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# Muscle Plasticity and Intramuscular signaling in the Insulin-resistant Obese Zucker Rat

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# MUSCLE PLASTICITY AND INTRAMUSCULAR SIGNALING IN THE INSULIN-RESISTANT OBESE ZUCKER RAT

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by
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## ABSTRACT

### MUSCLE PLASTICITY AND INTRAMUSCULAR SIGNALING IN THE INSULIN-RESISTANT OBESE ZUCKER RAT

**By Anjaiah Katta**

The ability to increase skeletal muscle mass may have important implications for the treatment of insulin resistance (IR) and diabetes [1-3]. Recent data suggest that IR muscle may adapt differently than normal muscle; however, molecular mechanism(s) responsible for this finding are not well understood [4]. Herein, we investigate the molecular mechanisms underlying the skeletal muscle remodeling in the IR Obese Zucker (OZ) rat.

The OZ rat is characterized by skeletal muscle insulin resistance, hyperglycemia, and hyperlipidemia. Compared to LZ rats, our data demonstrate that soleus muscle hypertrophy was significantly attenuated in the OZ rats after 3-weeks of muscle overload and that these findings appear to be accompanied by significant impairments in the ability of the soleus to undergo phosphorylation of mammalian target of rapamycin (mTOR), 70 kDa ribosomal protein S6 kinase (p70S6k), ribosomal protein S6 (rpS6) and protein kinase B (Akt).

Recent *in vitro* and *in vivo* studies have suggested a role for AMP-activated protein kinase (AMPK) and dsRNA-dependent protein kinase (PKR) in skeletal muscle adaptation and their interactions with mTOR related signaling [5, 6]. Our data suggest that IR attenuates overload-induced skeletal muscle hypertrophy through the activation of AMPK and PKR, which appears to be associated with an inhibition of mTOR and eIF2α phosphorylation. This finding is consistent with the possible depression of protein synthesis. Other data demonstrate that IR resistance is associated with the PKR-mediated activation of p38 MAP kinase, which would be predicted to lead to increased protein degradation. Further, we demonstrated that the regulation

of heat shock proteins (HSPs) and the mitogen-activated protein kinases (MAPKs) are altered during hypertrophy in OZ rat, which suggest that these molecules may play a role in explaining why IR may be associated with alterations in muscle plasticity.

In addition to traditional biochemical signaling cascades, recent data have strongly suggested that muscle-specific miRNAs may participate in the regulation of load-induced skeletal muscle remodeling [7]. To this end, we demonstrate for the first time that miR-1 and miR133 expression levels are lower in IR muscle. Further, we also observed that overload decreased mir-1 expression in the LZ muscle to a greater extent to that measured in the OZ muscle. Combined, these results are the first to report evidence that overload-induced skeletal muscle remodeling in IR OZ rat is associated with multiple level decrements including changes in mTOR signaling, hyperphosphorylation of AMPK and PKR and altered regulation of muscle-specific miRNAs.

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## ABBREVIATIONS

| | |
|---|---|
| 4E-BP1 | Eukaryotic translation initiation factor 4E-binding protein 1 |
| AAALAC | Association for assessment and accreditation of laboratory animal care |
| AICAR | 5-aminoimidazole-4-carboxamide ribonucleoside |
| ANOVA | One-way analysis of variance on ranks |
| AMPK | AMP-activated protein kinase |
| BSA | Bovine serum albumin |
| CaMKII | Calcium/calmodulin-activated protein kinase |
| DM | Diabetes mellitus |
| ECL | Enhanced chemiluminescence |
| eEF2 | eukaryotic elongation factor 2 |
| ERK | Extracellular regulated kinase |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GSK-3β | Glycogen synthase kinase 3 beta |
| HSP | Heat shock protein |
| IGF | Insulin-like growth factor |
| IR | Insulin resistance |

| | |
|---|---|
| IRS | Insulin receptor substrate |
| JNK | c-Jun N-terminal kinase |
| LZ | Lean Zucker |
| MAPK | Mitogen-activated protein kinase |
| miRNA | MicroRNA |
| MS | Metabolic syndrome |
| mTOR | Mammalian target of rapamycin |
| OZ | Obese Zucker |
| p70S6K | 70 kDa ribosomal protein S6 kinase |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline with 0.5% tween |
| PI3K | Phosphatidylinositol 3-kinases |
| PKB | Protein kinase B |
| PKR | dsRNA-dependent protein kinase |
| PLD | Phospholipase D |
| PRT | Progressive resistance training |
| PTEN | Phosphatase and tensin homolog deleted on chromosome 10 |

| | |
|---|---|
| Rheb | Ras-homolog enriched in brain |
| RISC | RNA-induced silencing complex |
| ROS | Reactive oxygen species |
| rpS6 | Ribosomal protein S6 |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| TAK1 | Transforming growth factor-β-activated kinase-1 |
| TBS | Tris buffered saline |
| TBST | Tris buffered saline with 0.5% tween |
| T2DM | Type 2 diabetes mellitus |
| TGF-β | Transforming growth factor beta |
| TNF-α | Tumor necrosis factor-α |
| T-PER | Tissue Protein Extraction Reagent |
| TSC2 | Tuberous sclerosis protein 2 |

# CHAPTER 1

## INTRODUCTION

The incidence of diabetes is growing at epidemic proportions and is rapidly becoming a global health problem. According to the World Health Organization, the number of diabetics throughout the world was 171 million in the year 2000 and is estimated to be 336 million by 2030. India is the diabetic capital of the world with the largest numbers of people with diabetes (40.9 million), followed by China (39.8 million), the United States (19.2 million), Russia (9.6 million) and Germany (7.4 million) (International Diabetes Federation, 2007). Diabetes affects 25.8 million people, or 8.3% of the United States population, including 7.0 million people who are unaware that they have diabetes (National Diabetic Fact Sheet, 2011). In 2010 it was estimated that 79 million American adults aged 20 years or older were prediabetic. Diabetes is the seventh leading cause of death in the United States and is associated with heart disease, stroke, peripheral vascular diseases, blindness, kidney failure and nervous system damage. The total cost of diabetes in the United States in 2007 was $218 billion (National diabetes statistics, 2007). Given this setting, it is clear that the scientific community must continue to search for more effective methods to treat and prevent this disease.

Skeletal muscle accounts for 80 to 90 percent of glucose disposal, and insulin sensitivity is thought to be a key factor in muscle glucose uptake [8]. A myriad of studies has shown that skeletal muscle insulin resistance is the key defect in maintaining glucose homeostasis and that skeletal muscle health is thought to play a central role in the pathogenesis of several components of the metabolic syndrome (MS) including obesity, diabetes, dyslipidemia, hypertension and atherosclerosis [9, 10]. Therefore, increasing skeletal muscle mass and improving function could

play an integral role in increasing glucose storage capacity and improving the regulation of glucose metabolism.

Previous studies demonstrated that different exercise modalities cause favorable metabolic adaptations in skeletal muscle, including significant increases in glucose transport and in the activities of enzymes involved in regulating glucose metabolism [11-14]. Recently we have demonstrated that diabetes affects contraction-induced signaling in skeletal muscle and have found differences in activation of MAPK signaling proteins between normal and diabetic rats immediately after and during the recovery phase of an acute bout of high-frequency electrical stimulation [15]. Further, we demonstrated that contraction induced activation of p70S6k signaling is altered in muscle of the insulin resistant obese Zucker rat [16].

Various overload models have been developed to induce skeletal muscle growth and adaptations in animal models. They include stretch hypertrophy, compensatory hypertrophy, resistance training, alteration of hormonal levels and dietary supplementation. Mechanical overload in the form of synergistic ablation provides a chronic stimulus that initiates skeletal muscle adaptation by increasing the workload of selected muscles by ablation of a few or all synergistic muscles [17]. How skeletal muscle tissue "senses" and adapts to altered loading conditions with insulin resistance is not known. Furthermore, it is not known if the adaptive potential of insulin resistant muscle differs from that of non-diabetic controls. A greater understanding of how insulin resistant muscle responds to hypertrophic stimuli may help to unravel new treatment modalities for use in the diabetic population.

Studies of exercise *in vivo* and stretch *in vitro* have established that increased loading of muscle cells induces growth [18, 19] and this growth is characterized by increases in protein synthesis [20, 21]. One critical signalling pathway controlling protein synthesis during

mechanically induced skeletal muscle growth involves the mammalian target of rapamycin (mTOR) [19, 22, 23]. mTOR protein lies downstream of Akt in the insulin signaling pathway, and is thought to integrate signals from metabolic, hormonal, nutritional and mechanical stimuli. The mTOR is also involved in regulating various physiological functions, including gene transcription, protein metabolism, the cell cycle, and cytoskeleton organization [24, 25]. When active, mTOR promotes an increase in the efficiency and capacity for translation of mRNA to protein by increasing, either directly or indirectly, the activity of several proteins, including 70 kDa ribosomal protein S6 kinase (S6k), ribosomal protein S6 (rpS6), eIF4E-binding protein 1 (4E-BP1), and eukaryotic elongation factor 2 (eEF2) [26, 27]. S6k promotes the translation of various ribosomal proteins and elongation factors (including eEF2), presumably via phosphorylation of rpS6 [28], and also promotes a general increase in protein elongation by an indirect activation of eEF2 [29]. On the other hand, mTOR phosphorylation of 4E-BP1 promotes translation initiation of 5′-cap mRNAs, including most eukaryotic mRNAs [22, 23, 30], and enhances muscle protein synthesis [19, 31, 32].

Recent *in vitro* and *in vivo* studies have suggested that 5'–AMP-activated protein kinase (AMPK) and the dsRNA-dependent protein kinase (PKR) can play a role in the inhibition of protein synthesis [6, 26, 33, 34]. It has been shown that AMPK may inhibit protein synthesis through its ability to suppress mTOR activation [22, 34]. The factors controlling the activity of AMPK have not been fully elucidated, however it is thought that protein tumor suppressor LKB1 kinase [35], calcium/calmodulin-activated protein kinase (CaMKII) [36] and transforming growth factor-β-activated kinase-1 (TAK1), a member of the mitogen-activated protein (MAP) kinase family, may be involved [37]. PKR, when activated, phosphorylates eIF2α at Ser-51 [6], which in turn leads to translational inhibition and presumably a decrease in protein synthesis

[38]. In addition to its potential effect on protein synthesis, PKR may also have a hand in controlling the ubiquitin proteolytic pathway through the activation of p38 MAP kinase [39, 40].

Stress responsive proteins like heat shock proteins (HSP) and Mitogen-activated protein kinases (MAPKs) are thought to be involved in the regulation of protein synthesis. Previous studies demonstrated the induction of HSPs following high force eccentric contraction, aerobic exercise, and during the development of muscle hypertrophy [41-43]. Similarly, several studies demonstrated that the extracellular regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) MAPK proteins are involved in the regulation of skeletal muscle hypertrophy following increased loading [44-47]. However, it is not clear whether the regulation of these molecules is altered with insulin-resistance.

In addition to these signaling cascades, recent data have strongly suggested the role of muscle-specific miRNAs (miR-1, miR-133a and miR-206) in load-induced skeletal muscle remodeling [7, 48, 49]. MicroRNAs are small endogenous ~ 22-nucleotide, non-coding RNAs and newly emerging class of trans-factors, which repress gene expression by inhibiting the translation of target mRNAs through interaction with the 3'- untranslated region (3'UTR) of target mRNAs [50, 51]. Among the muscle-specific miRNA's, miR-1 and miR-133a are important for embryonic muscle growth [52] and myoblast differentiation and proliferation by regulating the expression of SRF, MyoD and MEF2 [53]. miR-206 plays a role in myogenesis [54]. A recent study by McCarthy and Esser (2007) reported decreased expression of miR-1 and miR-133a following 7days of functional overload and suggested that this response acted to remove the repression of hypertrophic regulators [7]. How insulin resistance may affect the regulation of muscle-specific miRNAs during muscle hypertrophy is currently unclear.

**SPECIFIC AIMS**

Diabetes is growing in epidemic proportions and is becoming a global health problem. The Obese Zucker (fa/fa) rat (OZ) presents a range of metabolic aberrations such as hyperinsulinemia, hyperglycemia, and hyperlipedemia along with central obesity [55]. These animals have point mutations in the leptin receptor gene, leading to the development of hyperphagia and obesity. It is thought that the OZR closely models the progression of metabolic syndrome seen in humans [56].

Previous studies have shown decreased muscle mass in OZ rat compared to LZ rat as reflected by muscle wet-weight, protein content, DNA, and RNA [57-61]. These alterations in muscle weights are very important because these changes are highly correlated with hyperglycemia, given that skeletal muscle accounts for 80 to 90 percent of glucose disposal. Recent work by Paturi et al., has shown that the capacity of the OZ soleus muscle to undergo hypertrophy in response to increased loading is diminished compared to that observed in the LZ [4]. The molecular mechanism(s) responsible for the decreased adaptability of insulin resistant muscle is not well understood.

Our long-term goal is to improve our understanding of how insulin resistance may affect the ability of skeletal muscle to adapt to changes in contractile activity. The central *hypothesis* of this study is that insulin resistance will be associated with alterations in the activation of several signaling cascades in skeletal muscle in response to given hypertrophic stimuli and that these alterations in signaling pathway activation may be responsible for altered skeletal muscle remodeling with insulin resistance.

To test our central hypothesis and accomplish the objective of this study the following three specific aims are proposed:

**SPECIFIC AIM I:** To investigate whether insulin resistance affects the regulation of the mTOR signaling during muscle hypertrophy. (Paper I)

**SPECIFIC AIM II:** To investigate whether insulin resistance affects the regulation of the AMPK and PKR during muscle hypertrophy. (Paper II)

**SPECIFIC AIM III:** To investigate whether insulin resistance affects the regulation of the HSPs (HSP27, HSP60, HSP70 and HSP90), MAPKs (ERK1/2 and JNK) and muscle-specific miRNA's (miR-1 and miR133a) during muscle hypertrophy. (Paper III)

# CHAPTER II

## REVIEW OF THE LITERATURE

A review of the pertinent literature concerning the present study will be presented in the following chapter. The following areas will be addressed: 1. Diabetes and skeletal muscle insulin resistance; 2. Different animal models of Type 2 Diabetes Mellitus (T2DM); 3. Different overload models of skeletal muscle hypertrophy; and 4. Molecular mechanisms of skeletal muscle adaptation to increased loading.

### 2.1.1 Diabetes

Diabetes mellitus (DM) is a disease characterized by elevated levels of blood glucose resulting from defects in insulin production, insulin action, or both. Insulin is a polypeptide hormone, secreted by the β-cells of the pancreas and regulates body glucose homeostasis. A deficiency or the inability of cells to respond to insulin leads to consistent hyperglycemia, which if allowed to proceed unchecked can lead to several complications. There are two major types of diabetes, type 1 (also called juvenile-onset or insulin-dependent) and type 2 (adult-onset or non-insulin-dependent). Type 1 diabetes mellitus (T1DM) accounts for about five to ten percent of total diabetic cases and is characterized by inability of the pancreas to secrete insulin because of autoimmune destruction of the pancreatic β-cells [62]. Type 2 diabetes mellitus (T2DM) is the most common form of diabetes, which accounts for ninety to ninety five percent of all diagnosed diabetic cases [63]. T2DM is characterized by insulin resistance and/or abnormal insulin secretion, either of which may predominate. T2DM is considered a global health problem as it afflicts 171 million people worldwide (International Diabetes Federation, 2007). This epidemic is associated with increased mortality and morbidity, in particular due to cardiovascular

complications, and results in a number of complications that diminish the quality of life [64]. A better understanding of the mechanism(s) leading to the development of T2DM is needed to improve therapeutic and preventive strategies.

### 2.1.2 Skeletal muscle insulin resistance

It is a well known that skeletal muscle is the principal site for whole body glucose disposal and that insulin sensitivity is a key factor in the regulation of muscle glucose uptake. In healthy conditions, insulin tightly regulates glucose homeostasis by stimulating glucose uptake in peripheral tissues [65]. Insulin is released from β-cells of the pancreas in response to elevated levels of glucose, and its binding to insulin receptor leads to translocation of glucose transporters to the membrane and increased skeletal muscle uptake of glucose [65]. Insulin resistance (IR) refers to the decreased ability of insulin to stimulate glucose uptake in insulin sensitive tissues such as skeletal muscle and fat and to decrease glucose production in the liver. Skeletal muscle IR is the key defect in maintaining glucose homeostasis and is thought to play a central role in the pathogenesis of several components of the metabolic syndrome (MS) including obesity, diabetes, dyslipidemia, hypertension and atherosclerosis [9, 10]. Chronic hyperglycemia, a hallmark feature of diabetes, is shown to associate with reactive oxygen species (ROS) production, lipid peroxidation, and decreased antioxidant defenses [66]. However, the mechanisms responsible for development of IR and IR-mediated alterations in the adaptation of skeletal muscle to different components of the MS are poorly defined. Because of the key role of skeletal muscle IR in the pathogenesis of several components of the MS, it has been postulated that interventions that could enhance skeletal muscle mass and function may act to improve insulin action in different insulin sensitive tissues.

Different exercise modalities cause favorable metabolic adaptations in skeletal muscle and improve insulin action, including significant increases in glucose transport and activities of enzymes involved in glucose metabolism, by upregulating several proteins of insulin signaling [11-14]. The exact molecular mechanisms underlying the skeletal muscle remodeling with a given contractile stimulus are not yet understood. A review on the different molecular mechanisms of skeletal muscle adaptation to increased loading is presented in subsection 4 of chapter II.

### 2.1.3 Skeletal muscle mass

Skeletal muscle is the most abundant tissue in the human body. The maintenance of muscle mass is essential for overall health, functionality and quality of life. Skeletal muscle constitutes 55 percent of the body weight and is the primary tissue for glucose and lipid utilization. Attenuated muscle mass associated with obesity and T2DM could be particularly detrimental as blood glucose and lipid levels increase and the ability to utilize each is diminished [67]. The obese zucker rat (OZR) is characterized by significant reduction in muscle mass, even though its body weight is significantly higher compared to the LZ rat phenotype [15, 57, 68]. Identification of molecular mechanism(s) that are associated with altered skeletal muscle adaptation with T2DM may lead to prevention of the development of complications that are associated with insulin resistance.

### 2.1.4 Summary

Type 2 diabetes mellitus (T2DM) is considered as epidemic of the 21$^{st}$ century. Skeletal muscle plays an important role in glucose homeostasis and skeletal muscle insulin resistance is a

major metabolic defect leading to T2DM. A better understanding of the mechanism(s) leading to the development of T2DM is necessary to prevent the complications associated with this disease.

### 2.2 Animal models of Type 2 Diabetes Mellitus (T2DM)

Animal models of T2DM are excellent tools to study the pathophysiology of human disease. Knowledge about the manifestation of T2DM and the possible causes has expanded due to the utilization of animal models. Many of these animal models are developed using selective breeding, which typically produces specific genetic mutations in order to obtain a desired phenotype. Other methods for developing diabetic rodent models include gene targeting and transgenic techniques [69]. The following are some of the animal models that are commonly used in the field of diabetic research.

#### 2.2.1 ob/ob mouse

The ob/ob mouse was developed by mutation of chromosome 6 in the C57BL/6J strain [70]. The mutation is in the *ob* gene, which encodes for leptin protein and causes these mice to become hyperphagic and develop obesity. *Ob/ob* mice exhibit a diabetes like syndrome that is characterized by mild hyperglycemia, mildly impaired glucose tolerance, severe hyperinsulinemia, insulin resistance, and impaired wound healing [71].

#### 2.2.2 db/db mouse

The db/db mouse was developed by an autosomal recessive mutation on chromosome 4 in the C57BL/KSJ strain [72]. The mutation is in the *db* gene, which encodes for leptin receptors. These mice are spontaneously hyperphagic and develop insulin resistance within the first month.

In later stages, these animals exhibit nephropathy, neuropathy, vasculopathy and myocardial disease in a manner similar to that seen in human T2DM [72].

### 2.2.3 KK mouse

The KK mouse which was developed by inbreeding Japanese mice, displays moderate obesity, polyphagia, polyuria, persistent glucosuria and moderate hyperglycemia with hyperlipidemia [73]. These animals, in their later stages, develop renal lesions similar to human nephropathy including glomerular basement membrane thickening and proteinuria [74].

### 2.2.4 Nagoya-Shibata-Yasuda (NSY) mouse

The NSY mouse was developed by selective inbreeding and develops diabetes spontaneously in an age-dependent manner. These are non-obese and characterized by mild insulin resistance and marked impairment in insulin secretion. This model is particularly useful when considering age-related phenotypes (e.g., decline in β-cell function) [69].

### 2.2.5 New Zealand Obese (NZO) mouse

The NZO mouse is a polygenic model of diabetes and obesity developed by selective inbreeding and exhibits hyperphagia, obesity, mild hyperglycemia, hyperinsulinaemia, impaired glucose tolerance and insulin resistance. There is marked hyperplasia and hypertrophy with islets composed of up to 90 per cent of beta cells [72].

### 2.2.6 Goto Kakizaki (GK) rat

The GK rat is a non-obese model of T2DM. It was developed by selective breeding of the Wistar rats and is characterized by non-obesity with relatively stable hyperglycemia, hyperinsulinemia and insulin resistance [75]. The primary defect in these rats is in the beta cells

with “starfish-shaped” abnormalities. Later stage complications include nephropathy with thickened glomerular basement membrane, osteopathy, structural changes in peripheral nerves and altered retinal endothelial retinopathy [69, 76]. The GK rat serves as an ideal model to study diabetes without the contribution of dyslipidemia or obesity.

### 2.2.7 Zucker fatty rat

The Zucker (fa/fa) fatty or obese rat was developed by a simple autosomal recessive mutation on chromosome 5, which encodes the leptin receptor. The impaired leptin receptor mediated counter regulation leads to increased levels of circulatory leptin in these rats [77]. The classical orexigenic peptides such as neuropeptide Y, galanin, orexins and melanin-concentrating hormone are upregulated in OZ rats [78-80]. These animals show mild hyperglycemia, insulin resistance, mild glucose intolerance, hyperlipidemia, hyperinsulinemia, and moderate hypertension while they also mimic the early stage of human T2DM. The OZ rats present abnormalities similar to those seen in human metabolic syndrome and are a widely used model of insulin resistance [56].

### 2.2.8 Zucker diabetic fatty rat

This model is a substrain of Zucker fatty rat selectively inbred for hyperglycemia. In contrast to fa/fa rats, these rats have limited ability to show hyperinsulinemic responses to compensate for peripheral insulin resistance [71]. These animals exhibit impaired insulin response to hyperglycemia by the β-cells of pancreas, although the response remains intact to nonglucose secretogogues like arginine in a manner similar to human T2DM.

### 2.2.9 Otsuka Long Evans Tokushima Fatty (OLETF) rats

The OLETF rats exhibit hyperglycemia, insulin resistance, hypertension, obesity and hypertriglyceridemia with marked triglyceride infiltration into pancreatic islets [70, 81]. These rats are also characterized by diminished expression of GLUT-4,which plays a key role in skeletal muscle glucose transport,and the distribution of GLUT-4 in these rats is characteristic of human T2DM [82, 83].

### 2.2.10 Psammomys obesus gerbil

The Psammomys obesus (sand rat) gerbil is a normal inhabitant of the desert; when their native diet is substituted with laboratory diet for few weeks they develop insulin resistance with hyperinsulinemia [84, 85]. It has been shown that insulin resistance in these gerbils is associated with diminished insulin receptor action, increased serine phosphorylation of insulin receptor substrate (IRS) and increased in the activity of an enzyme of the protein kinase isoenzyme group, protein kinase C epsilon (PKCε) [86, 87].

### 2.2.11 Obese rhesus monkey

When maintained on an *ad libitum* diet, the obese rhesus monkey develops obesity, hyperinsulinemia, and insulin resistance over time [88]. The deposition of amylin/amyloid in β-cells in the later stages of diabetes is similar to human T2DM, where islet amyloid is commonly found in 90% of cases [89].

### 2.2.12 Summary

Various animal models have been developed to address the understanding of the pathology of T2DM. These animal models present with a diversified pathology and allow scientists to further explore the possible genetic, cellular, molecular, and environmental factors that contribute to this costly global epidemic.

## 2.3 Overload models of skeletal muscle hypertrophy

This section will summarize briefly various animal models of overload for skeletal muscle hypertrophy. These paradigms include stretch hypertrophy, compensatory hypertrophy, resistance training, alteration of hormonal levels and dietary supplementation.

### 2.3.1 Stretch hypertrophy

It is a well known fact that muscle when lengthened undergoes hypertrophy, and that muscle maintained in a shortened position will atrophy. The functional and hypertrophic responses from different animal models of stretch like chicken and quail wing muscles [90-94] rabbits [95] and rats [96] are quite similar. The stretch-induced muscle adaptations include muscle hypertrophy, hyperplasia, increased muscle fiber length and contractile function in both fast and slow myosin-containing fibers [97, 98]. The hypertrophic response to this model is shown to be much greater than the functional overload model [99, 100]. The main advantages of this model include that it can be performed in a non-surgical manner and that it also induces hyperplasia, thus providing an opportunity to study mechanisms of new fiber formation. In contrast, some of the disadvantages of utilizing this model include the stimulus provided and magnitudes of adaptations are clearly not the same as progressive resistance training (PRT), and connective tissue content increases,whereas it is hardly evident with PRT [98].

### 2.3.2 Compensatory hypertrophy

When one of the synergistic muscles is removed or made inactive, the remaining muscles must compensate for the loss and undergo hypertrophy. Three different procedures for compensatory overload include tenotomy, synergist ablation and denervation.

Tenotomy is the oldest method used to induce hypertrophy. It involves severing the tendon of synergist muscle. Goldberg et al., (1975) demonstrated that tenotomy of the gastrocnemius for 5 days resulted in a 40% increase in soleus muscle mass and a 20% increase in plantaris muscle mass [96]. This procedure produces a large initial hypertrophic response; however, most of it is due to edema due to surgery. Some of the main disadvantages of this procedure include tendon reattachment after a few days and lack of a recovery period from overload compared to normal hypertrophy from the resistance training model.

The synergist denervation method for overload involves denervating the synergist muscles. For example, denervation of branches of the tibial nerve that innervates gastrocnemius and soleus results in an increase in muscle mass of the plantaris by 40% after 21 days [101]. The advantage of the synergist denervation model includes lesser bleeding during the surgical procedure, decreased inflammatory response post- surgery, and a faster recovery compared to the surgical ablation procedure.

Synergistic ablation involves removal of the synergistic muscle completely, and large numbers of studies have utilized this model to study skeletal muscle hypertrophy. The hypertrophic response with this model was significant and persistent rather than transient [102, 103]. Synergist ablation involves an immediate short-term inflammatory response and much slower long-term response in which the muscle undergoes functional and true hypertrophy. The main advantages of the synergist ablation model for compensatory hypertrophy are utilizing the

contra lateral non-surgical limb as the control, a large and relatively rapid hypertrophic response, and freedom from complications of tendon reattachment as in the tenotomy method [98]. On the other hand, the disadvantages include post-surgical complications like inflammation and edema, and the lack of similarity with progressive resistance training as it lacks a recovery period from mechanical overload [98].

Various structural and functional adaptations of skeletal muscle hypertrophy by synergist ablation have been well defined [104-111]. Nonetheless, very little is known about adaptation in diabetic skeletal muscle with overload. Armstrong and Ianuzzo (1977) reported differences in the adaptation of skeletal muscle between normal and streptozotocin-induced diabetic rat muscles. This study revealed a 53% greater plantaris muscle weight in normal compared to diabetic rats, and after 60 days of ablation surgery, the muscles were hypertrophied and increased by 79% and 61% in normal and diabetic muscle compared to their respective contralateral control muscles. Another interesting finding revealed the slow-twitch fibers in diabetic rats were capable of responding to the overloaded condition but that the fast-twitch fibers had a reduced capacity to undergo compensatory growth [112]. These findings indicate that differences may exist in the response and adaptation to hypertrophy between the diabetic and normal muscle. The underlying mechanism(s) to explain the differences in these adaptations at cellular and molecular level remain to be elucidated.

