ROLES OF NR4A3 IN INSULIN SENSITIVITY IN SKELETAL MUSCLE

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ABSTRACT

For the past several decades, the prevalence of Type 2 Diabetes and the metabolic syndrome has been increasing. Insulin resistance is the central pathogenic event for these diseases. We previously reported that the NR4A family of orphan nuclear receptors augments insulin sensitivity in adipocytes. We now found that lentiviral mediated NR4A3 hyper-expression increased insulin stimulated glucose transport (ISGT) in C2C12 myocytes while NR4A3 knock-down exhibited opposite effect. We also examined the effects of prostaglandin A2 (PGA2) on insulin action and NR4A3 trans-activation in C2C12 myocytes. PGA2 augmented ISGT and insulin stimulated AKT phosphorylation in C2C12 myocytes. Importantly, PGA2 treatment led to increased ISGT in NR4A3 over-expressing C2C12 myocytes while the sensitizing effect of PGA2 was diminished in NR4A3 knock-down myocytes. These novel results suggest that a working model that PGA2 augments insulin action in myocytes via a mechanism involving NR4A3, which provides a proof of principle for future anti-diabetic medication development.

Exercise is used therapeutically to reverse insulin resistance. Interestingly, exercise was also reported to induce NR4A mRNA expression. Given the established role of NR4As in insulin sensitivity modulation, it was imperative to explore whether exercise regulates NR4A protein expression. Therefore, we measured muscle NR4A protein in 16 healthy young and 11 old males following acute (AE) and chronic (CE) resistance
exercise. Muscle biopsies were performed at baseline and 24 hr after both AE and CE. NR4A1 levels are similar in young and old at baseline, increased by AE in young only, and reduced in young and old following chronic resistance training. NR4A3 protein levels are diminished in elderly subjects but are not affected by AE or CE. Our data indicate differential effects of age and exercise on muscle NR4A protein expression, which may shed a light to address whether NR4As are involved in the beneficiary effects of exercise.

Keywords: NR4A3, MINOR, insulin sensitivity, prostaglandin A$_2$, glucose transport, skeletal muscle, C2C12 cells, insulin action, exercise, NR4A1
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INTRODUCTION
INSULIN RESISTANCE IN SKELETAL MUSCLE

For the past several decades, the prevalence of Type 2 Diabetes and the metabolic syndrome has been increasing. Insulin Resistance, which is defined as impaired peripheral tissue response to endogenously secreted insulin, is the central event in the pathogenesis of these diseases. Insulin Resistance is typically manifested as decreased insulin stimulated glucose transport in skeletal muscle and adipose tissue, together with impaired suppression of hepatic glucose production.

Among all the insulin target tissues, skeletal muscle plays a pivotal role in the development of Insulin Resistance. It is responsible for 85% of whole body insulin stimulated glucose transport. In skeletal muscle, insulin stimulates glucose transport by facilitating the translocation of glucose transporter molecules, mainly GLUT 4, from cytoplasm to surface. This signaling cascade begins with the binding of insulin to its receptor. Subsequently there is consecutive phosphorylation of cytosolic proteins including Insulin Receptor Substrate (IRS), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/AKT). IRS serves as the docking site for various signaling proteins such as PI3K. Four major IRS isoforms have been discovered as the insulin signaling mediators and IRS-1 and IRS-2 appear to be the predominant forms in skeletal muscle. Like IRS, four isoforms of PI3K in human skeletal muscle were also proposed recently by different groups. PKB/AKT and some Protein Kinase C (PKC) isoforms
have been shown to be important mediators of insulin signaling downstream of IRS and PI3K. Although the exact underlying molecular mechanism by which insulin exerts its biological action remains poorly understood despite decades of research effort, the overall outcome of this signaling cascade is the translocation of GLUT4 from intracellular reservoir compartments to the plasma membrane. Glucose is transported across GLUT4 to cytosolic compartments and further metabolized via aerobic and anaerobic pathways.

Currently, the mechanism causing insulin resistance in skeletal muscle is still poorly understood. Several hypotheses have been proposed and each of these hypotheses is supported by a considerable body of data despite the existence of negative evidence.

*Insulin resistance and inflammation*

Most commonly, insulin resistance in skeletal muscle is considered to be the outcome of a complex interaction involving several different tissues. Adipose tissue secretes various adipokines and inflammatory mediators, which have been implicated in the development of insulin resistance in skeletal muscle. TNF-α is one of the major inflammatory mediators secreted by adipose tissue. Upon activation of NF-κB, it suppresses many genes involved in glucose and free fatty acid utilization and triggers the release of free fatty acid from adipose tissue to blood circulation. It has been shown that free fatty acid release impairs insulin sensitivity in skeletal muscle. TNF-α can also act together with other cytokines such as IL-1. IL-1 and TNF are produced upon the same stimuli, mutually enhance each others’ production, and act synergistically. However,
much less is known about IL-1 in insulin resistance development compared to TNF, especially in skeletal muscle. In adipocytes, it is currently believed that IL-1α impairs insulin action by phosphorylation of serine residues on IRS-1, which partially inhibits IRS-1 tyrosine phosphorylation and PI3K activity. One the other hand, chronic IL-1β exposure significantly inhibits IRS-1 expression and subsequent AKT activity, which is associated with a decrease of GLUT4 expression and translocation. Interestingly, another cytokine derived from adipose tissue, IL-6, exerts acute beneficial effect on insulin action in cultured skeletal muscle cells via a mechanism involving IRS-1 recruitment to IL-6R complex. Moreover, it has been reported that IL-6 increases basal and insulin stimulated glucose transport in human myocytes and muscle fibers by activation of AMP-activated protein kinase. In contrast, evidence has shown that chronic IL-6 exposure decreases insulin stimulated glucose transport to muscle. Thus, a dual role of IL-6 on insulin sensitivity in skeletal muscle could be documented; it acutely promotes insulin sensitivity, while chronic exposure leads to an insulin resistant state by activating JNK, SOCS-3 and protein tyrosine phosphatase 1B.

*Insulin resistance and lipotoxicity*

Lipotoxicity, which is referred as the elevation of lipids or lipid metabolites within tissues or blood circulation, is a postulated mechanism for skeletal muscle insulin resistance. In humans, the physiological serum free fatty acid concentration ranges from 350 to 550 μmol/L and increases with insulin resistance and type 2 diabetes. Muscle biopsy data have shown that insulin resistance is strongly correlated with
intramyocellular lipids in undertrained subjects. Consistently, lipid infusion has been shown to increase intramyocellular lipid content\textsuperscript{20,21} and impair insulin stimulated glucose transport in both healthy and type 2 diabetes subjects.\textsuperscript{22} DeFronzo et al hypothesized that the increased free fatty acid availability provides abundant substrate for intermediate metabolism, leading to increased ratios of NADH/NAD\textsuperscript{+} and acetyl-CoA/CoA. It also facilitates beta-oxidation, which in turn impairs glucose transport and oxidation.\textsuperscript{23} The inhibition of insulin stimulated glucose transport and associated insulin signaling events such as IRS tyrosine phosphorylation, PI3K activity and PKB/AKT activity, are strongly correlated with increased serum fatty acid concentration elicited by lipid infusion.\textsuperscript{24} Moreover, human studies have shown that lipid infusion elicited insulin resistance is associated with an increase of PKC activity, an increased diacylglycerol concentration and a decrease of IκB-α, a physiological NF-κB inhibitor.\textsuperscript{25} In short, these data suggests that lipid exposure stimulates skeletal muscle to shift substrate use toward fatty acids, which induces skeletal muscle insulin resistance and inhibition of glucose transport.

However, some human studies have shown contradictory results. A prospective cohort study of 11380 human subjects reported that participants adhering to Mediterranean diet (low in saturated fat and rich in mono- and poly-unsaturated fat) have a lower incidence of diabetes. Even when focusing on saturated fat diet, there are abundant data which have not confirmed a clear correlation between a high saturated fat diet and increased insulin resistance by either insulin clamp or intravenous glucose tolerance test.\textsuperscript{20,26-30}
As a matter of fact, there is a growing interest that increased levels of fatty acid metabolites, rather than intramyocellular lipids per se, may be involved in underlying mechanism for lipid induced insulin resistance. Several lipid metabolites, including diacylglycerol\textsuperscript{25,31}, ceramide and long chain fatty acyl CoA\textsuperscript{32} have been proposed as molecules responsible for insulin resistance.

Diacylglycerol is produced via lipid hydrolysis or de novo synthesis and is reported to be elevated in obese patients compared with controls.\textsuperscript{33} It is generally believed that the diacylglycerol induced activation of PKC, particularly some novel PKC isoforms(PKC $\delta$, $\varepsilon$, $\eta$), is responsible for lipid induced insulin resistance. Human studies have shown that lipid infusion induced insulin resistance is strongly correlated with elevated muscle diacylglycerol concentration, elevated PKC activity and decreased IkB protein expression.\textsuperscript{25} A rat model study confirmed these observations and found an increased IRS-1 serine phosphorylation together with a decreased PI3K activity, which suggests that the insulin signaling cascade in skeletal muscle is impaired upon diacylglycerol accumulation.\textsuperscript{31} Consistent with the hypothesized role of diacylglycerol in insulin resistance, reduction of diacylglycerol’s effect, either via promoting fatty acid oxidation or increasing intramyocellular lipid synthesis, results in improvement of insulin sensitivity.\textsuperscript{34-36}

Ceramide is a by-product of long chain saturated fatty acid metabolism and it is also the prototype for more complex sphingolipids. Higher than physiological concentrations of ceramide have been observed in insulin resistant patients.\textsuperscript{37} Consistently, exercise training, which is well known for improving insulin sensitivity, is also associated with reduced ceramide.\textsuperscript{35,36} Lipid induced insulin resistance has been associated with
elevated total levels of ceramide in skeletal muscle. These data strongly support the role of ceramide in the development of skeletal muscle insulin resistance. The underlying mechanism is believed to involve disruption of important insulin signaling events such as inhibition of PKB/AKT activity and IRS-1 tyrosine phosphorylation.\textsuperscript{38,39}

There is a growing pool of evidence showing that saturated fatty acids and long fatty acids are associated with skeletal muscle insulin resistance. For instance, in a human study, Manco et al reported that increased saturated fatty acids and long chain fatty acyl CoA content in skeletal muscle are both associated with insulin resistance.\textsuperscript{40} Using different models, Chen et al confirmed an association between long chain fatty acyl CoA and insulin resistance.\textsuperscript{41} In cultured myocytes, short chain fatty acid exposure tends to generate less diacylglycerol and ceramide and cause less inhibition of PKB/AKT activity than long chain fatty acid exposure.\textsuperscript{42} Consistent with these results, Bajaj et al found that acipimox (a niacin related hypolipidemic agent) treatment reduces free fatty acid levels, improves insulin sensitivity and decreases total intramuscular long chain fatty acyl CoA concentration.\textsuperscript{43}

\textit{Mitochondrial defects and insulin resistance}

Numerous reports have shown that impaired mitochondrial function is associated with both type 2 diabetic patients\textsuperscript{44-51} and insulin resistant subjects (but not diabetic).\textsuperscript{4,51,52} Smaller mitochondrial size\textsuperscript{48}, down-regulated expression of oxidative phosphorylation genes\textsuperscript{46,47}, impaired mitochondrial enzyme activity and ATP production\textsuperscript{44,45}, lower ATP
synthetic flux rates and TCA cycle rates, and slower postischemic phosphocreatinine recovery have been observed in such subjects.

“Metabolic inflexibility”, referred as impaired ability to switch from predominant fat metabolism in fasting states to predominant glucose utilization in fed states, is another manifestation of reduced mitochondrial function. Metabolic inflexibility is also reported to be correlated with insulin resistance in both healthy subjects and subjects with type 2 diabetes family history.

However, the association between mitochondrial function impairment and insulin resistance has aroused considerable discrepancy and debate. For instance, Morino et al shown that there is a reduction in insulin signaling, mitochondrial density and cytochrome c oxidase activity in insulin resistant subjects. However, they failed to observe the concordant changes in succinate dehydrogenase expression, pyruvate dehydrogenase expression, mtDNA copy number, PGC-1α expression, PGC-1β expression and TFAM expression. Another group using in vivo MRS estimates of mitochondrial capacity found no difference between long term type 2 diabetic patients, prediabetic patients, recently diagnosed diabetic patients, and normal control subjects. A comparison between diabetic patients in North Europe and those in India revealed that Indian diabetic patients with higher insulin resistance tend to display elevated higher mitochondrial capacity, thus disproving a possible connection between mitochondrial function defects and insulin resistance.

NR4A Orphan Nuclear Receptors
Nuclear receptors represent a large family of transcription factors with a broad biological function spectrum. They have been reported to be involved in development, differentiation, metabolism, and cancer. Lipid sensing nuclear receptors, such as PPAR, RXR, LXR, FXR, have been found to play important roles in metabolic regulation. Orphan nuclear receptors, so called due to the lack of identified ligands, represent another important branch of nuclear transcription factors. The NR4A family is a subgroup of orphan nuclear receptors and is composed of NR4A1 (also Nur77, NGFI-B or Testicular Receptor 3, TR3), NR4A2 (also TINUR, NOT or Nurr1) and NR4A3 (NOR-1, TEC, CHN and Mitogen inducible Nuclear Orphan Receptor, MINOR).

