

MINOR (NR4A3) Overexpression in Mouse Skeletal Muscle Enhances Insulin Action

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Abstract

MINOR (Mitogen-Inducible Nuclear Orphan Receptor) is one member of the NR4A3 nuclear orphan receptor family which are immediate early gene products involved in neuroendocrine regulation, neural differentiation, liver regeneration, cell apoptosis, and mitogenic and inflammatory stimulation in different cell types. We have found that MINOR can modulate insulin action and the glucose transport system in 3T3-L1 adipocytes; however, MINOR is highly expressed in skeletal muscle and its function *in vivo* is not well understood.

To determine the role of MINOR *in vivo*, we have generated a mouse model that has the MINOR gene specifically expressed in the skeletal muscle using a muscle creatine kinase (MCK) promoter, and investigated whether the gene functions of MINOR would be linked to insulin action *in vivo* since skeletal muscle is one of the primary target tissues for insulin action.

We demonstrate that these MCK-MINOR transgenic mice have reduced body weight due to a reduction of fat mass inside the body. Mice with MINOR overexpression also have improved insulin and glucose tolerances, reduced plasma levels of triglyceride, cholesterol and free fatty acid as well as enhanced expression of genes which are related to insulin action and its signaling pathways. Thus, MINOR functions in skeletal muscle act to improve insulin sensitivity and glucose intolerances and regulate insulin action and lipid and energy expenditure process.

Keywords: NR4A Orphan Nuclear Receptors; Muscle; Insulin and glucose; Intolerances; Obesity; Diabetes

Introduction

Type 2 diabetes is a disease caused by defects in insulin secretion combined with the presence of insulin resistance in peripheral target tissues which mainly include skeletal muscle, adipose tissue, and liver. To better understand the molecular defects responsible for human insulin resistance, we previously assessed differential gene expression in human skeletal muscle biopsies from insulin-sensitive and -resistant individuals using cDNA microarray technology [1]. These studies identified one of the differentially expressed genes that are members of the NR4A (NGFI-B) family of orphan nuclear receptors within the greater steroid/thyroid receptor superfamily, namely, MINOR (Mitogen-Inducible Nuclear Orphan Receptor).

MINOR (also known as NR4A3, NOR-1, TEC, and CHN) was originally identified as a protein induced in primary cultures of rat embryonic forebrain neurons undergoing apoptosis [2]. Based on homology within the DNA binding domain, there are two additional members of the NR4A (NGFI-B) family of orphan nuclear receptors, Nurr1 (also known as NR4A2, TINUR, and NOT) and Nur77 (also known as, NR4A1, NGFI-B, and TR3). These NR4A/NGFI-B proteins are immediate early response gene products that are involved in neuroendocrine regulation, neural differentiation, liver regeneration, cell apoptosis, and mitogenic and inflammatory stimulation in different cell types [3-8].

Based on our unpublished data, MINOR is highly expressed in human skeletal muscle but almost undetectable in other tissues. We have recently overexpressed MINOR in a mouse skeletal muscle cell line, C2C12, and found that overexpression of MINOR in these cells can lead to a significant increase in glucose transport activity in the presence of insulin and insulin-mediated AKT phosphorylation was also increased by the MINOR overexpression in these cells [9]. These results and our previous data from adipocytes [10] confirmed that MINOR is able to enhance insulin sensitivity in these insulin target cells.

To further investigate whether MINOR has the same effects on increasing insulin sensitivity *in vivo*, we have generated a mouse model, MCK-MINOR, which has the MINOR gene specifically expressed in the skeletal muscle using a muscle creatine kinase (MCK) promoter. Our current studies have shown that MINOR overexpression in skeletal muscle results significant improved systemic insulin sensitivity and glucose tolerance *in vivo* and MINOR also enhances some key components of insulin signaling pathway for insulin action in skeletal muscle, suggesting that MINOR as a novel modulator to function in skeletal muscle for modulating insulin actions.