### 2.3.3 Resistance training

Several animal models mimicking progressive resistance training (PRT) have been used to study the effects of resistance training on muscle adaptation. High frequency electric stimulation model was used to induce eccentric contraction of rat soleus [18]. For example, using a modification of Booth's original model, Wong and colleagues [113] trained rat soleus

eccentrically as a countermeasure for non-weight bearing muscle atrophy. Another model used for weight training in animals is the ladder climbing exercise. Animals are been trained to climb vertically with weight attached to their tail [114-117]. Such studies have demonstrated a 5 - 26% increase in relative muscle mass. Results of these studies vary because of differences in training protocol, species and muscles studied. The main advantage of the resistance training model is that most of the characteristics of this model like magnitude, time course of muscle hypertrophy, and strength gains are similar to human PRT. On the other hand, some of the disadvantages include difficulty in exercising animals voluntarily, training results in bilateral hypertrophy so that internal control is not possible, and slower adaptations when compared to other models of hypertrophy.

### 2.3.4 Hormone induced hypertrophy

Growth factors are thought to be involved in muscle hypertrophy [118]. Important growth factors for skeletal muscle hypertrophy include insulin-like growth factor (IGF), fibroblast growth factor (FGF), and hepatocyte growth factor (HGF). These growth factors work in conjunction with each other to cause skeletal muscle hypertrophy. Song et al., (2005) reported that overexpression of IGF-I in mice skeletal muscle results in hypertrophy, which is likely mediated by the mTOR/p70S6K pathway, potentially via an Akt-independent signaling pathway [119]. Growth hormone (GH) is a peptide hormone that stimulates IGF in skeletal muscle, promoting satellite cell activation, proliferation and differentiation [120]. For example, GH administration to a hypophysectomized animals increased muscle mass, total protein, RNA content, ribosome content and enzymes related to protein synthesis compared to non-GH treatment group [121]. In addition to growth factors and GH, testosterone has anabolic effects and induces muscle hypertrophy by increasing the protein synthesis [122]. Further, Brown et al.,

(2009) showed that testosterone-induced muscle hypertrophy is associated with activation of notch signaling and the inactivation of JNK together with the activation of p38 MAPK [123].

### 2.3.5 Diet induced hypertrophy

Several studies have shown that creatine supplementation in conjunction with resistance training augments gains in muscle strength and size [124-127]. Recent work by Hulmi et al., (2010) reported that augmented whey protein intake along with resistance exercise led to significant rises in muscle protein synthesis and cross sectional areas [128, 129]. In addition, ingestion of essential amino acid (EAA) has been shown to have positive effects on muscle hypertrophy and architecture [130, 131]. Several other supplements such as clenbuterol, beta-hydroxy-betamethlbutyrate (HMB) and branched-chain amino acids (BCAA) have been utilized to induce skeletal muscle hypertrophy [132].

### 2.3.6 Summary

Different animal models of skeletal muscle hypertrophy have been employed to understand the different aspects of skeletal muscle adaptation. The functional and hypertrophic responses of most of these models are analogous to PRT in humans. Rapid and large degrees of persistent hypertrophy are induced by synergist ablation and provide an opportunity to investigate the mechanisms of muscle remodeling.

## 2.4 Molecular mechanisms in skeletal muscle adaptation to increased loading

Numerous possible signaling pathways involved in muscle adaptation have emerged as a consequence of the rapid progress in the understanding of the molecular mechanisms underlying skeletal muscle adaptation to increased load. This section is focused on reviewing some of the key molecular mechanisms in skeletal muscle adaptation including mammalian target of

rapamycin (mTOR) signaling, AMP-activated protein kinase (AMPK) signaling, dsRNA-dependent protein kinase (PKR) signaling, stress protein signaling and muscle specific miRNA regulation.

### 2.4.1 mTOR Signaling

The mammalian target of rapamycin (mTOR) receives and integrates signals exerted by hormonal factors, nutrients, and energy status, and plays critical roles in a wide variety of physiological functions, including gene transcription, growth, cell cycle, cytoskeletal organization, and the regulation of protein synthesis at both transcriptional and translational levels [24, 25]. mTOR is a Ser/Thr kinase that belonging to the phosphoinositol-3-kinase (PI3K) related family of kinases, with a molecular weight of 289 kDa [133]. It contains 2549 amino acids and has several structurally conserved domains. The C terminus consists of a FKBP-rapamycin binding (FRB) domain and a catalytic kinase domain, and the N terminus consists of two clusters of Huntington, elongation factor 3, the A subunit of protein phosphatase 2A, and TOR1 (HEAT) repeats [134].

#### 2.4.1.1 mTOR complex and function

mTOR exists in two complexes, mTORC1 and mTORC2. mTORC1 consists of mammalian lethal with SEC13 protein 8 (mLST8, also known as G protein beta subunit-like protein (GβL)), raptor and mTOR [135].

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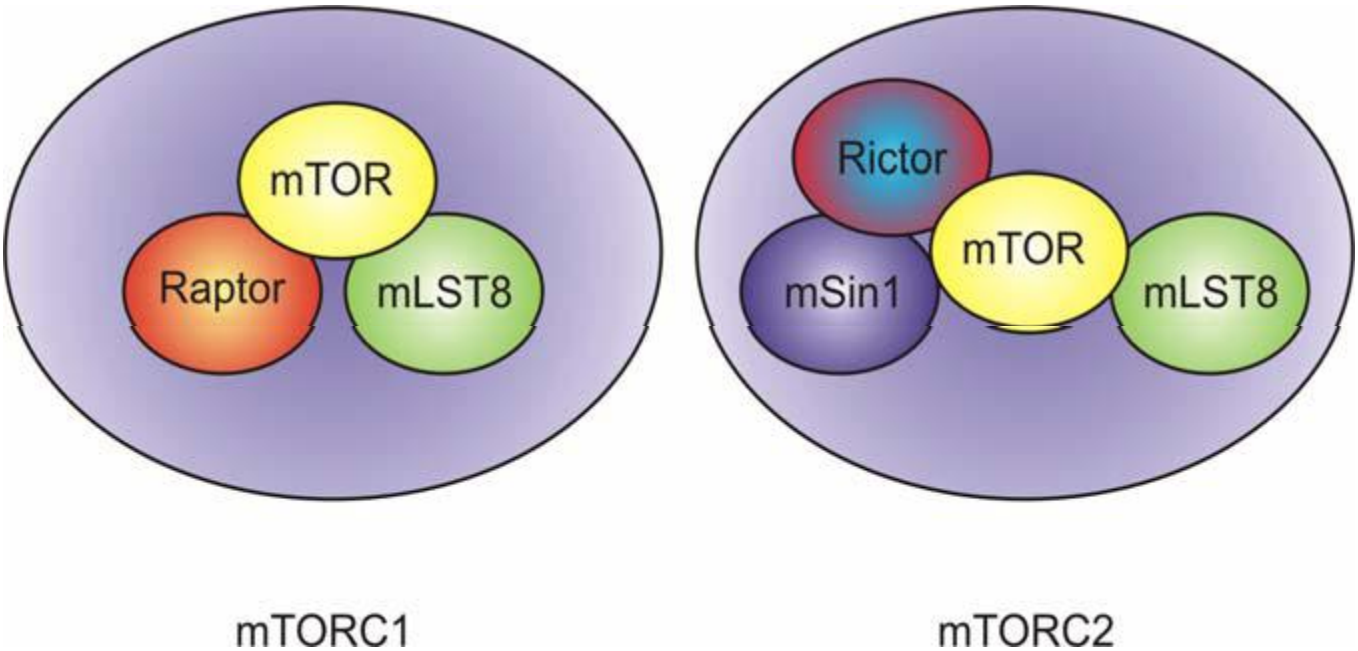


**Figure 1**: The mTOR complexes. In mTORC1, the mTOR catalytic subunit associates with raptor and mLST8 (also known as GβL). mTORC2 in contrast, contains instead of raptor a protein called rictor, but also other subunits such as mLST8 and mSin1.

This complex is rapamycin sensitive and mainly responsible for regulation of the size of the cell. mTORC2 consists of mLST8, rictor and mTOR. The mTORC2 is rapamycin insensitive plays a key role in actin cytoskeleton organization and regulation of the shape of the cell [135].

#### 2.4.1.2 Downstream targets of mTOR

The two important downstream targets of mTOR are p70 S-6 kinase (p70S6K) and eukaryotic initiation factor 4E- binding protein 1 (eIF4E-BP1), which are important factors in the protein synthetic pathway [133]. p70S6K and eIF4E-BP1 coordinate the translation of proteins, the regulation of eukaryotic initiation factors and ribosomes [136]. p70S6K is a Ser/Thr protein kinase that is required for cell growth and G1 cell progression is thought to be an important signaling intermediate that leads to the activation of protein synthesis and muscle hypertrophy [137]. The primary structure of p70s6k consists of four functional domains or modules and the

activation of p70s6k occurs in a hierarchal fashion through the sequential phosphorylation of each module [137]. Activation of this kinase causes activation or phosphorylation of the S6 protein of the 40S ribosomal subunit [138]. The activation of both kinase and its substrate accelerates the translation of specific mRNAs containing a terminal oligopolypyrimidine (TOP) track at the 5' end that encode mainly ribosomal proteins and elongation factors. This is important as the regulation of TOP containing proteins has been postulated to be a rate limiting step in protein synthesis [139]. Rapamycin (mTOR inhibitor) treatment inhibits p70S6K activation both *in vitro* and *in vivo* and is associated with inhibition of 5' TOP mRNA translation [22, 140]. Baar and Esser (1999) reported that p70S6K activation (phosphorylation) correlates highly correlated with muscle hypertrophy following resistance exercise [30].

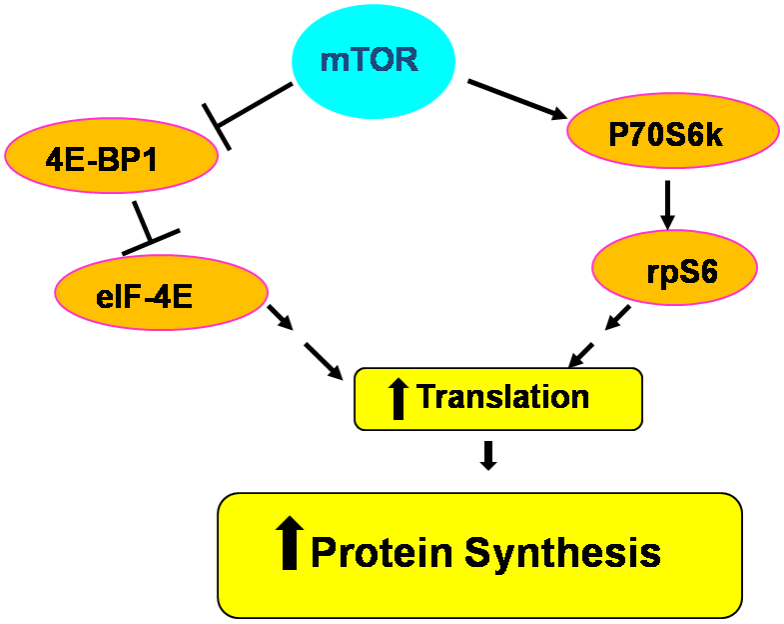


**Figure 2**: mTOR signaling: different downstream effectors

Translation repressor protein 4E-BP1 (also known as PHAS-1) is another important downstream effector of mTOR. 4E-BP1 normally exists in a dephosphorylated form, bind to and inhibit the

eukaryotic initiation factor 4E (eIF4E), and inhibits cap-dependent translation. When 4E-BP1 is phosphorylated by mTOR and other protein kinases it is dissociated from eIF4E resulting in increased formation of the eIF4F complex and increased translational initiation [141]. mTOR also regulates translational elongation along with translational intiation as discussed above by regulating the elongation factor, eEF2 [142]. The mTOR dependent regulation of eEF2 kinase involves its phosphorylation at several sites, including Ser366, Ser78 and Ser359 [142].

#### 2.4.1.3 Control of mTOR by PI3K/Akt signaling

mTOR signaling is regulated by growth factors and hormones, which is believed to be mediated through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. The interaction of insulin and growth factors with their receptors leads to the activation of phosphoinositide 3-kinase (PI3K). PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate, which results in the production of phosphatidylinositol-3,4,5-triphosphate [PtdIns(3,4,5)P3 ], that in turn leads to the activation of Akt or protein kinase B(PKB) [143]. Akt is a Ser/Thr kinase which lies upstream of mTOR signaling where it participates in regulation the expression of genes involved in protein synthesis and atrophy [144]. Complete activation of Akt requires phosphorylation of both Thr 308 and Ser 473 residues. Akt has been shown to be implicated in muscle hypertrophy [22], and overexpression of AKT in rat muscle by transfection [145] or genetic manipulation [146] increases muscle fiber diameter. Akt can influence the activity of mTOR through its ability to phosphorylate TSC2 (tuberous sclerosis complex 2) at Thr1462 [147, 148]. TSC2 acts as a GTPase-activator protein (GAP) for the small G-protein Rheb (Ras-homolog enriched in brain), and phosphorylation of TSC2 inhibits its GAP activity, allowing Rheb to accumulate in its active GTP-bound form where it stimulates the kinase activity of mTOR [149, 150].

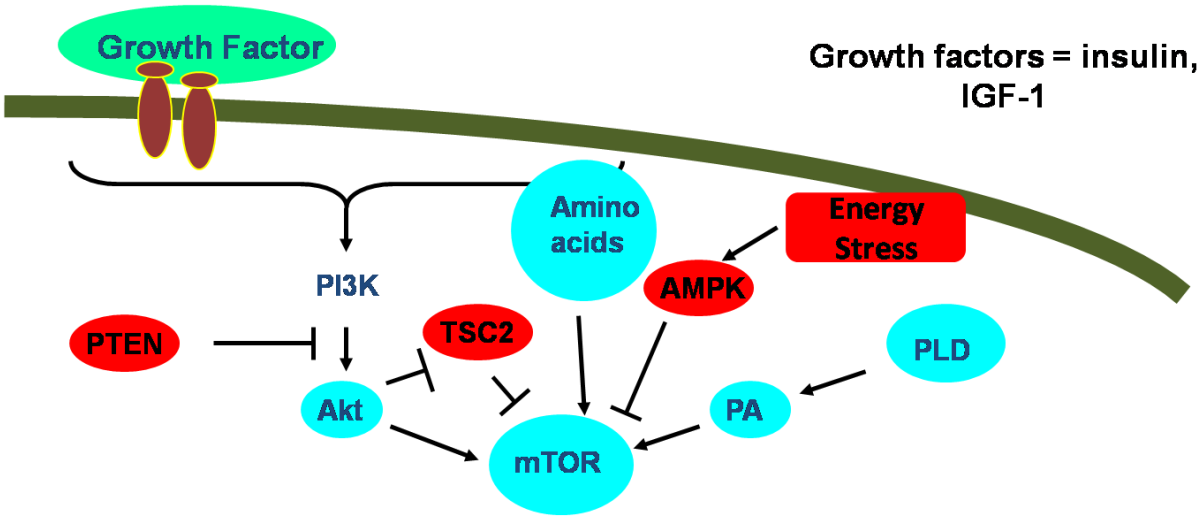


**Figure 3**: Scheme of different possible upstream regulators of mTOR signaling, based on the available knowledge in the literature.

In addition to positive regulators, these processes are also influenced by negative modulators. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a phosphatase and central negative regulator of the PI3K/AKT/mTOR signaling cascade that transduces signals regulating growth, survival and proliferation [151]. PTEN is thought to function by removing the phosphate from the D3 position of phosphatidylinositol 3,4,5-triphosphate (PIP3), thereby directly antagonizing the action of the phosphatidylinositol 3-kinases (PI3K) [152].

#### 2.4.1.4 Regulation of Hypertrophy by the mTOR signaling

A large part of tissue or organ hypertrophy is mediated through mTOR signaling. Different positive regulators of cell growth like growth hormone, insulin, IGF-I and nutrient availability are shown to stimulate hypertrophic muscle protein synthesis via the mTOR signaling pathway [153-156]. Negative hormonal factors such as glucocorticoids, pro-inflammatory cytokines, and the TGF-β superfamily member myostatin, are known to inhibit hypertrophic muscle protein synthesis via the mTOR signaling pathways [157, 158]. Skeletal

muscle hypertrophy is an adaptation to muscle overload and is the result of an increase in protein synthesis. An increase in protein synthesis allows new contractile filaments to be added to the muscle fiber, thus increasing its size. It is well accepted that increases in protein synthesis precede skeletal muscle growth and that this process is regulated, at least in part, by mTOR and its signaling intermediates [22, 23]. It is currently unclear how mTOR signaling may be regulated in insulin resistant skeletal muscle.

### 2.4.2 AMPK Signaling

AMP-activated protein kinase (AMPK) is a serine/threonine kinase and is considered a master regulator of cellular energy metabolism due to its role in the regulation of glucose, lipid, and protein metabolism. AMPK is responsible for down regulating protein synthesis, glycogen synthesis and cholesterogenesis [106, 159, 160]. AMPK is activated when there is decreased energy status of the cell that elevates the AMP/ATP ratio. This can occur through either increased ATP consumption or decreased ATP production. Several pharmacological agents like 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), metformin, and the peroxisome proliferator-activated receptor gamma PPAR-γ agonist rosiglitazone appear to activate AMPK [161-163]. Once activated, AMPK switches on several catabolic pathways such as glycolysis and fatty acid oxidation and switches off several ATP- consuming pathways such as lipogenesis and cholesterol biosynthesis [5].

#### 2.4.2.1 AMPK structure and function

AMPK is a heterotrimeric enzyme complex consisting of a catalytic subunit (α) and two regulatory subunits (β, γ). Each catalytic subunit is encoded by different genes. There are two isoforms of both the α- (α1 and α2) and the β-subunits (β1 and β2), and three isoforms of the γ-

subunits (γ1, γ2, and γ3). The α subunit (63 kDa) contains the kinase domain at the N terminus, plus a C-terminal regulatory domain containing an autoinhibitory region that inhibits the kinase in the absence of AMP [164]. The α1 isoform is predominantly found in cardiac and skeletal muscle while the α2 isoform is predominant in pancreatic islet β-cells [165]. The β subunits act as "scaffold" that binds the α and γ subunit via conserved *KIS* and *ASC* domains. The β2 subunit is predominantly expressed in skeletal muscle and β1 predominates in the liver. The γ3 isoform expression is predominant in glycolytic skeletal muscle and γ1 and γ2 show broad tissue distributions [166]. The γ subunit contains a core cystathionine β-synthase (CBS) domain that appears to function in the binding of AMP [167].

#### 2.4.2.2 Biochemical regulation of AMPK

The regulation of AMPK is complex and involves an AMP-dependent conformational change, phosphorylation by upstream kinases, and decreased dephosphorylation by protein phosphatases. Increases in AMP levels enhance the binding of AMP to the γ subunit, leading to conformational changes in the AMPK molecule and inhibit the dephosphorylation of AMPK by protein phosphatases [168]. Different possible upstream kinases have been identified for AMPK, including the protein tumor suppressor LKB1 kinase LKB1 complex [35, 169, 170], the calcium/calmodulin-activated protein kinase (CaMKII) [36, 171] and transforming growth factor-β-activated kinase-1 (TAK1), a member of the mitogen-activated protein (MAP) kinase family [37, 172]. LKB1 is a tumor suppressor that phosphorylates AMPK at site Thr172 of the α-subunit [170]. LKB1 knockout mice were reported to have diminished AMPK activation and suppressed glucose transport caused by both electrically stimulated contraction and treatment with AICAR and phenformin [35]. LKB1 can function as an AMPK kinase only when AMP enables the kinase access to the activating phosphorylation site of AMPK [173]. CaMKII

regulates AMPK in a Ca2+/ calmodulin-dependent, AMP-independent manner. The activity of CaMKII is $Ca2^{+}$dependent [174]. Lack of TAK1 in cardiac muscle was shown to prevent the phosphorylation of AMPK (Thr172) induced by treatment with metformin [172].

#### 2.4.2.3 Regulation of hypertrophy by the AMPK signaling

Activation of skeletal muscle AMPK during conditions of energetic stress is thought to act as a negative regulator of protein synthesis and may therefore modulate skeletal muscle mass and hypertrophy [5, 26, 34]. This negative regulation of protein synthesis by AMPK activation has also been reported in cardiac muscle [175, 176]. The inhibition of protein synthesis by AMPK appears to occur through the down regulation of mTOR and mTOR-dependent signaling proteins [5]. This down regulation of mTOR signaling by AMPK is likely due to direct phosphorylation of tuberous sclerosis complex 2 (TSC2) at Thr1227 and Ser1345, which subsequently inactivates the G-protein Rheb thereby blocking activation of mTOR [177, 178]. This notion is further extended by a recent study showing that AMPK deletion is associated with increase in p70S6K Thr389 and rpS6 Ser-235/236 phosphorylation, and an enhanced protein synthesis rate [179]. AMPK activation has been shown to have a negative correlation with overload induced hypertrophy in plantaris muscles of aged rats [180]. The regulation of AMPK signaling during muscle hypertrophy in insulin resistant skeletal muscle has, to our knowledge, not been studied.

### 2.4.3 PKR Signaling

#### 2.4.3.1 PKR structure and function

The dsRNA-dependent protein kinase (PKR) was first described as p68 kinase in human cells and as p65 kinase in murine cells on the basis of electrophoretic mobility in SDS [181].

PKR consists of a regulatory domain at the N-terminus, which contains the binding sites for dsRNA, and a protein kinase binding domain at the C-terminus [182, 183]. It is thought that the binding of dsRNA to PKR exposes the ATP binding domain and results in autophosphorylation of PKR on multiple serine and threonine residues [181]. Once activated, PKR phosphorylates exogenous substrates. The best characterized substrate of PKR is the small subunit of the eukaryotic initiation factor 2 (eIF2α). PKR phosphorylates eIF2α at Ser-51, which functions to inhibit translational initiation [6].

#### 2.4.3.2 Regulation of muscle mass by PKR signaling

Several *in vitro* and *in vivo* studies in the last few years strongly suggest a role for dsRNA-dependent protein kinase (PKR) in both protein synthesis and degradation processes [6, 33, 184, 185]. PKR regulates protein synthesis through phosphorylating eIF2α at Ser-51, thereby inhibiting translational initiation [6]. In addition, recent studies have reported that hyperglycemia, TNF-α and Ang II induced protein degradation are associated with caspase-3/-8 mediated activation of PKR [33, 186]. PKR is also thought to be involved in the regulation of protein degradation by its ability to influence the ubiquitin- proteasome pathway, most likely by mechanism involving the activation of p38 MAPK [40] and the transcription factor nuclear factor-κB (NF-κB) [39]. How insulin resistance may affect the regulation of PKR during muscle hypertrophy has, to our knowledge, not been investigated.

### 2.4.4 Stress signaling

#### 2.4.4.1 Heat shock protein (HSP) signaling and regulation of hypertrophy

HSPs, also called stress proteins, are important components of the cellular protective response and fulfill diverse functions, including chaperoning unfolded proteins, inhibiting

apoptosis, and stabilizing the cytoskeleton [187-191]. They are subdivided into several classes according to their molecular weights. The most commonly abundant HSPs in muscle are small HSPs like HSP27 and large molecular weight proteins like HSP60, HSP70 and HSP90. These stress proteins provide protection in response to a wide variety of stressors such as hypoxia, free radical stress, energy deprivation, increased temperature and exercise [192-194].

HSPs function as chaperones during protein synthesis by binding to the nascent polypeptide chains and preventing improper folding [191]. This chaperone function of HSPs make them good candidates for being involved in muscle hypertrophy, which includes adding new proteins to the existing muscle fibers. HSPs also appear to modulate actin dynamics and assist in the preservation of muscle mass at the tissue level [195]. Shue and Kohtz (1994) reported that HSP90 activates the muscle specific transcription factor MyoD and functions to promote increases in muscle mass through the activation of specific genes and satellite cells [196].

HSPs are thought to be regulated by phosphorylation at specific sites. For example, HSP27 is regulated by phosphorylation at two sites in the rat (Ser-15 and Ser-85) and the mouse (Ser-15 and Ser-86) and at three sites in humans (Ser-15, Ser-78, and Ser-82) [197]. These phosphorylation events are thought to be mediated by mitogen-activated protein kinases (MAPKs) [197]. Several previous studies demonstrated the stress response with high force eccentric contraction, the aerobic exercise model (cycling and running), and compensatory and stretch-induced hypertrophy [41-43]. No studies to date have investigated the response of HSPs in insulin-resistant skeletal muscle to functional overload.

### 2.4.4.2 MAPKs signaling and regulation of hypertrophy

Mitogen-activated protein kinases (MAPKs) are another class of stress responsive proteins, capable of regulating cellular processes such as protein synthesis, cellular proliferation, differentiation, and apoptosis [198-200]. The MAPK family of proteins are divided into four major subfamilies, including extracellular signal-regulated kinases (ERK), p38 MAPK, c-Jun NH2-terminal kinases (JNK), and extracellular signal-regulated kinase 5 [201]. The MAPK pathways consist of a cascade of kinase intermediates that phosphorylate and activate in specific order: MAPKKKK (Ras) → MAPKKK (Raf) → MAPKK (MEK) → MAPK.

The MAPKs are activated by dual phosphorylation and then translocated to the nucleus, where they are capable of regulating such diverse process such as gene expression, glucose uptake, cell replication, protein synthesis and apoptosis [202, 203]. The ERK signaling cascade plays a primary role in cell proliferation or mitogenesis and has been shown to regulate cell development, differentiation and survival [204]. Pearson et al., (2001) reported that ERK1/2 activation or phosphorylation at Thr202 and Tyr204 leads to activation of several transcriptional factors including cAMP response element binding protein (CREB), Elk, c-jun and c-fos, [205]. ERK may also play a role in modulating protein synthesis by regulating mTOR activity through inhibition of the TSC complex [206]. In T2DM, oxidative stress and inflammotry cytokines stimulate P38 and JNK MAPKs [66, 207]. JNK activation or phosphorylation is shown to modulate several transcriptional factors, including c-jun, signal transducer and activator of transcription (STAT3) and nuclear factor of activated T-cell (NFAT) [208-210]. Several downstream substrates of MAPK signaling have also been identified, including the Erk1/2-dependent protein MAPK-activated protein kinase-1 (MAPKAP-K1), also called p90 ribosomal S6kinase (p90RSK) [211] and the p38-dependent protein MAPK-activated protein kinase-2

(MAPKAP-K2) [212, 213]. Previous studies have demonstrated ERK1/2- and JNK- MAPK activation in skeletal muscle hypertrophy in response to increased loading in humans and rodents [44-47, 214]. The degree of activation of these MAPKs is affected by the mode, intensity and duration of the exercise. How muscle overload may regulate MAPK signaling in insulin resistant muscle has not been elucidated.

### 2.4.5 MicroRNA

#### 2.4.5.1 MicroRNA transcription, biogenesis and processing

MicroRNAs (miRNAs) are small ~ 22-nucleotide, non-coding RNAs which are a newly emerging class of trans-factors that repress gene expression post-transcriptionally by inhibiting translation of target mRNAs through interaction with the 3'- untranslated region (3'UTR) of target mRNAs [50, 51, 215]. miRNA were first discovered by the Ambros and Ruvkun laboratories during genetic screening of developmental progress in *C. elegans* [216, 217]. Most of these miRNAs are located in intergenic regions and are transcribed from their own promoter [218, 219]. miRNAs are transcribed as the primary transcript miRNAs (pri-miRNAs), which consists of cap structure, Poly (A) tails, and several uridine residues [220]. These pri-miRNAs are transcribed by polymerase II (Pol II) before being converted into hairpin intermediates called pre-miRNAs (~ 65-nucleotide) and subsequently processed into mature miRNAs. RNase III enzymes like drosha and dicer are involved in the maturation process. Drosha is localized in the nucleus and has a catalytic domain and a dsRNA-binding domain, whereas dicer is localized in the cytoplasm and contains two catalytic domains and additional helicase and PAZ motifs [221, 222]. After processing in the nucleus pre-miRNAs are exported to the cytoplasm in a process that is mediated by exportin-5 [223]. These pre-miRNAs in the cytoplasm are further processed

by dicer before the mature form (22-nucleotide) is incorporated into the RNA-induced silencing complex (RISC) [224].

