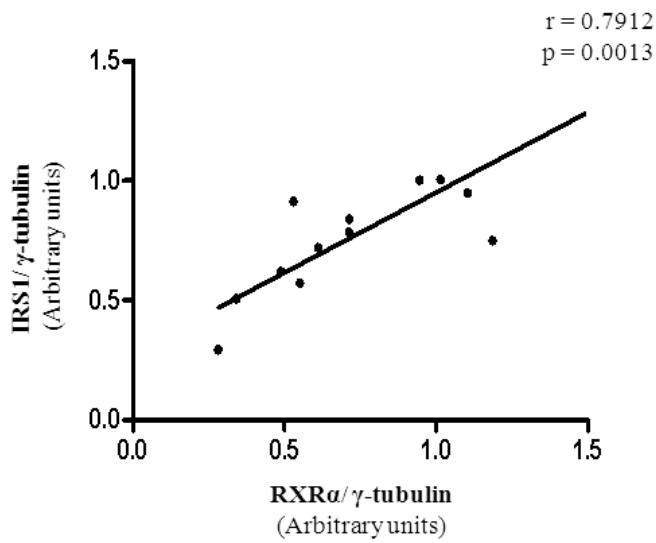


Figure 6. Correlation between the protein levels of RXRα and IRS1 in mice skeletal muscle tissues.

Spearman's rank correlation test was performed to examine correlation between RXRα and IRS1 proteins levels, based on the normalized quantity data in Figure 4. Correlation coefficient of the test was represented as 'r' right above the figure with its p value.

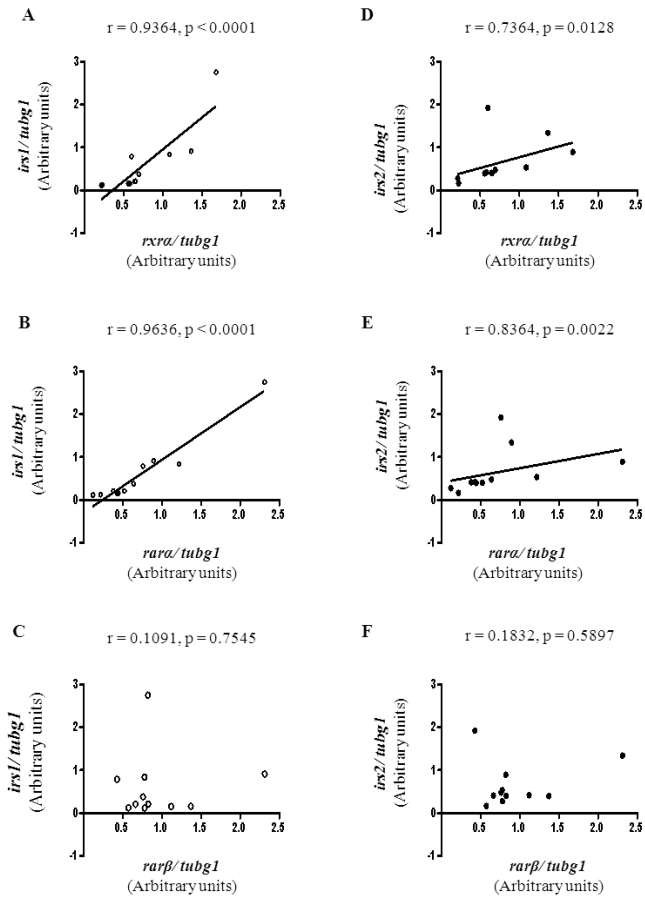


Figure 7. Correlation between the mRNA expression levels of RXRα and IRS1 in mice skeletal muscle tissues.

Spearman's rank correlation test was performed with the normalized mRNA quantity data in Figure 6. Correlation between the mRNA expression levels of **A.** RXRα and IRS1, **B.** RARα and IRS1, **C.** RARβ and IRS1, **D.** RXRα and IRS2, **E.** RARα and IRS2 and **F.** RARβ and IRS2. Correlation coefficient of each test was presented at each figure with its p value.