Among all three NR4A family members, NR4A1 is ubiquitously distributed in various highly metabolically active tissues, such as skeletal muscle, adipose, T-cells, brain and kidney etc. In contrast, NR4A3 is abundantly expressed in skeletal muscle and adipose, which are major peripheral glucose disposal tissues. The NR4A proteins bind to NGFI-B response element (NBRE) and putatively activate gene expression by a constitutive ligand-independent manner. In the presence of retinoic acid, NR4A1 and NR4A2, but not NR4A3 can heterodimerize with RXR and transactivate the DR5 response element composed of direct repeats separated by five nucleotides. NR4A response element sequences are shown in Figure 1.
Like other nuclear receptors, NR4A proteins are composed of a ligand binding domain, a DNA binding domain and an Activation Function (AF) domain. The NR4A members are well conserved in the DNA binding domain (~95%) and the ligand binding domain (~60%), but highly divergent in the AF domain.\textsuperscript{67} The N-terminal AF-1 domain is capable of recruiting cofactors by a ligand-independent manner.\textsuperscript{68} However, the C-terminal ligand binding domain is distinct from other nuclear receptors in that it lacks the classic hydrophobic cleft for recruitment of coactivators and corepressors.\textsuperscript{69} A schematic picture of NR4A structure is shown in Figure 2.
The NR4A nuclear receptors are widely distributed immediate early or stress response genes which can be induced by a wide range of physiological signals such as inflammatory cytokines\textsuperscript{70}, prostaglandins\textsuperscript{71}, growth factors\textsuperscript{72}, etc. A more detailed list of stimuli which triggers NR4A expression is available in Figure 3.\textsuperscript{66}

Figure 2. Schematic picture showing major domains of NR4A proteins.

Figure 3. Reported stimuli for NR4A expression. From reference 66.
Furthermore, this subgroup has been shown to be involved in various pathophysiological conditions, which include Parkinson’s disease\textsuperscript{73}, inflammation\textsuperscript{74}, atherosclerosis\textsuperscript{75} and cancer cell apoptosis\textsuperscript{76}. One or more NR4A receptors are reported to be involved in the neural differentiation regulation, liver regeneration, apoptosis, mitogenesis and inflammatory signaling cascades.\textsuperscript{72-76} The NR4A functions are listed as shown in Figure 4.\textsuperscript{66}

![Figure 4](image_url)

**Figure 4** List of physiological and pathophysiological functions of NR4As.

Recently, there is a growing interest in the role of NR4As in metabolism regulation. Given the fact that β-adrenergic signaling regulates lipid and glucose metabolism in skeletal muscle and that NR4As can be rapidly induced by β-adrenergic signaling\textsuperscript{81-84}, it is logical to hypothesize that NR4As play regulatory roles in β-adrenergic signaling in skeletal muscle. By knocking down NR4A1 expression in
C2C12 myocytes, Maxwell et al found reduced lipolysis and altered gene expression including AMPKγ3, fatty acid translocase and GLUT4, suggesting a role of NR4A1 in metabolic regulation. Consistently, Chao et al reported that adenovirus mediated NR4A1 overexpression results in profound gene expression changes associated with insulin action, glucose transport, glycolysis and glyconeogenesis. A more comprehensive list of NR4A target genes (either direct or indirect) is reviewed by Pearen et al.

Similarly, NR4A3 knock down in C2C12 myocytes led to reduced palmitate oxidation and increased lactate production and hypoxia-inducible factor-1α mRNA expression, indicating a predominant shift from aerobic to anaerobic metabolism. Furthermore, NR4A3 knock down also exerted a profound change in metabolic gene expression, including Lipin1, FOXO1, PGC1α, PDP1c and PDP1r. Interestingly, some NR4A3 target genes such as UCP2, UCP3, Lipin1 and PGC1α could also be induced by β-adrenergic signaling, indicating that NR4A3 may be a down-stream effector of β-adrenergic cascade.

It is worth noting that the spectrum of NR4A1 target genes and NR4A3 target genes are largely complementary rather than overlapping, suggesting a similar but not identical role of these two NR4A members. For instance, only one gene, caveolin 3 was reported to be down regulated by both NR4A1 and NR4A3 attenuation. However, UCP3 and UCP2 were reported to be suppressed by NR4A1 and NR4A3 attenuation, respectively.

So far, only NR4A1 has been manipulated in in vivo model in terms of metabolism in skeletal muscle. Electroporation mediated NR4A1 overexpression in rats
and attenuation in both rats and mice, NR4A1 null mice and NR4A1 agonist treatment models have been utilized in different studies. Consistent with the in vitro research data, many identical target genes have been found with in vivo models such as UCP3 and GLUT4.

In terms of phenotype, NR4A1 knockout mice did not exhibit major metabolic changes on normal diet. When challenged with high-fat diet, however, there is a panel of significant changes in knockout mice compared to the wild type control, including, but not limited to, increased weight gain, decreased energy expenditure (by indirect measurement of oxygen consumption), insulin resistance in skeletal muscle and slower blood glucose clearance. In skeletal muscle, there is a decreased expression of GLUT4 and other glucose disposal related genes, inhibited IRS-1 phosphorylation, elevated intramyocellular lipid level and lower glycolytic metabolites. These data strongly suggest impaired insulin sensitivity after NR4A1 knockout. Given the functional redundancy of NR4As and increased NR4A3 expression after NR4A1 knockout, it is reasonable to hypothesize that the NR4A1 knockout phenotype is partially masked by compensatory NR4A3 upregulation.

In addition, there is a considerable body of indirect evidence suggesting the role of NR4A in skeletal muscle metabolism. For instance, rats with low endurance capacity were reported to exhibit lower expression of NR4A1 and NR4A1 target genes such as UCP3, CD36 and AMPKγ3 compared to the high endurance group.(71) Furthermore, decreased β-adrenergic agonist mediated lipolysis and hormone sensitive lipase phosphorylation were observed in the low endurance group. Other reports showed that skeletal muscle NR4A1 expression was down-regulated after high fat diet challenged
mice and the expression of all three NR4As were decreased in obese and diabetic subjects. In contrast, exercise was reported to dramatically induce NR4A1 expression. All NR4As could be induced by restricted calorie intake in rodent models.

Currently, the role of NR4As in metabolism has been documented in both white adipose tissue and brown adipose tissue. Brown adipose tissue in mammals is well known for its thermogenic effect after cold exposure, which is mediated by a series of signaling cascade events involving adrenergic stimulation hormone sensitive lipase, and UCP1. In brown adipose tissue, NR4As were also found to be stimulated by adrenergic agonists and cold exposure, suggesting a possible role in nonshivering thermogenesis. However, NR4A1 knockout mice did not exhibit defects in nonshivering thermogenesis. This may be partially explained by compensatory NR4A3 induction, which masked the NR4A1 knockout phenotype. Consistently, the expression of dominant-negative NR4A1, which blocks all three NR4As’ activity, impaired β-adrenergic signaling mediated-UCP1 expression significantly. Further analysis with gel shift, chromatin immunoprecipitation and luciferase reporter techniques suggested that UCP1 could be a direct target NR4A3 in brown adipose tissue.

In white adipose tissue, it remains unclear whether NR4As are associated with adipogenesis. NR4As were reported to be induced in adipogenesis and regulate this process. Fumoto et al reported that chronic NR4A1 hyperexpression and knockdown in 3T3-L1 fibroblasts both inhibited adipogenesis. Interestingly, transient NR4A1 hyperexpression facilitated adipogenesis, which suggested that transient NR4A induction may be required in this process. However, Chao et al also demonstrated that viral mediated NR4A hyperexpression also inhibited adipogenesis in 3T3-L1 cells and this
inhibition could not be rescued through PPARγ pathway, which plays a pivotal role in adipogenesis. However, Au et al argued against these results by demonstrating that siRNA mediated NR4A1 attenuation in 3T3-L1 adipocyte showed no effect on adipogenesis. Consistent with this result, retrovirus mediated dominant negative NR4A1 expression, which, as described before, blocks all NR4A activity, inhibited NR4A induction during adipogenesis in 3T3-L1 fibroblasts without an effect on genes required for adipocyte differentiation or adipogenesis.

White adipose tissue is also an important target tissue of β-adrenergic action. Upon activation, β-adrenergic cascade stimulates lipolysis and fatty acid utilization. All NR4As were also reported to be induced by β-adrenergic cascade in epididymal white adipose tissue, but not in inguinal, visceral or sc adipose. β-adrenergic activation also resulted in a global metabolic gene expression changes in epididymal fat, which may indicates a role of NR4A in mediating such events.

Our interest on NR4As, particularly NR4A3 originated with a study from our group in which insulin induced gene expression changes was analyzed by micro-array analysis. A total number of 779 insulin responsive gene were identified and subsequent insulin induced NR4A1 up-regulation in L6 myocytes was documented. Moreover, NR4A1 was found to accumulate in the nucleus upon insulin stimulation without translocation to cytoplasm, which is consistent with the fact that it functions as a transcription factor.

Given the fact that NR4A1 is more ubiquitously expressed in different tissues and NR4A3 is abundantly in skeletal muscle and adipose tissue, we became more
interested in exploring the role of NR4A3 in insulin action and metabolism. In 3T3-L1 adipocytes, we demonstrated that both NR4A3 and NR4A1 could be induced by insulin and by pioglitazone and troglitazone, which are well known PPARγ agonists. Furthermore, gene expression of NR4A3 and NR4A1 was reduced in skeletal muscles and adipose tissue from in insulin resistant rodent models compared to control. Moreover, we observed that NR4A3 overexpression increases insulin stimulated glucose transport significantly via a mechanism involving increased GLUT4 translocation to the plasma membrane in 3T3-L1 adipocytes. It also resulted in an elevation of insulin-mediated IRS-1 tyrosine phosphorylation and Akt phosphorylation. Consistently, NR4A3 knockdown reduced insulin mediated glucose transport, IRS-1 phosphorylation and AKT/PKB phosphorylation. These data strongly support a role of NR4A3 in glucose metabolism and insulin action machinery in adipocytes. However, it remains unclear whether NR4A3 plays a role in insulin action and glucose utilization in skeletal muscle, which is the largest glucose disposal tissue in human. More importantly, if the potential role of NR4A3 in glucose metabolism and insulin action could be established in both adipose tissue and skeletal muscle, it would be imperative and interesting to find agonists which targets NR4A3 and sensitizes insulin action, either from scientific significance consideration or a pharmaceutical perspective of view.

NR4A agonists

As described before, NR4As do not possess a classic hydrophobic cleft for ligand binding. Endogenous/physiological ligands, referring to molecules with specific affinity
to NR4A receptors under physiological conditions and concentrations, have not been identified.\textsuperscript{105-108} However, several natural or synthetic compounds have been reported as “NR4A agonists” by different groups.

By high throughput screening, two independent groups discovered 6-mercaptopurine, a thiopurine metabolite as the first NR4A agonist.\textsuperscript{105,109,110} Moreover, some thiopurine molecules with similar structures were also shown to activate NR4A3.\textsuperscript{105} Unlike traditional ligand/nuclear receptor binding, which requires the ligand binding domain of the nuclear receptor, deletion studies have demonstrated that activation function domain (AF-1), but not the ligand binding domain is required in 6-mercaptopurine mediated NR4A2 and NR4A3 activation.\textsuperscript{105,109} Wansa et al reported that 6-mercaptopurin also modulate TRAP220 activity, which has been shown to be a NR4A coactivator.\textsuperscript{110} Previous studies have demonstrated that NR4A1 was involved in atherogenic lesion formation.\textsuperscript{111,112} Consistent with these observations, Pires et al found that local 6-mercaptopurine administration exerted a protective effect by inhibiting vascular smooth muscle cell proliferation and vascular lesion formation.\textsuperscript{113} 1,1-di(3’-indolyl)-1-(p-substituted phenyl)methane and several other related compounds have also been reported as NR4A1 agonists.\textsuperscript{114,115} These compounds have a common prototype molecule, diindolylmethane, which can be found in broccoli and cauliflower and are currently being studied as potential anti-carcinogen compound.\textsuperscript{114,115} Recent data have shown that the 1,1-di(3’-indolyl)-1-(p-substituted phenyl)methane induced proapoptotic signaling cascade is dependent upon NR4A1 both \textit{in vivo} and \textit{in vitro}\textsuperscript{115}, which is consistent with earlier reports.\textsuperscript{116,117} One of the most obvious advantages of these compounds is that they can be well tolerated without significant side
However, it would be hard to determine whether these compounds exert anti-carcinogenic effects through NR4A since they also activate other transcription factors such as PPARγ. Deletion studies revealed that 1,1-di(3′-indolyl)-1-(p-substituted phenyl)methane-mediated NR4A1 activation was dependent upon the ligand binding domain of NR4A1.

Additionally, the octaketide cytosporone B from HTF3 fungus was recently shown to be discovered as a new NR4A1 agonist. Deletion studies showed that cytosporone B directly binds to the ligand binding domain of NR4A1. Like 1,1-di(3′-indolyl)-1-(p-substituted phenyl)methane, cytosporone B was well tolerated in mice despite an obvious untoward effect of increasing fasting glucose.

Synthetic NR4A2 agonists have also been reported in two recent publications. Dubios et al found various benzimidazole related compounds as potent NR4A2 agonists with a low EC50 values of up to 8nm. Later, Hintermann et al reported a second class of NR4A2 agonists: isoxazolopyridinone related compounds. These compounds have been demonstrated to possess stronger activity (EC50 approximately 0.8nm) compared to benzimidazole related molecules. Given the fact that isoxazolopyridinone can penetrate the blood brain barrier and the established role of NR4A2 in dopamine metabolism, these compounds may have therapeutic applications in Parkinson Disease treatment.