#### 2.4.5.2 Mechanism of miRNA action

There are several mechanisms for protein suppression by miRNA. miRNA can bind to mRNA and cause cleavage, destabilization, or degradation of target mRNA. Several miRNAs may target a single or multiple mRNAs [225]. The second mechanism is translational repression, which is thought to be the main mechanism for repression in mammalian cells [226]. In addition to functioning as translational repressors, it has been suggested that miRNA might play a role in translational activation. Vasudevan et al., (2008) reported that miRNAs can induce the translational activation of target mRNAs in proliferating mammalian cells during cell cycle arrest, indicating that the translational regulation by miRNA can be either repressive or activating [227].

#### 2.4.5.3 MicroRNA functions

More than 600 human miRNAs have been identified, and it is estimated that they might target more than one third of human protein-coding genes [228-230]. Several lines of evidence indicate that miRNAs play a key role in a wide variety of biological processes including cell proliferation, differentiation, apoptosis as well as in important diseases such as diabetes, cancer, cardiovascular disease, HIV/AIDS and muscle wasting [53, 231-233].

miRNAs regulate the expression of several transcriptional factors and signaling mediators and are thought to play key roles in the development and function of skeletal and cardiac muscle [234, 235]. Chen et al., (2006) demonstrated that the muscle-specific miRNAs, miR-1 and miR-133, regulate skeletal muscle development in an antagonistic manner. miR-1 promotes muscle myogenesis by targeting histone deacetylase 4 (HDAC4), a transcriptional repressor of muscle

gene expression, where as miR-133 enhances myoblast proliferation by repressing serum response factor (SRF) [53]. Ivey et al., (2008) reported that muscle-specific miR-1 and miR-133 promote cardiac differentiation from embryonic stem cells [236]. Chen et al., (2010) showed that miR-1 and miR-206 are sharply up-regulated during satellite cell differentiation [237]. In addition, a recent study by Nakasa et al., (2010) demonstrated enhanced muscle regeneration by locally injecting double-stranded (ds) miR-1, miR-133 and 206 in a rat skeletal muscle injury model, likely through the up regulation of the myogenic markers MyoD1, myogenin and Pax7 at both mRNA and protein level [238].

MicroRNA-1 is highly conserved between both invertebrates and vertebrates, and it was shown to be highly expressed in muscle tissue [219]. Sokol and Ambros (2005) reported that miR-1 mutants of Drosophila have severely deformed musculature [52]. miR-1 has been shown to regulate muscle transcription factor MEF2 at the neuromuscular junction in *C. elegans* [239]. Liu et al., (2007) showed that intragenic MEF2-dependent enhancer directs the muscle specific expression of miR-1-2 and 133a-1 [240]. Oxidative stress was shown to increase miR-1, and miR-133 expression in cardiomyocytes. miR-1 and miR-133 have produce opposite effects on apoptosis. miR-1 promotes apoptosis by repressing the targets HSP60 and HSP70, where as miR-133 being anti-apoptotic by repressing the expression of caspase-9 [51].

Several lines of evidence suggest that miRNAs lead to aberrant gene expression and that these molecules may play a role in the pathophysiology of T2DM [241-247],cardiac hypertrophy [248, 249], muscular dystrophy [250-253] and HIV/AIDS [254-256]. For example, silencing miR-133 increases glucose uptake in cardiac muscle by increasing glut-4 expression, suggesting that miR-133 dysregulation may be involved in the development of T2DM [257]. Thum et al., (2008) reported mir-21 antagomir administration in mice with cardiac disease inhibits ERK-

MAPK activity and cardiac fibrosis [258]. He et al., (2007) reported up regulation of miR-29, miR-29a, miR-29b, and miR-29c in muscle, fat and liver of diabetic Goto-Kakizaki (GK) rats [259]. Taken together, these studies suggest that muscle-specific miRNAs may play a role in muscle development and functional remodeling.

#### 2.4.5.4 Role of muscle-specific miRNAs in hypertrophy

The function of muscle-specific miRNAs in embryonic but not adult development has been well established. McCarthy and Esser (2007) reported that the expression of muscle-specific miRNAs is decreased by 50% following 7 days of functional overload in rodent skeletal muscle. Interestingly, the transcript levels for both pri-miRNA-1-2 and pri-miRNA-133a-2 along with components of the miRNA biogenesis pathway like Drosha and Exportin-5 were significantly increased in response to functional overload. This loss of a direct relationship in the expression level of pri-miRNAs and their corresponding miR is suggestive of additional regulation of miRNA biogenesis during functional overload, which does not appear to involve the canonical pathway [7]. How insulin resistance may affect the regulation of these muscle-specific miRNAs during muscle hypertrophy has, to our knowledge, not been investigated.

### 2.4.6 Summary

Skeletal muscle adaptation to increased load is a complex process that involves activation of several intracellular signaling cascades to reprogram gene expression and to sustain muscle performance. mTOR and its downstream effectors play a critical role in load induced hypertrophy by regulating protein synthesis at multiple levels. AMPK and PKR are two important negative regulators of growth that have been shown to inhibit protein synthesis through the down regulation of mTOR signaling. In addition, PKR may also be involved in enhancing protein degradation. Stress responsive proteins like the HSPs and MAPKs are also

capable of regulating protein synthesis and are involved in skeletal muscle adaptation. miRNAs are non-coding RNAs that have been shown to play a role in muscle development, function and remodeling. The exact molecular mechanism(s) regulating the activity of these molecules is not well understood. Similarly, how insulin resistance may affect the regulation of these intracellular signaling proteins and muscle-specific miRNAs during muscle hypertrophy has to our knowledge, not been investigated. Elucidating the differences between normal and diabetic muscle tissue at the molecular level may be beneficial for exercise and pharmacological interventions designed for the treatment of T2DM.

# CHAPTER III

The following chapter includes three different research papers describing in detail the research experiments conducted to test our hypotheses set forth for specific aims I, II and III of this dissertation project.

**SPECIFIC AIM I:** To investigate whether insulin resistance affects the regulation of the mTOR signaling during muscle hypertrophy

## PAPER 1

The following paper corresponds to the specific aim I

**Impaired overload-induced hypertrophy is associated with diminished mTOR signaling in insulin resistant skeletal muscle of the obese Zucker rat**

**Abstract**

Recent data have suggested that insulin resistance may be associated with a diminished ability of skeletal muscle to undergo hypertrophy [4]. Here we examine the effects of insulin resistance using the OZ rat with increased muscle loading on the regulation of the mammalian target of rapamycin (mTOR) and its downstream signaling intermediates 70 kDa ribosomal protein S6 kinase (p70S6k), ribosomal protein S6 (rpS6), eukaryotic elongation factor 2 (eEF2), and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Compared to that observed in LZ rats, the degree of soleus muscle hypertrophy as assessed by changes in muscle wet weight (LZ: 35% vs. OZ: 16%) was significantly less in the OZ rats after 3-weeks of muscle overload ($P < 0.05$). This diminished growth in the OZ rats was accompanied by significant impairments in the ability of the soleus to undergo phosphorylation of mTOR (Ser2448), p70S6k (Thr389), rpS6 (Ser235/236) and Akt (Ser473 and Thr308) ($P < 0.05$). Taken together, these data suggest that impaired overload-induced hypertrophy in insulin resistant skeletal muscle may be related to decreases in the ability of the muscle to undergo mTOR-related signaling.

## Introduction

The obese Zucker (*fa/fa*) rat (OZ) is commonly used as an animal model for the investigation of metabolic syndrome given its proclivity to exhibit severe skeletal muscle insulin resistance, hyperglycemia, and hyperlipidemia [55]. Previous data from our laboratory have suggested that the capacity of the OZ soleus muscle to undergo hypertrophy in response to increased loading is diminished compared to that observed in the LZ rat. Why metabolic syndrome may affect the hypertrophic response of muscle is not clear, although we and others have noted that insulin resistance is associated with differences in the ability of skeletal muscle to activate intracellular signaling cascades in response to alterations in contractile activity [15, 57, 68, 260].

It is well accepted that increases in protein synthesis precede skeletal muscle growth [20, 21]. One critical signaling pathway that has been shown to play a role in controlling protein synthesis following increased muscle loading is the mammalian target of rapamycin (mTOR) [22, 23]. The regulation of mTOR signaling is complex and is likely influenced by several upstream molecules and pathways as previous data have suggested the participation of phosphoinositide 3-kinase (PI3K), phosphatase and tensin homologue deleted on chromosome 10 (PTEN), protein kinase B/ Akt, TSC2 / Tuberin, and raptor [22, 147, 148, 261-263]. The mTOR functions to regulate several physiological functions such as gene transcription, protein metabolism, cell cycle control, and cytoskeleton organization [24, 25]. When active (phosphorylated), mTOR is thought to promote protein translation by controlling the activity of several downstream effectors, including the 70 kDa ribosomal protein S6 kinase (p70S6k), ribosomal protein S6 (rpS6), eIF4E-binding protein 1 (4E-BP1), and eukaryotic elongation factor 2 (eEF2) [27, 264].

The primary purpose of this study was to determine whether insulin resistance affects the ability of skeletal muscle to activate mTOR signaling in response to increased loading. The second purpose was to examine the time course of mTOR signaling during the initial and latter phases of muscle adaptation. We hypothesized that overload-induced mTOR signaling would differ between normal and insulin resistant muscle. Our data suggest that insulin resistance or other co-morbidities may be associated with decreases in the ability of skeletal muscle to activate mTOR signaling. Whether these changes alone or in combination with other factors may explain why insulin resistance may lead to differences in the capacity of skeletal muscle to undergo growth are currently unclear.

## Materials and Methods

*Animal Care.*

All procedures were performed as outlined in the Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society and the institutional animal use review board of Marshall University. Young male LZ (n=12) and OZ (n=12) rats were obtained from the Charles River Laboratories. All animals were 12 wk of age at the completion of this study. Rats were housed two per cage in an AAALAC approved vivarium. Housing conditions consisted of a 12 H: 12 H dark-light cycle and temperature was maintained at 22° ± 2 °C. Animals were provided food and water *ad libitum* and allowed to recover from shipment for at least two weeks before experimentation. During this time the animals were carefully observed and weighed weekly to ensure none exhibited signs of failure to thrive, such as precipitous weight loss, disinterest in the environment, or unexpected gait alterations.

*Synergist ablation procedure.*

Unilateral overload of the soleus muscle for 1- and 3-weeks was achieved through the surgical ablation of the medial and the proximal two-thirds of the lateral head of the gastrocnemius [109].

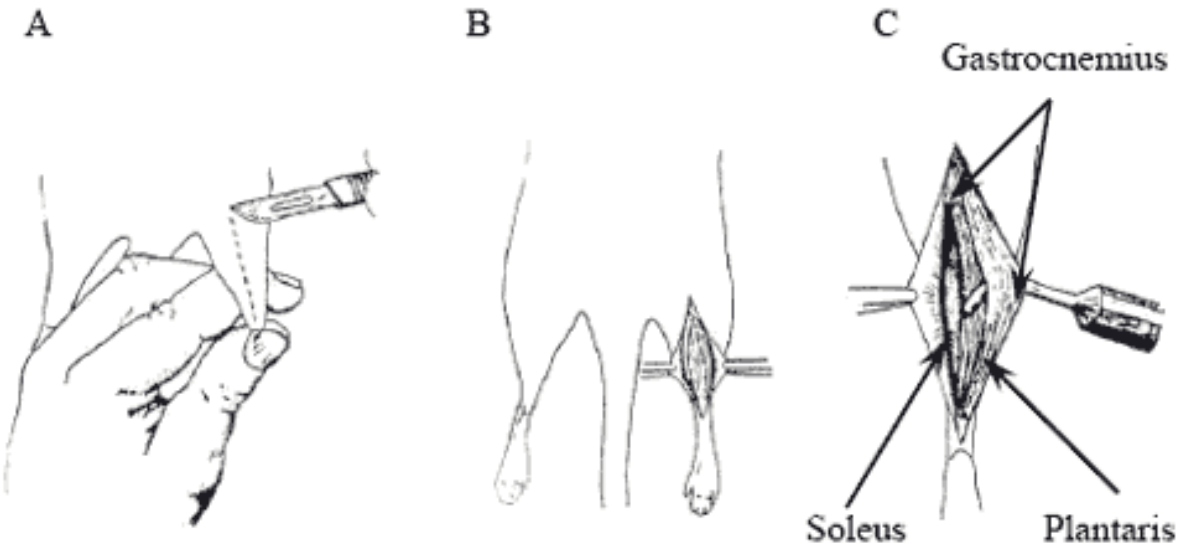


**Figure 4**: Synergistic ablation procedure: In ablation experiment, a muscle is surgically removed (A, B, and C). Here the ablated muscle is the gastrocnemius (C- both heads).

The unilateral ablation model allows within animal comparisons, thus eliminating bias due to systemic factors. Rats were anesthetized with a ketamine–xylazine (4 : 1) cocktail (50 mg/kg, I/P), and the distal two-thirds of the gastrocnemius muscle were surgically removed from the left hindlimb as previously described [109]. A sham (control) operation was performed on the right hindlimb. The sham procedure consisted of an incision through the skin, followed by blunt isolation of the Achilles tendon and gastrocnemius muscle prior to closure. Animals were active immediately after recovering from anesthesia and were checked twice daily during the 7-day postoperative period. No signs of postoperative complications (such as infection or undue distress) were observed.

*Measurement of blood glucose and serum insulin.*

Animals were fasted for 14-h and blood samples were collected directly just prior to animal death. Blood samples were immediately centrifuged at 2000 g for 10 min and the

supernatant serum was stored at −80 °C until use. Serum insulin concentration was determined using a rat/mouse insulin ELISA kit (Linco Research, Inc., St. Charles, MO, USA) as outlined by the manufacturer. Blood glucose was measured using blood glucose meter (Bayer Contour, Bayer HealthCare LLC, Tarrytown, NY, USA).

*Tissue collection.*

The soleus muscles were collected 7 days ($n$ = 6 LZ-7 and $n$ = 6 OZ-7) or 21 days ($n$ = 6 LZ-21 and $n$ = 6 OZ-21) after the synergist ablation. The animals were 12 wk old at the time of tissue collection. Rats were anesthetized with a ketamine–xylazine (4: 1) cocktail (50 mg/kg, I/P) and supplemented as necessary for reflexive response before tissue collections. The soleus muscles from both legs were quickly removed, trimmed of excess connective tissue, weighed on an analytical balance, frozen in liquid nitrogen, and stored at −80◦C until further analysis.

*Tissue homogenization and determination of protein concentration.*

Muscles were homogenized in a Pierce Tissue Protein Extraction Reagent (T-PER) (10 mL/g tissue; Rockford, IL, USA) that contained protease inhibitors (P8340, Sigma-Aldrich, Inc., St. Louis, MO, USA) and phosphatase inhibitors (P5726, Sigma-Aldrich, Inc., St. Louis, MO, USA). After incubation on ice for 30 min, the homogenate was collected by centrifuging at 12,000 g for 5 min at 4°C. The protein concentration of homogenates was determined via the Bradford method (Fisher Scientific, Rockford, IL, USA). Homogenate samples were boiled in a Laemmli 2× sample buffer (Sigma-Aldrich, Inc., St. Louis, MO, USA) for 5 min.

*SDS-PAGE and immunoblotting.*

Forty micrograms of total protein from each sample was separated on a 10% PAGEr Gold Precast gel (Lonza, Rockland, ME, USA) and then transferred to a nitrocellulose membrane. Visual verification of transfer and equal protein loading amongst lanes was

accomplished by Ponceau S staining of the membranes. For immunodetection, membranes were blocked for 1 h at room temperature in blocking buffer (5% non-fat dry milk in TBS-T (20mM Tris-base, 150mM NaCl, 0.05% Tween-20), pH 7.6), serially washed in TBS-T at room temperature, then probed with antibodies for the detection of Akt (#9272), phospho-Akt (Ser473) (#9271), phospho-Akt (Thr308) (#9275), mTOR (#2972), phospho-mTOR (Ser2448) (#2971), p70S6k (#9202), phospho-p70S6k (Thr389) (#9205), phospho-p70S6k (Thr421/Ser424) (#9204), rpS6 (#2217), phospho-rpS6 (Ser235/236) (#4858), 4E-BP1 (#9452), phospho 4E-BP1 (Thr37/46) (#9459), eEF2 (#2332), phospho-eEF2 (Thr56) (#2331), Raptor (#2280), Tuberin/TSC2 (#3612), phospho-Tuberin/TSC2 (Thr1462) (#3617), PTEN (#9552), phospho-PTEN (Ser380/Thr382/383) (#9552), PI3K (#4257), GAPDH (#2118) (from Cell Signaling Technology, Inc., Beverly, MA, USA) and myogenin (M-225) (sc-576), and myo-D (C-20) (sc-304) (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Membranes were incubated overnight at 4°C in primary antibody buffer (5% BSA in TBS-T, pH 7.6, primary antibody diluted 1:1000), followed by washing in TBS-T (3X 5 min each), and incubation with HRP-conjugated secondary antibody (anti-rabbit (#7074) or anti-mouse (#7076), Cell Signaling Technology, Inc., Danvers, MA, USA) in blocking buffer for 1 h. After removal of the secondary antibody, membranes were washed (3X 5 min each) in TBS-T and protein bands visualized on reaction with ECL reagent (Amersham ECL Western Blotting reagent RPN 2106, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Target protein levels were quantified by AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA, USA) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

*Statistical analysis.*

Results are presented as mean $\pm$ SEM. Data were analyzed using the Sigma Stat 3.5 statistical program. The effects of insulin resistance on protein phosphorylation were analyzed using a two-way ANOVA followed by the Student-Newman-Keuls *post-hoc* testing where appropriate. Differences were considered significant at $P < 0.05$.

## Results

*Insulin resistance is associated with skeletal muscle atrophy*

All the animals were 12 weeks of age at the end of the study. The OZ animals had significantly higher serum levels of insulin (1.75±.05 ng/ml *vs.* 0.48±.03 ng/ml; 3.64 fold), and blood glucose (226±11 mg/dL vs. 121±10 mg/dL; 1.85 fold) compared to their lean counterparts. These results are consistent with the notion that the OZ rats were hyperglycemic and hyperinsulinemic (Fig. 5).

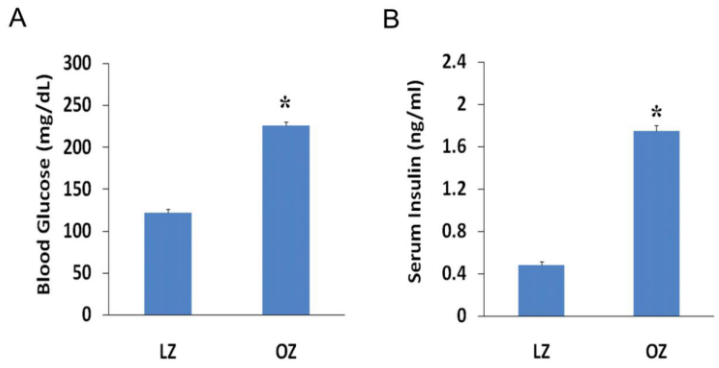


**Figure 5**: Biochemical determination of blood glucose and insulin levels in LZ and OZ rats.*Significantly different from LZ animals; *n* = 12.

The OZ rats exhibited a significantly higher body weight than the LZ at both 7 and 14 days of observation (464 ± 12 g *vs. 302* ± 3 g at 7 days; *P < 0.05*; 460±26 g *vs.* 289±10 g at 21 days; *P < 0.05*). Soleus muscle wet weights were significantly lower in the OZ compared to the LZ (128 ± 11 mg *vs.* 150±6 mg at 7 days; *P < 0.05*; 128.0±6.5 mg *vs.* 141.0±8.3 mg at 21 days; *P < 0.05*; Fig. 6).

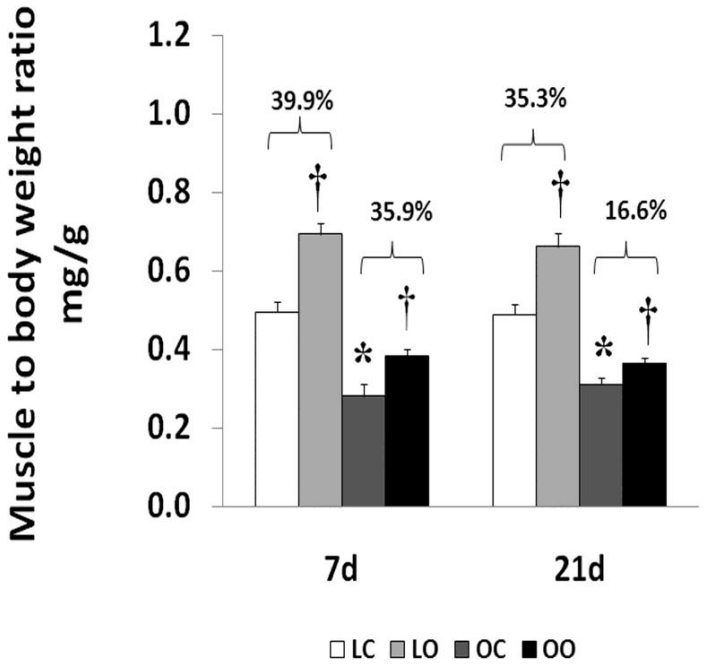


**Figure 6**: Muscle wet weight to-body weight ratios in control and loaded (7- or 21-days) soleus muscles of lean Zucker rat and obese Zucker rats; $n = 6$. *Significantly different from lean control value, $P < 0.05$. †Significantly different from contralateral control, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

*Insulin resistance is associated with a diminished hypertrophic response that is characterized by decreases in the activation of mTOR related signaling.*

Muscle overload increased soleus muscle mass to a similar extent in the LZ and OZ animals at 7 days (39% *vs.* 36%) but the degree of overload-induced muscle growth at 21days was greater in the LZ compared to OZ animals (35% *vs.* 16%; $P < 0.05$; Fig. 6). According to our immunoblot analysis, the phosphorylation of mTOR (Ser2448) and p70S6k (Thr389) in overloaded muscles was elevated relative to the contralateral control after 7 and 21 days of

overload in the LZ rats and after 7 days of overload in the OZ rats (*P < 0.05;* Figs. 7B, 8B). The phosphorylation of p70S6k (Thr421/Ser424) was significantly higher in overloaded muscles of both LZ and OZ rats after 7 and 21 days (*P < 0.05;* Fig. 8C). Overload increased the amount of rpS6 protein in both the LZ and OZ rats at 7 and 21 days of observation (*P < 0.05;* Fig. 9A). Conversely, the phosphorylation of rpS6 (Ser235/236) in overloaded muscles was elevated after 7 and 21 days of overload in LZ animals but only at the 7 day time point in the OZ rats (*P < 0.05;* Fig. 9B). The phosphorylation eEF2 (Thr56) was higher in both the LZ and OZ rats after 7 days of overload (*P < 0.05*) (Fig. 10B). Overload did not alter the amount of phosphorylated 4E-BP1 in either the LZ or OZ animals (Fig. 11B).