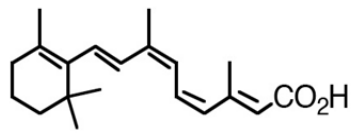
II. The quantitative recovery of IRS1 by RXRα activation in C2C12 myotubes

RXRα activation restores ATP production reduced by mitochondrial OXPHOS complex inhibition in C2C12 myotubes.

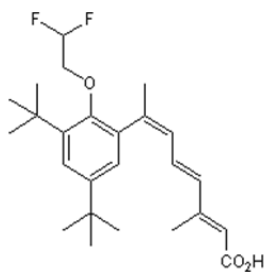
To induce activation of RXRα, three different RXR specific ligands, 9-cis retinoic acid (9cRA), LG101506 (LG1506) and LG100268 (LG268) (**Fig. 8**), were treated to the myotubes which had been impaired by mitochondrial OXPHOS complex I inhibitor. Firstly, the relative ATP contents produced by the cells were measured to examine the effect of RXRα activation on the recovery of mitochondrial function. RXRα activation with its specific ligands indeed restored cellular ATP production which was reduced by rotenone and the effects were statistically significant (**Fig. 9**).

RXRα activation restores IRS1 protein level down-regulated by mitochondrial OXPHOS complex inhibition in C2C12 myotubes.

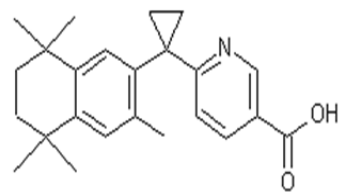
Activation of RXRα also restored the protein level of IRS1 which was down-regulated by mitochondrial OXPHOS complex I inhibition in C2C12 myotubes (**Fig. 10**).



9-cis retinoic acid (9cRA)



LG101506 (LG1506)



LG100268 (LG268)

Figure 8. RXR specific ligands.

Three types of RXR specific ligand used to induce RXRα activation.

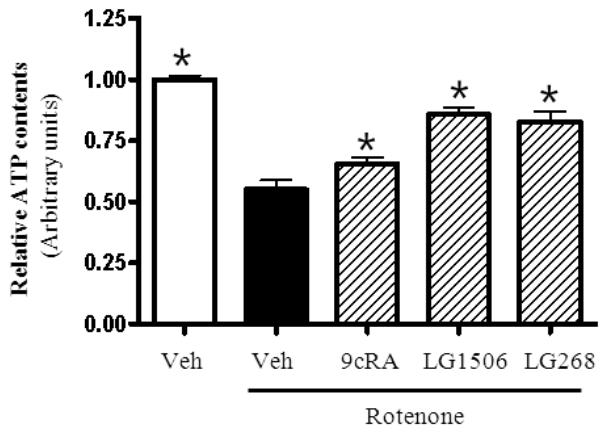


Figure 9. Effects of RXRα activation on ATP production in mitochondrial dysfunction-induced C2C12 myotubes.

C2C12 skeletal myocytes were induced to differentiate and the differentiated myotubes were treated with DMSO (vehicle) or OXPHOS complex I inhibitor, rotenone (3μM), for 24 hours. Then three RXR specific ligands, 9-cis retinoic acid (9cRA, 5μM), LG101506 (LG1506, 2μM) and LG100268 (LG268, 2μM), were treated for 18 hours. Cells were lysed with ATP releasing buffer and ATP bioluminescent assay was performed to measure relative ATP contents of the cells. Result was represented as means ± S.E. of three independent experiments *, $p < 0.05$ vs. the cells treated with rotenone only.

RXRα activation improves insulin signaling pathway impaired by mitochondrial OXPHOS complex inhibition in C2C12 myotubes.