Materials and Methods

Experimental mice

Fusion cDNAs, containing the full length MINOR coding sequence and a V5 epitope tag, were cloned into a MCK enhancer/promoter

cassette vector (a kind gift from Drs. Morris Birnbaum and Ron Kahn). Before microinjection into mouse embryos, the gene fragments (9.5 kb) were purified. Transgenic animals were created by injections of the appropriate DNA fragments into the pronucleus of inbred FVB single cell embryos. Three independent lines of MINOR transgenic mice were obtained and identified by Southern blotting or by PCR. Tissue specific expression was verified by RT-PCR with one primer designed in the V5 sequences and another located in the MINOR sequences. Animals were housed in colony cages and maintained on a 12 hours light/12 hours dark cycle. The animal care and procedures were approved by the Animal Resources Program (ARP) of the University of Alabama at Birmingham.

To investigate the metabolism of these MINOR transgenic mice, a regular diet or a high fat diet (60% kcal% fat) from the Research Diets Company (New Brunswick, NJ) was fed to control and transgenic mice from 4 weeks to 36 weeks for measuring mouse body weight and analyzing glucose metabolism and gene expression in skeletal muscle.

Immunoblots

Soleus muscles were carefully dissected from mice and quickly frozen in liquid nitrogen. Muscle tissues were homogenized with tissue lysis buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing freshly added protease inhibitor cocktail (Sigma). Twenty-five µg of protein per lane and known molecular weight markers from Bio-Rad (Hercules, CA) were separated by SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred onto nitrocellulose membranes and incubated overnight at 4°C with blocking solution (5% nonfat milk in TBS). The blocked membranes were incubated with gene specific antibodies, and then the horseradish peroxidase (HRP)-conjugated second antibody for 1 hour for each at room temperature, and washed three times for each with TBS buffer containing 0.1% Tween 20 for 15 minutes at room temperature with shaking. Immunodetection analyses were accomplished using the Enhance Chemiluminescence Kit (NEN Life Science Products, Boston, MA).

Glucose and insulin tolerance tests

Glucose tolerance tests were performed in mice after 12 hours fast. Insulin tolerance tests were performed in mice after 6 hours fast. Glucose and insulin solutions were injected into peritoneal cavity at the dose of 1.0 g/kg and 1.0 U/kg, respectively. Blood was collected via tail vein at different time points, and glucose levels were measured by the use of a glucometer (Precision). Serum lipid concentrations were determined by established assays as previously described [11].

Glucose transport activity assays

Glucose uptake assays in soleus muscle strips were performed as described before [12]. For measurement of glucose transport activity, muscle strips were treated with 100 nM insulin for one hour then the muscle strips were treated with 1 N NaOH at 70°C for 5 min; the aliquots of the supernatant were centrifuged and added to the scintillation mixtures and then counted for the isotope activities. In these experiments, the distribution space of radiolabeled L-glucose was used to correct for nonspecific carryover of radioactivity with the cells and uptake of hexose by simple diffusion.

Statistics

Experimental results are shown as mean ± SE. Statistical analyses were performed by unpaired Students' *t*-test assuming unequal variance unless otherwise indicated. Significance was defined as $p < 0.05$.