Prostaglandins

The formation of Prostaglandins begins with the peroxidation of an unsaturated lipid, arachidonic acid under oxidative stress. AA can be peroxidized enzymatically
either by Lipoxygenase to yield hydroperoxyeicosatetraenoic acids or by Cyclooxygenase
to generate the unstable intermediate molecule, prostaglandin H₂ (PGH₂). PGH₂ can be
further metabolized by various enzymes to synthesize PGF₂α, PGD₂, PGE₂, PGI₂ (also
known as prostacyclin) and Thromboxane A₂ (TXA₂). PGJ₂, PGA₂ and the corresponding
metabolites, which all belong to the cyclopentenone prostaglandin family, are formed by
dehydration of the cyclopentane ring of PGD₂ and PGE₂ respectively. An unsaturated
carbonyl group that is electrophilic and reactive is formed by this dehydration. The
mechanism by which cyclopentenone Prostaglandins exert their biological activity relies
on their covalent binding to target cellular proteins. This reaction between electrophilic
carbons and target proteins is highly specific.¹²³

In contrast, the parent PGs such as PGE and PGD exert their biological activity by
interacting with corresponding G protein coupled receptors.¹²⁴

PGA₂ was discovered in semen in 1966.¹²⁵ Subsequently, the hypotensive
substance “medulin”, which was extracted from the rabbit kidney medulla turned out to
be PGA₂.¹²⁶ PGA₂ infusion into rat lowered blood pressure due to arteriole dilation.¹²⁷
Human studies also showed that PGA₂ and the related compound PGA₁ possessing an
anti-hypertensive effects in essential hypertension patients. The other cyclopentenone
member, PGJ₂ was first discovered as the dehydration product of PGD₂.¹²⁸ The PGJ₂
metabolic product, 15-deoxy-Δ¹²,¹⁴-PGJ₂, was found to be a strong PPAR-γ ligand and
agonist.¹²⁹ This discovery is exciting since it is reasonable to propose that
cyclopentenone prostaglandins may play a regulatory role in insulin sensitivity and
glucose metabolism based on the interaction between cyclopentenones and PPAR-γ
or other nuclear receptors. This hypothesis was confirmed by Kagaya et al when they
screened a comprehensive pool of arachidonic acid molecule/s and found that only PGA_1 and PGA_2 can transactivate the NR4A3-dependent transcription activity. By mutational assay, they also showed that this transactivation was largely dependent on the interaction between PGAs and the ligand binding domain and/or Activation Function domain of NR4A3.\textsuperscript{71}

As the next step, it would be interesting to explore whether PGA_2 can increase insulin sensitivity and if so, whether this effect is dependent on NR4A3. As previously described our group has documented the role of NR4A3 in insulin sensitivity in an adipocyte model.\textsuperscript{60} However, we did not report NR4A3 agonism by PGs in that model. These results will shed lights to the effort to find pharmacologic modulation of NR4A3 activity.

In addition, it is imperative to investigate whether other prostaglandins can improve insulin sensitivity given the similarities between PGA_2 and these molecules. We first investigated the effects of 15-deoxy-\(\Delta^{12,14}\)-PGA_2, a metabolite of PGA_2, because that the corresponding molecule, 15-deoxy-\(\Delta^{12,14}\)-PGJ_2 is a PPAR\(\gamma\) agonist, which is known for its insulin sensitization effect.\textsuperscript{129,130} Interestingly, Rossi et al and Straus et al reported that both PGA_2 and 15-deoxy-\(\Delta^{12,14}\)-PGJ_2 modulated inflammation by blocking NFkB activity and this inhibition was mediated by I\(\kappa\)B alkylation on its cysteine residues.\textsuperscript{131,132} We also investigated another class of lipid molecules which need to be investigated is cyclopentenone isoprostane, which share similar structures with PGA_2 and PGJ_2. Unlike PGA_2 and PGJ_2, they are synthesized under condition of oxidative stress in vivo through free radical induced membrane arachidonate peroxidation. 15-A\(_2\)-IsoP, is one of the major components of cyclopentenone isoprostanes.\textsuperscript{133} It is highly active with
glutathione and albumin and is protective in oxidative stress by decreasing IL-6 and TNFα production in RAW macrophages. Even though it is synthesized in a pathway different from PGA₂ and PGJ₂, the structural similarity warrants further investigation in terms of NR4A3 agonism. Finally, we also investigated the effect of PGE₂, which is the parental molecule of PGA₂ also need to be investigated despite the fact that they possess distinct signaling cascades. Careful analysis of these data will provide a structural point of view by which pharmacological modulators of NR4A3 activity can be developed in the future.

SIGNIFICANCE

Type 2 diabetes, Pre-Diabetes and the Metabolic Syndrome represent a cluster of metabolic diseases which have caused huge burdens and social costs in the world. Among all the pathophysiological characteristics, insulin resistance plays a pivotal role in the pathogenesis of these diseases and remains to be the main target for therapeutic intervention.

Currently, exercise is well known for its insulin sensitization effect and has been widely used as part of the lifestyle modification regimen to treat or prevent Type 2 Diabetes, Pre-Diabetes and the Metabolic Syndrome. Given the fact that NR4As can be readily induced by exercise in skeletal muscle, we sought to determine whether NR4As are involved in the insulin sensitization effect of exercise.

Thiazolidinediones, a series of PPARγ agonists, appear to be the only available medication which exerts therapeutic benefits by improving insulin sensitivity in adipose
and skeletal muscle. Furthermore, the success of Thiazolidinedione provides a proof of principal that insulin sensitivity could be improved by modulations of nuclear transcription factor activity.

However, the utility of Thiazolidinedione medication has been restricted by its untoward effects such as weight gain, increased risk of Congestive Heart Failure and the development of atherosclerosis. Thus, a new insulin sensitization medication category, which possesses a safer side effect spectrum is avidly needed in the prevention and treatment of this cluster of diseases.

Our interest on NR4A3 is based upon its restrictive expression in skeletal muscle and adipose tissue, which happen to be major insulin target tissues and our preliminary data showing its role in insulin sensitivity in both tissues. The Characterization of role of NR4A3 in metabolism is important in order to establish its rationale as a pharmacological target in Diabetic treatment. Furthermore, we endeavored to search for the small compounds which could be potential pharmacological modulators of NR4A3. We have demonstrated that PGA₂ is a potential agonist since it enhances insulin sensitivity via a NR4A3 dependent mechanism, which can be instrumental in the future development of novel insulin sensitization medication.
Prostaglandin A₂ Enhances Cellular Insulin Sensitivity via a Mechanism that Involves the Orphan Nuclear Receptor NR4A3

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Abstract

We have previously reported that members of the NR4A family of orphan nuclear receptors can augment insulin’s ability to stimulate glucose transport in adipocytes. In the current study, we endeavored to test for an insulin-sensitizing effect in muscle cells and to identify a potential trans-activator. Lentiviral constructs were used to engineer both hyper-expression and shRNA silencing of NR4A3 in C2C12 myocytes. The NR4A3 hyper-expression construct led to a 30% (p<.05) increase in glucose transport rates in the presence of maximal insulin while the NR4A3 knock-down exhibited a 35% (p<.05) reduction in insulin-stimulated glucose transport rates. Consistently, insulin-mediated AKT phosphorylation was increased 40% by NR4A3 hyper-expression and decreased by 50% following shRNA NR4A3 suppression. Then we examined effects of prostaglandin A$_2$ (PGA$_2$) on insulin action and NR4A3 trans-activation. PGA$_2$ augmented insulin-stimulated glucose uptake in C2C12 myocytes by 30% (p<.05) and AKT phosphorylation by 80% after 12 hour treatment, without significant effect on basal transport or basal AKT phosphorylation. More importantly, we demonstrated that PGA$_2$ led to a greater improvement in insulin-stimulated glucose rates (42%, p<.05) in NR4A3 over-expressing C2C12 myocytes, when compared with Lac-Z controls stimulated with insulin and PGA$_2$. Moreover, the sensitizing effect of PGA$_2$ was significantly diminished in NR4A3 knock-down myocytes compared to scramble controls. These results show for the first time that: (i) PGA$_2$ augments insulin action in myocytes as manifested by enhanced stimulation of glucose transport and AKT phosphorylation; and (ii) the insulin sensitizing effect is dependent upon the orphan nuclear receptor NR4A3.
**Key Words:** NR4A3, MINOR, insulin sensitivity, prostaglandin A₂, glucose transport, skeletal muscle, C2C12 cells, insulin action
Introduction

For the past several decades, the global prevalences of Type 2 Diabetes Mellitus (T2DM) and the Metabolic Syndrome have been increasing. Insulin resistance is central to the pathogenesis of these diseases, and involves impaired insulin action in skeletal muscle. Even so, the mechanisms causing insulin resistance have not been fully elucidated, and optimal strategies for pharmacological intervention have not been developed.

Recently, there has been a growing interest on NR4A orphan nuclear receptors as potential targets for insulin sensitivity regulation. There are three members in NR4A family: NR4A1 (TR3, NGFI-B, N10, DHR38, NAK-1, TIS1 or Nur77), NR4A2 (HZF-3, RNR-3, TINUR, NOT or Nurr1) and NR4A3 (NOR-1, TEC, CHN or MINOR). NR4A family members differ from other nuclear receptors in that they lack the classic hydrophobic cleft for recruitment of coactivators and corepressors on their C-terminal ligand-binding domain (1). They bind the NGFI-B response element (NBRE) and putatively activate gene expression in a ligand-independent manner (2). NR4As are immediate early stress response genes that can be induced by a wide range of physiological signals such as inflammatory cytokines (3), prostaglandins (4), and growth factors (5). This subgroup has also been shown to be involved in various pathological conditions, including Parkinson’s disease (6), inflammation (7), atherosclerosis (8) and cancer (9). Furthermore, all three NR4A members are inducible by chronic caloric restriction in rat liver and skeletal muscle (10). This finding is particularly interesting since both chronic and intermittent caloric restriction have been shown to enhance insulin sensitivity in numerous species (11). Finally, NR4A1 null
mice exhibit insulin resistance in skeletal muscle and liver together with an impaired fat metabolism (12).

Our laboratory has reported that NR4A3, a member of NR4A orphan nuclear receptor family, can enhance insulin sensitivity and glucose transport stimulation in 3T3-L1 adipocytes, and is down-regulated in insulin resistant or diabetic rodent models (13). While these results suggest a potential role for NR4A3 as a therapeutic target for modulation of insulin action, it will be important to demonstrate that NR4A3 enhances insulin sensitivity in skeletal muscle which is responsible for the bulk of insulin-mediated glucose uptake in vivo. It would also be imperative to show that a small molecular agonist of the orphan receptor could produce an increase in insulin action. In our current study, we have examined whether alterations in NR4A3 expression affect insulin sensitivity in C2C12 muscle cells. Furthermore, we have studied prostaglandin A₂ (PGA₂) as a potential transactivator of NR4A3 in muscle cells following the report by Kagaya et al (4) that PGA₂ enhances NR4A3-dependent transcriptional activity. This transactivation was largely dependent on a direct physical interaction between PGA₂ and the ligand binding domain of NR4A3. We have shown for the first time that PGA₂ exerts an insulin-sensitizing effect, and that this action is dependent upon NR4A3. These results are consistent with our previous report in adipocytes (13), and confirmed the importance of NR4A3 in the regulation of insulin action in skeletal muscle cells. Further, our findings highlight the potential role of PGA₂ or other small molecule agonists of NR4A3 as therapeutic modulators of insulin action.
Materials and Methods

Reagents—Mouse C2C12 myoblast cells were purchased from American Type Culture Collection (Manassas, VA). Tissue culture media were products of Invitrogen. Akt and phosphor-Akt antibodies were purchased from Invitrogen. 2-Deoxy-D-[3H]- and L-[1-3H] glucose were purchased from Amersham Biosciences. All other reagents were purchased from Sigma unless specified.

Cell culture and stimulation—The C2C12 are maintained as growing myoblasts in Dulbecco’s minimal essential medium (DMEM, Mediatech) containing 1 g/l glucose, and 10% fetal bovine serum (GIBCO). Mass cultures reached 70%-80% confluence within 48 hours. Differentiation of C2C12 myoblasts was induced by reducing the serum concentration (2% horse serum, Hy-Clone). The cultures were fed with 2% horse serum DMEM daily thereafter, and then serum-starved overnight preceding experiments.

Recombinant lentiviruses and lentiviral-transduced cell lines—Lentiviral-transduced NR4A3 over-expression and shRNA construct cloning procedures have been described in our previous report (12). Briefly, the full-length human NR4A3 cDNA coding sequence and a V5 epitope tag were cloned into a Vira-Power-CMV vector (Invitrogen). The NR4A3 construct and a control LacZ gene plasmid were transfected into HEK293 cells. Western blots were performed to confirm successful transfection, and infectious virus particles were produced according to the manufacturer’s protocol (Invitrogen). To establish stable C2C12 myocytes that over-express NR4A3 or LacZ genes, recombinant NR4A3 or LacZ lentiviral stocks were
used to infect C2C12 myoblasts with Polybrene (Specialty Media, Phillipsburg, NJ) at a final concentration of 6 µg/ml. Seventy two hours after transfection, cells were placed under blasticidin selection (30 µg/ml) for 20 days. Western blot analyses were performed to test for stable NR4A3 or LacZ gene expression after antibiotic selection.