The quantitative recovery of total IRS1 protein achieved by the activation of RXRα consequently led to the increase of IRS1 Tyr^{612} phosphorylation in the myotubes impaired by mitochondrial OXPHOS complex I inhibition (**Fig. 11**). Then the increase of phophorylation of IRS1 triggered Akt activation as well. The result meant that insulin signaling pathway can be restored through the activation of RXRα even after the cellular mitochondrial OXPHOS function was inhibited.

RXRα activation restores IRS1 mRNA expression levels down-regulated by mitochondrial OXPHOS complex inhibition in C2C12 myotubes.

RNA samples were also prepared in the same condition above (**Fig. 10**) and subjected to real-time PCR. RXRα activation again restored the mRNA expression levels of IRS1 which was down-regulated by OXPHOS complex inhibition (**Fig. 12**). The effect of 9-cis retinoic aicd or LG101506 on the IRS1 mRNA expression levels was statistically significant, but the effect of LG268 was not.

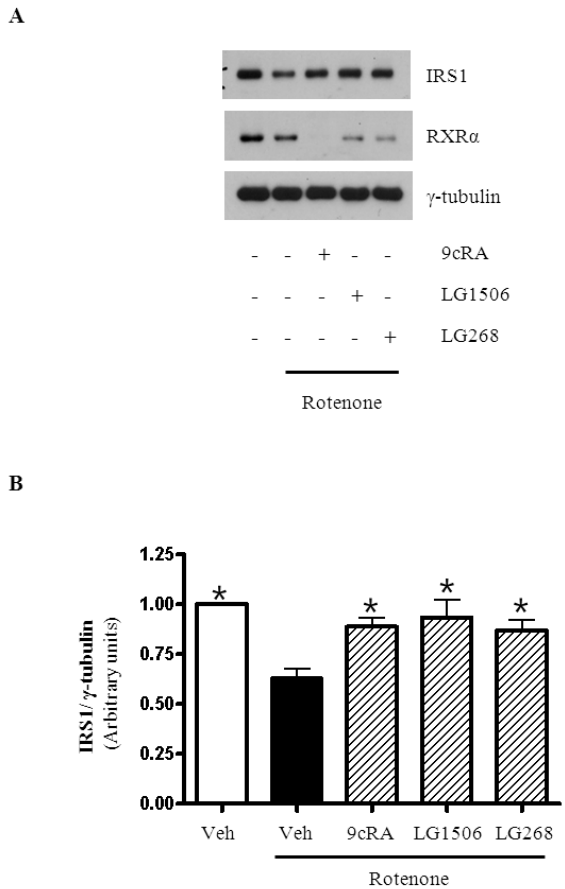


Figure 10. Effects of RXRα activation on IRS1 protein level in mitochondrial dysfunction-induced C2C12 myotubes.

Proteins were isolated from the same condition presented in Figure 10. **A.** IRS1 and RXRα proteins detected by Western blot, **B.** IRS1 protein level was quantitated. The result was represented as means ± S.E. of three independent experiments *, $p < 0.05$ vs. the cells treated with rotenone only.

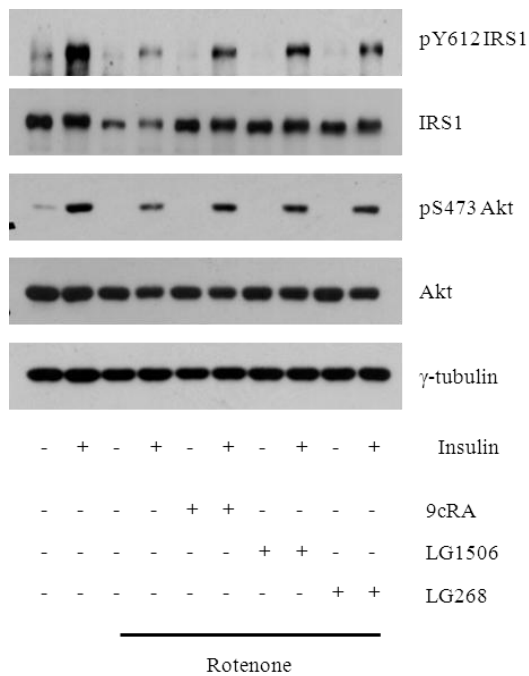


Figure 11. Effects of RXRα activation on insulin signaling pathway in mitochondrial dysfunction-induced C2C12 myotubes.