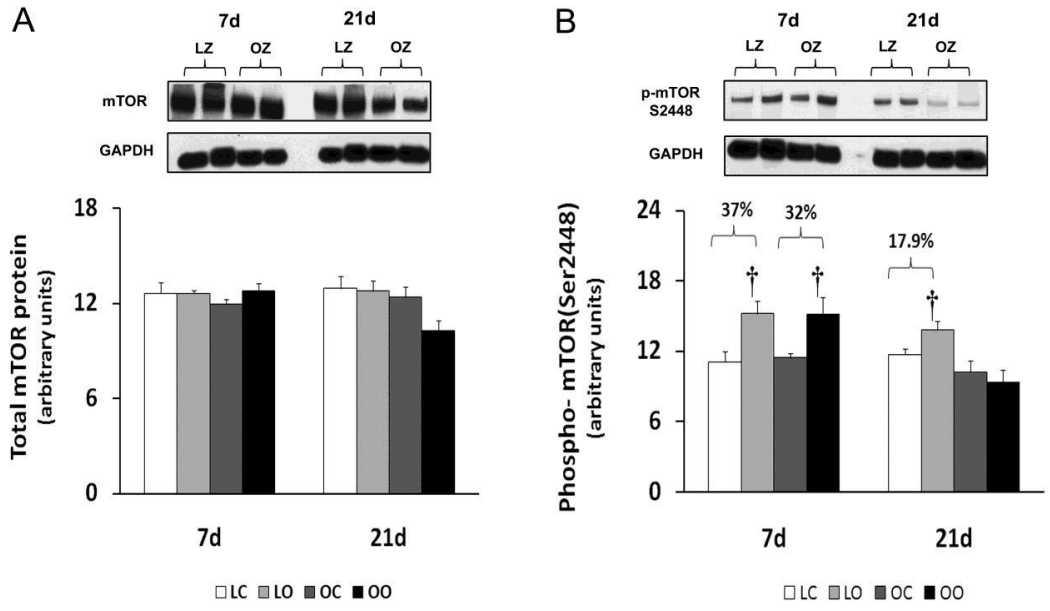


**Figure 7**: Immunoblot analysis of the mammalian target of rapamycin (mTOR) (A) protein content and (B) phosphorylation at $Ser^{2448}$ in control and overloaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of

loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

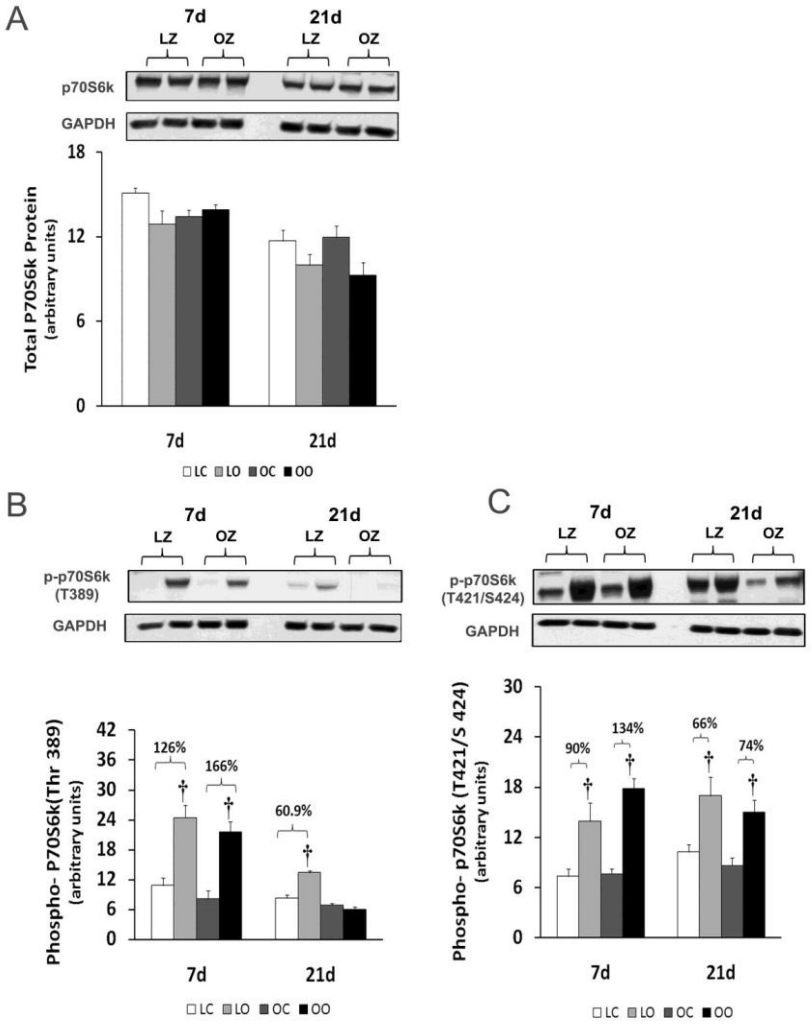


**Figure 8**: Immunoblot analysis of the 70 kDa ribosomal protein S6 kinase (p70S6k) (A) protein content, (B) phosphorylation at $Thr^{389}$, and (C) phosphorylation at $Thr^{421}/Ser^{424}$ in control and overloaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

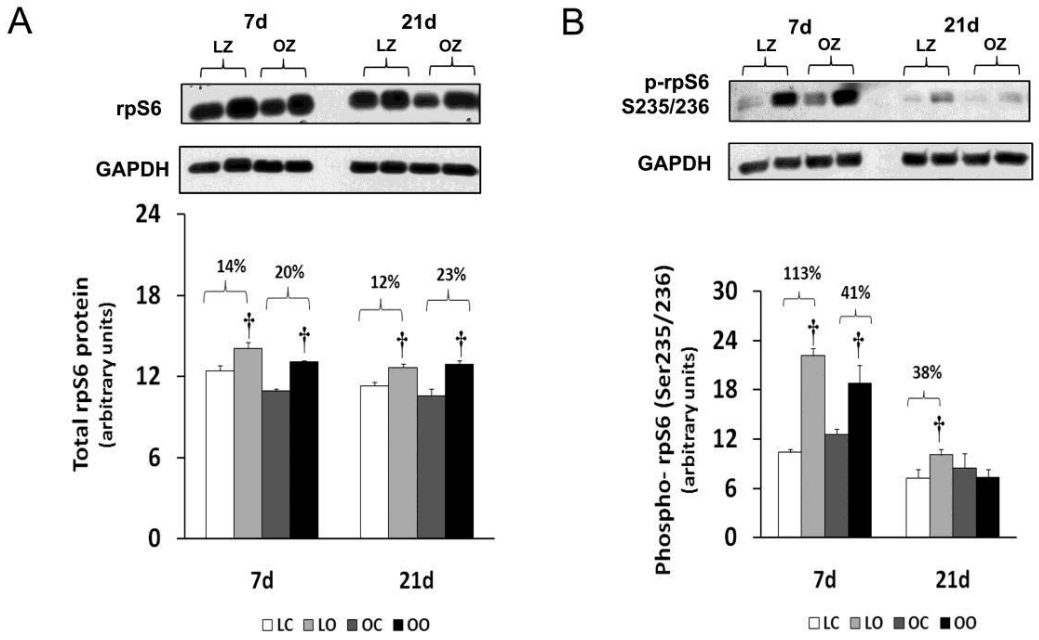


**Figure 9**: Immunoblot analysis of the ribosomal protein S6 (rpS6) (A) protein content and (B) phosphorylation at $Ser^{235/236}$ in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

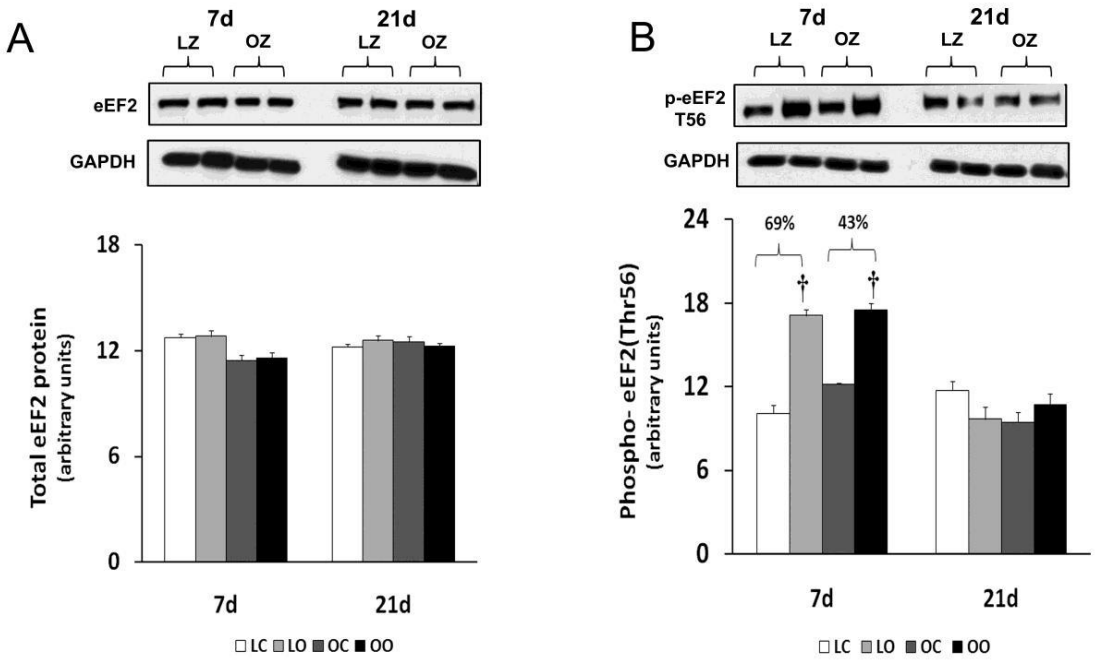


**Figure 10**: Immunoblot analysis of the eukaryotic factor 2 (eEF2) (A) protein content and (B) phosphorylation at $Thr^{56}$ in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

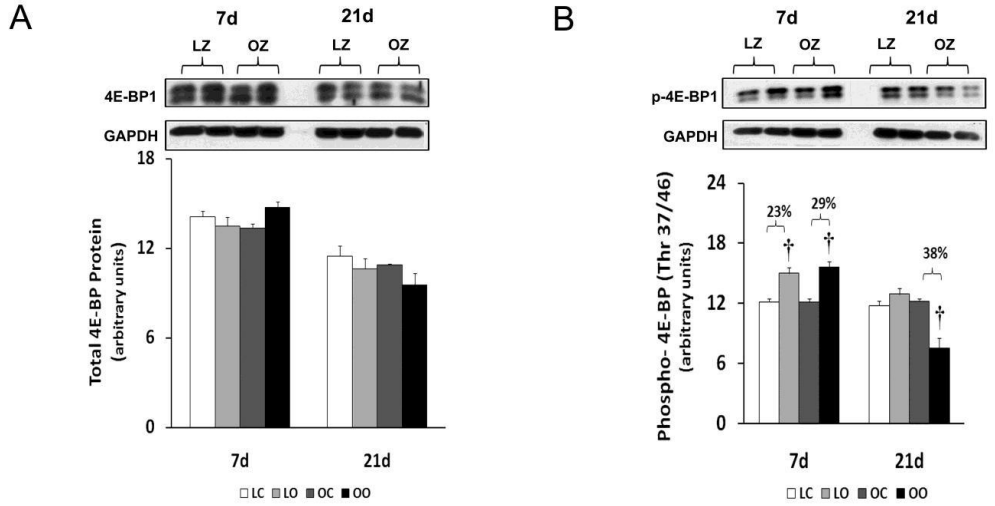


**Figure 11**: Immunoblot analysis of the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (A) protein content and (B) phosphorylation at $Thr^{37/46}$ in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH. $n = 6$. †Significantly different from contralateral control muscle, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

*Alterations in regulation of different possible upstream regulators of mTOR signaling in the soleus muscle of OZ with overload.*

To examine the effect of insulin resistance on the activation of different mTOR regulators with overload, we compared the protein content and phosphorylation of Akt, PTEN, TSC2 / Tuberin, and raptor between control and overloaded muscles. Similar to our findings for mTOR, the amount of phosphorylated Akt (Thr308) and Akt (Ser473) was increased at 7 and 21 days of overload in the LZ rats, where as it was only elevated after 7 days of overload in the OZ rats ($P < 0.05$; Fig. 12). The muscle content and phosphorylation of PTEN was increased by muscle overload in the LZ and OZ animals at both 7 and 21 days ($P < 0.05$; Fig. 13). The protein

content of Tuberin / TSC2 was higher in LZ and OZ animals after 7 days of overload ($P < 0.05$) and at 21 days in the OZ rats ($P < 0.05$; Fig. 14). The phosphorylation of Tuberin / TSC2 (Thr1462) was diminished in LZ rats at 7 and 21 days of overload and in the OZ animals after 7 days of increased loading ($P < 0.05$; Fig. 14). Muscle loading did not alter the expression of raptor in either the LZ or OZ animals (Fig. 15).

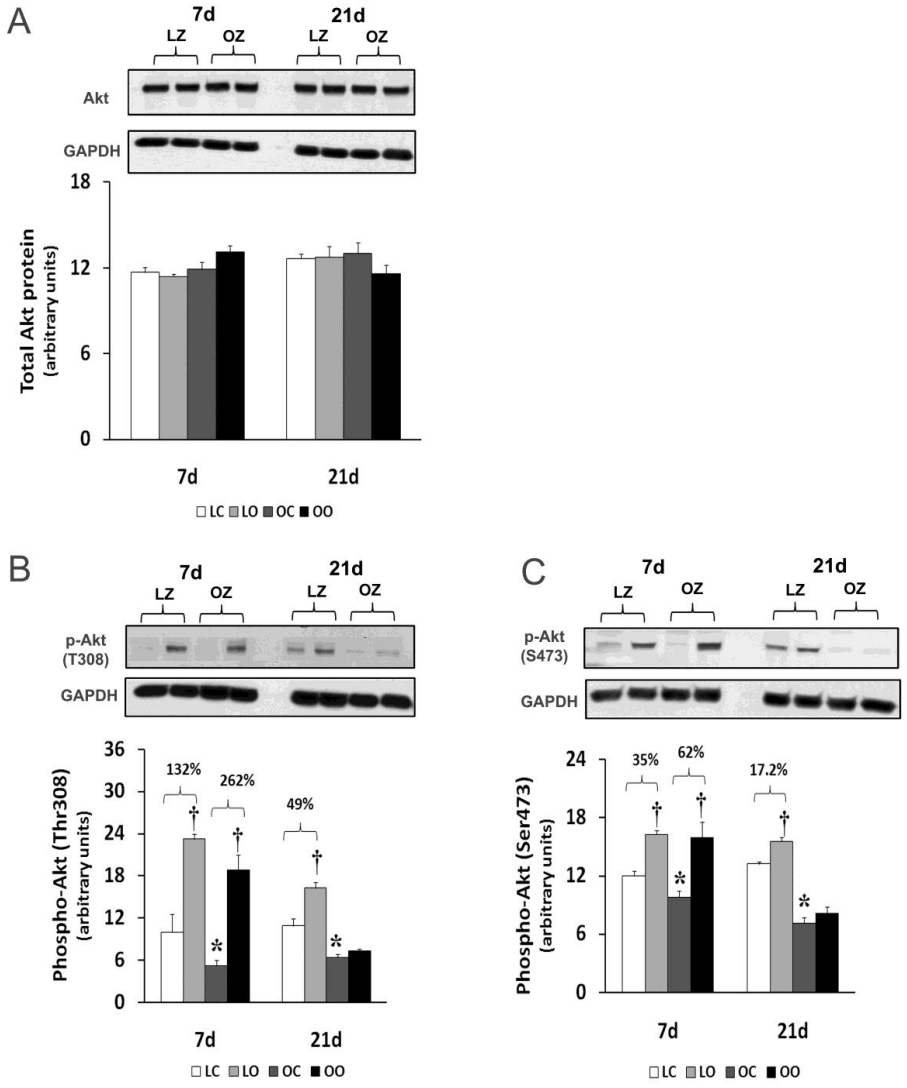


**Figure 12**: Immunoblot analysis of the Akt (A) protein content, (B) phosphorylation at $Thr^{308}$, and (C) phosphorylation at $Ser^{473}$ in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units

relative to the amount of GAPDH; *n* = 6.*Significant difference from respective LZ control muscle, *P < 0.05*. †Significantly different from contralateral control muscle, *P < 0.05*.

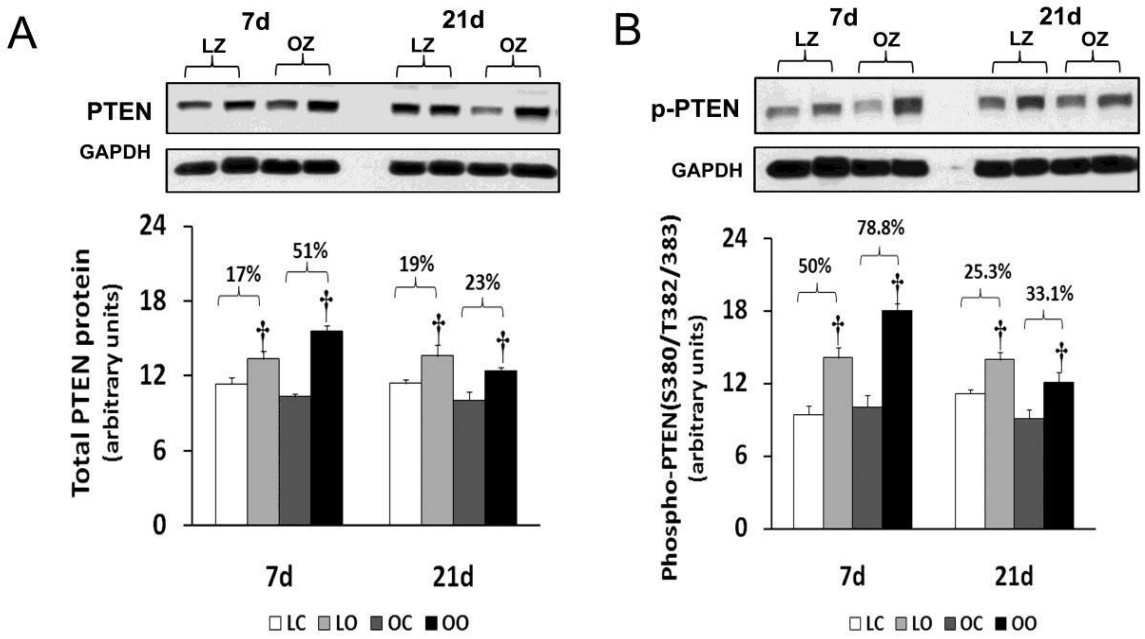


**Figure 13**: Immunoblot analysis of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (A) protein content and (B) phosphorylation at $Ser^{380}/Thr^{382/383}$, in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; *n* = 6. †Significantly different from contralateral control muscle, *P < 0.05*. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

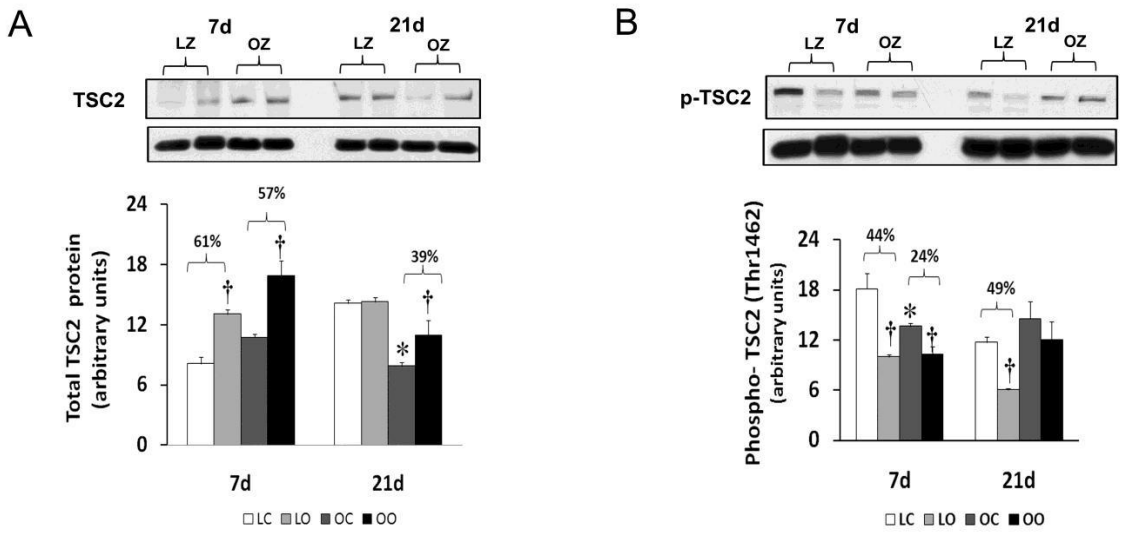


**Figure 14**: Immunoblot analysis of the Tuberin/TSC2 (A) protein content and (B) phosphorylation at $Thr^{1462}$, in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$.*Significant difference from respective LZ control muscle, $P < 0.05$. †Significantly different from contralateral control muscle, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

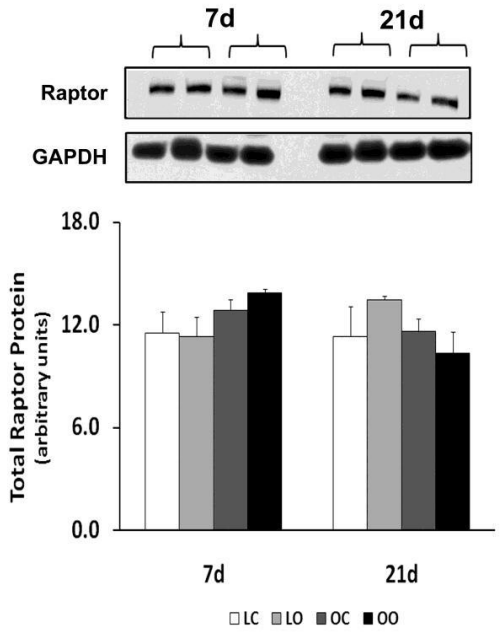


**Figure 15**: Immunoblot analysis of raptor protein content in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$.

*Overload did not alter expression of myogenic regulatory factors (MRFs).*

Given the potential role that MRF may play in the regulation of satellite activation and muscle hypertrophy [57, 265], we next examined the regulation of myogenin and myoD with overload. Overload of the soleus muscles did not alter the myogenin and myoD expression in either the lean or obese Zucker rats (Fig. 16).

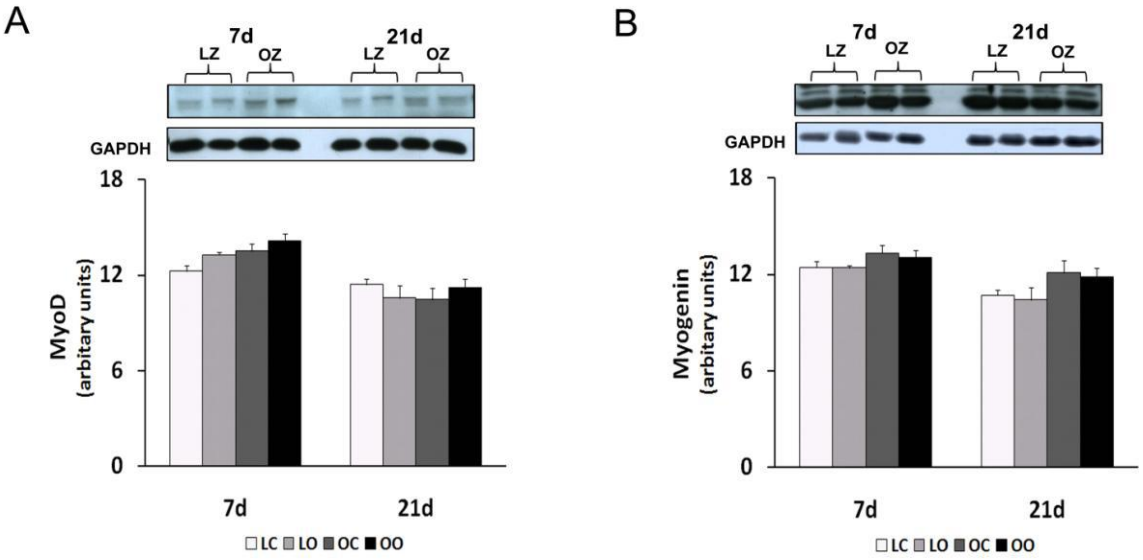


**Figure 16:** Supplemental Figure - Immunoblot analysis of myoD and myogenin expression in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$.

**Discussion**

Previous work by our laboratory has demonstrated that the degree of soleus muscle hypertrophy following eight weeks of compensatory overload appears to be blunted in the insulin resistant OZ rat compared to its lean counterpart [4]. Here we examine the time course of muscle growth and the activation of mTOR and mTOR related signaling at one- and three-weeks of overload in an effort to better understand why muscle hypertrophy may be diminished in the OZ rat. Our data suggest that insulin resistance may affect the ability of slow muscle to maintain activation of mTOR related signaling after the first week of loading.

mTOR has been suggested to be an important regulator of muscle growth as studies showing inhibition of mTOR by the drug rapamycin almost completely inhibit the hypertrophic response [22]. Here we demonstrated that the phosphorylation (activation) of mTOR appears to be similar after one week of overload but significantly less in the overloaded OZ rat compared to LZ rat after three weeks of overload (Fig. 7). This latter finding is consistent with our data demonstrating that the insulin resistant soleus exhibits a reduced ability to undergo hypertrophy following eight weeks of mechanical overload [4]. To examine how this defect in mTOR signaling might affect the regulation of molecules thought to be involved in controlling protein translation we next examined how insulin resistance affected the phosphorylation of eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1) and p70S6k in response to increased muscle loading. As expected from our mTOR data, the phosphorylation of the mTOR substrates, 4E-BP1 and p70S6k, was significantly less in the OZ rat compared to that observed in the LZ rat (Figs. 8, 11). How these differences in the activation (phosphorylation) of 4E-BP1 and p70S6k may affect the hypertrophic response of the insulin resistant soleus is currently unclear. However, other work has demonstrated convincingly that the phosphorylation of these molecules

is highly correlated with the degree of muscle hypertrophy [30, 266]. The underlying molecular mechanisms for these alterations remain unclear. However, it is interesting to note that other studies have demonstrated that the phosphorylation of p70S6k in response to insulin [267-269] and increased muscle loading [68] may be altered in the diabetic rat model.

To confirm these data, we also examined the regulation of ribosomal protein S6 (rpS6), which is a substrate of p70s6k [270]. It has been shown that phosphorylation of rpS6 by p70S6k correlates with an increase in translation, particularly of mRNAs with an oligopyrimidine tract in their 5' untranslated regions [271]. Consistent with our findings for p70S6k, we found that the degree of rpS6 phosphorylation at Ser 235/236 following three weeks of overload was diminished in the OZ compared to their lean counterparts (Fig. 9). Further, we examined the regulation of eukaryotic elongation factor 2 (eEF2) which is a GTP-binding protein that functions to repress the translocation step of elongation when in its nonphosphorylated state [176]. We found similar changes in the expression and phosphorylation of eEF2 following overload in both lean and obese animals (Fig. 10). These data suggest that the attenuation of hypertrophy in insulin resistant muscle may not be due to differences in the regulation of the elongation phase of the protein translation process.

Like mTOR and p70S6k, the activation of protein kinase B / Akt is thought to be required for muscle hypertrophy as the over expression of constitutively active Akt results in increased p70S6k phosphorylation, glycogen accumulation, and muscle fiber hypertrophy [272]. Previous data has demonstrated that phosphorylation of serine and threonine residues within the carboxyl terminal hydrophobic domain (Ser473) and catalytic domain (Thr308) are necessary for full activation of Akt kinase activity [273]. Compared to that seen in the LZ, the ability of the soleus to activate (phosphorylation at both Ser473 and Thr308) Akt in the OZ appears to be diminished

(Fig. 12). How this might affect the activation of other signaling molecules is currently unclear; however, it is likely that Akt can influence the activity of mTOR through its ability to phosphorylate the product of the tuberous sclerosis complex TSC2 gene, also termed tuberin. Tuberin is a tumor suppressor that inhibits mTOR when unphosphorylated and is involved in the regulation of cell proliferation and tumor development [147, 148]. Here we demonstrate that the Akt-dependent phosphorylation of tuberin (Thr1462) is diminished in the LZ rat while it is unaltered in OZ rat following three weeks of overload (Fig. 14). Because decreased tuberin phosphorylation should lead to a decrease in mTOR activity, it is likely that the differences in mTOR phosphorylation we see between models is not due to alterations in TSC2 regulation. Similar to tuberin, raptor is also thought to interact with mTOR where it acts to increase the phosphorylation of p70S6k and 4E-BP1 [274]. Unlike with tuberin, we did not observe any difference in the amount of raptor between models or with overload (Fig. 15) suggesting that the attenuation of mTOR activity in insulin resistant muscle may not be due to differences in raptor regulation.

The activity of PI3K/Akt/mTOR signaling is thought to be negatively regulated by the tumor suppressor protein, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) [263]. Like our findings for raptor, we found similar increases in the amount of PTEN phosphorylation following overload in both the lean and obese rats (Fig. 13). This finding is quite interesting as it suggests that PTEN is unlikely to play a role in explaining why insulin resistant muscle exhibits differences in its ability to activate Akt and mTOR following increased muscle loading. Why the magnitude of load-induced mTOR signaling may differ with insulin resistance is not clear. In addition to Akt, raptor and TSC2, the activity of mTOR is also influenced by a myriad of other molecules including AMP-activated protein kinase (AMPK),

rictor, REDD2, phospholipase D (PLD), and possibly others [275-280]. Recent *in vitro* and *in vivo* data has suggested that AMPK may inhibit protein synthesis through its ability to suppress mTOR activation [22, 34, 135]. Consistent with this finding, work by Thomson and Gordon has demonstrated that the diminished overload-induced hypertrophy seen in aged skeletal muscle is associated with AMPK hyperphosphorylation [180]. Further experiments to investigate whether a similar relationship between AMPK activation and the degree of muscle growth is present in insulin-resistant muscle will likely add to our understanding of what role this molecule may play in modulating muscle growth. In an effort to explore other possible mechanisms for the attenuated hypertrophy we observe in the OZ animals, we next examined if muscle overload was associated with alterations in the amount of myogenic regulatory factors, MyoD and myogenin, which have been posited to be involved in regulating satellite cell activation. Consistent with previous work examining the myogenin and myoD levels in rat soleus muscle after 4 weeks of surgical overload [281], we observed no changes in the amount of myogenin and myoD with overload in either the LZ and OZ animals (Fig. 16). It is also possible that differences in the degree of animal activity may also have played a role in the differential hypertrophic response between the LZ and OZ rats. Because the hypertrophy stimulus in the synergistic ablation model are dependent, at least in part, on animal activity, it is possible that the soleus muscles of the less active obese animals [282] were loaded to a lesser degree than their lean counterparts. Whether this was actually the case cannot be determined from our data. Nonetheless, it is also worthy to point out that the greater body mass of the obese Zucker rats likely produced a greater overload stimulus for muscle growth on the weight-bearing muscles. To address these possibilities, future studies employing other animal models that do not differ in their amount of activity or body weight may be warranted.