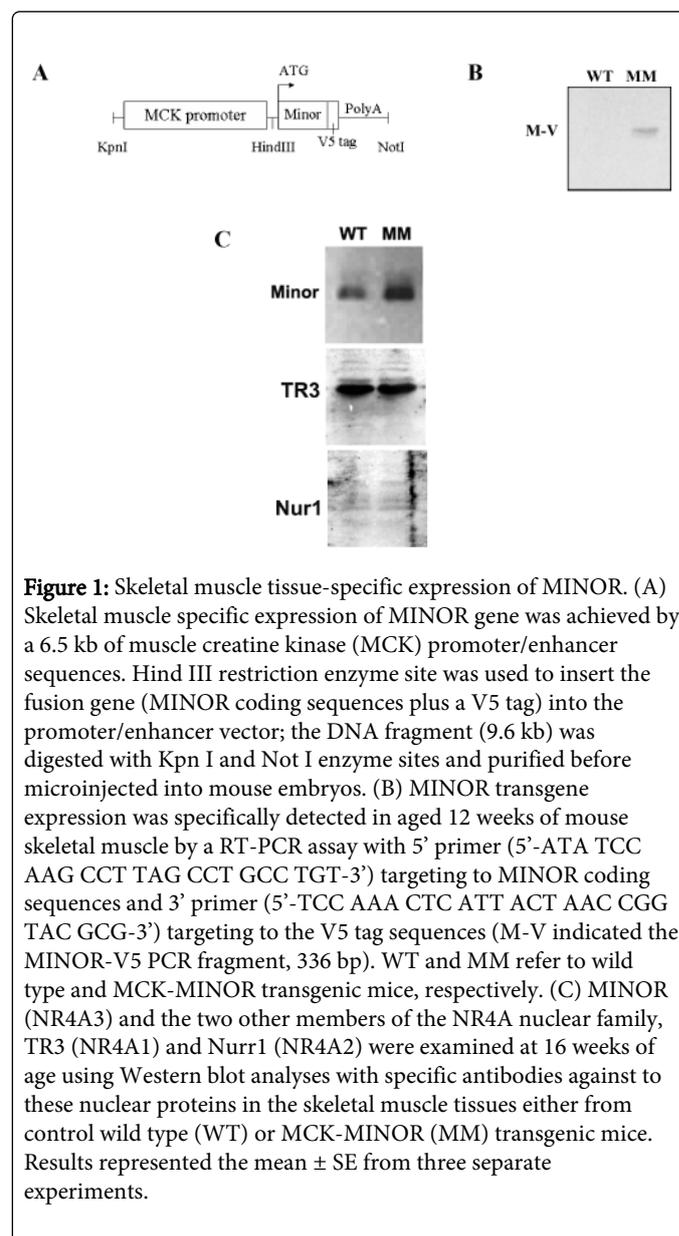


Figure 1: Skeletal muscle tissue-specific expression of MINOR. (A) Skeletal muscle specific expression of MINOR gene was achieved by a 6.5 kb of muscle creatine kinase (MCK) promoter/enhancer sequences. Hind III restriction enzyme site was used to insert the fusion gene (MINOR coding sequences plus a V5 tag) into the promoter/enhancer vector; the DNA fragment (9.6 kb) was digested with Kpn I and Not I enzyme sites and purified before microinjected into mouse embryos. (B) MINOR transgene expression was specifically detected in aged 12 weeks of mouse skeletal muscle by a RT-PCR assay with 5' primer (5'-ATA TCC AAG CCT TAG CCT GCC TGT-3') targeting to MINOR coding sequences and 3' primer (5'-TCC AAA CTC ATT ACT AAC CGG TAC GCG-3') targeting to the V5 tag sequences (M-V indicated the MINOR-V5 PCR fragment, 336 bp). WT and MM refer to wild type and MCK-MINOR transgenic mice, respectively. (C) MINOR (NR4A3) and the two other members of the NR4A nuclear family, TR3 (NR4A1) and Nurr1 (NR4A2) were examined at 16 weeks of age using Western blot analyses with specific antibodies against to these nuclear proteins in the skeletal muscle tissues either from control wild type (WT) or MCK-MINOR (MM) transgenic mice. Results represented the mean ± SE from three separate experiments.

Results

Generation of MINOR skeletal muscle transgenic mice

Overexpression of MINOR was selectively performed in mouse skeletal muscle using the MCK promoter. Skeletal muscle specific expression of MINOR gene was achieved by ligating 6.5 kilobases (kb) of the MCK promoter/enhancer to the MINOR coding sequences (Figure 1A). MINOR transgene expression was restricted to skeletal muscle (Figure 1B) and its expression level was 2.35-fold ($p < 0.05$) when comparing to endogenous gene expression in the control wild

type mice (Figure 1C). The tissue specificity and high efficiency of MINOR transgene activity were not observed in other metabolic tissues, such as, liver and adipose tissue. Furthermore, other two members of the NR4A nuclear family, TR3 (NR4A1) and Nurr1 (NR4A2) expression levels showed no significant changes in skeletal muscle due to the overexpression of MINOR in this tissue (Figure 1C), suggesting that there are no functional compensations occurred among MINOR and other two nuclear receptor family members in skeletal muscle.