**Lentiviral based endogenous NOR-1 gene hypo-expression**— Three shRNA hairpin oligonucleotides (sense 5'-CAC CGC TGT TTG TCC TCA GAC TTT CCG AAG AAA GTC TGA GGA CAA ACA GC-3', antisense 5'-AAA AGC TGT TTG TCC TCA GAC TTT CTT CGG AAA GTC TGA GGA CAA ACA GC-3'; sense 5'-CAC CGC TGA GCA TGT GCA ACA ATT CCG AAG AAT TGT TGC ACA TGC TCA GC-3', antisense 5'-AAA AGC TGA GCA TGT GCA ACA ATT CTT CGG AAT TGT TGC ACA TGC TCA GC-3'; sense 5'-CAC CGC TCT TGT CCG AGC TTT AAC ACG AAT GTT AAA GCT CGG ACA AGA GC-3', antisense 5'-AAA AGC TCT TGT CCG AGC TTT AAC ATT CGT GTT AAA GCT CGG ACA AGA GC-3') that are complementary to the mouse NR4A3 gene coding sequences were synthesized by Integrated DNA Technologies (Coralville, IA), and subcloned into the pENTR™/U6 lentiviral vectors (Invitrogen) to create the NR4A3 shRNA constructs following the manufacturer's instructions. Three specific target sequences in the gene coding region for the knockdown experiments were 5'-GCT GTT TGT CCT CAG ACT TTC-3', 5'-GCT GAG CAT GTG CAA CAA TTC-3', and 5'-GCT CTT GTG CGA GCT TTA ACA-3' for the three synthesized short oligonucleotides. The recombinant shRNA-NR4A3 lentiviral plasmid or control scramble construct was transfected into HEK293 cells to generate lentiviruses. Thereafter shRNA lentiviruses were transfected into C2C12 myoblasts to generate cell lines exhibiting NR4A3 suppression in parallel with the
scramble control cell lines. Stable knockdown cell lines were selected under the same conditions as the NR4A3 over-expression cell lines described above.

**Western blot analyses**—Myocytes were treated with cell lysis buffer (1x phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing freshly added protease inhibitor mixture (Sigma). Fifty micrograms of protein per lane and known molecular weight markers from Bio-Rad 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 Tris-buffered saline). The blocked membranes were incubated with Akt or phospho-Akt antibodies (Zee Biologic) and then peroxidase-conjugated second antibody (1:1000 or 1:5000 dilution with 1% nonfat milk in Tris-buffered saline (TBS)) for 1 h for each at room temperature and washed 3 times each with TBS buffer containing 0.1% Tween 20 for 15 min at room temperature with rocking. Immunodetection analyses were performed using the Enhance Chemiluminescence Kit (PerkinElmer Life Sciences). Typical data were shown after similar results were obtained in three or more independent experiments.

**Modified glucose uptake assay**—Glucose transport was assayed in monolayers as initial rates of 2-deoxy-[³H]-glucose uptake as we have described previously (15). Modification was made in order to enhance insulin response in C2C12 myotubes as described (16). Fully differentiated C2C12 myotubes were starved using low glucose (1g/L), serum-free DMEM for 4 h. The cells were then washed 3 times with glucose-free KRPH buffer (20 mM HEPES, 120 mM NaCl, 1.2 mM MgSO4, 2 mM CaCl2, 2.5 mM KCl, 1 mM NaH2PO4, and 1 mM sodium pyruvate) and stimulated
with or without 100 nM insulin. 15 minutes following insulin stimulation, glucose was added to KRPH buffer to a final concentration of 25mM. Cells were allowed to incubate for additional 45 minutes. Glucose transport was determined by the incubation of 2-deoxy-[3H]glucose for three minutes (0.1 mM, 0.2μCi/ml; PerkinElmer Life and Analytical Science). Cells were then placed on ice and washed 3 times with ice-cold KRPH buffer to remove any 2-deoxy-[3H]glucose remaining in the buffer. Finally, 2-deoxy-[3H]glucose uptake was assessed by counting β-emissions from the cells.

Statistics—Experimental results are shown as the mean ± S.D. Statistical analyses were performed by unpaired Students t test or ANOVA assuming unequal variance unless otherwise indicated. Significance was defined as *p<0.05, or ** p<0.01.

Results

Our group has shown that NR4A3 is induced during differentiation of 3T3-L1 adipocytes (13). In C2C12 myocytes, we now similarly observe that NR4A3 expression is induced during differentiation (Figure 1). In order to elucidate the role of NR4A3 in insulin action in C2C12 myocytes, we made lentiviral constructs for both NR4A3 stable hyperexpression and knockdown, together with transduced LacZ and scramble control cells. The hyperexpression construct results in approximately doubled NR4A3 protein expression compared to LacZ control, and the knockdown construct decreases NR4A3 protein by approximately 75% compared to scramble control (Figure 2). It is important to note that there was no NR4A1 and NR4A2 protein expression
change after NR4A3 hyperexpression or knock down (data not shown). Our initial observation was that the extent and temporal progression of muscle cell differentiation were not affected by lentivirus-mediated NR4A3 hyperexpression, nor by shRNA-mediated NR4A3 repression (data not shown). Thus, NR4A3 does not appear to influence differentiation from a histological perspective.

Even so, our data did indicate that NR4A3 was involved in achieving a fully insulin-responsive functional phenotype in myocytes as we had previously demonstrated in adipocytes (13). As a consequence of NR4A3 hyperexpression, we observed a significant elevation in insulin-stimulated glucose transport in C2C12 myocytes compared to LacZ controls (35%, p<0.05), while no significant effects on basal glucose transport were observed. We also found that NR4A3 hyperexpression significantly enhanced insulin-mediated Akt/PKB ser473 phosphorylation (Figure 3), without effecting GSK-3β ser9 phosphorylation. Consistent with our hyperexpression results, NR4A3 knockdown led to a state of relative insulin resistance with a significant decline in insulin’s ability to stimulate glucose transport activity (p<0.01) and Akt/PKB phosphorylation (Figure 4). Again, no basal glucose transport change was observed after NR4A3 silencing.

A previous report indicated that PGA₂ is able to bind NR4A3 and activate NR4A3-dependent transcription (4). Based on this discovery and the fact that NR4A3 regulates insulin action and downstream signaling, we hypothesized that PGA₂ and other cyclopentenone prostaglandins would also increase insulin sensitivity through NR4A3 agonism. In wild-type C2C12 cells, we did not observe any effects of PGE₂ or PGA₂-isoprostanes on insulin-stimulated glucose transport. The PGA₂ metabolite
15-deoxy-Δ-12,14- PGA₂ increased mean insulin-stimulated glucose transport, but this effect did not reach statistical significance. (Data not shown) In contrast, PGA₂ did significantly enhance insulin sensitivity without altering basal glucose uptake in wild-type C2C12 myocytes (p<0.01). We also observed increased Akt/PKB ser473 phosphorylation (Figure 5) when cells were incubated in the presence of both insulin and PGA₂, with no changes in GSK3β phosphorylation. Interestingly, the insulin sensitization effect of PGA₂ could not be attributed to decreased insulin EC₅₀. Our data suggested that PGA₂ enhances maximal insulin action. (Figure 5C) Additional data indicated that the insulin sensitization effect of PGA₂ is both time and concentration related, with a 12-hour incubation in 10μM PGA₂ producing the maximal increase in insulin-stimulated glucose transport (Figure 6).

We hypothesized that the insulin-sensitizing effect of PGA₂ was mediated via NR4A3 in muscle cells. To address this question, NR4A3 knockdown and scramble control C2C12 myocyte lines were pre-treated with or without PGA₂, and then acutely stimulated by insulin. As expected, NR4A3 knockdown not only reduced insulin-mediated glucose transport but also abrogated the insulin sensitizing effect of PGA₂. Likewise, NR4A3 hyperexpression significantly augmented both insulin-mediated glucose transport in wild type cells and the effect of with PGA₂ to improve insulin sensitivity (Figure 7). Taken together, we were able to conclude that NR4A3 is required for the insulin sensitizing effect of PGA₂.
Discussion

NR4A nuclear receptors appear to exert diverse influences on cell physiology, and have been shown to modulate metabolism, adrenergic signaling, neurodevelopment, and inflammation. Our group identified NR4A3 as an insulin-responsive gene (13), and observed decreased NR4A3 protein expression in skeletal muscle from various models of diabetes and insulin resistance (13). Since adipose tissue and skeletal muscle are highly insulin-sensitive, we sought to determine the role of NR4A3 in these tissues. In cultured adipocytes, we had previously shown that NR4A3 overexpression increases insulin-stimulated glucose transport by increasing GLUT4 translocation, with no change in total cellular GLUT4 content. The data presented in this report extend our previous findings to skeletal muscle. As observed in adipocytes, NR4A3 overexpression significantly enhances insulin-stimulated glucose transport in C2C12 muscle cells, while shRNA suppression of NR4A3 reduces insulin-stimulated glucose transport.

NR4A family members can be functionally redundant in specific instances. NR4A3 and NR4A1 have been implicated in metabolic regulation in liver, and adenoviral overexpression of NR4A3 or NR4A1 in primary mouse hepatocytes increases expression of both fructose bisphosphatase-2 (a.k.a. phosphofructokinase 2) and enolase-3 and increases pyruvate-derived glucose production (17). With respect to metabolism, NR4A3 has not been as well studied; however, when the current data are examined in light of previous publications concerning NR4A1, it is clear that both NR4A family members can overlap in their metabolic effects. Importantly,
overexpression of NR4A1 in C2C12 cells enhances basal glucose transport, with no change in Glut1 expression (18). It is also interesting to note that overexpression of NR4A1 increases expression of GLUT4, phosphofructokinase, and glycogen phosphorylase in C2C12 cells and in rat muscle (18). Furthermore, NR4A1 null mice are no different from wild-type mice when fed standard diet, but are more susceptible to metabolic dysfunction when maintained on high fat diet; thus, when fed high fat diet, NR4A1-/- mice become more obese and insulin resistant, with impaired insulin signaling in skeletal muscle driving these changes. Interestingly, NR4A1 knockout mice also displayed increased intramyocellular lipid content and hepatic steatosis, but maintained normal hepatic insulin sensitivity (12). These data, along with ours, suggest that NR4A3 and NR4A1 modulate cell-type specific metabolic functions, and enhance metabolic functions and insulin actions in muscle. The current effects on insulin action occurred as a result of specific modulation of NR4A3 expression, without changes in NR4A1(data not shown), indicating that the induced hyperexpression and suppression of NR4A3 predominate in the modulation of insulin sensitivity under these experimental conditions.

Cyclopentenone prostaglandins are known to modulate the transcriptional activity of other nuclear receptors, including PPAR-γ, NF-κB, AP-1, Nrf2, HIF1- α (19), and estrogen receptor-α (20). These prostaglandins include PGA₂, PGA₁, and PGJ₂, and are formed by dehydration of the cyclopentane rings of PGE₂, PGE₁, and PGD₂, respectively, resulting in an unsaturated carbonyl group that is electrophilic and reactive (14,21). Unlike receptor-binding prostaglandins, cyclopentenone prostaglandins covalently bind specific target proteins, and these adducts result from
covalent interaction between protein cysteine thiols and the α,β-unsaturated carbonyl moiety in the cyclopentenones (20). The reaction between the electrophilic carbons and their target proteins is non-promiscuous, highly specific, and can alter protein activities (14, 21-24). Because the ligand-binding domain of NR4A proteins lacks a hydrophobic cleft, it was thought that these receptors may function constitutively in the absence of ligand. However, Kagaya et al have identified PGA2 as a specific transactivator of NR4A3 (4). This group showed that PGA2 activated NR4A3-dependant transcription, and that this PGA2 action was dependent upon interaction with the NR4A3 ligand binding domain (4). Based on these results, we hypothesized that PGA2 would improve skeletal muscle insulin sensitivity through transactivation of NR4A3.

In the current studies, we did, in fact, observe an increase in insulin-stimulated glucose uptake in wild-type C2C12 cells pre-treated with PGA2. This is the first time that PGA2 has been reported to augment insulin sensitivity. While we observed that PGA2 enhanced insulin action in C2C12 muscle cells, it was not clear whether this effect was mediated by NR4A3. To address this question, we tested for the effect of PGA2 following both suppression and hyperexpression of NR4A3. PGA2 treatment failed to affect insulin sensitivity following shRNA suppression of NR4A3, while PGA2 had a greater insulin-sensitizing effect in cells overexpressing NR4A3, when compared with controls. Thus, the insulin sensitizing effect of PGA2 was dependent upon NR4A3.

The improvement in insulin action due to the interaction between a prostanoid (PGA2) and a nuclear transcription factor (NR4A3) is analogous to the effects of PGJ2
metabolites interacting with PPARγ. PGJ2 was discovered as a dehydration product of PGD2 in studies assessing their anti-neoplastic properties (25), and can undergo albumin-assisted catalysis to $\Delta^{12}$-PGJ2 and 15-deoxy-$\Delta^{12,14}$-PGJ2 (26). In 1995, 15-deoxy-$\Delta^{12,14}$-PGJ2 was found to enhance insulin action as a high affinity ligand and agonist for PPARγ (27,28). In light of the current results, it is important to note that PGA2 is not known to interact with PPARγ (14). Kagaya et al have comprehensively screened a large number of naturally-occurring arachidonic acid and glycerolipid metabolites from the KEGG database, and found that only PGA2 and PGA1 have the unique ability to activate NR4A3-dependent transcription using a GAL4-based reporter system (4). Furthermore, using mutational analyses and a Biacore plasmon resonance sensor, they demonstrated that PGA2 action was dependent upon binding of PGA2 to the ligand binding domain of NR4A3. Even so, the possible biochemical mechanism by which PGA2 interacts with NR4A3 in muscle cells remains unclear even though our data indicate that NR4A3 is necessary for the insulin-sensitizing affect of PGA2.