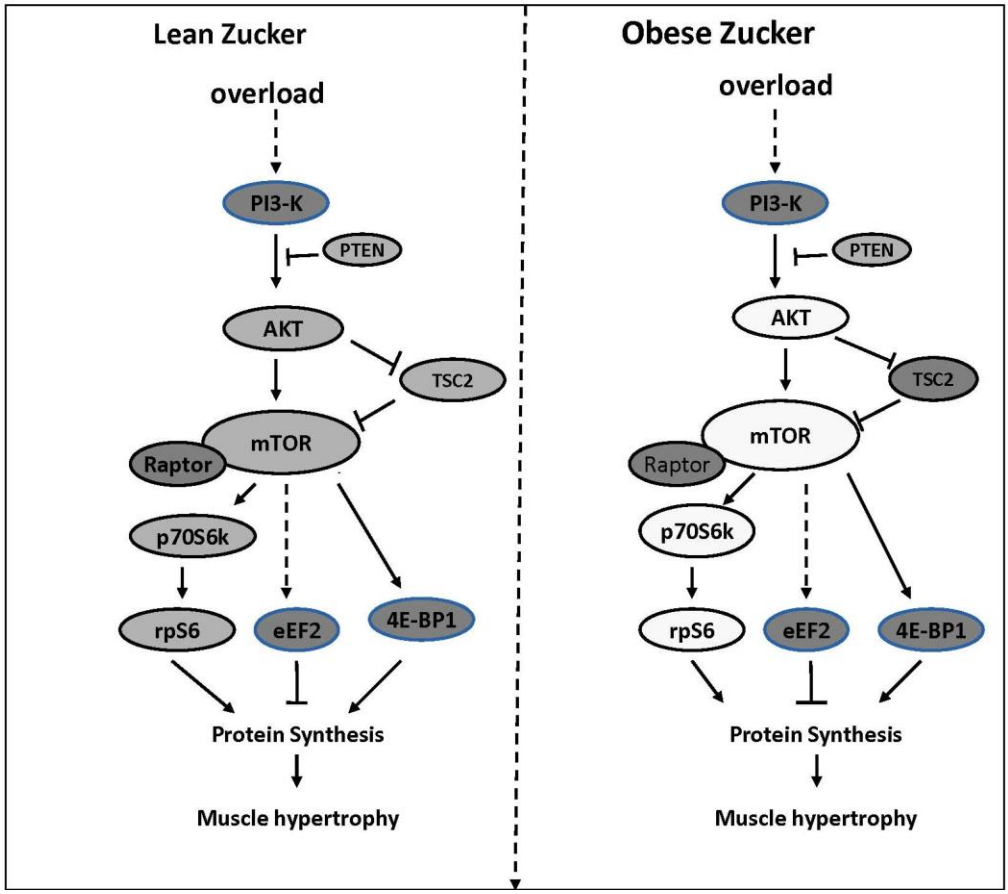


**Figure 17**: Schematic summarizing the differences in load-induced signaling between the lean and obese Zucker rats after 21 days of overload. Molecules shown in the dark-shaded, medium-shaded, and empty circles represent no change, complete activation or inactivation with respect to control leg, and differences in activation between animal models, respectively. Impaired hypertrophy in obese Zucker rats is associated with an attenuated activation of Akt, mTOR, p70S6k, and rpS6

In summary, the data of the present study suggest that insulin resistance may be associated with a decrease in the ability of the soleus muscle to undergo muscle hypertrophy and that this finding may be related to differences in mTOR, p70S6k, Akt, and rpS6 signaling (Fig. 17). These findings are novel, as they are the first to demonstrate impaired mTOR related

signaling in direct conjunction with attenuated overload induced hypertrophy in insulin-resistant skeletal muscle. Additional studies designed to directly inhibit or activate these signaling molecules, as well as to explore the other possible mechanism(s) e.g. AMPK signaling during muscle adaptation, will be needed to increase our understanding of the exact mechanism(s) involved and the clinical ramifications for an ever increasing diabetic population.

**SPECIFIC AIM II:** To investigate whether insulin resistance affects the regulation of the AMPK and PKR during muscle hypertrophy.

**PAPER 2**

The following paper corresponds to the specific aim II.

**Attenuation in overload-induced hypertrophy in insulin- resistant muscle is associated with hyperphosphorylation of AMPK and dsRNA-dependent protein kinase**

**Abstract**

Previous data have suggested that diminished hypertrophy in insulin-resistant skeletal muscle may result, at least in part, from decrements in mammalian target of rapamycin (mTOR) signaling [283]. Here we investigate whether alterations in AMP-activated protein kinase (AMPK) and dsRNA-dependent protein kinase (PKR)-dependent signaling may also play a role in this process. Compared to that observed in LZ rats, the phosphorylation of AMPKα at Thr172 and its upstream activator calcium/calmodulin-activated protein kinase (CaMKII) at Thr286 were higher in the insulin resistant obese Zucker (OZ) soleus ($P < 0.05$). The activation (autophosphorylation) levels of PKR, PKR dependent phosphorylation of eIF2α at Ser51 and p38 MAP kinase were significantly higher in OZ rats compared to LZ rats ($P < 0.05$). Taken together, these results suggest that insulin resistance attenuates overload-induced skeletal muscle hypertrophy through the activation of AMPK and PKR leading to inhibition of mTOR signaling and phosphorylation of eIF2α and depression of protein synthesis, together with PKR mediated p38 MAP kinase activation and increased protein degradation.

**KEY WORDS:** Insulin resistance; obese Zucker rat; skeletal muscle; AMPK; PKR

**Introduction**

Skeletal muscle is a primary consumer of blood glucose and is thought to participate in the control of blood glucose levels, where as other studies have demonstrated that increases in muscle mass or muscle hypertrophy are associated with blood glycemic control [284-286]. It has been hypothesized that the regulation of muscle growth is dependent upon the balance between protein synthesis and degradation with increases in muscle mass occurring when protein accretion is favored over its breakdown. The molecular mechanism(s) controlling muscle hypertrophy are not well understood.

Previous studies from our laboratory using the obese Zucker (OZ) rat have demonstrated that insulin resistance is associated with a diminished ability of skeletal muscle to undergo hypertrophy that may be caused, at least in part, by decrements in activation of the mammalian target of rapamycin (mTOR) [4, 287]. Why insulin resistance may be associated with differences in the regulation of mTOR signaling is currently unclear but it is possible that these differences may be related to events thought to reside upstream of the mTOR molecule. In this regard, one protein of potential interest may be the 5'–AMP-activated protein kinase (AMPK). Recent data suggest that AMPK may function as a master sensor of cellular energy balance and that it may be a negative regulator of protein synthesis [26, 34]. AMPK is a heterotrimeric complex consisting of one catalytic subunit (α) and two non catalytic regulatory (β and γ) subunits, whose activation is controlled through phosphorylation of the α subunit at Thr172 [168]. It has been shown that AMPK may inhibit protein synthesis through its ability to suppress mTOR activation [22, 34, 135, 288, 289]. The factors controlling the activity of AMPK have not been fully elucidated,

however, it is thought that protein tumor suppressor LKB1 kinase [35, 169, 170], calcium/calmodulin-activated protein kinase (CaMKII) [36, 171], and transforming growth factor-β-activated kinase-1 (TAK1), a member of the mitogen-activated protein (MAP) kinase family, may be involved [37, 172]. In addition to AMPK signaling, other recent *in vitro* and *in vivo* studies have suggested that dsRNA-dependent protein kinase (PKR) can also play a role in the inhibition of protein synthesis [33, 185, 290, 291]. PKR when activated phosphorylates eIF2α at Ser-51 [290], which in turn leads to translational inhibition and presumably, a decrease in protein synthesis [38]. In addition to its potential effect on protein synthesis, PKR may also have a hand in controlling the ubiquitin proteolytic pathway through the activation of p38 MAP kinase [39, 40]. How insulin resistance may affect the regulation of PKR during muscle hypertrophy has to our knowledge not been investigated.

Using the same animals and tissues used in our previous work [287], here we investigate whether insulin resistance affects the regulation of AMPK and PKR, during muscle hypertrophy. We hypothesize that the attenuation of muscle growth and mTOR activation following increased muscle loading observed with insulin resistance may be related to alterations in AMPK and PKR signaling.

**Materials and Methods**

*Materials.*

Primary antibodies against AMPKα (#2532), AMPKα1 (#2795), AMPKα2 (#2757), AMPKβ1/2 (#4150), phospho-AMPKα (Thr172) (#2535), LKB1 (#3050), phospho-LKB1 (Ser428)(#3482), CaMKII (#3362), phospho-CaMKII (Thr286)(#3361), TAK1 (#4505), phospho-TAK1 (Thr184/187) (#4531), eIF2α (#9722), phospho-eIF2α (Ser51)(#3597), GSK-3β (#9315), phospho-GSK-3β (Ser9) (#9336), p38 MAP kinase (#9212), phospho-p38 MAP kinase (Thr180/Tyr182)(#9216), Ubiquitin (#3936), glyceraldehyde 3-phosphate dehydrogenase (GAPDH,#2118), secondary antibodies conjugated with horseradish peroxidase (HRP) (anti-rabbit (#7074) or anti-mouse (#7076)) were purchased from Cell Signaling Technology (Beverly, MA). PKR (sc-708) and phospho-PKR (Thr446) (sc-101783) were from Santa Cruz Biotechnology (Santa Cruz, CA). The Laemmli 2 × sample buffer was from Sigma-Aldrich (St. Louis, MO). Pierce Tissue Protein Extraction Reagent (T-PER), Pierce 660nm protein assay reagent (#22660), GE Healthcare Amersham ECL™ Western Blotting Detection Reagents (RPN2106) and Advance Western Blotting Detection Kit (RPN2135) were from Thermo Fisher Scientific Inc. (Rockford, IL). The PAGEr Gold Precast gels (10%) were purchased from Lonza (Rockland, ME).

*Animal Care.*

All procedures were performed as outlined in the Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society and the institutional animal use review board of Marshall University. The animals and tissues used in this study have been previously examined [287]. Young male LZ (n=12) and OZ (n=12) rats were obtained from the Charles River Laboratories. All animals were 12 wk of age at the

completion of this study. Rats were housed two per cage in an AAALAC approved vivarium. Housing conditions consisted of a 12 H: 12 H dark-light cycle and temperature was maintained at 22° ± 2°C. Animals were provided food and water *ad libitum* and allowed to recover from shipment for at least two weeks before experimentation. During this time the animals were carefully observed and weighed weekly to ensure none exhibited signs of failure to thrive, such as precipitous weight loss, disinterest in the environment, or unexpected gait alterations.

*Synergist ablation procedure.*

Unilateral overload of the soleus muscle for 1 and 3 weeks was achieved through the surgical ablation of the medial and the proximal two-thirds of the lateral head of the gastrocnemius [109]. The unilateral ablation model allows within animal comparisons, thus eliminating bias due to systemic factors. Rats were anesthetized with a ketamine–xylazine (4 : 1) cocktail (50 mg/kg, I/P) and, the distal two-thirds of the gastrocnemius muscle were surgically removed from the left hindlimb as previously described [109]. A sham (control) operation was performed on the right hindlimb. The sham procedure consisted of an incision through the skin, followed by blunt isolation of the Achilles tendon and gastrocnemius muscle prior to closure. Animals were active immediately after recovering from anesthesia, and were checked twice daily during the 7 day postoperative period. No signs of infection or other complications were observed postoperatively.

*Tissue collection.*

Soleus muscles were collected 7 days ($n$ = 6 LZ-7 and $n$ = 6 OZ-7) or 21 days ($n$ = 6 LZ-21 and $n$ = 6 OZ-21) after the synergist ablation procedure. Animals were 12 wk old at the time of tissue collection. Rats were anesthetized with a ketamine–xylazine (4: 1) cocktail (50 mg/kg, I/P) and supplemented as necessary for reflexive response. Soleus muscles from both legs were

quickly removed, trimmed of excess connective tissue, weighed on an analytical balance, frozen in liquid nitrogen, and stored at −80°C until further analysis.

*Tissue protein extraction.*

Muscles were homogenized in a Pierce Tissue Protein Extraction Reagent (T-PER) (10 mL/g tissue; Rockford, IL, USA) that contained protease inhibitors (P8340, Sigma-Aldrich, Inc., St. Louis, MO, USA) and phosphatase inhibitors (P5726, Sigma-Aldrich, Inc., St. Louis, MO, USA). After incubation on ice for 30 min, the homogenate was collected by centrifuging at 12,000 g for 5 min at 4°C. The protein concentration of the homogenates was determined via the Bradford method (Fisher Scientific, Rockford, IL, USA). Homogenate samples were boiled in Laemmli 2× sample buffer (Sigma-Aldrich, Inc., St. Louis, MO, USA) for 5 min prior to SDS-PAGE.

*SDS-PAGE and immunoblotting.*

Forty micrograms of total protein from each sample was separated on a 10% PAGEr Gold Precast gel (Lonza, Rockland, ME, USA) and then transferred to a nitrocellulose membrane. Visual verification of transfer and equal protein loading amongst lanes was accomplished by Ponceau S staining of the membranes. Immunodetection of antigens was performed as described previously [16, 68]. Briefly, membranes were blocked for 1 h at room temperature in blocking buffer (5% non-fat dry milk in TBS-T (20mM Tris-base, 150mM NaCl, 0.05% Tween-20), pH 7.6), serially washed in TBS-T at room temperature, then incubated overnight at 4°C in primary antibody buffer (5% BSA in TBS-T, pH 7.6, primary antibody diluted 1:1000), followed by washing in TBS-T (3X 5 min each), and incubation with HRP-conjugated secondary antibody (anti-rabbit (#7074) or anti-mouse (#7076), Cell Signaling Technology, Inc., Danvers, MA, USA) in blocking buffer for 1 h. After removal of the secondary

antibody, membranes were washed (3X 5 min each) in TBS-T and protein bands visualized on reaction with ECL reagent (Amersham ECL Western Blotting reagent RPN 2106, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Target protein levels were quantified by AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA, USA) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

*Statistical analysis.*

Results are presented as mean $\pm$ SE. Data were analyzed using the Sigma Stat 3.5 statistical program. The effects of insulin resistance on protein phosphorylation were analyzed using a two-way ANOVA followed by the Student-Newman-Keuls *post-hoc* testing where appropriate. Values of $P < 0.05$ were considered to be statistically significant.

## Results

*Insulin resistance is associated with a diminished hypertrophic response that is characterized by hyperphosphorylation of AMP-activated protein kinase (AMPK)*

As outlined previously, the OZ rat exhibited a significantly higher body weight than the LZ at both 7 and 14 days of observation (464 ± 12 gm *vs. 302* ± 3 gm at 7 days; $P < 0.05$; 460±26 *vs.* 289±10 mg at 21 days; $P < 0.05$) and soleus muscle wet weights were significantly lower in the OZ compared to the LZ (128 ± 11 mg *vs.* 150±6 mg; at 7 days; $P < 0.05$; 128.0±6.5 vs. *141.0*±8.3 mg at 21 days; $P < 0.05$) [287]. To examine the effect of insulin resistance on the regulation of AMPK with overload, we compared the protein content of different AMPK isoforms and phosphorylation of AMPKα at Thr172. Muscle loading did not alter the expression of AMPK isoforms (α1, α2, β1 and β2) in either the LZ or OZ animals (Fig. 18). The phosphorylation of AMPKα at Thr172 was diminished in both LZ and OZ animals after 7 days of increased loading, while it was significantly increased in OZ rats after 21 days of overload ($P < 0.05$; Fig. 18).

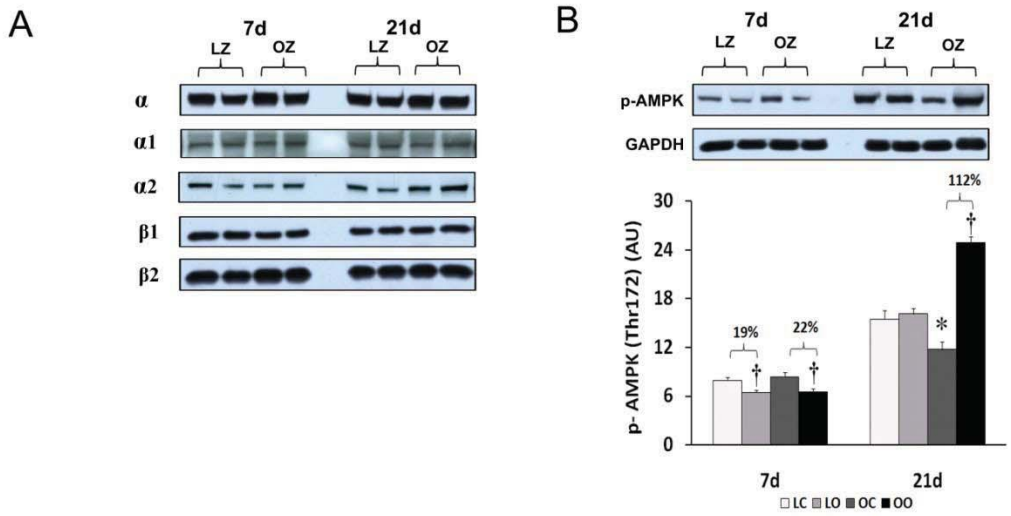


**Figure 18**: (A) AMP-activated protein kinase (AMPK) subunit protein content and (B) Immunoblot analysis of the AMPKα at Thr172 in control and overloaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, *Significantly different from lean control value, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

*Effect of overload on different potential upstream regulators of AMPK signaling in the soleus muscle of LZ and OZ rats*

To examine the effect of insulin resistance on the activation of different potential upstream regulators of AMPK with overload, we compared the protein content and phosphorylation of LKB1, CaMKII, and TAK1 between control and overloaded muscles. The protein expression for LKB1 was increased by muscle overload in the LZ and OZ animals at 7 days, and the phosphorylation of LKB1 at Ser428 was increased by muscle overload in both LZ and OZ animals at both 7 and 21 days ($P < 0.05$; Fig. 19, Panels A, B). The protein expression for CaMKII was unaltered with muscle overload in both LZ and OZ animals, where as the

phosphorylation levels of CaMKII was significantly higher in OZ rat with overload at both 7 and 21 days (*P < 0.05;* Fig. 19, Panels C, D). The amount of TAK1 protein was increased by muscle overload in the LZ and OZ animals at 7 days and the amount of phosphorylated TAK1 at Thr184/187 was increased at 7 and 21 days of overload in the LZ rats, while it was elevated only after 7 days of overload in the OZ rats (*P < 0.05;* Fig. 19, Panels E, F).

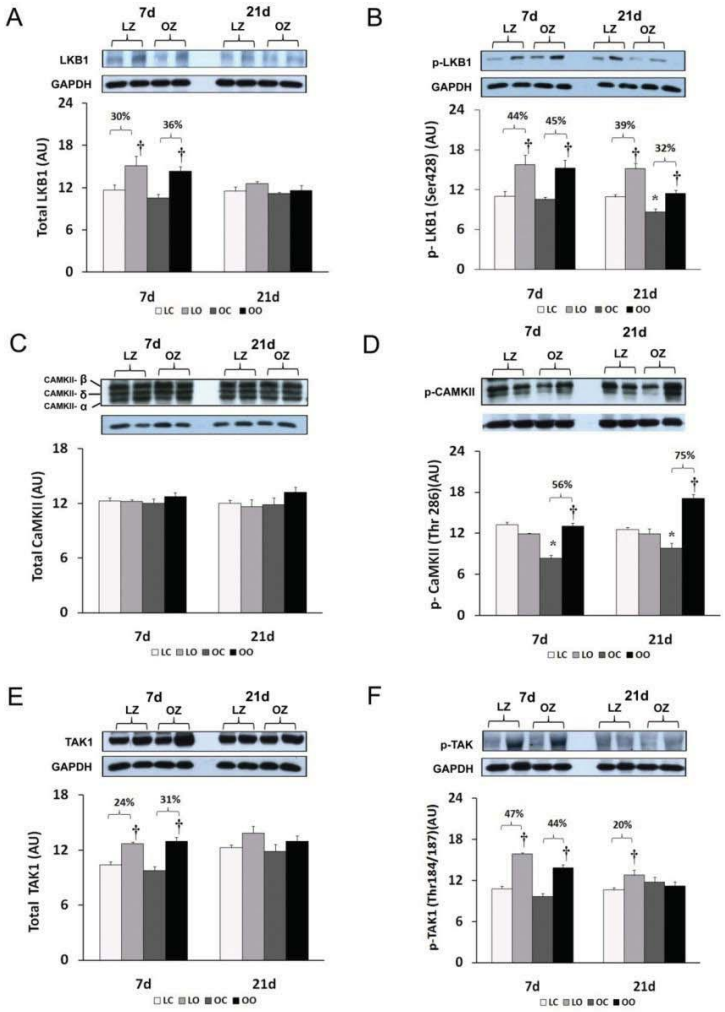


**Figure 19**: Immunoblot analysis of the different upstream regulators of AMPK , LKB1 (A) protein content and (B) phosphorylation at Ser428, CaMKII (C) protein content and (D) phosphorylation at Thr286, and CaMKII (E) protein content and (F) phosphorylation at Thr184/187 in control and overloaded soleus muscles of LZ and OZ. Data are expressed as

integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, * Significantly different from lean control value, $P < 0.05$.

*The diminished hypertrophic response seen in insulin resistant animals is associated with the activation of PKR dependent signaling*

To examine the effect of insulin resistance on the activation of PKR dependent signaling with overload, we compared the protein content and phosphorylation of PKR, eIF2α, and p38 MAP kinase between control and overloaded muscles. The total amount of PKR protein in overloaded muscles was elevated relative to the contralateral control after 7 days of overload in the LZ rats and after 7 and 21 days of overload in the OZ rats ($P < 0.05$; Fig. 20). Conversely, the phosphorylation of PKR at Thr446 was decreased at both 7 and 21 days of overload in the LZ rats, where as it was decreased after 7 days of overload and significantly elevated after 21 days overload in the OZ rats ($P < 0.05$; Fig. 20). Similar to our findings for PKR, the amount of phosphorylated eIF2α at Ser51 was decreased at both 7 and 21 days of overload in the LZ rats while it was decreased after 7 days of overload but significantly elevated after 21 days overload in the OZ rats ($P < 0.05$; Fig. 21). Muscle loading did not alter the expression of eIF2α and p38 MAP kinase in either the LZ or OZ animals (Figs. 21 and 23). The phosphorylation levels of p38 MAP kinase at Thr180/Tyr182 in overloaded muscles were elevated relative to the contralateral control after 7 days of overload in the LZ rats and after 7 and 21 days of overload in the OZ rats ($P < 0.05$; Fig. 23). The ubiquitinated protein levels were increased in both LZ and OZ rats after 7 days and significantly elevated only in the OZ rats after 21 days overload ($P < 0.05$; Fig. 24).

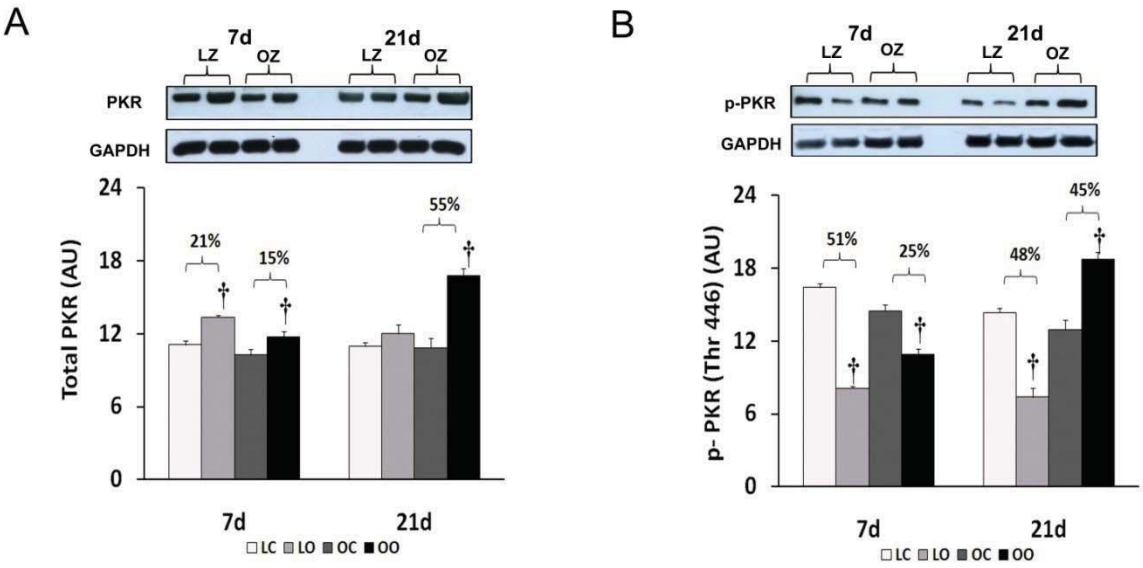


**Figure 20**: Immunoblot analysis of the dsRNA-dependent protein kinase (PKR) (A) protein content and (B) phosphorylation at Thr446, in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, $P < 0.05$.

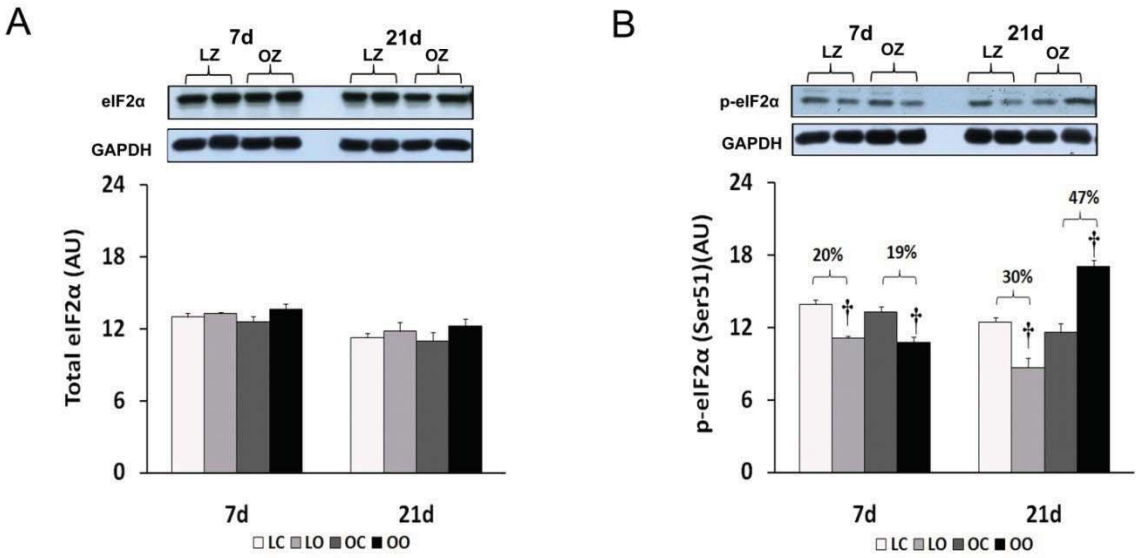


**Figure 21**: Immunoblot analysis of the eIF α (A) protein content and (B) phosphorylation at Ser51, in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, $P < 0.05$.

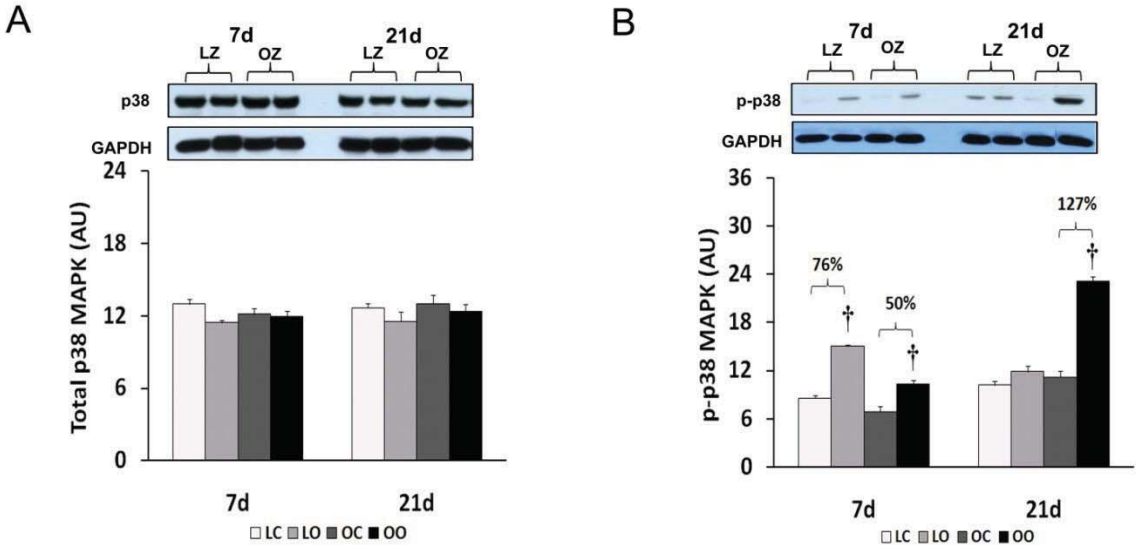


**Figure 223:** Immunoblot analysis of the p38 MAP kinase (A) protein content and (B) phosphorylation at Thr180/Tyr182, in control and loaded soleus muscles of LZ and OZ. Data are

expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, $P < 0.05$.