Reduction of whole body weight in transgenic mice

Although there was no gross apparent abnormality in reproduction, growth, and development of MCK-MINOR transgenic mice under standard laboratory conditions in either sex, we observed that transgenic mice exhibited a reduction (15%) in total body weight between 5 to 11 weeks on high fat diet when compared to control wild type animals despite normal axial growth (Figure 2A), suggesting that overexpression of MINOR in skeletal muscle might influence adiposity in these animals. To assess whether this reduction in total body weight gain in MCK-MINOR transgenic mice is related to local and systemic alterations in adiposity, we dissected these mice for analyzing abdominal fat pads and also performed dual energy X-ray absorption (DEXA) analyses for total body composition. Both of dissection and DEXA analyses of MCK-MINOR mice demonstrated significantly reduced total body adipose mass in these mice when compared to control wild type animals (Figure 2B), suggesting that overexpression of MINOR in skeletal muscle can significantly affect adiposity, especially adipose tissue accumulation in the body under high fat diet condition.

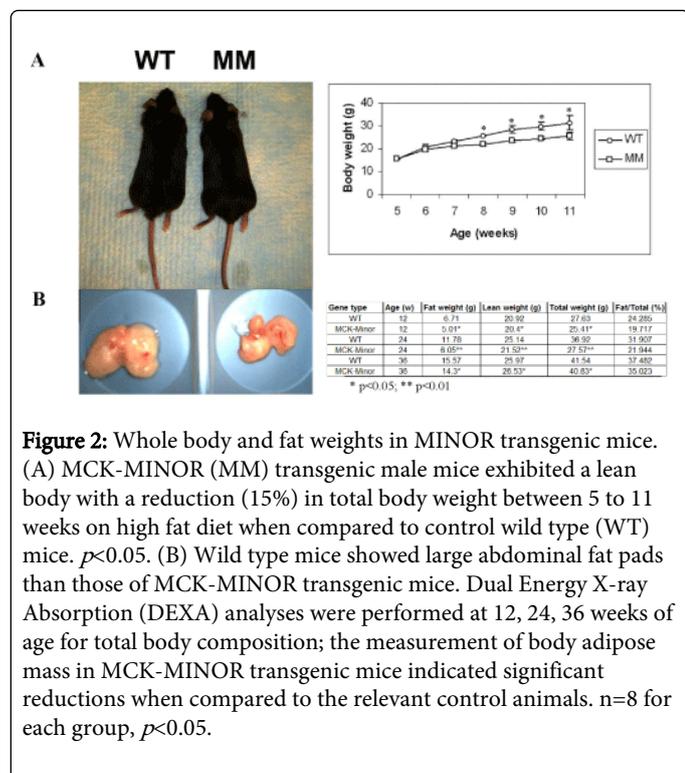


Figure 2: Whole body and fat weights in MINOR transgenic mice. (A) MCK-MINOR (MM) transgenic male mice exhibited a lean body with a reduction (15%) in total body weight between 5 to 11 weeks on high fat diet when compared to control wild type (WT) mice. $p < 0.05$. (B) Wild type mice showed large abdominal fat pads than those of MCK-MINOR transgenic mice. Dual Energy X-ray Absorption (DEXA) analyses were performed at 12, 24, 36 weeks of age for total body composition; the measurement of body adipose mass in MCK-MINOR transgenic mice indicated significant reductions when compared to the relevant control animals. $n = 8$ for each group, $p < 0.05$.

Improved glucose and insulin tolerances in transgenic mice

Next, to further investigate systemic insulin sensitivity *in vivo*, we performed Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT) in MCK-MINOR mice and control wild type animals. Glucose tolerance tests revealed a higher degree of hyperglycemia in wild type animals through the experiments when compared to MCK-MINOR mice either on regular diet or high fat diet (Figure 3A). As shown in Figure 3B, the increase in insulin sensitivity in insulin tolerance tests was most dramatic in MCK-MINOR mice on either regular diet or high fat diet when compared to the wild type control animals. These results demonstrated that MINOR overexpression in skeletal muscle resulted in a significant protection from systemic hyperglycemia and insulin resistance.