Half an hour before harvesting myotubes under the same condition as described in Figure 10, 100nM of insulin was additionally treated. Proteins were collected, and then the levels of IRS1 and Akt, and their phosphorylation forms (pY612 IRS1 and pS473 Akt) were detected by western blot.

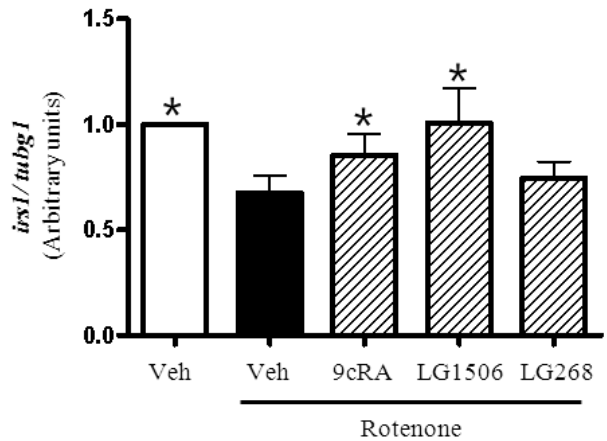


Figure 12. Effects of RXRα activation on IRS1 mRNA expression levels in mitochondrial dysfunction-induced C2C12 myotubes.

Total RNAs were isolated from the same conditions as mentioned in Figure 9 and subjected to real-time PCR with specific primers for IRS. The result was represented as means ± S.E of three independent experiments *, $p < 0.05$ vs. the value of only rotenone treated.

III. The mechanism for the induction of IRS1 expression by RXRα activation

The mouse IRS1 promoter is activated by RXRα and LG101506.

To reveal the mechanism by which RXRα activation affects IRS1 expression, mouse IRS1 promoter-luciferase constructs containing various length of the promoter and an expression vector for RXRα were co-transfected into Cos7 cells. Four different promoter fragments inserted into luciferase vectors were prepared for this inspection (**Fig. 13**). Two RXR specific ligands, 9-cis retinoic acid and LG101506 were treated afterwards. Luciferase activity of the three promoter fragments, -4674bp to +375bp, -1875bp to -875bp and -1155bp to -875bp, showed almost the same pattern while the fragment, -998bp to -875bp, did not (**Fig. 14**). Luciferase activities of the three IRS1 promoters were increased by RXRα overexpression or LG1506 treatment.

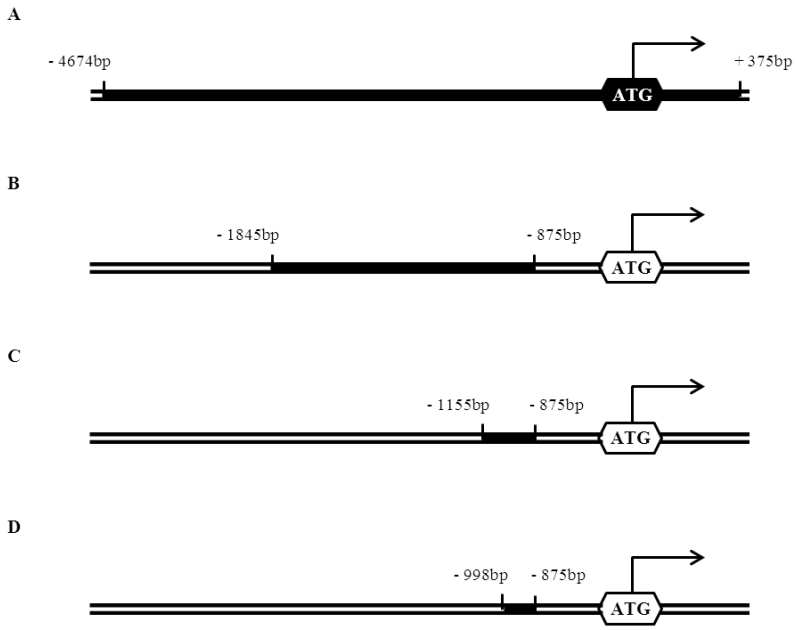


Figure 13. Mouse IRS1 promoter regions inserted into luciferase vectors.