In closing, our results demonstrate that NR4A3 mediates an increase in insulin-stimulated glucose transport and AKT phosphorylation in muscle cells. Thus, the NR4A3 nuclear receptor is an attractive pharmacologic target for the modulation of insulin sensitivity, and diseases characterized by insulin resistance such as Type 2 Diabetes and the Metabolic Syndrome. We have also shown for the first time that PGA2 enhances insulin sensitivity, and this occurs via a mechanism involving NR4A3. This provides proof-of-principle that a small molecule mediator can have an insulin-sensitizing effects through an interaction with NR4A3.
Acknowledgments

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Figure Legends

Figure 1. NR4A3 Protein is induced during Differentiation of C2C12 Cells. Wild type C2C12 myoblasts were allowed to differentiate as described in materials and methods. A sequential increase of NR4A3 protein expression was observed after two days and four days of differentiation.

Figure 2. Characterization of lentiviral transduced cell lines. A) Lentiviral mediated NR4A3 over-expression in C2C12 myocytes. C2C12 myoblasts was transformed with NR4A3 lentiviral particles and allowed to grow in 10% FBS containing DMEM for 72 hours before a 20-day blasticidin selection. Transformed C2C12 myoblasts were allowed to differentiate for 6 days before experiment. A one fold increase of NR4A3 protein expression was observed in NR4A3 overexpression cell line compared to LacZ control cell line. B) shRNA mediated silencing of NR4A3 in C2C12 myocytes. C2C12 myoblasts was transformed with indicated shRNA particles and allowed to grow in 10% FBS containing DMEM for 72 hours before a 20-day blasticidin selection. Transformed C2C12 myoblasts were allowed to differentiate for 6 days before experiment. A 75% decrease of NR4A3 protein expression was observed in NR4A3 knockdown cell line compared to scramble control cell line.

Figure 3. NR4A3 hyperexpression augments insulin sensitivity in C2C12 myocytes. A) NR4A3 hyperexpression enhances insulin stimulated glucose transport. Stable C2C12 myoblasts were allowed to differentiate 5-6 days before experiment. Glucose transport was measured as $^3$H radioactivity intake in the initial 3 minutes as described in materials and methods. A 30% (p<.05) increase in glucose transport rates in the presence of 100nM insulin was observed without significant effects on basal transport, when compared to control cells. B) NR4A3 hyperexpression enhances insulin stimulated AKT Ser473 phosphorylation in C2C12 myocytes. C2C12 myoblasts were allowed to differentiate for 5-6 days before cellular protein was harvested. Western blot analysis was performed to measure NR4A3 protein expression. A Typical blot data was shown after similar results
were obtained in three independent experiments. C) The quantification of insulin stimulated AKT phosphorylation enhancement after NR4A3 overexpression.

**Figure 4.** NR4A3 silencing reduces insulin sensitivity in C2C12 myocytes. A) NR4A3 knockdown suppresses insulin stimulated glucose transport activity. C2C12 myoblasts were transformed with shRNA particles targeting on NR4A3 silencing in parallel with scramble control. Stable C2C12 myoblasts were allowed to differentiate for 5-6 days before experiment. Glucose transport was measured as \(^{3}\)H radioactivity intake in the initial 3 minutes as described in materials and methods. A 35% (p<.05) decrease in glucose transport rates in the presence of 100nM insulin was observed without significant effects on basal transport, when compared to control cells  B) NR4A3 knockdown reduces insulin mediated AKT phosphorylation. C2C12 myoblasts were allowed to differentiate for 5-6 days before cellular protein was harvested. Western blot analysis was performed to measure NR4A3 protein expression. A Typical blot data was shown after similar results were obtained in three independent experiments. C) The quantification of insulin stimulated AKT phosphorylation impairment after NR4A3 knockdown.

**Figure 5.** PGA\(_2\) augments insulin sensitivity in C2C12 myocytes. A) 10\(\mu\)M PGA\(_2\) 12 hour treatment significantly enhances insulin stimulated glucose transport. Wild type C2C12 myoblasts were allowed to differentiate for 5-6 days. Cells was then starved in low glucose DMEM(1g/L) with or without 10\(\mu\)M PGA\(_2\) 12 hours before experiment. Glucose transport was measured as \(^{3}\)H radioactivity intake in the initial 3 minutes as described in materials and methods. A 30% (p<.05) increase in glucose transport rates in the presence of 100nM insulin was observed without significant effects on basal transport, when compared to control cells  B) 10 \(\mu\)M PGA\(_2\) 12 hour treatment enhances insulin stimulated AKT Ser473 phosphorylation. Wild type C2C12 myoblasts were allowed to differentiate for 5-6 days before cellular protein was harvested. Western blot analysis was performed to measure NR4A3 protein expression. A Typical blot data was shown after similar results were obtained in three independent experiments. C) The quantification of insulin stimulated AKT phosphorylation enhancement after PGA\(_2\) treatment. D) 10 \(\mu\)M PGA\(_2\) 12 hour treatment did not change insulin EC50 but increases maximal insulin
stimulated glucose transport. Wild type C2C12 myoblasts were allowed to differentiate for 5-6 days. Cells was then starved in low glucose DMEM(1g/L) with or without 10μM PGA2 12 hours before experiment. Glucose transport was measured as ³H radioactivity intake in the initial 3 minutes as described in materials and methods. No insulin EC50 change was calculated based upon current data. PGA2 treatment significantly enhances insulin (with concentrations > 5nM) mediated glucose transport activity in C2C12 cells. (P<0.01)

**Figure 6.** PGA2 enhances insulin action in C2C12 myocytes in a concentration and time related manner. A) PGA2 treatment enhances insulin stimulated glucose transport in a concentration related manner. C2C12 myoblasts were allowed to differentiate and glucose transport was measured as previously described. Only 10 μM treatment significantly enhances insulin stimulated glucose transport (30%, p<.05) without a significant impact on basal glucose transport. B) 10μM PGA2 treatment of different time length enhances insulin mediated glucose transport. 12 hour treatment enhances insulin stimulated glucose transport by 20% (p<.05) and 24 hour treatment by 15% (p<.05).

**Figure 7.** The insulin sensitization effect of PGA2 is dependent on the full presence of NR4A3. C2C12 myoblasts differentiation was induced and glucose transport was measured as previously described. A) PGA2 (10μM,12 hour) enhances insulin stimulated glucose transport by 30% (p<.05) in control cells but not in NR4A3 knockdown cell lines (p>.05). B) PGA2 (10μM,12 hour) and NR4A3 hyperexpression enhances insulin stimulated glucose transport additively. PGA2 (10μM,12 hour) treatment enhances insulin stimulated glucose transport by 28% (p<.05) and NR4A3 hyperexpression enhances by 17% (p<.05). PGA2 treatment and NR4A3 hyperexpression together enhances insulin stimulated glucose transport by 42% (p<.05), which is significantly higher than PGA2 treatment alone (p<.05) and NR4A3 hyperexpression alone (p<.05).
**Figure 1**

<table>
<thead>
<tr>
<th>Days after Differentiation</th>
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<th>2</th>
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<tbody>
<tr>
<td>NR4A3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>beta actin</td>
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</tbody>
</table>
Figure 2A

Lac Z       NR4A3

NR4A3

β-actin

Figure 2B

Scramble     NR4A3

NR4A3

β-actin
Figure 3A

Glucose Uptake (mmol/µg/3min)

NR4A3  -  -  +  +  +
Insulin  -  +  -  +

* p<0.05

Figure 3B

<table>
<thead>
<tr>
<th>NR4A3</th>
<th>Insulin(min)</th>
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<tbody>
<tr>
<td>-</td>
<td>0  30  60</td>
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<tr>
<td>-</td>
<td>0  30  60</td>
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</tbody>
</table>

P-AKT

total-AKT
Figure 3C

![Graph showing relative AKT phosphorylation](image)

- Insulin (100nM)
  - Time (min)
    - 0
    - 30
    - 60

- Lac Z control
- NR4A3

**p < 0.01
Figure 4A

![Graph showing glucose uptake with bars for different conditions.

Figure 4B

<table>
<thead>
<tr>
<th>sh NR4A3</th>
<th>Insulin (min)</th>
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<tr>
<td>-</td>
<td>0 10 30 60</td>
</tr>
<tr>
<td>-</td>
<td>0 10 30 60</td>
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P-AKT

Total-AKT
Figure 4C

The figure shows the relative AKT phosphorylation over time with different insulin treatments. The x-axis represents time in minutes (0, 10, 30, 60), while the y-axis represents relative AKT phosphorylation. Two conditions are compared: Scramble Control and Sh NR4A3. The graph indicates a significant decrease in relative AKT phosphorylation in the Sh NR4A3 condition compared to the Scramble Control at 30 and 60 minutes. The data is presented with error bars, and the significance is marked with ** indicating p<0.01.
Figure 5A

![Graph showing glucose uptake with different conditions of Insulin and Prostaglandin A2](image)

**p < 0.01

Figure 5B

<table>
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<tr>
<th>PG A2</th>
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<th>60</th>
<th>120</th>
<th>0</th>
<th>30</th>
<th>60</th>
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</tbody>
</table>

P-AKT

![Phosphorylated AKT Western Blot](image)

Total-AKT

![Total AKT Western Blot](image)
Figure 5C

Relative AKT phosphorylation

Insulin (100nM) - - + + + +
Time (min) 0 30 60 120

** p<0.01

- MOCK - PGA2

**
Figure 5D
Figure 6A

![Bars showing glucose uptake with different treatments](image)

Figure 6B

![Bars showing glucose uptake with different time points](image)
Figure 7A

![Graph A]

** p<0.01  ** p<0.001

Figure 7B

![Graph B]

* p<0.05  ** p<0.01
EXERCISE INDUCED NR4A PROTEIN EXPRESSION CHANGE IN HUMAN SKELETAL MUSCLE

By

XIAOLIN ZHU, JIARONG LIU, R GRACE WALTON, ANNA THALACKER-MERCER, LING TIAN, MARCAS M BAMMAN, W TIMOTHY GARVEY

Format adapted for dissertation
ABSTRACT

Skeletal muscle is a major metabolic tissue which accounts for 80% of insulin stimulated glucose disposal in the human body. Glucose metabolism dysregulation in skeletal muscle plays a pivotal role in the pathogenesis of Type 2 Diabetes. Physical exercise has been used as part of the therapeutic regiment to reverse glucose utilization defects, leading to the prevention of T2DM.

There was a growing interest in the potential role of NR4A orphan nuclear receptors in exercise induced glucose utilization. Exercise induced NR4A1 and NR4A3 mRNA up-regulation in skeletal muscle has been reported by various groups. However, these observations are limited by the fact that only NR4A mRNA levels were measured and only young subjects were enrolled into these studies. Moreover, the effect of chronic exercise on NR4A protein expression remains unclear.

In the current study, we try to elucidate exercise induced NR4A protein expression changes in skeletal muscle after acute or chronic exercise in young and elderly men. Eleven healthy elderly males (64.8±4 year old) and sixteen healthy young males (25.8±4.8 year old) participated in the study. All subjects underwent a single bout (Acute Exercise, AE) and a 16 week resistance training program (3days/wk, CE) that focused primarily on knee extensors. Each exercise was performed for three sets of 8-12 repetitions. Muscle biopsy was performed at baseline, 24 hr after AE and CE. Metabolic parameters were recorded. Relative NR4A protein expression was measured with western blot analysis.
Results showed that NR4A1 protein expression was up-regulated after AE only in young men (+50%, p<0.01). In contrast, NR4A1 expression was down-regulated by AE in older men (-35%, p<0.01). There was no significant difference in baseline NR4A1 expression between young and old men, except after AE, with young men showing 90% higher NR4A1 levels. Interestingly, there is significant down-regulation of NR4A1 after CE in both young (-30%, p<0.05) and old (-75%, p<0.01). No significant AE- or CE-induced NR4A3 expression changes were observed. However, NR4A3 expression is higher in young people compared to old at both basal (+180%, p<0.01) and CE (+120%, p<0.05) time points.

Our results demonstrated that age and exercise exerted differential effects on NR4A protein expression in human skeletal muscle. NR4A1 expression was similar at baseline in both young and old, induced by AE in young subjects only and down-regulated by CE in both young and old subjects. On the other hand, NR4A3 expression was lower in elderly and did not show an exercise dependent expression change. These results expand previous observations to the protein level and suggest that different mechanisms may be involved in NR4A regulation in different age groups.
INTRODUCTION

Insulin Resistance, which is referred as impaired peripheral tissue response to endogenously secreted insulin, is the central event in the pathogenesis of a cluster of diseases including Type 2 Diabetes, Pre-Diabetes and Metabolic Syndrome. Insulin Resistance is typically manifested as decreased insulin stimulated glucose transport in skeletal muscle and adipose tissue, together with impaired suppression of hepatic glucose production. Among all the insulin target tissues, skeletal muscle has been well documented to be the most important one. It makes up 40% of human body weight and accounts for over 80% of insulin stimulated glucose utility. Thus, amelioration of insulin resistance in skeletal muscle plays a pivotal role in the prevention and treatment of Type 2 Diabetes, Pre-Diabetes and Metabolic Syndrome.

To date, the underlying mechanism by which insulin resistance develops in skeletal muscle remains poorly understood. Generally, insulin resistance in skeletal muscle is associated with an imbalance manifested by increased uptake of free fatty acids and defects in fatty acid oxidation, which leads to lipid metabolite accumulation in skeletal muscle.

Exercise has been part of the therapeutic regimen to reverse insulin resistance for decades. Exercise improves insulin sensitivity in both healthy and diabetic patients, and this effect is manifested by increased fatty acid uptake in skeletal muscle, enhanced fatty acid oxidation capacity, increased glucose transport and up-regulated Glycogen synthase activity. It was shown that modest exercise such as brisk walking, together with a mild caloric restriction was more potent in insulin resistance prevention in patients with obesity and Type 2 Diabetes. However, the
molecular mechanism of exercise induced insulin sensitization in skeletal muscle has not been fully elucidated.