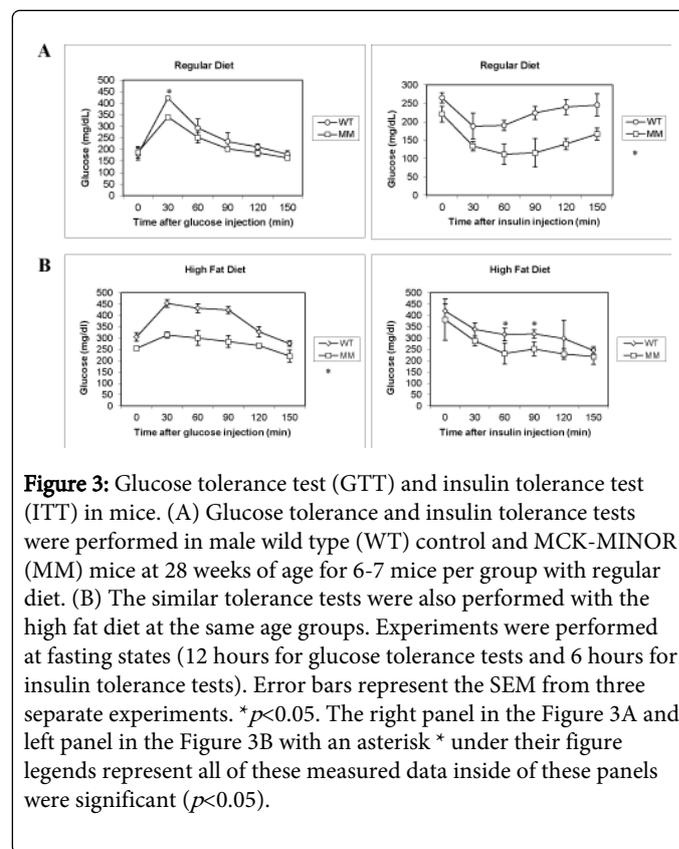
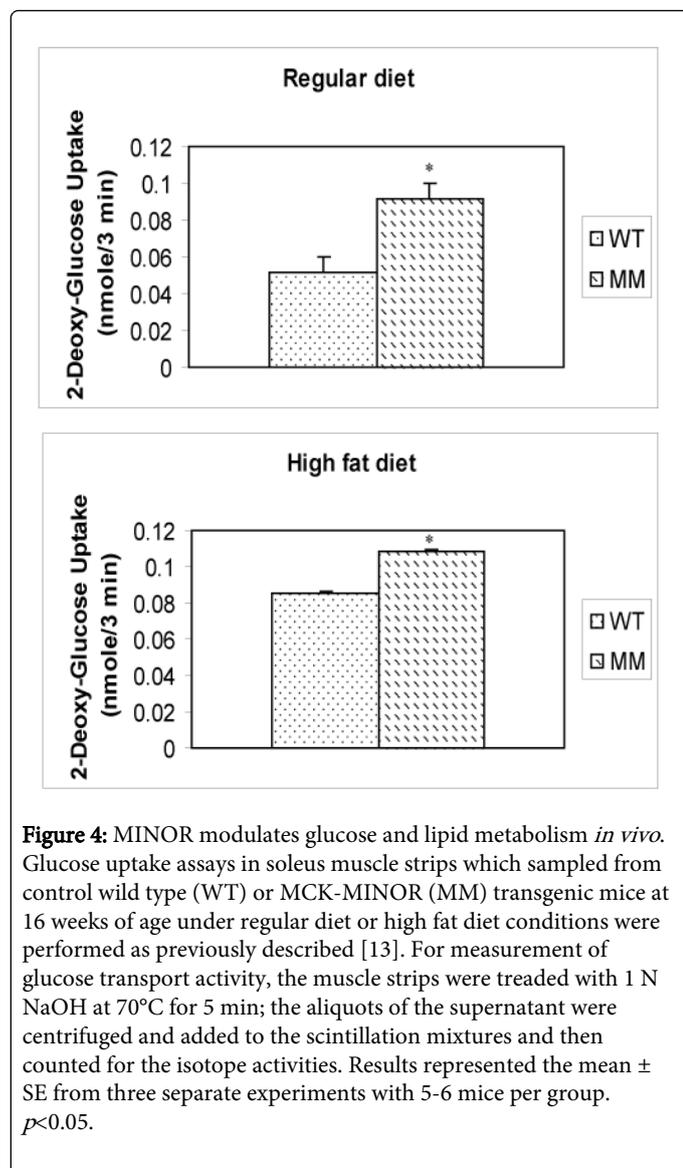