The mouse IRS1 promoter fragments from -4674bp to +375bp, from -1845bp to -875bp, from -1155bp to -875bp and from -998bp to -875bp were inserted into the region upstream of the luciferase gene of the pGL2-Basic vector or pGL3-Basic vector.

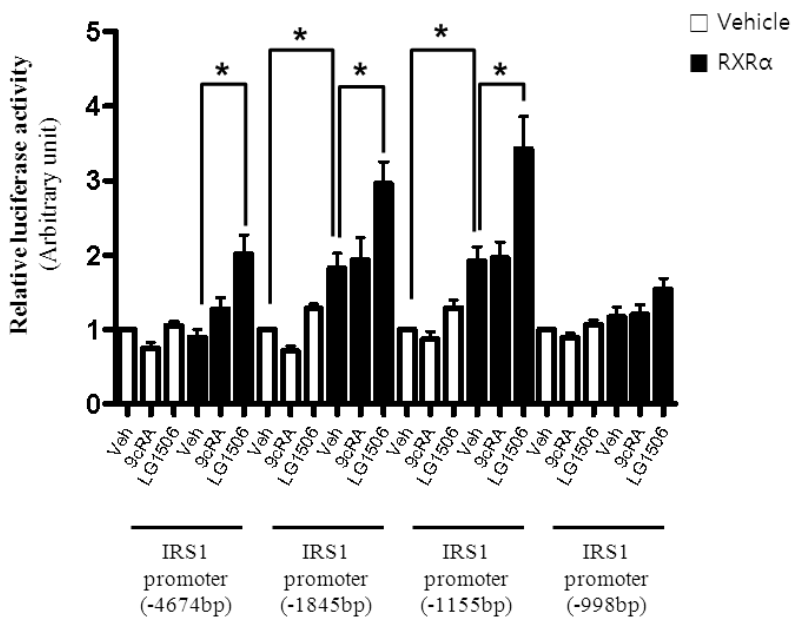


Figure 14. Effects of RXRα overexpression and RXR specific ligands on IRS1 promoter activity.

Various mouse IRS1 promoter-luciferase constructs and expression vectors of RXRα were co-transfected into Cos7 cells. And RXR specific ligands, 9-cis retinoic acid (9cRA, 1μM) and LG101506 (LG1506, 1μM), were treated for 18 hours. β-galactosidase activity was used for normalization of transfection efficiency. Luciferase activity of the IRS1 promoter in the absence of RXRα expression vector with vehicle treatment was set to one and other activities were expressed relative to this value. The result was represented as means ± S.E. of three independent experiments *, $p < 0.05$

Discussion

In this study, the expression levels of RXRα and IRS1 were decreased when mitochondrial functions were impaired by mitochondrial OXPHOS complexes inhibitors. In addition, the expression levels of RXRα were significantly correlated with those of IRS1 in mice skeletal muscles. RXRα knock-down decreased IRS1 expression in myotubes. RXRα activation by its ligands restored IRS1 expression which was down-regulated by OXPHOS complex inhibition in C2C12 myotubes. In the transient transfection system, the mouse IRS1 promoter fragment from -1155bp to -875bp was maximally activated by RXRα over-expression and the ligand, LG101506.