Recently, there is growing interest in the role of the NR4A orphan nuclear receptor family in insulin action and glucose and lipid metabolism in skeletal muscle. NR4A family members include Nur77 (TR3, NGFI-B, N10, DHR38, NAK-1, TIS1, NR4A1), Nurr1 (HZF-3, RNR-3, TINUR, NOT, NR4A2) and Mitogen-inducible nuclear orphan receptor (MINOR, also NOR-1, TEC, CHN, NR4A3). NR4A family members are expressed in numerous metabolically active tissues including brain,[26] skeletal muscle,[27] kidney,[28] liver, certain leukocytes, and adipose.[27] They are early response genes whose expression is induced in a cell-type specific manner by numerous stimuli, including inflammation, inflammatory cytokines,[29] prostaglandins,[30] growth factors,[31] and many others. NR4A members modulate expression of steroidogenic genes in both the HPA-axis and target tissues, with known mutual transcriptional repression between NR4A members and the glucocorticoid receptor.[32] Finally, NR4A members are thought to have a high degree of transcriptional and functional redundancy.

Previously, our group has reported that both NR4A1 and NR4A3 were down-regulated in skeletal muscle of diabetic and insulin resistant rodent models.[27] We have also demonstrated that lentiviral mediated NR4A3 overexpression increased insulin stimulated glucose transport and GLUT-4 translocation.[27] Consistently, NR4A1 and/or NR4A3 knockdown significantly attenuate expression of genes that are involved in glucose and fat metabolism, such as fatty acid translocase, PPARα/βcoactivator-1, lipin and GLUT-4.[33-35] These data strongly suggest that NR4As may play regulatory roles in insulin action and glucose metabolism in skeletal muscle.
The link between NR4As and exercise was demonstrated by Mahoney et al in 2005. They showed that 3 hour endurance exercise significantly stimulated NR4A mRNA expression in human *vastus lateralis* muscle, suggesting that NR4As may be involved in exercise induced glucose and fatty acid metabolism. Given the fact that NR4A expression can be robustly induced by β-adrenergic signaling cascades and exercise stimulated sympathetic nervous system activation in skeletal muscle, it is logical to hypothesize that NR4A induction may partially explain exercise induced glucose and fatty acid disposal in skeletal muscle.

Using a rat model, Kawasaki et al demonstrated that 3 hour low intensity exercise, as well as 1 hour acute electric stimulation, induced NR4A1 and NR4A3 mRNA expression, implicating the role of skeletal muscle contraction in NR4A induction. Moreover, they also showed that NR4A1 and NR4A3 were up-regulated by treatment with AICAR, a well known AMPK activator. These results strongly suggest that AMPK activation may also be involved in the muscle contraction stimulated NR4A mRNA induction.

However, these observations are limited by the fact that only NR4A mRNA levels were quantified and only young subjects were involved in the studies. Whether the observed NR4A mRNA induction can be translated to protein expression changes remains unclear. Furthermore, the effect of age and chronic exercise (CE) on NR4A protein expression needs to be clarified given the well documented notion that long term resistance exercise training improves insulin sensitivity in skeletal muscle.

In the current study, we endeavored to elucidate exercise induced NR4A protein expression changes in humans in two different age groups. Also, we examined the effect
of chronic exercise on skeletal muscle NR4A protein expression by quantifying NR4A levels using western blot.

**Materials and Methods**

*Experimental subjects*

Eleven healthy old males and sixteen healthy young males from metropolitan area of Birmingham, Alabama were involved in the current study as a part of a larger 16-week exercise training clinical trial project. Surveys about health histories and physical activity were completed by all participants. The physical characteristics of involved subjects are shown in Table 1. All elderly subjects passed a comprehensive physical examination and a 12-lead EKG monitored exercise stress test by a cardiologist before being enrolled into the current study. All participants was enrolled into a screening test before this study to exclude any possible musculoskeletal disorders which could lead to poor resistance training intolerance. Also, subjects with any of the following characteristics were also excluded from this study: obesity (body mass index > 30 kg/m²), knee extensor resistance training within the past five years, the past history of taking exogenous testosterone or any other pharmacological stimulants which regulates muscle mass or muscle recovery. Approvals from the Institutional Review Boards of both the University of Alabama at Birmingham (UAB) and the Birmingham Department of Veterans Affairs (VA) Medical Center were obtained. All subjects were instructed with the potential risks of this study and written informed consent was obtained before participation.
Unaccustomed resistance loading bout and progressive resistance training

All participants were provided with the user instructions of knee extensor device until familiarization was achieved. The contractions of knee extensor muscle group against resistance were achieved by a standardized resistance loading bout given that subjects were untrained and unaccustomed to the resistance loading. Each of the resistance loading episodes was made up by 9 sets of 10 repetitions of bilateral, concentric-eccentric knee extension movements against a fixed external load on a weight-stack knee extension machine. Chronic Exercise program was made up of knee extensions 3 days/week for 16 weeks. Specific instructions were given to the participants and they were encouraged to perform the concentric phase of each contraction with the maximal possible velocity. Then they were informed to control the eccentric lowering phase. The external resistance loading value for each subject was determined as 40% of bilateral maximum voluntary isometric contraction (MVC) force by a load cell and regression procedures as a previous report [39]. The loading of each contraction was considered to be moderate based upon the extent of external load would. However, key parameters of this protocol that could increase the overall intensity of this exercise program consisted of the explosive concentric phase of each repetition and brief 90 second rest periods between sets. Generally, 10 successive repetitions were sufficient to induce velocity-dependent muscle fatigue as measured by electrogoniometry, especially among elderly participants.
**Muscle biopsy and tissue preparation procedure**

As previously reported, after local anesthesia with 1% lidocaine, percutaneous needle biopsy of the vastus lateralis with a 5-mm Bergstrom biopsy needle. Muscle specimens were taken in the morning after an overnight fast at baseline (before resistance loading) and 24 h after resistance loading in the resting stage. The post-resistance loading muscle samples were extracted from the contralateral leg to avoid any impacts from previous procedure. Each specimen was quickly blotted with gauze and all visible connective and/or adipose tissue were dissected from the specimen. Muscle samples were dissected into 30 mg particles, snap frozen in liquid nitrogen, and stored at 80°C. 10 ml venous blood was drawn from an antecubital vein in the fasting state simultaneously with muscle biopsy, then serum was isolated and stored at 80°C.

**Immunoblotting**

Standard western blot protocol was used for quantification of NR4A protein expression as previously described on samples from 27 subjects (16 young and 11 elderly). Only samples from males were used in this study. Muscle protein lysate was extracted from frozen muscle specimens (approximately 30 mg) as previously described. Protein concentrations were measured by the bicinchoninic acid (BCA) technique with bovine serum albumin as a standard. Samples were run on 10% Bis-Tris (Biorad) SDS-PAGE gel matrices with a total protein load of 30 μg per
lane. Samples within subjects across time were loaded in adjacent lanes. Proteins were transferred to Nitrocellulose membranes at 12 V for 1.5 hours using Bio-Rad semi-dry transfer cassette. NR4A1 polyclonal antibody was obtained from Santa Cruz and NR4A3 monoclonal antibody was obtained from Pierce. Optimal primary antibody concentrations (1:1000, volume dilution) were determined by preliminary experiments and data sheet from manufacturer. Horseradish peroxidase-conjugated secondary antibody was used at 1:2,000 (volume dilution), followed by ECL detection. Relative protein expression was determine by density and area scanning using Image One software normalized by amido-black staining on all membranes. Parameters for image development were consistent across all membranes by predefined saturation criteria as described previously[42].

Statistics

All statistical work was accomplished under Sigma Plot software. Repeated-measure ANOVAs were used to identify effects of time and age. Tukey HSD tests were performed post hoc. Data are presented as mean± standard deviation. Significance was accepted only if P <=0.05 for all tests.
Results

The fundamental metabolic parameters are shown in Table 1. No statistically significant differences were observed in the following: body weight (mean 88.9kg in elderly and 81.6kg in young, p>0.05), height (1.79m in elderly and 1.78m in young, p>0.05), body mass index (27.7kg/m2 in elderly and 25.7 in young, p>0.05), fasting blood glucose (105mg/dL in elderly and 100 mg/dL in young, p>0.05), fasting insulin (11.6mg/dL in elderly and 9.4mg/dL in young, p>0.05), or lean mass (57739G in elderly and 59246G in young, p>0.05).

However, there was a trend toward metabolic syndrome in elderly subjects, manifested by higher systolic blood pressure (132.9 in elderly and 116.8 in young, p<0.01), higher total trunk fat mass (15624 in elderly and 10351 in young, p<0.01), higher waist conference (98cm in elderly and 90 in young, p<0.05). Furthermore, we also found slightly higher blood glucose, insulin, triglyceride levels, body weight, body mass index and diastolic blood pressure, as well as a slightly reduced lean mass in elderly subjects. These observations were consistent with the previous notion that the prevalence of metabolic syndrome clusters is positively correlated with aging, as reviewed by Solano et al in 2009.[45]

Previously, reports demonstrated that exercise induces NR4A mRNA expression in rodent models.[36, 37] Surprisingly, our results (Figure 1) showed that NR4A1 protein expression was down-regulated by both Acute Exercise (-35%, p<0.01) and Chronic Exercise (-75%, p<0.01) in elderly subjects. Unlike the protocols in previous publications,
in which animals were sacrificed immediately after exercising $^{[36]}$, we performed muscle biopsy 24 hours after resistance loading. Thus it would be possible that NR4A1 mRNA/protein was transiently induced after exercising, and decreased to baseline levels before muscle biopsies were performed. Moreover, chronic resistance loading-induced NR4A1 down-regulation suggests that it may be involved in muscle adaptations to continuous training.

In contrast, NR4A1 protein expression was transiently stimulated by acute resistance loading by 50% in young subjects as shown in Figure 2. These observations seem to be consistent with the previous report showing that NR4A1 mRNA expression is up-regulated after acute resistance loading$^{[37]}$, suggesting a translation from mRNA to protein expression. However, we also observed a 30% reduction in NR4A1 protein expression after chronic exercise in young subjects, which was similar to the results obtained from elderly subjects, and may reflect muscle physiological adaptations to continuous exercise.

Comparing the NR4A1 protein expression, we found that there was a significant difference between elderly subjects and young subjects only after acute resistance loading as shown in Figure 3 (p<0.05). There were no differences between the old and young subjects at baseline or after chronic exercise (p>0.05).

Interestingly, we did not observe a significant effect of exercise on NR4A3 protein expression after acute resistance loading or after chronic resistance loading, compared to baseline as shown in Figure 4. However, there was a consistent age dependent effect on NR4A3 protein expression manifested by higher expression at base
(+180%, p<0.01) and after chronic exercise (+120%, p<0.05) in young subjects. Thus it appears that NR4A3 protein expression is diminished with aging.

**Discussion**

Previously, our group reported that the mRNA expression of NR4A1 and NR4A3 are suppressed in diabetic rodent models.\[^{27}\] Furthermore, lentiviral mediated NR4A3 hyperexpression significantly enhances insulin mediated GLUT-4 translocation to the plasma membrane and insulin stimulated glucose transport in 3T3-L1 adipocytes.\[^{27}\]

Consistently, we also found that lentiviral mediated NR4A3 overexpression enhances insulin stimulated glucose transport in C2C12 myocytes. (data unpublished, see chapter 1) Thus, NR4A1 and NR4A3 appear to play regulatory roles in insulin sensitization in both skeletal muscle and adipocytes.

As described before, exercise improves insulin sensitivity in both healthy subjects and diabetic patients,\[^{10, 11}\] and it has been part of diabetic therapy regimens to reverse insulin resistance.\[^{12, 13}\] However, the mechanism by which exercise improves insulin sensitivity remains poorly understood despite decades of research effort. Recently, Mahoney et al reported that NR4A mRNAs were robustly induced 3 hours after endurance exercise in human vastus lateralis muscle, implicating a potential role of NR4As in mediating exercise induced insulin sensitization.\[^{36}\] However, it was still unclear whether NR4A mRNA up-regulation was induced by contractility or circulating neurotrophic factors. Later Kawasaki et al proposed that the contractile activity was involved in NR4A mRNA upregulation after resistance loading by showing that electrical...
stimulation of isolated skeletal muscle, which eliminated the impact of circulating factors, induced NR4A mRNA expression.\textsuperscript{[37]} These results suggested the possibility that NR4As may be involved in exercise mediated insulin sensitization. Thus, measuring NR4A protein expression before and after exercise training appears to be imperative in elucidating the role of NR4As in exercise and insulin sensitization in skeletal muscle.

Our results indicate that age and exercise exerte differential effects on NR4A protein expression in human skeletal muscle. NR4A1 expression is similar at baseline in both young and old, and is induced by AE in young subjects only. On the other hand, baseline NR4A3 expression is lower in elderly and does not show an exercise induced expression change.

To date, this is the first report illustrating that NR4A protein expression is age dependent. We still do not know the underlying mechanism of differential expression of NR4A protein in different age groups. However, evidence indicates that NR4A expression is induced by β-adrenergic (β2 and β3 adrenergic receptors) signaling cascades in skeletal muscle.\textsuperscript{[34]} In a later report, the same group showed that β2 adrenergic agonists robustly induce NR4As, particularly NR4A3 expression in slow-twitch and fast-twitch skeletal muscles.\textsuperscript{[35]} Consistent with this notion, downstream adrenergic effector molecules, such as PKA, CREB and MAPK are also involved in β-adrenergic signaling induced NR4A up-regulation.\textsuperscript{[35]} This NR4A induction does not seem to be unique in skeletal muscle because they also found that the β adrenergic agonist isoprenaline stimulates NR4A expression in several tissues of cardiovascular, endocrine and gastrointestinal origin.\textsuperscript{[46]} Since we observed differential expression of NR4A1 in old versus young men, exercise induced β-adrenergic signaling cascades may
be impaired with aging. This hypothesis seems to be consistent with a report showing that Isoproterenol, a β-adrenergic agonist, mediated responses in skeletal muscle is impaired with aging.\cite{47} Blaak EE et al also demonstrated an age dependent impairment of β-adrenergic signaling induced energy expenditure and lipid oxidation.\cite{48,49} Moreover, numerous reports demonstrate diminished β-adrenergic responses in aging cardiomyocytes.\cite{50,51} Thus, it appeared that diminished β-adrenergic signaling may partially explain the higher acute exercise NR4A1 induction in young subjects compared to elderly subjects. However, this hypothesis needs to be validated by further studies.