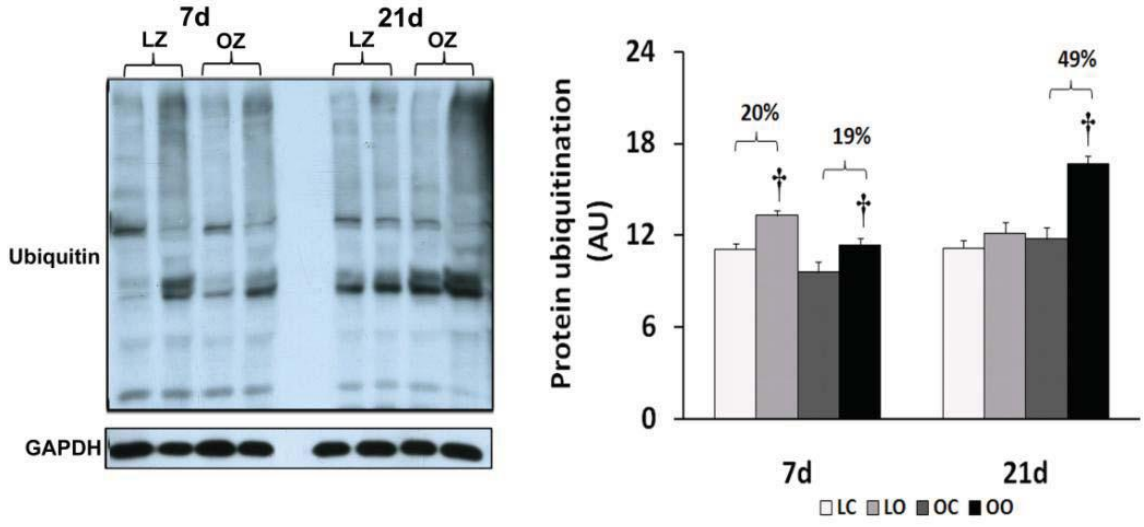


**Figure 234:** Immunoblot analysis of the ubiquitinated protein levels, in control and loaded soleus muscles of LZ and OZ rats. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, $P < 0.05$.

*Effect of overload on regulation of GSK-3β in the soleus muscle of LZ and OZ rats*

Given the role of GSK-3β in suppression of protein synthesis through its ability to inhibit the translational initiation complex [292], we next examined the regulation of GSK-3β with overload. The muscle content and phosphorylation of GSK-3β on Ser9 was increased by muscle overload in the LZ and OZ animals at 7 days. Conversely, after 21 days of overload the phosphorylation of GSK-3β on Ser9 (Ser235/236) was elevated only in LZ animals ($P < 0.05$; Fig. 22)

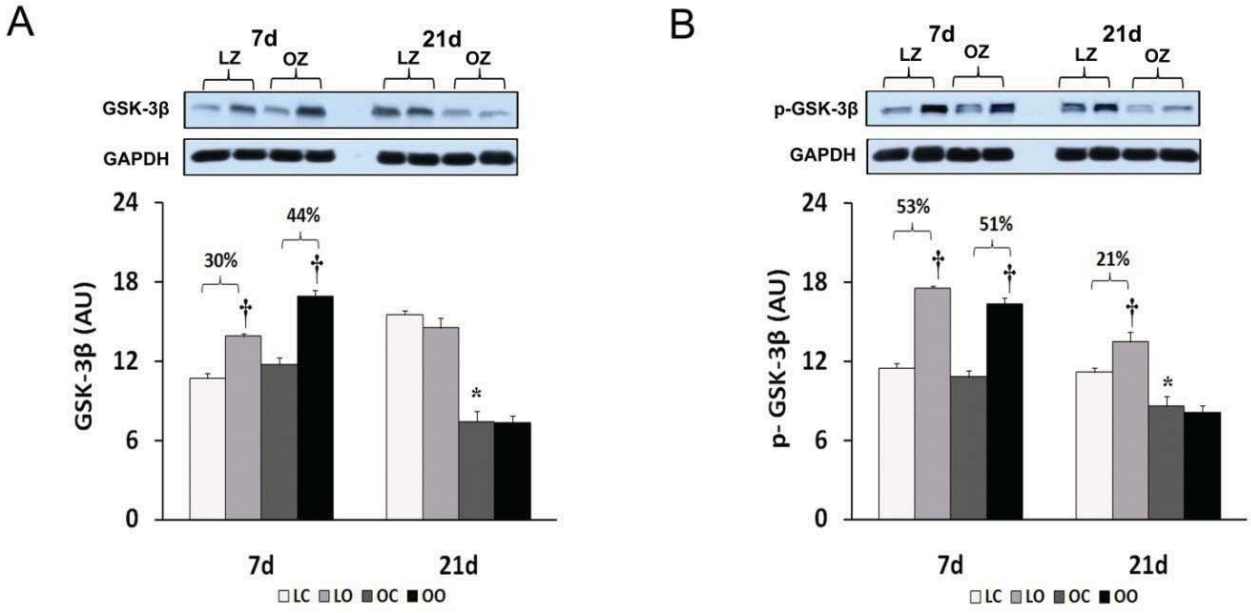


**Figure 242:** Immunoblot analysis of the GSK-3β (A) protein content and (B) phosphorylation at Ser9, in control and loaded soleus muscles of LZ and OZ. Data are expressed as integrated optical density (IOD) × band area and expressed in arbitrary units relative to the amount of GAPDH; $n = 6$. †Significantly different from contralateral control muscle, * Significantly different from lean control value, $P < 0.05$.

**Discussion**

The phosphorylation of AMPK at Thr172 is thought to lead to an inhibition of protein synthesis and has been found to be negatively correlated with the degree of muscle hypertrophy in the overloaded rat [180]. Here we found that the phosphorylation of Thr172 appears to be similar after one week of overload in the LZ and OZ animals but that it is significantly higher in the overloaded OZ rat compared to LZ rat after three weeks of overload (Fig. 18). These latter data are consistent with our previous report demonstrating a decrease in the ability of the OZ to undergo muscle hypertrophy following overload [4]. In addition, because the AMPK is thought to inhibit the activity of mTOR [34, 135], the increased AMPK phosphorylation (activation) observed in the OZ animals may help to explain why mTOR signaling may be decreased in the OZ soleus following muscle overload.

The mechanism(s) whereby insulin resistance may lead to an increase in AMPK phosphorylation during muscle overload is currently unknown. Recent studies have suggested that the activity of AMPK may be regulated by its upstream kinases, LKB1 [170, 293], CaMKII [294, 295], and TAK1 [296]. Here we demonstrated that the phosphorylation of CaMKII at Thr286 (Fig. 19, Panels C, D) but not LKB1 (Fig. 19, Panels A, B) or TAK1 (Fig. 19, Panels E, F) was significantly higher in the insulin-resistant skeletal muscle of OZ rat compared to LZ rats (Fig. 19). Although not investigated here, it is thought that CaMKII activation is stimulated by elevated calcium and the increased association of CaMKII with calcium/calmodulin [297]. Whether the increased phosphorylation of CaMKII we observe here is responsible for the increased AMPK phosphorylation found in insulin resistant soleus of OZ rat will require further experimentation.

Recent studies have suggested that the phosphorylation (activation) of PKR may be involved in the depression of protein synthesis while also causing an increase in protein degradation [290, 298]. PKR is regulated by double-stranded RNA (dsRNA), which initiates autophosphorylation at multiple Ser and Thr residues resulting in an increase in enzyme activity. Here we demonstrated that the phosphorylation (activation) of PKR appears to be decreased after one week of overload in both LZ and OZ rats, but that the degree of PKR phosphorylation appears to be significantly higher in the overloaded OZ after three weeks of overload (Fig. 20). Why insulin-resistance might be associated with differences in the regulation of PKR is currently unclear.

To examine how changes in the amount of activated PKR might influence the activation of molecules thought to be important in regulating protein synthesis, we next examined the phosphorylation of eIF2α. It has been hypothesized that PKR activation gives rise to the phosphorylation of eIF2α at Ser51, which in turn results in the inactivation of eIF2α and the inhibition of translational initiation [299]. As expected from our PKR data, the PKR dependent eIF2α phosphorlyation at Ser51 was significantly higher in overloaded OZ rat compared to LZ rat after three weeks of overload (Fig. 21). In addition to eIF2α, the glycogen synthase kinase-3β (GSK-3β) has also been proposed to negatively regulate cellular growth, with inhibition of this protein by protein phosphorylation considered an important mechanism of hypertrophy in cardiac tissue [300]. Similar to our data for CaMKII and PKR, we found increases in phosphorylation (inactivation) of GSK-3β (Ser9) following 7 days overload in both the lean and obese rats, but no increase in phosphorylation of GSK-3β at Ser9 in obese rats after 3 weeks of overload (Fig. 22). Whether this change in GSK-3β activation directly contributes to the

diminished hypertrophic response we observe in the insulin resistant soleus muscle is not known and cannot be determined from the present experimental design.

To further explore the potential effects of PKR activation on protein degradation, we next examined the phosphorylation of p38 MAP kinase. It is postulated that that PKR activation can lead to the phosphorylation of p38 MAP kinase, which in turn, has been associated with the activation of the ubiquitin–proteasome pathway [33, 186]. Consistent with our PKR data, the phosphorylation of p38 MAP kinase (Thr180/Tyr182) was significantly higher in the overloaded OZ rat compared to LZ rat after three weeks of overload (Fig. 23). To investigate the potential downstream effects of elevated p38 MAP kinase activation, we next measured the degree of protein ubiquitination. Ubiquitin is a conserved polypeptide that when covalently linked to cellular proteins acts to target that protein for degradation by the 26S proteosome [301]. As expected, we found that the level of ubiquitination was much higher in insulin-resistant skeletal muscle compared to normal muscle after 3 weeks overload (Fig. 24).

Figure 25 summarizes results from the present study in concert with previous conclusions on the roles of AMPK, CaMKII, PKR, eIF2α, GSK-3β, and p38 MAP kinase signaling in various cell types.

The findings support our hypothesis that the attenuation of muscle growth and mTOR activation following increased muscle loading observed with insulin resistance may be related to alterations in AMPK and PKR signaling. Considered in the context of our previous data showing diminished muscle hypertrophy with insulin resistance [287], the results suggest that muscle overload in the OZ soleus muscle is associated with alterations in AMPK, PKR, eIF2α and p38 MAP kinase phosphorylation.

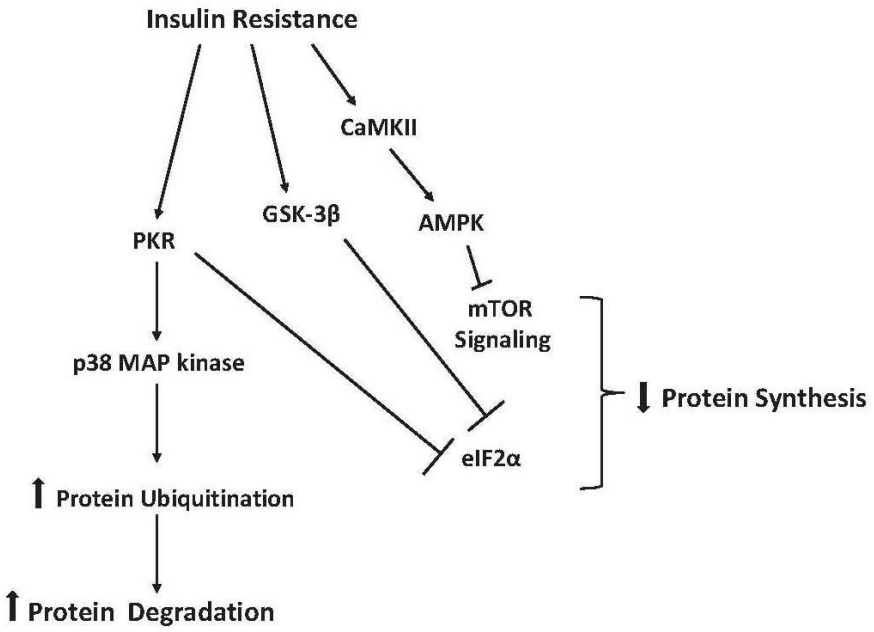


**Figure 25**: Schematic representation of possible mechanism(s) for attenuation of hypertrophy in obese insulin-resistant skeletal muscle

With the exception of the latter, each of these changes would be predicted to decrease protein synthesis. Conversely, the increased activation of p38 MAP kinase activation, and the increase in protein ubiquination occurring in parallel to this finding, would be thought to lead to increases in the amount of protein degradation. Although our experimental design precludes the investigation of cause and effect, this study nonetheless provides new insight into why insulin resistance may be associated with differences in the ability of skeletal muscle to adapt following an overload stimulus.

**SPECIFIC AIM III:** To investigate whether insulin resistance affects the regulation of the HSPs (HSP27, HSP60, HSP70 and HSP90), MAPKs (ERK1/2 and JNK) and muscle-specific miRNA's (miR-1 and miR133a) during muscle hypertrophy.

**PAPER 3**

The following paper corresponds to the specific aim III.

**Overload induced heat shock proteins (HSPs), MAPK and miRNA (miR-1 and miR133a) response in insulin-resistant skeletal muscle**

**Abstract**

Heat shock proteins (HSPs), mitogen-activated protein kinases (MAPKs) and muscle-specific miRNA have been reported to be associated with skeletal muscle adaptation to physiological stimuli. Little is known about regulation of these with insulin resistance. The purpose of this study was to determine the overload induced alterations in HSPs, MAPKs and miRNA in insulin-resistant muscle. To this end, we examined the regulation of HSPs (HSP27, HSP60, HSP70 and HSP90), MAPKs (ERK1/2 and JNK) and muscle-specific miRNAs (miR-1 and miR133a) in 7-day and 21-day overloaded soleus muscles of OZ *versus* LZ rats. Overload regulates the expression of HSPs in a similar pattern in both LZ and OZ rat. The phosphorylation of HSP27 at Ser82 was increased in both LZ and OZ animals after 7 days overload, where as it was significantly increased only in LZ rats after 21 days overload. The basal phosphorylation levels of p42 MAPK were significantly higher in insulin-resistant soleus muscle compared to normal soleus muscle. The phosphorylation levels of p44-, p42-, P46- MAPK were significantly higher in both LZ and OZ rat with overload, with higher magnitudes in LZ compared to OZ. The miR-1 and miR133 expression levels were lower in insulin-resistant muscle, and overload decreased the mir-1 expression in both LZ and OZ, with much higher magnitudes in normal muscles. Combined, these results are the first to report alterations in the regulation of HSPs, MAPKs and muscle-specific miRNAs with overload in insulin-resistant skeletal muscle and suggest that might play a role in muscle adaptation to insulin resistance.

## Introduction

The adaptation of skeletal muscle to physiological and pathological stimuli is thought to be governed by the activation of intracellular signaling cascades. Previous data from our lab have suggested that insulin resistant muscle is characterized by a diminished hypertrophic response and that this response is associated with decrements in the activation of the mammalian target of rapamycin (mTOR) signaling [4, 287]. Whether other signaling systems may also play a role in this response is not well understood.

It is thought that the heat shock proteins (HSPs) are important components of the cellular protective response and have been implicated in the chaperoning of unfolded proteins, the inhibition of apoptosis, and in stabilization of the cytoskeleton [187-191]. Previous studies demonstrated the induction of HSP proteins following high force eccentric contraction, aerobic exercise, and during the development of muscle hypertrophy [42, 43, 302]. However, it is not clear whether the regulation of these molecules is altered with insulin-resistance.

Mitogen-activated protein kinases (MAPKs) are another class of stress responsive proteins that are thought to be involved in the regulation of protein synthesis and cellular proliferation [198-200]. The MAPK proteins are activated by dual phosphorylation prior to their translocation to the nucleus, where they are capable of regulating various transcriptional factors [202, 203]. Previous studies have demonstrated that the extracellular regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) MAPK proteins are involved in the regulation of skeletal muscle hypertrophy following increased loading [44-47, 214]. If or how insulin resistance may affect MAPK signaling in response to increased muscle loading has, to our knowledge, not been investigated.

In addition to these signaling cascades, recent data have strongly suggested the involvement of muscle-specific miRNAs (miR-1, miR-133a and miR-206) in load induced skeletal muscle remodeling [7, 303, 304]. MicroRNAs are small endogenous ~ 22-nucleotide, non-coding RNAs and a newly emerging class of trans-factors, which repress gene expression by inhibiting the translation of target mRNAs through interacting with the 3'- untranslated region (3'UTR) [50, 51, 305]. Among the muscle-specific miRNA's, miR-1 and miR-133a are important for embryonic muscle growth [52], myoblast differentiation and proliferation by regulating the expression of SRF, MyoD and MEF2 [53], and miR-206 plays a role in myogenesis [54]. A recent study by McCarthy and Esser (2007) reported decreased expression of miR-1 and miR-133a following 7days of functional overload and suggested this response acted to remove the repression of hypertrophic regulators [7]. How insulin resistance may affect the regulation of muscle-specific miRNAs during muscle hypertrophy is currently unclear.

Using the same animals and tissues from in our previous work [287], here we investigate whether insulin resistance affects the regulation of the HSPs (HSP27, HSP60, HSP70 and HSP90), MAPKs (ERK1/2 and JNK) and muscle-specific miRNA's (miR-1 and miR133a), during muscle hypertrophy. Our data suggest that the alterations in the regulation of HSPs, MAPKs and muscle-specific miRNAs may be associated with the diminished ability of insulin resistant muscle to undergo hypertrophy following muscle overload.

## Materials and Methods

*Materials.*

Primary antibodies against HSP27 (#2442), phospho-HSP27 (Ser82) (#2401), HSP60 (#4870), HSP70 (#4872), HSP90 (#4877), phospho-HSP90 (Thr5/7) (#3488), p44/42 MAPK (ERK1/2) (#9102), phospho-p44/42 MAPK (Thr202/Tyr204)(#4377), JNK/SAPK (#9252), phospho-JNK/SAPK (Thr183/Tyr185) (#9251), glyceraldehyde 3-phosphate dehydrogenase (GAPDH,#2118) and secondary antibody conjugated with horseradish peroxidase (HRP) (anti-rabbit (#7074) were purchased from Cell Signaling Technology (Beverly, MA). The Laemmli 2 × sample buffer was from Sigma-Aldrich (St. Louis, MO). Pierce Tissue Protein Extraction Reagent (T-PER), Pierce 660nm protein assay reagent (#22660), GE Healthcare Amersham ECL™ Western Blotting Detection Reagents (RPN2106) and Advance Western Blotting Detection Kit (RPN2135) were from Thermo Fisher Scientific Inc. (Rockford, IL). The PAGEr Gold Precast gels (10%) were from purchased from Lonza (Rockland, ME).

*Animal Care.*

All procedures were performed in accordance with the Marshall University Institutional Animal Care and Use Committee (IACUC) guidelines, using the criteria outlined by the American Association of Laboratory Animal Care (AALAC). The animals and tissues used in this study have been previously examined [287]. Young male LZ (n=12) and OZ (n=12) rats were obtained from the Charles River Laboratories. All animals were 12 wk of age at the completion of this study. Rats were housed two per cage in an AAALAC approved vivarium. Housing conditions consisted of a 12 H: 12 H dark-light cycle and temperature was maintained at 22° ± 2°C. Animals were provided food and water *ad libitum* and allowed to recover from shipment for at least two weeks before experimentation. During this time the animals were

carefully observed and weighed weekly to ensure none exhibited signs of failure to thrive, such as precipitous weight loss, disinterest in the environment, or unexpected gait alterations.

*In vivo* model of muscle hypertrophy.

Unilateral overload of the soleus muscle for 1 and 3 weeks was achieved through the surgical ablation of the medial and the proximal two-thirds of the lateral head of the gastrocnemius [109]. The unilateral ablation model allows within animal comparisons, thus eliminating bias due to systemic factors. Rats were anesthetized with a ketamine–xylazine (4 : 1) cocktail (50 mg/kg, I/P) and, the distal two-thirds of the gastrocnemius muscle were surgically removed from the left hindlimb as previously described [109]. A sham (control) operation was performed on the right hindlimb. The sham procedure consisted of an incision through the skin, followed by blunt isolation of the Achilles tendon and gastrocnemius muscle prior to closure. Animals were active immediately after recovering from anesthesia, and were checked twice daily during the 7 day postoperative period. No signs of infection or other complications were observed postoperatively.

*Tissue collection.*

Soleus muscles were collected 7 days ($n$ = 6 LZ-7 and $n$ = 6 OZ-7) or 21 days ($n$ = 6 LZ-21 and $n$ = 6 OZ-21) after the synergist ablation procedure. Animals were 12 wk old at the time of tissue collection. Rats were anesthetized with a ketamine–xylazine (4: 1) cocktail (50 mg/kg, I/P) and supplemented as necessary for reflexive response. Soleus muscles from both legs were quickly removed, trimmed of excess connective tissue, weighed on an analytical balance, frozen in liquid nitrogen, and stored at $-80^{\circ}$C until further analysis.

*Tissue protein extraction.*

Muscles were homogenized in a Pierce Tissue Protein Extraction Reagent (T-PER) (10 mL/g tissue; Rockford, IL, USA) that contained protease inhibitors (P8340, Sigma-Aldrich, Inc., St. Louis, MO, USA) and phosphatase inhibitors (P5726, Sigma-Aldrich, Inc., St. Louis, MO, USA). After incubation on ice for 30 min, the homogenate was collected by centrifuging at 12,000 g for 5 min at 4°C. The protein concentration of homogenates was determined via the Bradford method (Fisher Scientific, Rockford, IL, USA). Homogenate samples were boiled in Laemmli 2× sample buffer (Sigma-Aldrich, Inc., St. Louis, MO, USA) for 5 min prior to SDS-PAGE.

*SDS-PAGE and immunoblotting.*

Forty micrograms of total protein from each sample was separated on a 10% PAGEr Gold Precast gel (Lonza, Rockland, ME, USA) and then transferred to a nitrocellulose membrane. Visual verification of transfer and equal protein loading amongst lanes was accomplished by Ponceau S staining of the membranes. Immunodetection of antigens was performed as described previously [15, 68]. Briefly, membranes were blocked for 1 h at room temperature in blocking buffer (5% non-fat dry milk in TBS-T (20mM Tris-base, 150mM NaCl, 0.05% Tween-20), pH 7.6), serially washed in TBS-T at room temperature, then incubated overnight at 4°C in primary antibody buffer (5% BSA in TBS-T, pH 7.6, primary antibody diluted 1:1000), followed by washing in TBS-T (3X 5 min each), and incubation with HRP-conjugated secondary antibody (anti-rabbit (#7074) or anti-mouse (#7076), Cell Signaling Technology, Inc., Danvers, MA, USA) in blocking buffer for 1 h. After removal of the secondary antibody, membranes were washed (3X 5 min each) in TBS-T and protein bands visualized on reaction with ECL reagent (Amersham ECL Western Blotting reagent RPN 2106, GE Healthcare

Bio-Sciences Corp., Piscataway, NJ, USA). Target protein levels were quantified by AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA, USA) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

*miRNA expression analyses.*

For miRNA isolation, 25 mg pieces of soleus muscle tissue from each animal were pulverized with mortar and pestle in liquid nitrogen. Then miRNA was isolated from these pulverized tissues using miRVana miRNA Isolation Kit according to manufacturer's directions (Ambion, Austin, TX). cDNA synthesis from miRNA was performed using the QuantiMir RT kit (System Bioscience) according to the manufacturer's protocol. SYBR green-based real-time qPCR was performed by using a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) and gene-specific primers for the miRNA of interest designed according to the QuantiMir RT kit's guidelines and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Melt analysis was used after each PCR run to ensure amplification of only a single product. To account for possible differences in the amount of starting miRNA, all samples were normalized to miR-206 as the expression of this molecule was not different between animal models or following overload. Relative fold changes in miRNA were determined from the $C_t$ values after normalization to housekeeping gene using the $2^{-\Delta ct}$ method (modified Levak method) [306].

*Statistical analysis.*

Results are presented as mean $\pm$ SE. Data were analyzed using the Sigma Stat 3.5 statistical program. Data were analyzed using two way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc testing where appropriate. Values of $P < 0.05$ were considered to be statistically significant.

**Results**

*The impaired hypertrophic response of insulin resistant muscle is associated with diminished HSP27 phosphorylation.*

As outlined previously, the OZ rat exhibited a significantly higher body weight than the LZ at both 7 and 14 days of observation (464 ± 12 gm *vs. 302* ± 3 gm at 7 days; $P < 0.05$; 460±26 *vs.* 289±10 mg at 21 days; $P < 0.05$) while soleus muscle wet weights were significantly lower in the OZ compared to the LZ (128 ± 11 mg *vs.* 150±6 mg; at 7 days; $P < 0.05$; 128.0±6.5 vs. *141.0*±8.3 mg at 21 days; $P < 0.05$) [287]. To examine the effect of insulin resistance on the regulation of HSP molecules with overload, we compared the expression and phosphorylation levels of HSP27, HSP60, HSP70 and HSP90. Muscle loading did not alter the expression of HSP60, HSP70 and HSP90 in either the LZ or OZ animals (Fig. 27 and 28A). The expression of the small heat shock protein HSP27 was increased in both LZ and OZ animals after 7 and 21 days of overload ($P < 0.05$; Fig. 26A). The phosphorylation of HSP27 at Ser 82 was increased in both LZ and OZ animals after 7 days of increased loading, while it was significantly increased only in the LZ rats after 21 days of overload ($P < 0.05$; Fig. 26B).