Similar to our previous results of cybrids carrying mtDNA A3243G mutation, RXRα expression was remarkably down-regulated in the myotubes treated with mitochondrial OXPHOS complex inhibitors in this study (**Fig. 1, 2**). One study has revealed that the activation of RXRα improves fatty acid metabolism in 3T3L1 adipocytes treated with rotenone [56]. Even though the study did not confirm the expression level of RXRα in those adipocytes, it suggested that the function of RXRα had been inhibited in the adipocytes having mitochondrial dysfunction. In addition, we have previously confirmed that reactive oxygen species (ROS) formation also down-regulates RXRα. These suggest that RXRα is

possibly down-regulated by oxidative stress which is generated by mitochondrial dysfunction and this phenomenon seems to appear through many cells. Perhaps either genetic mutation or environmental toxins causing mitochondrial dysfunction also results in the decreased expression of RXRα thus impairs various intracellular signaling pathways in which RXRα is involved. Moreover, IRS1 has been found decreased in the cells with oxidative stresses however its mechanism has been understood differently by each study [30, 55]. One showed IRS1 degradation is mediated by microRNA targeting IRS1 when the cells were treated with mitochondrial OXPHOS complex inhibitors [30]. The other one showed the increased reactive oxygen species facilitates serine phosphorylation of IRS1, which eventually leads to ubiquitin-mediated degradation of IRS1 [55]. In this study, the decreased expression levels of RXRα also down-regulated IRS1 expression (**Fig. 3**). Since IRS1 mediates not only the metabolic signaling pathway but also the cell proliferation signaling pathway [57], there can be a more than one factor that regulates IRS1 expression. Our study showed that decreased RXRα at least in part contributes to decreased IRS1 expression in mitochondrial dysfunction-induced muscle cells.

Decreased IRS1, induced by mitochondrial dysfunction, was restored by RXRα activation in this study (**Fig. 10, 12**). Regardless of the mechanism involved in IRS1 degradation in mitochondrial dysfunction-induced states, RXRα activation was enough to restore IRS1 expression. RXRα ligand treatment increased mRNA expression levels of IRS1 and increased IRS1 promoter activity. Since retinoid X receptors (RXRs) conserve not only ligand-binding domain (LBD) but also DNA-

binding domain (DBD) for anchoring the protein to specific DNA sequences [39], RXRα may anchor to specific DNA sequences on IRS1 promoter so to activate the transcription. Unfortunately, this study only confirmed the effect of RXRα activation in a specific mitochondrial dysfunction-induced condition when mitochondrial oxidative phosphorylation is inhibited. However, RXRα activation would be expected to restore IRS1 expression levels in other mitochondrial dysfunction-induced states as well if this study additionally reveals the exact binding site of RXRα on IRS1 promoter.

To reveal the mechanism by which 9-cis retinoic acid and LG101506 affect IRS1 expression, IRS1 promoter-luciferase construct containing various length of the promoter was co-transfected with the expression vector for RXRα and then treated with 9-cis retinoic acid and LG101506. The results of reporter assays indicate that the effect of RXRα activation on IRS1 expression can be achieved at least in part by direct regulation of IRS1 promoter activity by RXRα (**Fig. 14**). However, there are still remained questions of exact RXRα activation 'partners' to increase IRS1 expression levels and of exact RXRα 'binding site' on the IRS1 promoter which may be located between -1155bp and -998bp according to the reporter assays. Specifically, only the LG101506 showed the effect to activate IRS1 promoter while the 9-cis retinoic acid did not in this study. This may doubt the direct regulation of RXRα on IRS1 induction by 9-cis retinoic acid. However there should be further investigations first to reveal the partners of RXRα. They are all RXR specific yet LG101506 has higher selectivity to induce the conformation of RXRs:PPARs while 9-cis retinoic acid recognizes a broad range

of RXR dimeric forms [39]. This implies 9-cis retinoic acid needs much more effective RXR dimeric forms so that it can recognize. In other words, when the partners of RXR is found, it needs to be confirmed again if 9-cis retinoic acid shows effect to activate IRS1 promoter, in the presence of the partners. In addition, since the over-expression of RXRα could activate IRS1 promoter in this assay, it also needs to be verified that the effect of RXRα activation is comparable with the effect of RXRα over-expression in mitochondrial dysfunction-induced myotubes.