It was interesting to see that chronic exercise leads to diminished NR4A1 protein expression in both elderly and young subjects. This may be an effect of training since chronic resistance training was shown to abolish acute exercise induced muscle stress and damage.\cite{52} Moreover, regular exercise also decreases pro-inflammatory cytokine production in various tissues.\cite{53-55} Steensberg et al demonstrated that this muscle contraction dependent inhibitory effect on pro-inflammatory cytokines, such as TNF-α, was mediated by IL-6.\cite{56,57} On the other hand, exercise training can enhance the IL-10/TNF-α ratio, an indicator of the inflammatory status\cite{54,58} causing inhibition of Toll-like receptor 4 (TLR-4) signaling, which promotes inflammatory cytokine production.\cite{59} The degree of anti-inflammatory effect appears to be correlated with exercise duration, intensity, and specific muscle mass involved.\cite{57} Coincidently, NR4As have been reported to be induced by TLR-4\cite{60-62} and TNF-α.\cite{61} Thus exercise mediated NR4A up-regulation may be partially dependent upon activation of inflammatory cytokine mediated signaling cascade involving TLR-4 and TNF-α.

On the other hand, we observed that expression of NR4A3 protein at baseline and
after chronic exercise diminishes with aging. Given our previous data showing that NR4A3 was down-regulated in insulin resistant rodent models,\cite{27} and the fact that elderly subjects were more insulin resistant than young subjects in this study, it seems that NR4A3 may be inversely correlated with metabolic health in the elderly subjects. Given that other groups have reported that exercise induced mRNA up-regulation of NR4A3 was more robust than that of NR4A1, we were surprised to observe no effect of exercise on NR4A3 protein expression in either young or elderly subjects.\cite{37} However, our experimental protocol differed from previous reports in that we performed muscle biopsy 24 hours after resistance loading. It could also be possible that statistical significance could be achieved with a larger sample size.

In any event, our data demonstrate differential effects of age and exercise on NR4A protein expression in human skeletal muscle. In terms of NR4A1, levels are similar in young and old at baseline, increased by acute exercise in young only, and reduced in young and old following chronic resistance training. On the other hand, NR4A3 protein levels are substantially diminished in elderly subjects at baseline and after chronic exercise, and are not significantly affected by exercise. These results are at odds with the reports that exercise augments mRNA expression for NR4A3 and call for a re-examination of the role of NR4A in exercise. Additional study is needed to elucidate the functional significance of NR4A3 depletion with aging, and its potential role in insulin sensitivity and muscle mass and/or function.
Acknowledgement

We thank UAB Diabetes Research and Training Center for providing facility and equipment for this study. We thank participants for their effort.

References


Figure Legends

**Figure 1. Exercise induced NR4A1 protein expression change in elderly subjects.**

11 old (64.8±4.0 yr) males were selected. Muscle biopsies were performed at baseline, 24hrs after acute exercise or chronic exercise. Acute exercise contained a single bout of 3 X 8-12 sets of knee extension while chronic exercise indicated a 3day/week continuous training program for 16 weeks. NR4A protein expression was measured with western blot and amido-black staining was used as loading control. NR4A protein expression level was expressed as NR4A protein band density quantification divided by amido-black stain density quantification. ANOVA analysis was performed to comparison between different groups. Results showed that NR4A1 protein expression was down-regulated by both Acute Exercise (-35%, p<0.01) and Chronic Exercise (-75%, p<0.01) in elderly subjects. NR4A1 protein expression was significantly lower after Chronic Exercise than after Acute Exercise.(p<0.05)

**Figure 2. Exercise induced NR4A1 protein expression change in young subjects.** 16 healthy young (25.8±4.8 yr) males were selected. Muscle biopsies were performed at baseline, 24hrs after acute exercise or chronic exercise. Acute exercise contained a single bout of 3 X 8-12 sets of knee extension while chronic exercise indicated a 3day/week continuous training program for 16 weeks. NR4A protein expression was measured with western blot and amido-black staining was used as loading control. NR4A protein expression level was expressed as NR4A protein band density quantification divided by amido-black stain density quantification. ANOVA analysis was performed to
comparison between different groups. NR4A1 protein expression was transiently stimulated by acute resistance loading by 50% (p<0.01) and suppressed by 35% (p<0.05) by chronic resistance loading in young subjects.

Figure 3. Comparing Exercise induced NR4A1 protein expression change in young subjects and old subjects. The young and old subjects described previously were involved. Muscle biopsies were performed at baseline, 24hrs after acute exercise or chronic exercise. Acute exercise contained a single bout of 3 X 8-12 sets of knee extension while chronic exercise indicated a 3day/week continuous training program for 16 weeks. NR4A1 protein expression was measured with western blot and amido-black staining was used as loading control. NR4A1 protein expression level was expressed as NR4A protein band density quantification divided by amido-black stain density quantification. ANOVA analysis was performed to comparison between different groups. NR4A1 protein expression significantly different only after acute resistance loading.(p<0.05)

Figure 4. Comparing Exercise induced NR4A3 protein expression change in young subjects and old subjects. The young and old subjects described previously were involved. Muscle biopsies were performed at baseline, 24hrs after acute exercise or chronic exercise. Acute exercise contained a single bout of 3 X 8-12 sets of knee extension while chronic exercise indicated a 3day/week continuous training program for 16 weeks. NR4A3 protein expression was measured with western blot and amido-black staining was used as loading control. NR4A3 protein expression level was expressed as
NR4A protein band density quantification divided by amido-black stain density quantification. ANOVA analysis was performed to comparison between different groups. NR4A3 protein expression in old subjects was significantly lower at base line (p<0.01) and after chronic exercise than that of young subjects (p<0.05).
Table 1.

Basal metabolic parameters of all participants

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Elderly</th>
<th>Young</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years old)</td>
<td>64.8±3.99</td>
<td>25.8±4.75</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.79±0.08</td>
<td>1.78±0.085</td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>88.9±10.42</td>
<td>81.64±12.2</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>27.72±2.24</td>
<td>25.66±3.28</td>
</tr>
<tr>
<td>Waist Conference (cm)*</td>
<td>97.95±8.48</td>
<td>89.57±9.4</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)**</td>
<td>132.91±7.71</td>
<td>116.75±6.11</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>80.91±2.26</td>
<td>76.31±9.0</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>136±83.69</td>
<td>113.94±60.95</td>
</tr>
<tr>
<td>Blood Glucose (mg/dL)</td>
<td>105±13.2</td>
<td>100.15±11.27</td>
</tr>
<tr>
<td>Total Trunk Fat Mass (G)**</td>
<td>15624±3942</td>
<td>10351±4916</td>
</tr>
<tr>
<td>Lean Mass (G)</td>
<td>57739±6514</td>
<td>59246±7225</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>11.58±7.2</td>
<td>9.42±5.72</td>
</tr>
</tbody>
</table>

Results were recorded from 16 young male subjects and 11 old male subjects. Values are represented as mean±standard deviation. * indicates p<0.05. ** indicates p<0.01.
Figure 1  Exercise induced NR4A1 protein expression change in elderly subjects.

![Bar graph showing protein expression levels across basal, acute exercise, and chronic exercise conditions for elderly subjects.]

Figure 2  Exercise induced NR4A1 protein expression change in young subjects.

![Bar graph showing protein expression levels across basal, acute exercise, and chronic exercise conditions for young subjects.]

* p<0.05  ** p<0.01
Figure 3. The comparison of NR4A1 protein expression between young subjects and elderly subjects after acute or chronic exercise.
Figure 4. The effect of age on NR4A3 protein expression.
SUPPLEMENTARY DATA

NR4A ORPHAN NUCLEAR RECEPTORS MODULATE INSULIN ACTION AND THE GLUCOSE TRANSPORT SYSTEM: POTENTIAL ROLE IN INSULIN RESISTANCE

by

FU YUCHANG, LUO LIEHONG, LUO NANLAN, ZHU XIAOLIN, W.TIMOTHY GARVEY


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ABSTRACT

Prior to current study, we observed that expression of two NR4A orphan nuclear receptors, NR4A3 and NR4A1, was altered by insulin in cDNA microarray analyses of human skeletal muscle, we studied whether these receptors could modulate insulin sensitivity. We found that both NR4A3 and NR4A1 were induced by insulin and by thiazolidinedione drugs (pioglitazone and troglitazone) in 3T3-L1 adipocytes. Furthermore, gene expression of NR4A3 and NR4A1 was reduced in skeletal muscles and adipose tissues from multiple rodent models of insulin resistance. To determine whether NR4A3 could modulate insulin sensitivity, 3T3-L1 adipocytes were stably transduced with NR4A3 or LacZ (control) lentiviral vectors. Compared with LacZ expressing cells, hyperexpression of NR4A3 increased the ability of insulin to augment glucose transport activity, and the mechanism involved increased recruitment of GLUT4 glucose transporters to the plasma membrane. NR4A3 hyperexpression also led to an increase in insulin-mediated tyrosine phosphorylation of insulin receptor substrate-1 as well as Akt phosphorylation. Suppression of NR4A3 using lentiviral short hairpin RNA constructs reduced the ability of insulin to stimulate glucose transport and phosphorylate Insulin receptor substrate-1 and Akt. Thus, NR4A3 and NR4A1 are attractive novel therapeutic targets for potential amelioration of insulin resistance, and treatment and prevention of type 2 diabetes and the metabolic syndrome.
FIGURE LEGEND

FIGURE 1. Effects of insulin on NR4A3 and NR4A1 gene expression in 3T3-L1 adipocytes. A and B, fully differentiated 3T3-L1 adipocytes were treated with 100 nM of insulin (treated) or 0 nM insulin (control) from 0 to 8 h. The control and treated cells were lysed and the mRNAs were extracted for cDNA synthesis. A, quantitative real-time PCR was used to measure expression of NR4A3. B, quantitative real-time PCR was used to measure expression of NR4A1. C and D, fully differentiated 3T3-L1 adipocytes were treated with 100 nM insulin alone or plus 10 nM LY294002 (phosphatidylinositol 3-kinase inhibitor), 10 nM SB203580 (p38 MAPK inhibitor), and 50nM Ro3 18220 (protein kinase C inhibitor) for 1 h. The control and treated adipocytes were lysed, and themRNAs were extracted for cDNAs synthesis. C, quantitative real-time PCR was performed for detecting the expression levels of NR4A3. D, quantitative real-time PCR was performed for detecting the expression levels of NR4A1. Results represented the mean ± S.E. from three separate experiments. p < 0.05 (*) and p < 0.01 (**) compared with base line.

FIGURE 2. Thiazolidinediones stimulate NR4A3 and NR4A1 gene expression in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated with 10 μM concentrations of the indicated thiazolidinedione (pioglitazone or troglitazone) from 0 to 48 h. Control cells were treated with vehicle alone. The control and treated adipocytes were lysed, and the mRNAs were extracted for cDNA synthesis. A and B, quantitative
real-time PCR was used to measure expression levels of NR4A3 gene induced with pioglitazone or troglitazone. C and D, quantitative real-time PCR was used to measure expression levels of the NR4A1 gene induced with pioglitazone or troglitazone. Results represented the mean ± S.E. from three separate experiments. \( p < 0.05 \) (*) and \( p < 0.01 \) (**) compared with base line.

**FIGURE 3.** NR4A3 modulates insulin-responsive glucose transport activity. A, in fully differentiated stably transduced 3T3-L1 adipocytes, control cells expressing LacZ and cells overexpressing NR4A3 were incubated in the absence (basal) and presence of insulin (100 nM) for 30 min at 37 °C. Measurements of 2-deoxyglucose transport were then performed. Results represented the mean±S.E. from three separate experiments; **, \( p<0.01 \) for comparing insulin-stimulated control and insulin-stimulated NR4A3 overexpressing cells. B, fully differentiated 3T3-L1 adipocytes were infected with shRNA-NR4A3 or control scramble lentiviruses 3 days before performing insulin-stimulated glucose uptake experiments as described above. Results represented the mean±S.E. from three separate experiments; *, \( p<0.05 \) for comparing insulin-stimulated control cells and insulin-stimulated NR4A3 inhibited cells.