Figure 3: Glucose tolerance test (GTT) and insulin tolerance test (ITT) in mice. (A) Glucose tolerance and insulin tolerance tests were performed in male wild type (WT) control and MCK-MINOR (MM) mice at 28 weeks of age for 6-7 mice per group with regular diet. (B) The similar tolerance tests were also performed with the high fat diet at the same age groups. Experiments were performed at fasting states (12 hours for glucose tolerance tests and 6 hours for insulin tolerance tests). Error bars represent the SEM from three separate experiments. * $p < 0.05$. The right panel in the Figure 3A and left panel in the Figure 3B with an asterisk * under their figure legends represent all of these measured data inside of these panels were significant ($p < 0.05$).

Minor modulates glucose and lipid metabolism *in vivo*

As one of the primary target tissues for insulin action, skeletal muscle is the main site of insulin stimulated glucose uptake [13,14]. To determine the role of MINOR in glucose homeostasis, we assessed insulin stimulated glucose transport activity in both skeletal muscle tissues of MCK-MINOR and wild type mice either on regular diet or high fat diet since glucose transport is the rate-limiting step in insulin's ability to stimulate glucose uptake and metabolism (Figure 4). Our results indicated that insulin augmented glucose uptakes in MCK-MINOR mice were averagely increased by 38% ($p < 0.05$) on regular diet and 20% ($p < 0.01$) on high fat diet compared to their wild type controls. These results indicated that MINOR can significantly enhance insulin stimulated glucose transport capacity in skeletal muscle, suggesting a novel role of MINOR in improving insulin sensitivity in this target tissue.