Different effects of RXRα ligands may complicate the exploration to find the underlying mechanism. In this study, the activation of RXRα by its three ligands, 9-cis retinoic acid, LG101506, LG100268, restored the protein level of IRS1 which was down-regulated by mitochondrial OXPHOS complex inhibition. Unfortunately, however, the mRNA expression levels of IRS1 were not restored by LG100268 treatment, which means the recovery of IRS1 protein expression by LG100268 might not be mediated by the transcriptional activation of RXRα. One study has demonstrated that LG100268 improves mitochondrial OXPHOS complex function in beta cells [58]. This study also showed that LG100268 restored mitochondrial function which was impaired by mitochondrial OXPHOS complex inhibitor (**Fig. 9**). The other RXR specific ligands, 9-cis retinoic acid and LG101506, which had restored IRS1 expression in mRNA levels as well as in protein levels, also restored mitochondrial function as LG100268 did. Kim Ravnskjaer et al have described the activation of RXRα:PPARδ by LG100268 up-regulates the gene expressions of CPT1b, UCP2 and CD36, and they have observed the improvement of mitochondrial substrate utilization by LG100268

treatment [58]. In skeletal muscles, peroxisome proliferator-activated receptor delta (PPARδ) and RXRα, both have been known to play a central role in the regulation of fatty acid oxidation [59, 60]. Oxidative stresses impair fatty acid metabolism [41] and increases IRS1 degradation [30, 55]. In this study, LG100268 might have restored RXRα:PPARδ activity and up-regulated the gene expressions associated with mitochondrial function, which eventually attenuated further generation of reactive oxygen species to increase IRS1 degradation. Also for 9-cis retinoic acid and LG101506 treatment, this might have worked synergistically. In addition, the ligands treated in this study can induce different phenomena, depending on the cell types and its states [39]. LG100268 normally elevates triglycerides as a side effect [39] while 9-cis retinoic acid restores fatty acid metabolism so to reduce triglyceride accumulation in the adipocytes treated with rotenone [58]. Therefore the other genes related with fatty acid metabolism or mitochondrial functions should be examined further to clarify the different or shared characters of each ligand and to select the best way RXRα can be activated.

Lastly, I confirmed the protein levels of RXRα and IRS1 were down-regulated in gastrocnemius skeletal muscles by high fat/ high sucrose diet (**Fig. 4**). mRNA levels of these two proteins tended to decrease, but not statistically significant in the same condition (**Fig. 5**). Nevertheless, the statistically significant correlation between IRS1 and RXRα appeared both in protein levels and mRNA levels (**Fig. 6, 7**). These were interesting because many studies have showed that mitochondrial OXPHOS complexes were down-regulated in skeletal muscle tissues of high fat diet when insulin sensitivity was also decreased [28, 61]. Moreover,

insulin resistance in the skeletal muscle of insulin-resistant offspring of patients with type 2 diabetes is also found to be associated with dysregulation of intramyocellular fatty acid metabolism which is caused by an inherited defect in mitochondrial oxidative phosphorylation [33]. These data suggest that high fat diet induced mitochondrial dysfunction and intramyocellular accumulation of lipid, resulting in insulin resistance. Many studies have reported that NADH-coenzyme Q oxidoreductase (mitochondrial OXPHOS complex I) is impaired in skeletal muscles of long-term high fat/ high sucrose diet and type 2 diabetes [29, 62]. This study confirmed that the association between the expression levels of RXRα and IRS1 in the myotubes treated with mitochondrial OXPHOS complex I inhibitor as well as in the skeletal muscles of high fat/ high sucrose diet. Perhaps in this study, high fat/ high sucrose diet had impaired mitochondrial OXPHOS complex I, resulting in the decrease of RXRα in skeletal muscle. Interestingly, in this study, the correlation between IRS1 and RARα, IRS2 and RXRα and IRS2 and RARα also showed statistically significant p values. Yun Chau Long et al have explained IRS1 and IRS2 coordinate skeletal muscle growth and metabolism via the Akt and AMPK pathways [63]. Bouzakri K et al have showed 9-cis retinoic acid induces IRS2/PI3-kinase signaling pathway and increases glucose transport in human skeletal muscle cells [64]. To presume, RXRα may associate the regulation of IRS1 with IRS2 in skeletal muscles and RARα seems to be involved in those regulations as a partner for RXRα, especially in high fat/ high sucrose diet.