**FIGURE 4.** NR4A3 and NR4A1 expression in skeletal muscle and adipose tissue of diabetic and insulin-resistant rats and mice. Skeletal muscle (gastrocnemius) and adipose tissue (white fat) from diabetic and insulin-resistant rats or mice and respective insulin sensitive controls were homogenized, and mRNAs were extracted for cDNA syntheses. Quantitative real-time PCR was used to measure the expression of
NR4A3 and NR4A1 genes. A, NR4A3 and NR4A1 gene expression in skeletal muscles from streptozotocin-induced diabetic rats (*STZ Rat*) and in skeletal muscles and adipose tissues from Zucker diabetic fatty rats (*ZDF Rat*) and in respective control rats. Shown are NR4A3 and NR4A1 gene expression in skeletal muscles and adipose tissues from ob/ob mice (*B*) and db/db mice (*C*) as well as in the respective control mice. All results represent the mean±S.E. from three separate experiments. $p<0.05$ (*) and $p<0.01$ (**) for comparing control and obese/diabetic animals.
Figure 3
Figure 4

A Skeletal muscle

Adipose tissue

B Skeletal muscle

Adipose tissue

C Skeletal muscle

Adipose tissue
SUMMARY

For the past several decades, Type 2 Diabetes Mellitus and the Metabolic Syndrome have been increasing health challenge in the United States and worldwide. A comprehensive survey estimated that approximately eight percent of American adults were affected by this disease, which has exerted heavy social burden and economic cost.\textsuperscript{135} Moreover, the prevalence of metabolic syndrome, a risk factor for the development of Type 2 Diabetes, was estimated to be 21.7\% during 1988-1994 by using NCEP/ATP\textsuperscript{III} criteria.\textsuperscript{136}

Among all the manifestations, insulin resistance is believed to be the central pathogenic event for both Type 2 Diabetes and Metabolic Syndrome.\textsuperscript{137} Amelioration of insulin resistance and improving insulin sensitivity, especially in those major glucose metabolism tissues such as adipose tissue and skeletal muscle, have been long term targets for therapeutic interventions for Type 2 Diabetes and metabolic syndrome.

As described before, skeletal muscle, adipose tissue and liver are three major insulin target tissues. Skeletal muscle has been demonstrated to be the predominant glucose disposal tissue and makes up 85\% of insulin stimulated glucose transport in human.\textsuperscript{4} Thus, amelioration of insulin resistance in skeletal muscle appears to be a cornerstone in Type 2 Diabetes.
Currently, the therapeutic regimen of Type 2 Diabetes includes lifestyle modification and medication therapy. Based upon the pharmacological action, anti-diabetic reagents could be divided into the following categories: insulin sensitizer with predominant action in the liver (Bioguanides), insulin sensitizer in peripheral insulin target tissues (Thiazolidinediones), reagents enhancing insulin secretions (Sulfonylureas), Carbohydrate Absorption Inhibitors (Acarbose and Miglitol) and insulin.138-141

Among all these anti-diabetic medications, the thiazolidinedione class (TZDs) represents the only category which acts primarily by improving insulin sensitivity in skeletal muscle, the major glucose disposal tissue. The pharmacological effect of TZDs is largely attributed to PPAR-γ agonism, which regulates the expression of a panoply of glucose metabolism genes.142 TZDs progressively improve insulin sensitivity and reduce free fatty acid over weeks to months.142

TZDs provide a good proof-of-principle that insulin sensitivity can be improved by transcriptional regulators. However, various side effects, such as weight gain143, increased risk for Congestive Heart Failure144 and liver dysfunction145 have been reported, which limited the clinical utilization of TZD medications. Thus, a new category of insulin sensitizing drug, which has a novel pharmacological target and may have a distinct side effect spectrum, is avidly needed in the combat against Type 2 Diabetes and metabolic syndrome, especially for those patients with poor TZD tolerance.

Recently, there is a growing interest of NR4A orphan nuclear receptors as novel targets for metabolism regulation. As described before, NR4As were implicated in β-adrenergic signaling81-84 and were involved in the regulation of a panoply of genes.
which were important in glucose and lipid metabolic signaling pathways such as AMPKγ3, fatty acid translocase, GLUT4, Lipin1, FOXO1, PGC1α, etc.

Our interest on NR4As as regulators of insulin action and glucose metabolism dates back to our micro-array experiment five years ago when we found that NR4A mRNA expression could be induced by insulin in human subjects. Using cultured L6 myocytes, we confirmed that NR4A1 was significantly up-regulated by insulin. Furthermore, insulin caused NR4A1 accumulation in the nucleus, which was consistent with its potential role as a transcriptional regulator of insulin action. In a later report, we demonstrated that NR4A3 enhanced insulin sensitivity and glucose transport stimulation in 3T3-L1 adipocytes, and was down-regulated in insulin resistant or diabetic rodent models. These data strongly suggested that NR4A3 may play a regulatory role in the regulation of insulin action and glucose metabolism.

In the current study, we tried to explore whether an insulin-sensitizing effect in muscle cells and to identify a potential trans-activator. First, lentiviral technology was used to engineer both overexpression and shRNA knockdown of NR4A3 in C2C12 stable myocytes. The overexpression construct caused approximately one fold increase in NR4A3 protein expression, and these cells exhibited a 30% (p<.05) increase in insulin mediated glucose transport, without significant effects on baseline, when compared to control cells transduced with lentiviral Lac-Z control. In contrast, the knockdown construct suppressed NR4A3 protein expression by 75%, and these myocytes exhibited a 35% (p<.05) reduction in insulin-stimulated glucose transport rates compared with scramble control cells. Consistently, insulin-stimulated PKB/AKT phosphorylation was increased 50% by NR4A3 overexpression and decreased by 20% following NR4A3
attenuation. The next experiments examined effects of prostaglandin A$_2$ (PGA$_2$) on insulin action and NR4A3 trans-activation. PGA$_2$ enhanced insulin-stimulated glucose uptake in C2C12 myocytes by 30% (p<.05) and AKT phosphorylation by 80% following 24 hour treatment, with no significant effects on basal transport or basal AKT phosphorylation.

To determine whether NR4A3 was involved in mediating the action of PGA$_2$, we showed that PGA$_2$ caused greater improvement in insulin-stimulated glucose rates (45%, p<.05) in NR4A3 overexpressing C2C12 myocytes, when compared with Lac-Z controls stimulated with insulin and/or PGA$_2$. Moreover, the sensitizing effect of PGA$_2$ was significantly diminished (p>.05) in myocytes transduced with NR4A3 knockdown cell lines compared to scramble controls. Other prostaglandin family members, including PGE$_2$, PGA$_2$-IsoP did not significantly enhance insulin sensitivity in either cultured myocytes or adipocytes. These findings extended our previous report to skeletal muscle.

The function of different NR4A members could be either redundant or compensatory to each other. For instance, Both NR4A3 and NR4A1 have been implicated to play regulatory roles on metabolism related gene expression such as fructose bisphosphatase-2 (a.k.a. phosphofructokinase 2) and enolase-3 in liver. In contrast, the role of NR4A3 was less understood compared with that of NR4A1. However, the consistency between previous report about NR4A1 and our data suggested that both NR4A family members could overlap in their metabolic effects.

As a matter of fact, NR4A1 hyperexpression in C2C12 myocytes enhanced basal glucose transport and up-regulated a panoply of glucose disposal gene expression such as
GLUT4, phosphofructokinase, and glycogen phosphorylase, without significant effect on GLUT1 expression.\textsuperscript{85} Moreover, NR4A1 knockout mice exhibited no difference from wild-type mice when fed standard diet, but are more obese and insulin resistant when maintained on high fat diet. Also, NR4A1 null mice exhibited increased intramyocellular lipid level and hepatic steatosis, but maintained normal hepatic insulin sensitivity.\textsuperscript{89}

Taken together, these data suggest that NR4A3 and NR4A1 modulate cell-type specific metabolic functions, and enhance metabolic functions and insulin actions in muscle. The current effects on insulin action indicated that the manipulation of NR4A3 expression predominated in the modulation of insulin sensitivity under these conditions specified in our report.

Previous reports demonstrated that cyclopentenone prostaglandins modulated the transcriptional activity of other nuclear receptors such as PPAR-\(\gamma\), NF-\(\kappa\)B, AP-1, Nrf2, HIF1- \(\alpha\),\textsuperscript{147} and estrogen receptor-\(\alpha\).\textsuperscript{148} These prostaglandins include PGA\(_2\), PGA\(_1\), and PGJ\(_2\) possess an unsaturated carbonyl group that is electrophilic and reactive.\textsuperscript{123,149} Thus, cyclopentenone prostaglandins covalently bind specific target proteins via the interaction between cysteine thiols and the \(\alpha,\beta\)-unsaturated carbonyl moiety in the cyclopentenones.\textsuperscript{148} The reaction was shown to be non-promiscuous, highly specific, and can alter protein activities.\textsuperscript{123,149-152}

Because of the absence of hydrophobic cleft, NR4A receptors were thought to function constitutively without physiologic ligands. However, Kagaya et al showed that PGA\(_2\) activated NR4A3-dependant transcription, and by comparing truncated mutants and wild type NR4A3 protein, they demonstrated that this PGA\(_2\) action was dependent upon interaction with the NR4A3 ligand binding domain.\textsuperscript{71} Thus, we hypothesized that
PGA$_2$ would improve skeletal muscle insulin sensitivity through transactivation of NR4A3.

In the current studies, we did, for the first time, report that PGA$_2$ augmented insulin sensitivity. However, it remained unclear whether this effect was dependent upon the full presence of NR4A3. Therefore, we studied the effect of PGA$_2$ following both attenuation and overexpression of NR4A3. PGA$_2$ failed to augment insulin sensitivity following NR4A3 attenuation, whereas it had a greater insulin-sensitizing effect in myocytes overexpressing NR4A3. Thus, we conclude that the insulin sensitizing effect of PGA$_2$ was dependent upon NR4A3.

The interaction between PGA$_2$ and a nuclear transcription factor (NR4A3) is analogous to that between PGJ$_2$ metabolites and PPAR$_{\gamma}$ nuclear receptor. PGJ$_2$ was first reported as a dehydration product of PGD$_2$.\textsuperscript{153} $\Delta^{12}$-PGJ$_2$ and 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ were metabolites of PGJ$_2$ following albumin mediated catalysis.\textsuperscript{154} Importantly, 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ was found to enhance insulin action via PPAR$_{\gamma}$ agonism.\textsuperscript{129} In light of the current results, it is important to note that PGA$_2$ is not known to interact with PPAR$_{\gamma}$.\textsuperscript{123} After comprehensively screening a large number of natural arachidonic acid and glycerolipid metabolites from the KEGG database, Kagaya et al showed that only PGA$_2$ and PGA$_1$ have the unique ability to activate NR4A3-dependent transcription.\textsuperscript{71} Furthermore, using mutational analyses and a Biacore plasmon resonance sensor, they showed that PGA$_2$ action was dependent upon binding of PGA$_2$ to the ligand binding domain of NR4A3. Even so, the underlying mechanism by which PGA$_2$ interacts with NR4A3 in muscle cells remains unclear even though our data suggests that the full presence of NR4A3 is necessary for the insulin-sensitizing affect of PGA$_2$. 
In short, our results demonstrated a working model that an arachidonic acid molecule regulates insulin action in muscle cells via a nuclear receptor. This provides proof-of-principle that future pharmacological compound may exert insulin-sensitizing effects by interacting with NR4A3, which could be largely summarized with the following model figure.

![Diagram](image)

Figure 1. Schematic figure illustrating PGA2 augments insulin sensitivity in skeletal muscle via a NR4A3 dependent manner.

Given the obvious importance of skeletal muscle in glucose metabolism, physical exercise has been part of the therapeutic regimen to reverse insulin resistance and to prevent Type 2 Diabetes.

Currently, the underlying mechanism by which exercise facilitates glucose disposal and GLUT4 translocation remains poorly understood. It was proposed that acute
or chronic exercise mediated beneficiary effect on glucose transport may be mediated by AMPK\textsuperscript{155-157} or PGC-1α\textsuperscript{158,159}, respectively.

Our group has demonstrated the role of NR4As in a previous publication\textsuperscript{60} and manuscript 1. Also, exercise induced NR4A1 and NR4A3 mRNA up-regulation in skeletal muscle has been reported by various groups.\textsuperscript{160,161} These results suggested that NR4As may be involved in exercise facilitated glucose disposal. However, these observations did not address whether NR4A protein expression could be altered by acute or chronic exercise. Moreover, the age dependent differential expression of NR4A proteins remains unclear.

In the second manuscript, we endeavored to explore the exercise and age dependent NR4A expression in human skeletal muscle. Eleven healthy elderly males (64.8±4 year old) and sixteen healthy young males (25.8±4.8 year old) participated in the study. All subjects underwent a single bout (Acute Exercise, AE) and a 16 week resistance training program (3days/wk, CE) focused primarily on knee extensors. Each exercise was performed for three sets of 8-12 repetitions. Muscle biopsy was performed at baseline, 24 hr after AE and CE. Metabolic parameters were recorded. Relative NR4A protein expression was measured with western blot.

Results showed that NR4A1 protein expression was up-regulated after AE only in young people (+50%, p<0.01). AE/Basal NR4A1 ratio was significantly correlated with basal BMI (p<0.01), total trunk fat mass (p<0.05) and waist hip ratio (p<0.05). In contrast, NR4A1 expression was down-regulated by AE in old people (-35%, p<0.01)) and no significant correlation are observed. There was no significant difference of NR4A1 expression between young and old people except after AE where young people showed a
90% higher NR4A1 level. Interestingly, there is a significant down-regulation of NR4A1 after CE in both young (-30%, p<0.05)) and old (-75%, p<0.01)). No significant AE or CE induced NR4A3 expression change is found. On the other hand, NR4A3 expression is higher in young people compared to old in both basal (+180%, p<0.01) and CE (+120%, p<0.05) groups.

Our results demonstrated that age and exercise played differential regulatory roles on NR4A protein expression in skeletal muscle. NR4A1 expression was similar at baseline in both young and old subjects, induced by AE in young subjects only and suppressed by CE in both age groups. In contrast, NR4A3 expression was lower in elderly and was not induced by either AE or CE. These results expand previous observations to protein level and suggest that different mechanisms may be involved in NR4A regulation in different age groups. Additional data is needed to elucidate the role of NR4As in exercise facilitated glucose metabolism and insulin sensitivity.