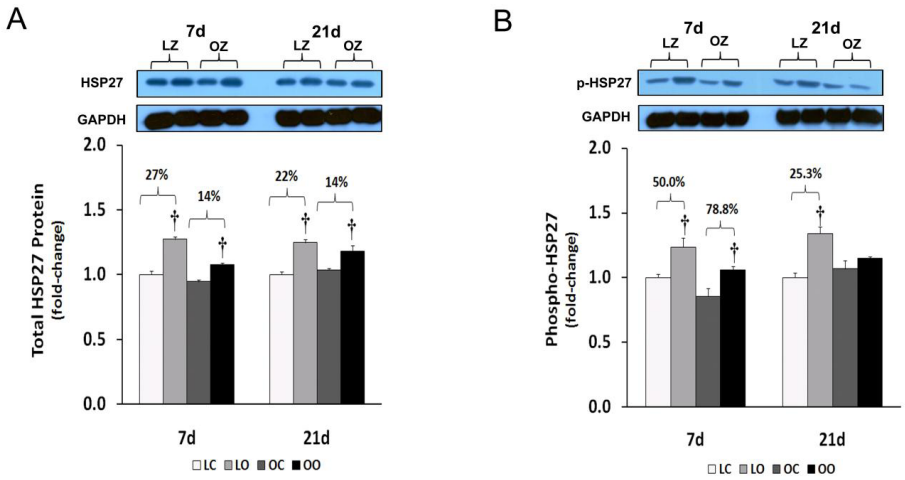


**Figure 26:** Immunoblot analysis of the small heat shock protein, HSP27 (A) protein content and (B) phosphorylation at Ser82, in control and loaded soleus muscles of LZ and OZ. All the protein quantifications were done after normalization by abundance of GAPDH protein; $n = 6$. †Significantly different from contralateral control muscle, * Significantly different from lean control value, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

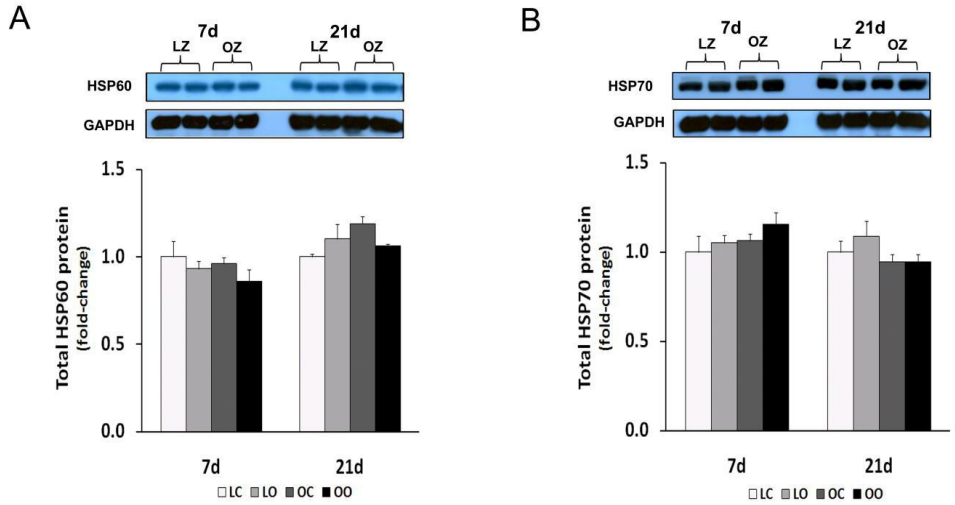


**Figure 27**: Immunoblot analysis of the expression levels of heat shock proteins,(A) HSP60 and (B) HSP70 in control and overloaded soleus muscles of LZ and OZ. All the protein

quantifications were done after normalization by abundance of GAPDH protein; n = 6. †Significantly different from contralateral control muscle, * Significantly different from lean control value, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

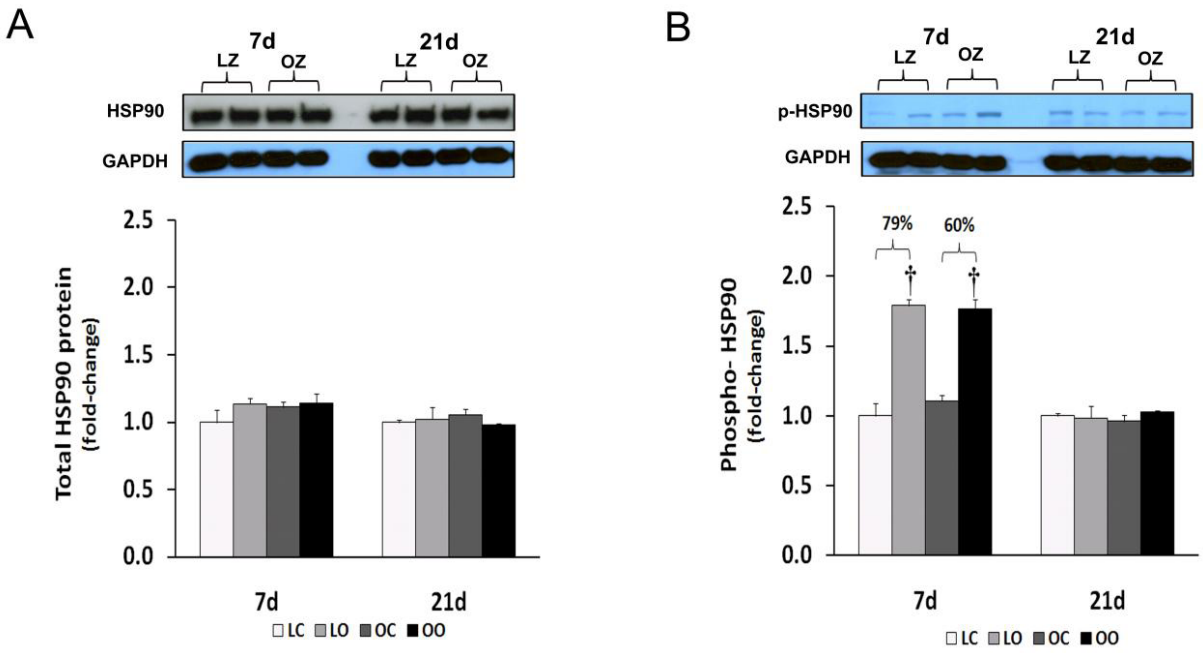


**Figure 28**: Immunoblot analysis of the heat shock protein, HSP90(A) protein content and (B) phosphorylation at Thr5/7, in control and loaded soleus muscles of LZ and OZ. All the protein quantifications were done after normalization by abundance of GAPDH protein; *n* = 6. †Significantly different from contralateral control muscle, * Significantly different from lean control value, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

*Insulin resistance alters the overload-induced phosphorylation of MAPK proteins in slow twitch soleus muscle.*

To examine the effect of insulin resistance on the activation of MAPK proteins with overload, we compared the protein content and phosphorylation of p44/42 MAPK (ERK1/2) and JNK / SAPK between control and overloaded muscles. The amount of ERK1/2 was unaltered with muscle overload in both LZ and OZ animals, where as the phosphorylation levels of p44 and p42 MAPK at Thr202 / Tyr204 was significantly higher in overloaded LZ and OZ muscles at

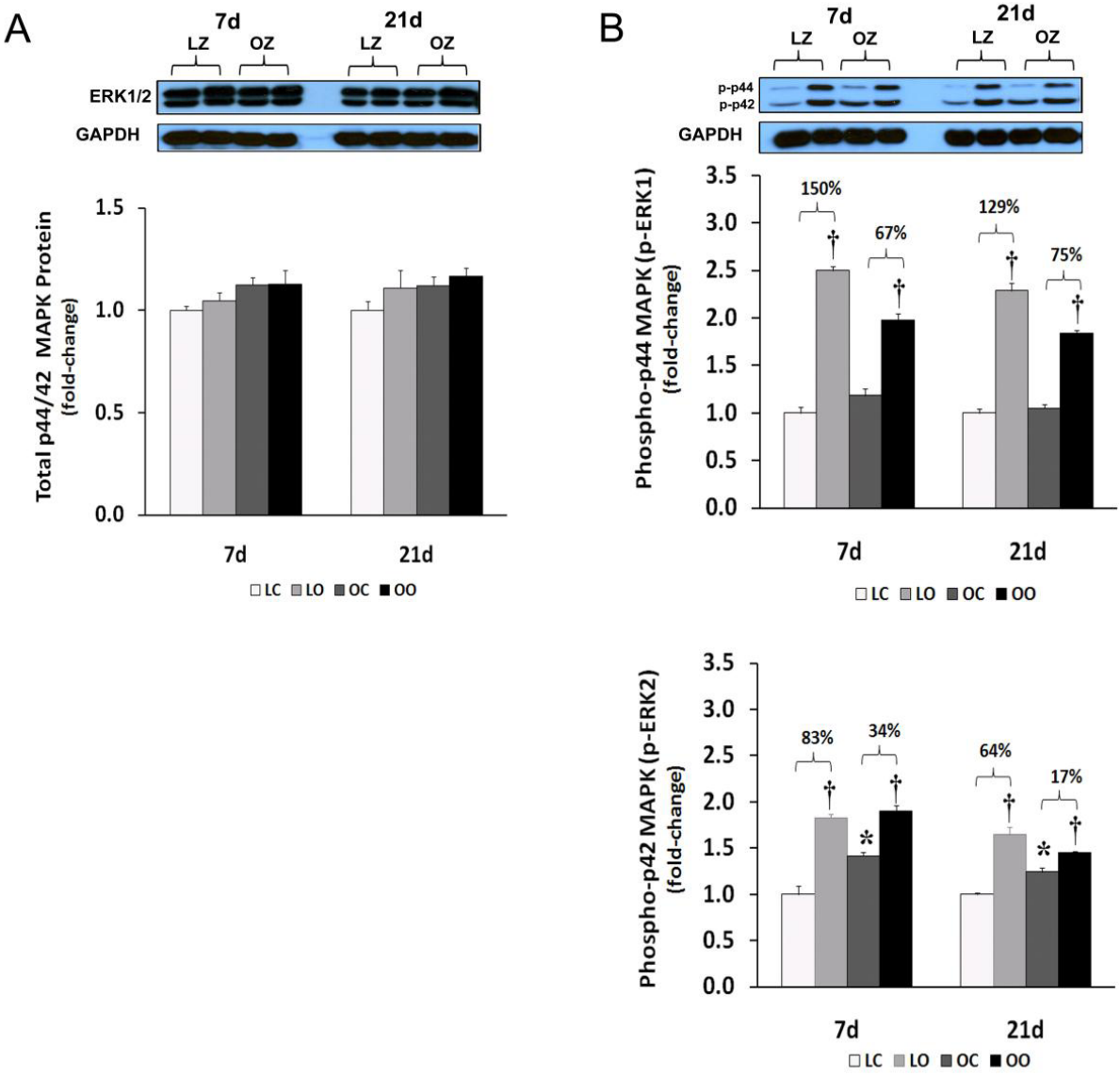


**Figure 29**: Immunoblot analysis of the p44/42(ERK1/2) (A) protein content and (B) phosphorylation at Thr202/Tyr204, in control and loaded soleus muscles of LZ and OZ. All the protein quantifications were done after normalization by abundance of GAPDH protein; $n = 6$. †Significantly different from contralateral control muscle, * Significantly different from lean control value, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

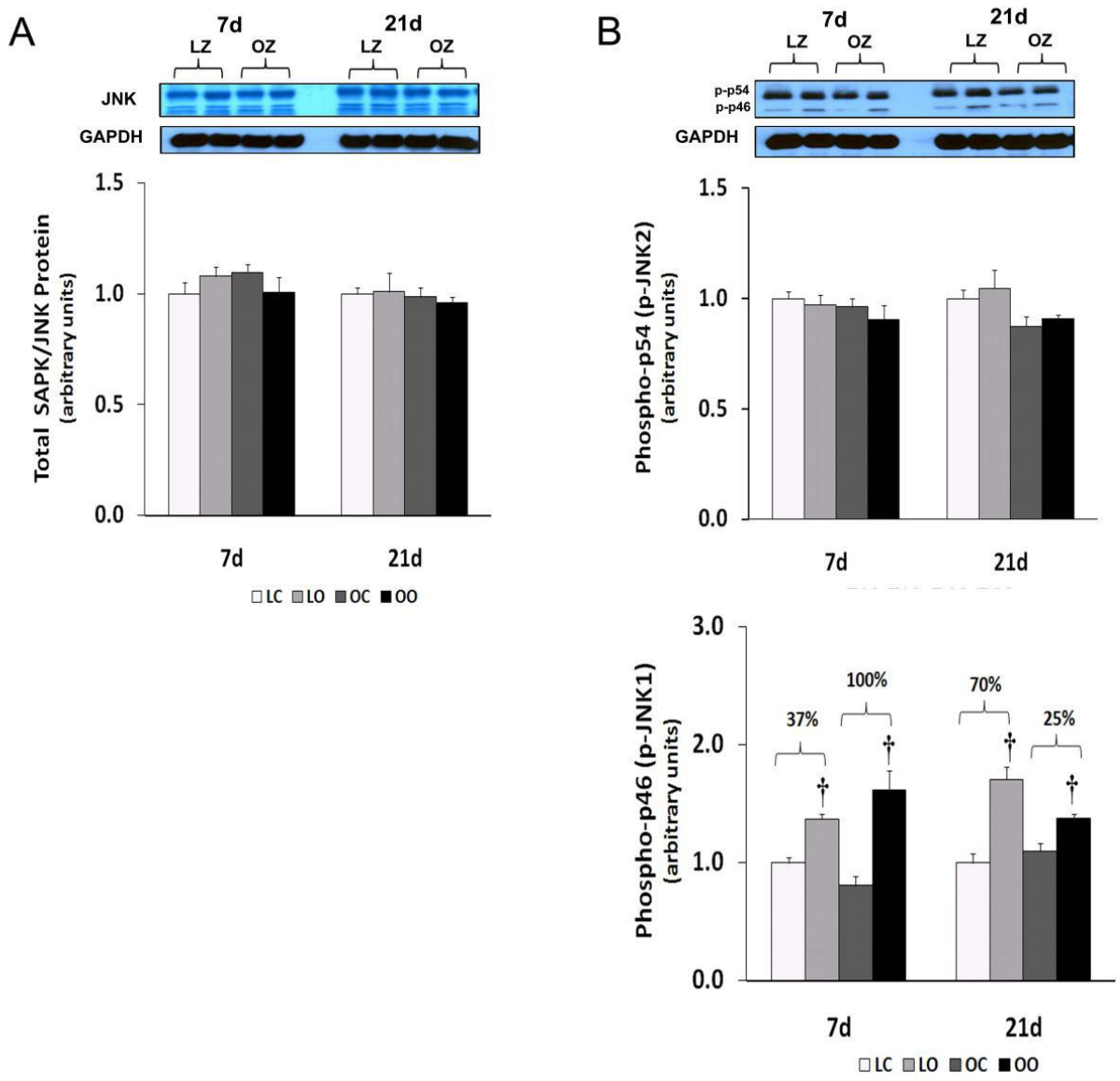


**Figure 30**: Immunoblot analysis of the SAPK/JNK (A) protein content and (B) phosphorylation at Thr183/Tyr185, in control and loaded soleus muscles of LZ and OZ. All the protein quantifications were done after normalization by abundance of GAPDH protein; $n$ = 6. †Significantly different from contralateral control muscle, * Significantly different from lean control value, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

both 7 and 21 days (*P < 0.05;* Fig. 29). The magnitude of ERK1/2 phosphorylation was higher in LZ compared to OZ rats. The basal phosphorylation levels of p42 MAPK were significantly higher in insulin resistant soleus muscle compared to normal soleus muscle (*P < 0.05;* Fig. 29). Muscle loading did not alter the expression of JNK/SAPK in either the LZ or OZ solei muscles (Fig. 30). The phosphorylation level of p46 (JNK1) at Thr183/Tyr185 was significantly higher in both LZ and OZ rat with overload at both 7 and 21 days, while the degree of phosphorylation was higher in the LZ compared to OZ soleus (*P < 0.05;* Fig. 29).

*Muscle specific miRNA expression following muscle overload is influenced by insulin resistance.*

The amount of mir-1 and miR133 in insulin resistant soleus muscles was less than that observed in the normal soleus muscle ($P < 0.05$; Fig. 31 and 32). Both 7 days and 21 days overload decreased mir-1 levels in both LZ and OZ, but the magnitude of decrease was higher in normal muscle compared to insulin-resistant muscle ($P < 0.05$; Fig. 31). The expression levels of miR-133a decreased significantly in the LZ animals with overload at both 7 and 21 days ($P < 0.05$; Fig. 32). In contrast, miR-133a levels in the OZ rat were increased significantly with overload at both 7 and 21 days ($P < 0.05$; Fig. 32).

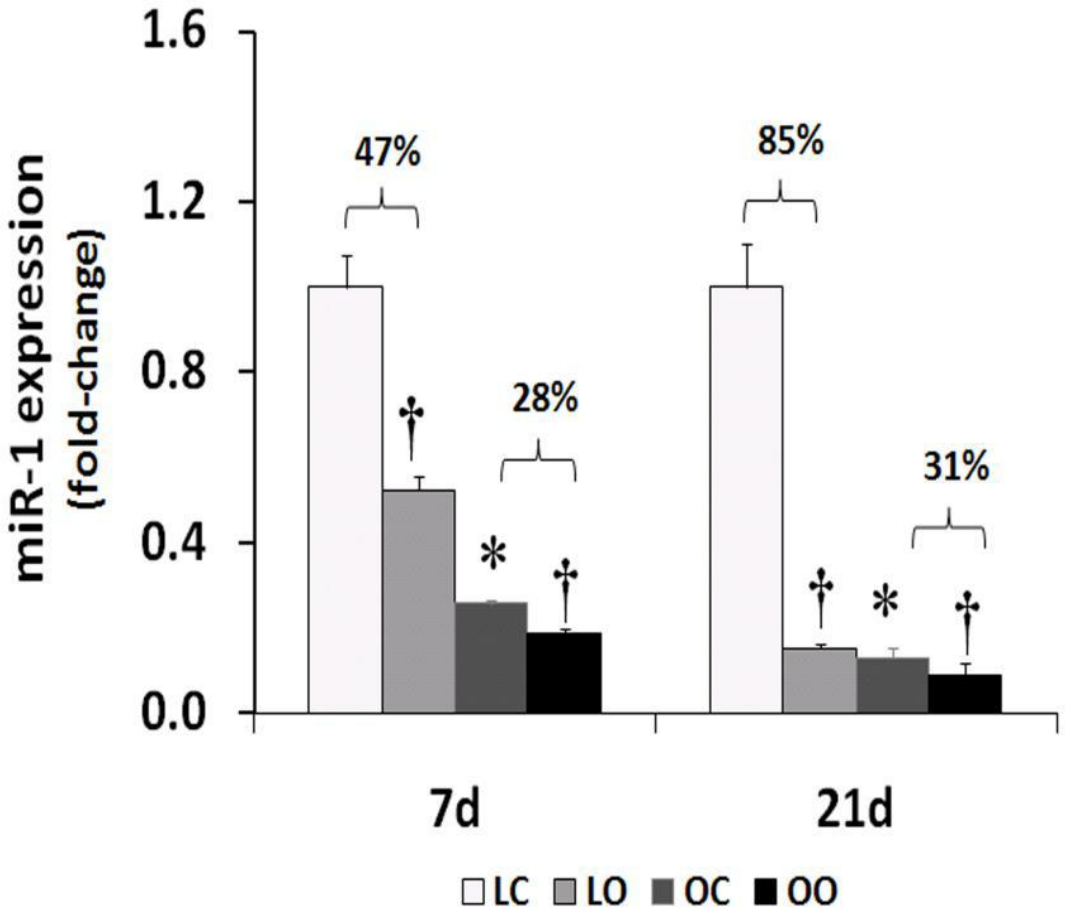


**Figure 31**: *Insulin resistance alters the regulation of miR-1 with overload.* Quantitative RT-PCR analyses of miR-1 expression in control and loaded soleus muscles of LZ and OZ rats using SYBR green I. miR-1 expression was normalized to miR-206 expression, which was found to be not change with either animal model or with functional overload. $n = 6$. †Significantly different from contralateral control muscle, * Significantly different from lean control value, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

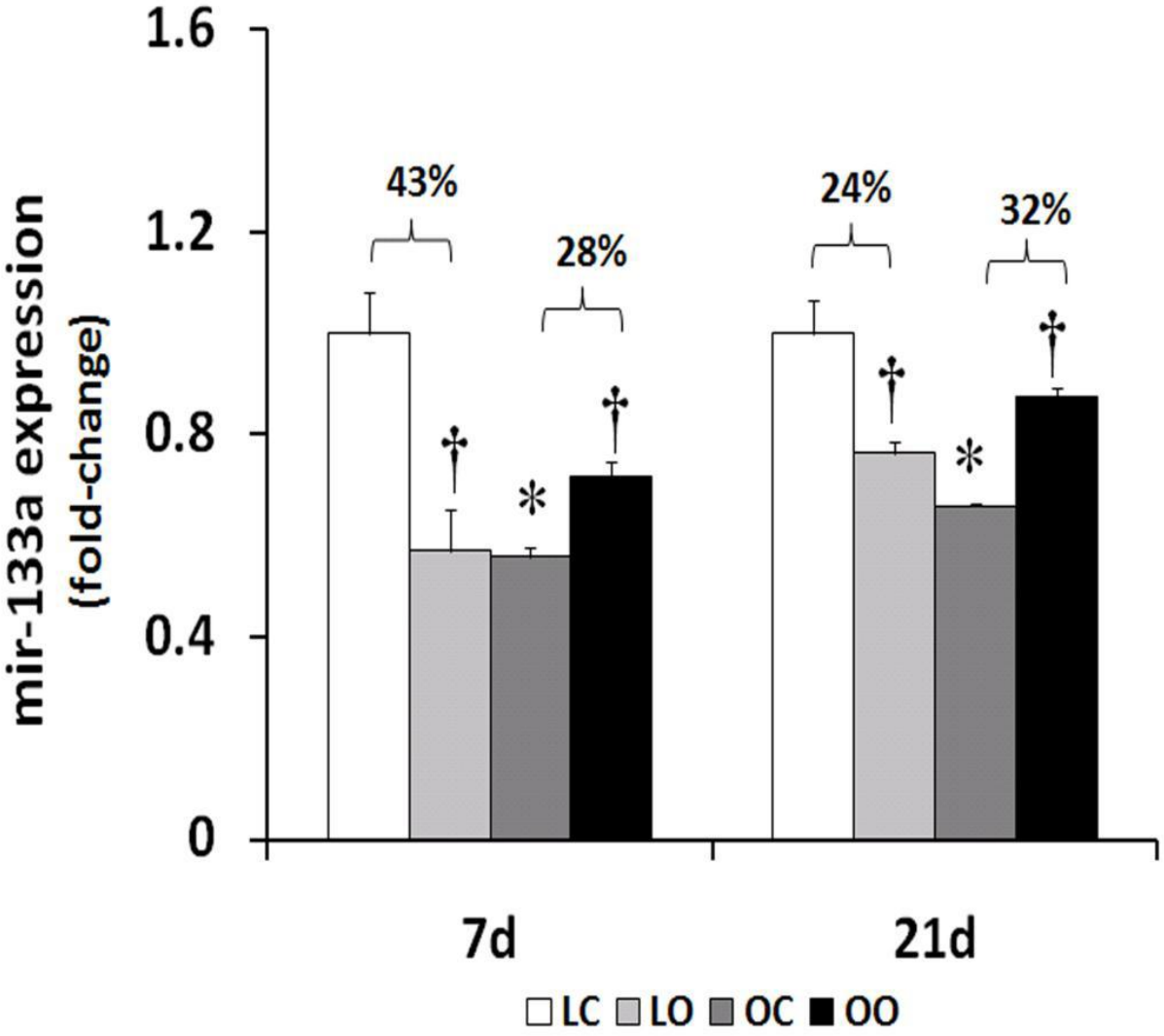


**Figure 32**: *Insulin resistance alters the regulation of miR-133a with overload.* Quantitative RT-PCR analyses of miR-133a expression in control and loaded soleus muscles of LZ and OZ rats using SYBR green I. miR-133a expression was normalized to miR-206 expression, which was found to be not change with either animal model or with functional overload. $n$ = 6. †Significantly different from contralateral control muscle, * Significantly different from lean control value, $P < 0.05$. Abbreviations: 7d, 7 days of loading; 21d, 21 days of loading; LZ, lean Zucker; OZ, obese Zucker; LC, lean control; LO, lean overload; OC, obese control; OO, obese overload.

**Discussion**

Skeletal muscle adaptation to increased load is characterized by increases in muscle mass (hypertrophy) that is thought to be regulated by the activation of several signaling cascades. Previously we demonstrated that the degree of muscle hypertrophy following an overload stimulus was decreased in the insulin-resistant obese Zucker (fa/fa) rat (OZ) when compared to that observed in the LZ rat, and that this attenuation was associated with multiple-level decrements in mammalian target of rapamycin (mTOR) and its downstream translational signaling intermediates, p70S6k , rpS6 and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) [287]. Here we extend upon those findings and demonstrate that overload in the OZ rat soleus is associated with diminished HSP27 phosphorylation, decreased MAPK phosphorylation and alterations in the regulation of muscle specific miRNAs.

*Insulin resistance is associated with alterations in HSP27 phosphorylation following muscle overload.*

Previous reports have demonstrated that Hsp27 expression increases in response to increased muscle load in both human and animal muscles [43, 307, 308]. Consistent with these data, here we show that Hsp27 expression was increased with overload in both the LZ and OZ soleus muscles. Overload also led to a significant increase in the phosphorylation or activation of Hsp27 at Ser82 in normal muscle; however, this response appeared to be attenuated in the insulin-resistant soleus. *In vitro* studies have suggested that the increased phosphorylation of Hsp27 is involved in maintaining cytoskeletal protein integrity. Whether HSP27 exhibits a similar function *in vivo* is currently unclear and beyond the scope of the present study. Additionally, it is also unknown why insulin resistance was associated with diminished overload-induced activation (phosphorylation) of Hsp27. Nonetheless, given that Hsp27 is a substrate for

the MAPK proteins [214, 309, 310], it is possible that the decreased activation of MAPK with overload seen in the OZ soleus (Figures 29 & 30) may be related. Future experiments designed to directly test this possibility will no doubt be useful in establishing cause and effect.

*Insulin resistance is associated with decreases in overload-induced MAPK phosphorylation.*

Several lines of evidence suggest that MAPKs play an integral role in the adaptation of skeletal muscle to alterations in contractile loading [214, 311-314]. It is thought that the phosphorylation (activation) of MAPK proteins is important in regulating such diverse processes as gene expression, glucose uptake, cell replication, and protein synthesis [315, 316]. The basal phosphorylation levels of p42- MAPK in the insulin-resistant soleus muscle was significantly higher compared to that observed in the normal soleus. Overload was found to increase the activation of p44- and p42-MAPK in both normal and insulin resistant soleus, however the magnitude of activation was higher in normal compared to that observed in insulin resistant soleus. Given the possibility that ERK1/2 activation is associated with skeletal muscle hypertrophy [317, 318], the differences in magnitude of activation of ERK1/2 between lean and obese rats with overload may help to explain the decreased hypertrophic response seen in the OZ animals. Although the exact mechanism for ERK1/2 involvement in muscle hypertrophy has yet to be elucidated, it has been hypothesized that ERK1/2 activation is associated with elevations in translational initiation, which may occur through increased phosphorylation of the eukaryotic initiation factor eIF4E [319].

*Muscle-specific miRNAs and insulin-resistance*

It has been suggested that muscle-specific miRNAs may play a key role in the adaptation of skeletal muscle. For example, the expressions of mir-1and miR133 are decreased in response to functional overload [7], chronic endurance training [304], and resistance exercise [320]. Here

we investigated whether insulin-resistance affects the expression and regulation of muscle-specific miRNAs with overload. Our semi-quantitative PCR data demonstrated that insulin resistance was associated with a lower expression of mir-1 and mir-133 compared to that observed in the non-diabetic soleus. The physiological significance and mechanism(s) for loss of muscle-specific miRNAs with insulin resistance are currently unclear. However, recent data suggest that the expression of mir-1 and mir-133, at least in C2C12 cells, may be regulated by the myogenic regulatory factors (MRFs) myogenin and MyoD [321]. Whether a similar finding exists *in vivo* is currently unclear. Previous work from our laboratory failed to demonstrate differences in the regulation of MRFs with overload in OZ rat [287]. These findings are consistent with the possibility that these MRFs may play a diminished role in regulating the expression of mir-1 and mir-133 in LZ and OZ animals. Similarly, like that observed for most of the known miRNA species, the exact role that these molecules may play *in vivo* has yet to be fully elucidated.