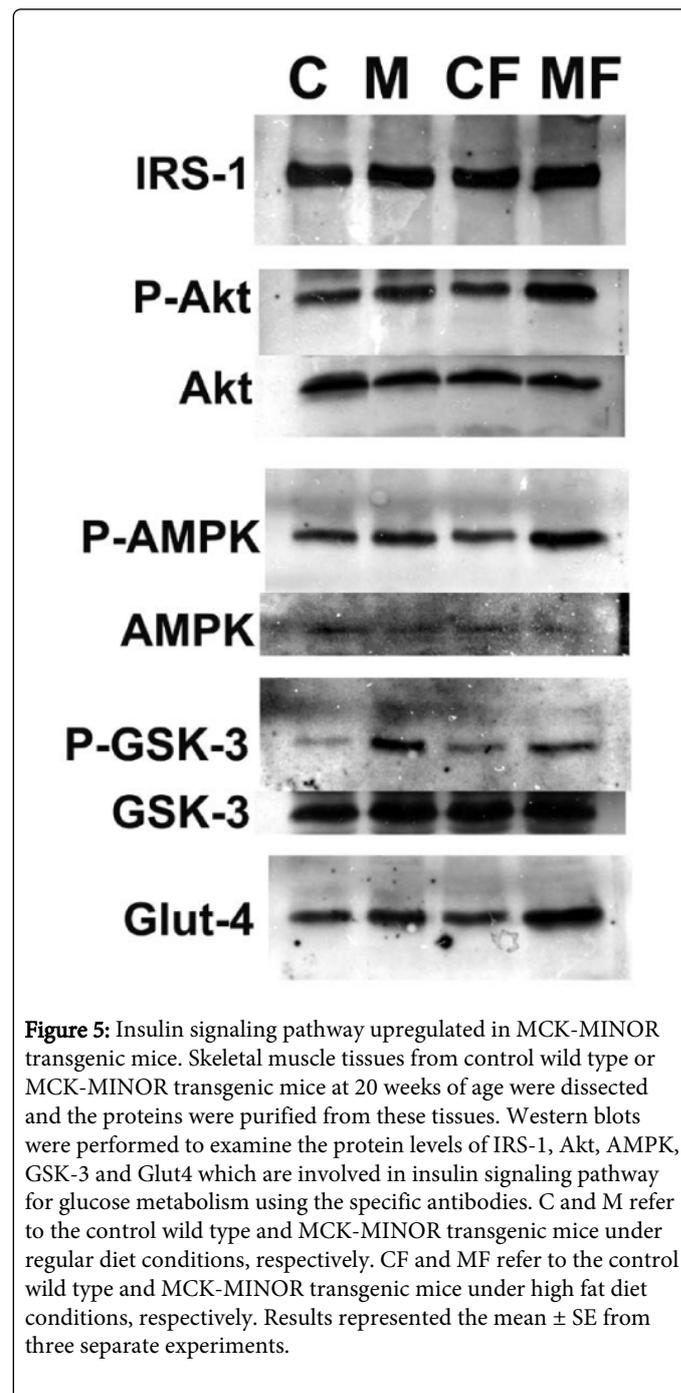


Enhanced insulin signaling pathways in transgenic mice

We next asked whether the MINOR enhanced insulin action in skeletal muscle alters specific molecules in insulin signaling pathway. To investigate this question, we examined the insulin stimulated phosphorylation levels of Akt^{Ser473}, AMPK^{Thr172}, GSK-3^{Ser21/Ser9} and expression of IRS-1 and Glut4 in soleus skeletal muscle tissues from the MCK-MINOR and control wild type mice on both regular diet and high fat diet (Figure 5). Our data showed that all of these insulin stimulated phosphorylation levels in skeletal muscle of MCK-MINOR mice were highly favorable for insulin action than their controls on either regular diet or high fat diet. In addition, we also observed higher expression levels of IRS-1 and Glut4 in skeletal muscle of the MCK-MINOR transgenic mice than those from their wild type control animals.

Reduction of lipid accumulation in transgenic mice

Since altered adiposity and increased tissue insulin sensitivity often influence lipid profiles *in vivo* [15,16], we wondered whether lipid levels in plasma from these transgenic animals will be affected due to the MINOR overexpression in skeletal muscle. Our results demonstrated that the levels of triglyceride, cholesterol and free fatty acids in plasma from MCK-MINOR transgenic mice were significantly lower than those from the wild type control animals (Figure 6). We reasoned that this lipid environment would be favorable to insulin action *in vivo*.



Discussion

The present results demonstrate for the first time that the MINOR overexpression in skeletal muscle results a reduction of body weight with reduced of fat deposit; these transgenic mice have significant improved insulin sensitivity and glucose tolerance in addition of reduced plasma levels of triglyceride, cholesterol and free fatty acids. Moreover, MINOR overexpression increased insulin-mediated phosphorylations of Akt, AMPK, and GSK-3, which are critical components of insulin signal pathway. These data implicated a role for MINOR in the modulation of insulin action and in the pathogenesis of insulin resistance.