To summarize, in the current study, the association between the expression levels of IRS1 and RXRα were examined in mitochondrial dysfunction-induced

myotubes as well as in skeletal muscles of high fat/ high sucrose diet. Moreover, the activation of RXRα by its ligands restored the expression level of IRS1 which was down-regulated in the myotubes having reduced activity of mitochondrial OXPHOS complex I. These data support that RXRα is at least partially involved in the regulation of IRS1 in those mitochondrial dysfunction-induced states but has a great potential to restore the levels of IRS1 only by its ligands. Further studies are needed to reveal the exact mechanism and to assess the different or shared characters of its ligands to determine in which way RXRα can be activated adequately.

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국문 초록

미토콘드리아는, 주요 영양소인 포도당과 지방의 대사 과정에서 에너지를 생산하는, 잘 알려진 세포 기관 중 하나이다. 이러한 미토콘드리아 기능 이상은 췌장 베타세포에서의 인슐린 분비 기능 손상과 간과 근세포와 같은 인슐린 표적세포에서의 인슐린 저항성을 유발한다고도 알려져 있다. 본 연구실에서는, 이전에 시행한 미토콘드리아 DNA A3243G 돌연변이에 대한 연구에서 리간드 자극에 의해 활성화되는 전사인자 중 하나인 retinoid X receptor α (RXRα)의 자극이 미토콘드리아의 기능을 회복함을 발견하였기에, 미토콘드리아 기능 이상으로 손상된 인슐린 신호 전달 과정에 이것의 자극이 어떠한 영향을 미칠 수 있는지 본 연구과정을 통해 더 알아보고자 하였다. 특히, 본 연구에서 RXRα는, 인슐린이 표적세포에 도달했을 때 세포 내 신호 전달 과정들을 자극시키는 주요 물질인 insulin receptor substrate 1(IRS1)과 함께 미토콘드리아 산화적 인산화 (OXPHOS) 복합체 기능이 저해된 근육 세포와 고지방/고자당 식이 생쥐의 골격근 조직에서 줄어있음이 확인되었다. 그러나, 리간드에 의한 RXRα 자극은 미토콘드리아 OXPHOS 복합체 기능이 저하된 근육세포에서 줄어있던 IRS1의 단백질 및 mRNA 발현 양을 회복시켰으며, 이것은 미토콘드리아 OXPHOS 복합체가 매개하는 ATP 생산의 회복 또한 동반하였다. 더불어, Transient transfection 과 luciferase assay system을 통해,

RXRα 의 과 발현 혹은 자극에 의해 IRS1 promoter의 활성이 증가됨을 확인하면서, 아마도 RXRα가 직접적으로 promoter에 붙게 됨에 따라 IRS1의 발현을 조절할 것임을 보였다. 이러한 결과는 RXRα가 미토콘드리아 기능이상으로 유도된 인슐린 저항성 상태에서 인슐린 신호전달체계를 개선시키는 데에 있어 잠재적 치료 표적이 될 수 있음을 시사하고 있다.

Key words: Insulin receptor substrate 1, Retinoic X receptor α, RXR 특이적 리간드, 미토콘드리아 기능 이상, 인슐린 저항성, C2C12 근육세포

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