In addition to the possibility that insulin resistance may affect miRNA expression profiles, we also observed that muscle overload is associated with diminished mir-1 expression in both the LZ and OZ rats (Figure 31). These results are in agreement with recent findings by McCarthy and Esser, who reported a 50% decrease in expression of mir-1 after 7-days functional overload in the mouse plantaris muscle [7]. Interestingly, the overload associated decreases observed in the present study appeared to be higher in the LZ compared to OZ after 7- and 21-days of overload (Figure 31). Similar results to what we had observed with mir-1 were seen when we examined mir-133a expression in the LZ animals (Figure 32). Conversely, in the OZ animals overload appeared to increase the expression of this molecule (Figure 32). Whether these findings are directly related to the diminished overload-induced hypertrophy we see in the OZ rat

soleus is currently unclear and beyond the scope of the present study. Similarly, why insulin-resistance might be associated with differences in how overload regulates miRNA expression is not known.

*Summary and conclusions*

The findings of the present study suggest that the impaired hypertrophic response seen in insulin resistant muscle appears to be associated with alterations in HSP and MAPK phosphorylation along with changes in the way muscle overload regulates muscle specific miRNA expression. These data extend our previous work showing that insulin resistance is characterized by differences in the overload induced activation of mTOR signaling pathways. Future studies perhaps using pharmacological and genetic manipulation will be useful in assessing the cause and effect of our findings and may offer therapeutic insight for the maximization of muscle growth in diabetic individuals.

# CHAPTER IV

## GENERAL DISCUSSION

The primary purpose of this study was to investigate why insulin resistance may be associated with a diminished ability of skeletal muscle to adapt to an overload stimulus. To address this purpose, we examined the regulation of mTOR signaling, AMPK signaling, PKR signaling, HSPs (HSP27, HSP60, HSP70 and HSP90), MAPKs (ERK1/2 and JNK) and muscle-specific miRNA's (miR-1 and miR133a), during the initial and latter phases of muscle hypertrophy in insulin resistant soleus muscles of the OZ rat.

Diabetes is considered a global health problem that afflicts 171 million people worldwide (International Diabetes Federation, 2007). This epidemic is associated with increased mortality and morbidity which negatively affects quality of life [64]. The OZ rat presents abnormalities similar to those seen in human metabolic syndrome and is a widely used model of insulin resistance [56]. Skeletal muscle insulin resistance is the key defect in maintaining glucose homeostasis and is thought to play a central role in the pathogenesis of metabolic syndrome [9, 10]. Skeletal muscle is a primary consumer of blood glucose and is thought to participate in the control of blood glucose levels, where as other studies have demonstrated that increases in muscle mass or muscle hypertrophy are associated with improvements in glycemic control [284-286]. Previous data from our laboratory has suggested that the capacity of the OZ soleus muscle to undergo hypertrophy in response to increased loading is diminished compared to that observed in the lean Zucker rat (LZ) [4]. The exact molecular mechanism(s) for these alterations in skeletal muscle adaption with different hypertrophic stimuli are not known.

**Regulation of mTOR signaling with insulin resistance during muscle hypertrophy**

It is well accepted that the regulation of muscle growth is dependent upon the balance between protein synthesis and degradation with increases in muscle mass occurring when protein accretion is favored over its breakdown. One critical signaling pathway that has been shown to play a role in controlling protein synthesis following increased muscle loading is the mammalian target of rapamycin (mTOR) [22, 23]. The regulation of mTOR signaling is complex and is likely influenced by several upstream molecules and pathways as previous data has suggested the participation of phosphoinositide 3-kinase (PI3K), phosphatase and tensin homologue deleted on chromosome 10 (PTEN), protein kinase B/ Akt, TSC2 / Tuberin, and raptor [22, 147, 148, 261-263]. The mTOR functions to regulate several physiological processes including gene transcription, protein metabolism, cell cycle control, and cytoskeleton organization [24, 25]. When active (phosphorylated), mTOR is thought to promote protein translation by controlling the activity of several downstream effectors, including the 70 kDa ribosomal protein S6 kinase (p70S6k), ribosomal protein S6 (rpS6), eIF4E-binding protein 1 (4E-BP1), and eukaryotic elongation factor 2 (eEF2) [27, 264].

In the present study we demonstrated that the phosphorylation (activation) of mTOR with insulin resistance appears to be similar after one week of overload before becoming significantly decreased by three weeks of overload. This latter finding is consistent with our data demonstrating that the insulin resistant soleus exhibits a reduced ability to undergo hypertrophy following eight weeks of mechanical overload [4]. To examine how this defect in mTOR signaling might affect the regulation of molecules thought to be involved in controlling protein translation , we next examined how insulin resistance affected the phosphorylation of eukaryotic initiation factor (eIF) 4E binding protein-1 (4E-BP1) and p70S6k in response to increased muscle

loading. As expected from our mTOR data, the phosphorylation of the mTOR substrates, 4E-BP1 and p70S6k were significantly less in the OZ rat compared to that observed in the LZ rat.

To confirm these data, we also examined the regulation of the p70S6k substrate, ribosomal protein S6 (rpS6) [270]. Consistent with our findings for p70S6k, we found that the degree of rpS6 phosphorylation at Ser 235/236 following three weeks of overload was diminished in the OZ compared to their lean counterparts. Further, we demonstrated the ability of the soleus to activate (phosphorylation at both Ser473 and Thr308) Akt in the OZ appears to be diminished compared to that observed in the LZ rat. How this might affect the activation of other signaling molecules is currently unclear; however, it is likely that Akt can influence the activity of mTOR through its ability to phosphorylate the product of the tuberous sclerosis complex TSC2 gene, also termed tuberin. The phosphorylation of tuberin (Thr1462) is diminished in the lean Zucker rat although it is unaltered in OZ rat following three weeks of overload. Because decreased tuberin phosphorylation should lead to a decrease in mTOR activity, it is likely that the differences in mTOR phosphorylation we see between models is not due to alterations in TSC2 regulation. In an effort to explore other possible mechanisms for the attenuated hypertrophy we observe in the OZ animals, we next examined if muscle overload was associated with alterations in the amount of myogenic regulatory factors, MyoD and myogenin, which have been posited to be involved in regulating satellite cell activation. Consistent with previous work examining the myogenin and myoD levels in rat soleus muscle after 4 weeks of surgical overload [281], we observed no changes in the amount of myogenin and myoD with overload in either the LZ and OZ animals. Taken together, these data suggest that insulin resistance may be associated with a decrease in the ability of the soleus muscle to undergo

muscle hypertrophy and that this finding may be related to differences in mTOR, p70S6k, Akt, and rpS6 signaling.

**Regulation of AMPK and PKR -dependent signaling with insulin resistance during muscle hypertrophy**

AMP-activated protein kinase (AMPK) is a serine/threonine kinase and master regulator of cellular energy metabolism that is thought to act as a negative regulator of protein synthesis [26, 34]. It has been shown that AMPK may inhibit protein synthesis through its ability to suppress mTOR activation [22, 34, 135, 288, 289]. The factors controlling the activity of AMPK have not been fully elucidated; however, it is thought that protein tumor suppressor LKB1 kinase [35, 169, 170], calcium/calmodulin-activated protein kinase (CaMKII) [36, 171], and transforming growth factor-β-activated kinase-1 (TAK1), a member of the mitogen-activated protein (MAP) kinase family, may be involved [37, 172].

In the present study, we found that the phosphorylation of Thr172 appears to be similar after one week of overload in the LZ and OZ animals, but that it is significantly higher in the overloaded OZ rat compared to LZ rat after three weeks of overload. These latter data are consistent with our previous report demonstrating a decrease in the ability of the OZ to undergo muscle hypertrophy following overload [4]. In addition, because AMPK is thought to inhibit the activity of mTOR [34, 135], the increased AMPK phosphorylation (activation) observed in the OZ animals may help to explain why mTOR signaling may be decreased in the OZ soleus following muscle overload. We have also found that the phosphorylation of CaMKII at Thr286 but not LKB1 or TAK1, was significantly higher in the insulin-resistant skeletal muscle of OZ rat compared to LZ rats. Whether the increased phosphorylation of CaMKII we observe here is

responsible for the increased AMPK phosphorylation found in insulin resistant soleus of OZ rat will require further experimentation.

Recent studies have suggested that the phosphorylation (activation) of PKR may be involved in the depression of protein synthesis while also causing an increase in protein degradation [290, 298]. Here we demonstrated that the phosphorylation (activation) of PKR appears to be decreased after one week of overload in both LZ and OZ rats but that the degree of PKR phosphorylation appears to be significantly higher in the overloaded OZ after three weeks of overload. To examine how changes in the amount of activated PKR might influence the activation of molecules thought to be important in regulating protein synthesis, we next examined the phosphorylation of eIF2α. As expected from our PKR data, the PKR dependent eIF2α phosphorlyation at Ser51 was significantly higher in overloaded OZ rat compared to LZ rat after three weeks of overload. To further explore the potential effects of PKR activation on protein degradation, we next examined the phosphorylation of p38 MAP kinase. Consistent with our PKR data, the phosphorylation of p38 MAP kinase (Thr180/Tyr182) was significantly higher in the overloaded OZ rat compared to LZ rat after three weeks of overload. To investigate the potential downstream effects of elevated p38 MAP kinase activation, we next measured the degree of protein ubiquitination. As expected, we found that the level of ubiquitination was much higher in insulin-resistant skeletal muscle compared to normal muscle after 3 weeks overload. Taken together, these results suggest that insulin resistance attenuates overload-induced skeletal muscle hypertrophy through the activation of AMPK and PKR, leading to inhibition of mTOR signaling and phosphorylation of eIF2α and depression of protein synthesis, together with PKR mediated p38 MAP kinase activation and increased protein degradation.

**Regulation of HSPs and MAPKs with insulin resistance during muscle hypertrophy**

It is thought that the heat shock proteins (HSPS) are important components of the cellular protective response and have been implicated in the chaperoning of unfolded proteins, the inhibition of apoptosis, and the stabilization of the cytoskeleton [187-191]. Previous studies demonstrated the induction of HSP proteins following high force eccentric contraction, aerobic exercise, and during the development of muscle hypertrophy [42, 43, 302]; however, it is not clear whether the regulation of these molecules is altered with insulin resistance. Here, we demonstrated that overload led to a significant increase in the phosphorylation or activation of Hsp27 at Ser82 in normal muscle; however, this response appeared to be attenuated in the insulin resistant soleus.

Mitogen-activated protein kinases (MAPKs) are another class of stress responsive proteins that are thought to be involved in the regulation of protein synthesis and cellular proliferation [198-200]. Previous studies have demonstrated that the extracellular regulated kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK) MAPK proteins are involved in the regulation of skeletal muscle hypertrophy following increased loading [44-47, 214]. If or how insulin resistance may affect MAPK signaling in response to increased muscle loading has, to our knowledge, not been investigated. In the present study we demonstrated that overload was found to increase the activation of p44- and p42-MAPK in both normal and insulin resistant soleus, however the magnitude of activation was higher in normal compared to that observed in insulin resistant soleus. Given the possibility that ERK1/2 activation is associated with skeletal muscle hypertrophy [317, 318], the differences in magnitude of activation of ERK1/2 between lean and obese rats with overload may help to explain the decreased hypertrophic response seen in the OZ animals. Taken together, these results suggest that the impaired hypertrophic response

seen in insulin resistant muscle appears to be associated with alterations in HSP and MAPK phosphorylation.

**Regulation of muscle-specific miRNAs with insulin resistance during muscle hypertrophy**

MicroRNAs (miRNAs) are small ~ 22-nucleotide, non-coding RNAs that are a newly emerging class of trans-factors that repress gene expression post-transcriptionally by inhibiting translation of target mRNAs through interaction with the 3'- untranslated region (3'UTR) of target mRNAs [50, 51, 215]. Previous studies have strongly suggested a role for muscle-specific miRNAs (miR-1, miR-133a and miR-206) in load induced skeletal muscle remodeling [7, 303, 304]. Among the muscle-specific miRNA's, miR-1 and miR-133a are important for embryonic muscle growth [52], myoblast differentiation and proliferation by regulating the expression of SRF, MyoD and MEF2 [53],and miR-206 plays a role in myogenesis [54]. How insulin resistance may affect the regulation of muscle-specific miRNAs during muscle hypertrophy is currently unclear.

In the present study we observed that muscle overload is associated with diminished mir-1 expression in both the LZ and OZ rats. These results are in agreement with recent findings by McCarthy and Esser, who reported a 50% decrease in expression of mir-1 after 7-days of functional overload in the mouse plantaris muscle [7]. Interestingly, the overload associated decreases observed in the present study appeared to be higher in the LZ compared to OZ after 7- and 21-days of overload. Similar results to what we had observed with mir-1 were seen when we examined mir-133a expression in the LZ animals. Conversely, in the OZ animals overload appeared to increase the expression of this molecule. Whether these findings are directly related to the diminished overload-induced hypertrophy we see in the OZ rat soleus is currently unclear.

Similarly, why insulin-resistance might be associated with differences in how overload regulates miRNA expression is not known.

## CONCLUSIONS

1. The OZ rat was hyperglycemic and hyperinsulinemic compare to the LZ rat.
2. The degree of hypertrophy following three weeks of compensatory overload was attenuated in insulin resistant OZ rat compared to its lean counterpart.
3. Impaired overload-induced hypertrophy in insulin resistant skeletal muscle may be related to decreases in the ability of the muscle to activate mTOR and its downstream effectors.
4. Impaired overload-induced hypertrophy in insulin resistant skeletal muscle was associated with hyperphosphorylation of AMPK and dsRNA-dependent protein kinase, which leads to the inhibition of mTOR signaling and phosphorylation of eIF2α and depression of protein synthesis, together with PKR mediated p38 MAP kinase activation and increased protein degradation.
5. Skeletal muscle adaptation to increased load in the OZ rat is associated with diminished HSP27 phosphorylation and decreased MAPK phosphorylation.
6. Insulin resistance alters the regulation of muscle-specific miRNAs during muscle hypertrophy.

| Molecule | 7d LZ | 7d OZ | 21d LZ | 21d LZ |
|---|---|---|---|---|
| p-mTOR (S2448) | ↑ | ↑ | ↑ | ↔ |
| p-p70S6k (T389) | ↑ | ↑ | ↑ | ↔ |
| p-p70S6k (T421/S424) | ↑ | ↑ | ↑ | ↑ |
| p-rpS6 (S235/236) | ↑ | ↑ | ↑ | ↔ |
| p-eEF2 (T56) | ↑ | ↑ | ↔ | ↔ |
| p 4E-BP1 (T37/46) | ↑ | ↑ | ↔ | ↓ |
| p-Akt (T308) | ↑ | ↑ | ↑ | ↔ |
| p-Akt (S473) | ↑ | ↑ | ↑ | ↔ |
| p-PTEN (S380/T382/383) | ↑ | ↑ | ↑ | ↑ |
| p-TSC2 (T1462) | ↓ | ↓ | ↓ | ↔ |
| Raptor | ↔ | ↔ | ↔ | ↔ |
| myo-D | ↔ | ↔ | ↔ | ↔ |
| myogenin | ↔ | ↔ | ↔ | ↔ |
| p-AMPKα (T172) | ↓ | ↓ | ↔ | ↑ |
| p-PKR (T446) | ↓ | ↓ | ↓ | ↑ |
| p-eIF2α (S51) | ↓ | ↓ | ↓ | ↑ |
| p-p38 MAPK (T180/T182) | ↑ | ↑ | ↔ | ↑ |
| Ubiquitin | ↑ | ↑ | ↔ | ↑ |
| p-GSK-3β (Ser9) | ↑ | ↑ | ↑ | ↔ |
| p-HSP27 (S82) | ↑ | ↑ | ↑ | ↔ |
| HSP60 | ↔ | ↔ | ↔ | ↔ |
| HSP70 | ↔ | ↔ | ↔ | ↔ |
| HSP90 | ↔ | ↔ | ↔ | ↔ |

Table 1 Summary of results. Alterations in expression or phosphorylation of signaling proteins in skeletal muscle adaptation with overload. ( ↑ = Increased, ↓ = decreased and, ↔ = unchanged compared to respective control).

# FUTURE DIRECTIONS

The present study focused on understanding the different molecular mechanisms in which insulin resistance may alter skeletal muscle remodeling with hypertrophy and thus may lead to treatment options aimed at improving skeletal muscle function. This dissertation has provided evidence that overload-induced skeletal muscle remodelling in the insulin-resistant OZ rat is associated with multiple level decrements in mTOR signaling, hyperphosphorylation of AMPK and PKR and altered regulation of muscle-specific miRNAs. Unfortunately, these experiments do not demonstrate the direct cause and effect relationship between insulin resistance and alterations in signaling events. As such, complementary experiments designed to directly inhibit or activate these signaling molecules will further enhance our understanding of mechanisms of skeletal muscle remodeling. For example, using genetic models of AMPK deficiency/ functional titration and looking at the regulation of mTOR signaling may improve our understanding of interaction between these two signaling pathways.

Recent studies demonstrated the interaction of myostatin signaling with Akt/mTOR signaling [322, 323]. Overexpression of myostatin was associated with decreased phosphorylation of Akt as well as reduced phosphorylation of other components of Akt/mTOR signaling such as ribosomal protein S6, p70S6K and 4E-BP1 [323, 324], and blocking myostatin activity increased protein synthesis and ribosomal protein S6 and p70S6K phosphorylation [322]. Whether insulin resistance alters the regulation of myostatin signaling during muscle hypertrophy is not known and is worthy of investigation.

The findings from this study demonstrated that IR is associated with altered skeletal muscle remodeling with contractile stimuli. However, besides IR, other co-morbidities (i.e.

obesity, leptin resistance) may also affect the muscle adaptation. To determine whether obesity affects the skeletal muscle adaptation during muscle hypertrophy, this study design should be repeated using a non obese insulin resistance model replacing the obese insulin resistance model. In addition, future studies are required to understand the leptin-mediated effect on the muscle remodeling.

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# CURRICULUM VITAE

**Anjaiah Katta**

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## OBJECTIVE

I am a DVM, PhD candidate who wants to contribute to the growth of the organization I work at by applying my current knowledge/experience of veterinary medicine and by learning and adapting to new technologies/approaches to achieve mutual goals.

## EDUCATION

**PhD in Biomedical Sciences (August 2011)**

Joan C**.** Edwards School of Medicine, Marshall University**;** Huntington, WV
Major: Physiology, Pharmacology & Toxicology (expected to graduate in June-2011)
Dissertation: "**Muscle plasticity and intramuscular signaling in the insulin- resistant obese Zucker rat"**

**MS in Biology (Jan 2006 – July 2007)**

College of Science, Marshall University; Huntington, WV
Major: Molecular Physiology
Thesis: **"Lean and Obese Zucker Rats Exhibit Different Patterns of p70S6kinase Regulation in the Tibialis Anterior Muscle in Response to High Force Muscle Contraction**"

**Master's in Veterinary Sciences (MVSc) (Nov 2001 – Nov 2003)**

College of veterinary Science, ANGRAU, Tirupati, A.P, India.
Major: Clinical Veterinary Medicine
Thesis:"**Studies on Subclinical Ketosis in Buffaloes"**

**Bachelor of Veterinary Science and Animal Husbandry (BVSc & AH; DVM equivalent) (Nov 1996 – Nov 2001)**

College of veterinary Science, ANGRAU, Tirupati, A.P, India.

**LICENSURE** **NC State Veterinary License (# 7026)**

**CERTIFICATIONS** **ECFVG certification, NOV 2010**

## PROFESSIONAL EXPERIENCE

### *CLINICAL EXPERIENCE*

Mar, 2010- Jun, 2010 Volunteer at Proctorville Animal Clinic, Proctorville, OH
Sep, 2009- Jan, 2010 Volunteer at Tri-State Veterinary Center, Huntington, WV
Sep, 2009- Jan, 2010 Volunteer Equine Medical Center, Chesapeake, OH
Nov, 2003- July, 2005 Lead Veterinarian at Veterinary polyclinic, Vizag, A.P, India
July, 2001- Nov, 2001 Intern, Veterinary Polyclinic, Kakinada, A.P, INDIA.

**Duties performed as a Veterinarian**

- Responsible for primary care including diagnosis and treatment of sick animals
- Spay/neuter of dogs
- Performed reproductive soundness exams in dairy cattle, artificial insemination, pregnancy diagnosis, handled dystocia cases and caesarian section in needy situations
- clinical laboratory sample processing
- Trained technicians about animal handling, workplace safety, work protocols, equipment handling and client interaction
- Organized rural animal health camps
- Supervised fifteen employees
- Responsible for the instruction of rotating senior veterinary students

### *RESEARCH EXPERIENCE*

Jan 2006- Present: Research Assistant, Molecular Physiology, Marshall University, Huntington, WV-25705

## RESEARCH SKILLS

Immunohistochemistry
Flourescent and Confocal Microscopy
Differential staining procedures
Sample preparation from Laboratory animals
SDS gel electrophoresis
Protein Quantitation using Bradford assay
Immunoprecipitation and Westerns
Quantitation using Densitometry
DNA, RNA and microRNA isolation

PCR, RT-PCR
DNA and Protein gel electrophoresis
Small animal surgery
Cell culture
Handling of Laboratory animals

**PUBLICATIONS**

**2011**

1. Al-Rousan RM, Rice KM, **Katta A**, Laurino J, Walker EM, Wu M, Triest WE, Blough ER. *Deferasirox protects against iron-induced hepatic injury in Mongolian gerbil.* Transl Res. 2011 Jun; 157(6):368-77. Epub 2011 Jan 13.

**2010**

2. **Katta A**, Sudarsanam K, Karkala SK, Wu M, Jacqueline Fannin, Paturi S, Hua Liu, Hari S. Addagarla, and Eric R. Blough. *Impaired overload-induced hypertrophy is associated with diminished mTOR signaling in insulin resistant skeletal muscle of the obese Zucker rat.* Am J Physiol Regul Integr Comp Physiol; Epub 2010 Oct 13.
3. Wang Y, Wu M, Al-Rousan R, Liu H, Fannin J, Paturi S, Arvapalli R, **Katta A**, Kakarla S, Rice K, Triest WE, Blough ER. *Iron-induced cardiac damage: role of apoptosis and deferasirox intervention.* J Pharmacol Exp Ther. ; Epub 2010 Oct 14.
4. **Katta A**, Kakarla S, Wu M, Paturi S, Gadde MK, Arvapalli R, Kolli M, Rice KM, Blough ER. *Altered regulation of contraction-induced Akt/mTOR/p70S6k pathway signaling in skeletal muscle of the obese Zucker rat*. Exp Diabetes Res. 2009; 2009:384683. Epub 2010 Mar 30.
5. Kakarla SK, Fannin JC, Keshavarzian S, **Katta A**, Paturi S, Nalabotu SK, Wu M, Rice KM, Manzoor K, Walker EM Jr, Blough ER. *Chronic acetaminophen attenuates age-associated increases in cardiac ROS and apoptosis in the Fischer Brown Norway rat.* Basic Res Cardiol. 2010 Jul;105(4):535-44.
6. Paturi S, Gutta AK, Kakarla SK, **Katta A**, Arnold EC, Wu M, Rice KM, Blough ER. *Impaired overload-induced hypertrophy in obese Zucker rat slow-twitch skeletal muscle.* J Appl Physiol. 2010 Jan;108(1):7-13
7. Miaozong Wu, Hua Liu, Jacqueline Fannin, **Katta A**, Yeling Wang, Ravi Kumar Arvapalli, Satyanarayana Paturi, Sunil K. Karkala, Kevin M. Rice, and Eric R. Blough. *Acetaminophen Improves Protein Translational Signaling in Aged Skeletal Muscle.* Rejuvenation Res. 2010 Jun.
8. Kolli MB, Day BS, Takatsuki H, Nalabotu SK, Rice KM, Kohama K, Gadde MK, Kakarla SK, **Katta A**, Blough ER. *Application of poly (amidoamine) dendrimers for use in bionanomotor systems.* Langmuir. 2010 May 4; 26(9):6079-82.
9. Arvapalli RK, Paturi S, Laurino JP, **Katta A**, Kakarla SK, Gadde MK, Wu M, Rice KM, Walker EM, Wehner P, Blough ER. *Deferasirox decreases age-associated iron accumulation in the aging F344XBN rat heart and liver.* Cardiovascular Toxicol. 2010 Jun; 10(2):108-16.

10. Paturi S, Gutta AK, **Katta A**, Kakarla SK, Arvapalli RK, Gadde MK, Nalabotu SK, Rice KM, Wu M, Blough E. *Effects of aging and gender on muscle mass and regulation of Akt-mTOR-p70s6k related signaling in the F344BN rat model.* Mech Ageing Dev. 2010 Mar; 131(3):202-9.
11. Kakarla SK, Rice KM, **Katta A**, Paturi S, Wu M, Kolli M, Keshavarzian S, Manzoor K, Wehner PS, Blough ER. *Possible molecular mechanisms underlying age-related cardiomyocyte apoptosis in the F344XBN rat heart*. J Gerontol A Biol Sci Med Sci. 2010 Feb;65(2):147-55
12. Rice KM, Kakarla SK, Mupparaju SP, Paturi S, **Katta A**, Wu M, Harris RT, Blough ER. Shear stress activates Akt during vascular smooth muscle cell reorientation. Biotechnol Appl Biochem. 2010 Feb 18; 55(2):85-90.

**2009**

13. **Katta A**, Sunil K. Kakarla, Wu M, Meduru S, Desai DH, Rice KM, Blough ER . *Lean and obese Zucker rats exhibit different patterns of p70s6 kinase regulation in the tibialis anterior muscle in response to high-force muscle contraction.* Muscle Nerve. 2009 Apr; 39(4):503-11.
14. Wu M, **Katta A**, Gadde MK, Liu H, Kakarla SK, Fannin J, Paturi S, Arvapalli RK, Rice KM, Wang Y, Blough ER. *Aging-associated dysfunction of Akt/protein kinase B: S-nitrosylation and acetaminophen intervention*. PLoS One. 2009 Jul 29;4(7):e6430
15. Wu M, Desai DH, Kakarla SK, **Katta A**, Paturi S, Gutta AK, Rice KM, Walker EM Jr, Blough ER. *Acetaminophen prevents aging-associated hyperglycemia in aged rats: effect of aging-associated hyperactivation of p38-MAPK and ERK1/2.* Diabetes Metab Res Rev. 2009 Mar; 25(3):279-86.

**2008**

16. **Katta A**, Preston DL, Karkala SK, Asano S, Meduru S, Mupparaju SP, Yokochi E, Rice KM, Desai DH, Blough ER. *Diabetes alters contraction-induced mitogen activated protein kinase activation in the rat soleus and plantaris.* Exp Diabetes Res. 2008; 2008:738101.

**2007**

17. Asano S, Rice KM, Kakarla S, **Katta A**, Desai DH, Walker EM, Wehner P, Blough ER. *Aging influences multiple indices of oxidative stress in the heart of the Fischer 344/NNia x Brown Norway/BiNia rat.* Redox Rep. 2007; 12(4):167-80.

**2006**

18. Rice KM, Desai DH, Kakarla SK, **Katta A**, Preston DL, Wehner P, Blough ER. *Diabetes alters vascular mechanotransduction: pressure-induced regulation of mitogen activated protein kinases in the rat inferior vena cava.* Cardiovasc Diabetol. 2006 Sep 8; 5:18.

## PROFESSIONAL AFFILIATIONS

American College of Sport Medicine (ACSM)
Center for Diagnostic Nanosystems (CDN)
Association of Young scientists (AYS)
Indian Veterinary Council
Andhra Pradesh Veterinary Association
Indian Association of Veterinary Laboratory Diagnosticians

## HONORS AND AWARDS

2010- **Best Graduate student Research performance award**; Joan C. Edwards School of Medicine, Marshall University ($2000 international travel grant)
2009- **Best Graduate student Research performance award**; Joan C. Edwards School of Medicine, Marshall University ($1500 national travel grant)
2001 - 2003 Graduate Research Assistantship; College of veterinary Science, Tirupati.

## COMPUTER EXPERTISE

Proficient in MS-office applications: MS-word, Excel and Power point applications, and preparation of charts and presentations for scientific meetings.

## REFERENCES

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