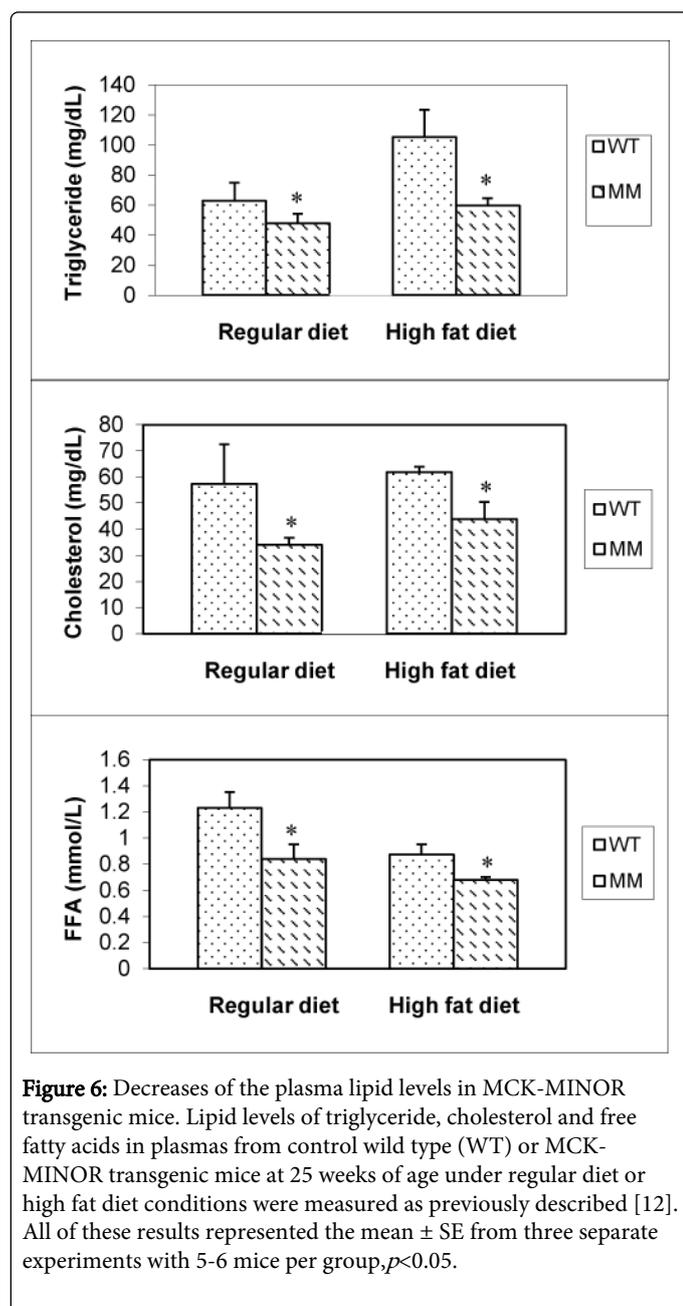


Figure 6: Decreases of the plasma lipid levels in MCK-MINOR transgenic mice. Lipid levels of triglyceride, cholesterol and free fatty acids in plasmas from control wild type (WT) or MCK-MINOR transgenic mice at 25 weeks of age under regular diet or high fat diet conditions were measured as previously described [12]. All of these results represented the mean \pm SE from three separate experiments with 5-6 mice per group, $p < 0.05$.

Numerous studies have identified NR4A orphan nuclear receptors as regulators for metabolic function in a cell- and tissue-specific manner [17]. Skeletal muscle is one of important target tissues for

insulin function and also is a major tissue for lipid and glucose metabolism *in vivo*. Previous studies have shown that Nur77 (NR4A1), another member of the NR4A orphan nuclear receptors, knockout mice exhibited metabolic changes under high fat diet condition [18,19]. These Nur77 knockout mice showed increased weight gain, insulin resistance in skeletal muscle, and slower blood glucose clearance with decreased expression of Glut4 gene, suggesting that Nur77 receptor play an important role in regulating the expression of metabolic genes specifically involved in glucose transport, insulin signaling. Conversely, our MINOR skeletal muscle tissue specific transgenic mice presented very similar results with reduced body weight, improved insulin and glucose intolerances, reduced plasma levels of triglyceride, cholesterol and free fatty acid as well as enhanced expression of genes which are related to insulin action and its signaling pathways. Interestingly, in these Nur77 knockout mice there were the dramatic compensatory increases in MINOR expression detected [20,21], however, we have not observed detectable changes of Nur77 or Nurr1 genes in the MINOR transgenic mice when compared to WT mice, probably due to the differences between global knockout and tissue specific overexpression. Whichever, these studies have indicated that expression and function of MINOR and Nur77 nuclear receptors are strongly associated with metabolic activities in skeletal muscle.

In summary, we have first time found that MINOR overexpression in skeletal muscle results a reduction of body weight with reduced of fat deposit; these transgenic mice have significant improved insulin sensitivity and glucose tolerance in addition of reduced plasma levels of triglyceride, cholesterol and free fatty acids. MINOR also enhances some key components of insulin signaling pathway for insulin action in skeletal muscle. Thus, MINOR as a novel insulin enhancer to function in skeletal muscle increases insulin action and energy expenditure process *in vivo*.

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