1-1-2011

Altered regulation of mitogen-activated protein kinases and GLUT4 expression in skeletal muscle, liver, heart and kidney of obese Zucker rat

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Altered regulation of mitogen-activated protein kinases and GLUT4 expression in skeletal muscle, liver, heart and kidney of Obese Zucker rat

A thesis submitted to the

Graduate College of Marshall University

In partial fulfillment of the requirements for the degree of

Master of Science

Department of Biological Sciences

By

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December 2011

ACKNOWLEDGMENTS

It is of immense pleasure to thank my mentor and guide Dr. Eric R. Blough for supporting me throughout my master's degree and without whose support I wouldn't have completed this thesis. Dr. Blough has been supportive and helpful throughout my master's program and I am blessed to have a wonderful guide like him. With his enthusiasm, inspiration, and great efforts to explain things clearly and simply, he made research very interesting to me. Throughout my thesis-writing period, he provided encouragement, sound advice, good teaching, and lots of good ideas. I would have been lost without him. I would also like to thank my committee member Dr. Simon Collier whose valuable suggestions and comments helped me in completing my thesis. I also would like to thank Dr. David S. Mallory for his valuable thoughts about the research work and comments on the thesis work. Last but not the least I would like to extend my heartfelt thanks to my lab members and colleagues Sunil, Kevin, Anjaiah, Miaozong, Thulluri, Arvapalli, Madhukar, Hari, Siva, Paturi, Para, Prasanna, Geeta, Sravanthi and Eli. Lastly, and most importantly, I wish to thank my parents K.V.Ramanaiah, Adilakshmi, my wife and kids for all the support and encouragement they have given me.

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ABBREVIATIONS

BSA	Bovine serum albumin
CDC	Center for Disease Control and Prevention
DM	Diabetes mellitus
ECL	Enhanced chemiluminiscence
PI-3 Kinase	Phosphoinositide 3-kinase
ERK1/2	Extra signal regulatory kinase
GLUT 4	Glucose transporter type 4
JNK	c-Jun N-terminal kinase
LH	Lean Zucker heart
LK	Lean Zucker kidney
LL	Lean Zucker liver
LM	Lean Zucker skeletal muscle
MAPK	Mitogen activated protein kinase
MEF2	Myocyte enhancement factor-2
NIH	National Institute of Health
OH	Obese Zucker heart
OK	Obese Zucker kidney
OL	Obese Zucker Liver
OM	Obese Zucker skeletal muscle
PKB	Protein kinase B
SEM	Standard Error of Mean
SDS-PAGE	Sodium dodecyl Sulfate-polyacrylamide gel electrophoresis

TBS	Tris buffered saline
TBST	Tris buffered saline with 0.5% tween

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ABSTRACT

Background: Metabolic syndrome is characterized by insulin resistance and hyperglycemia. The molecular mechanism(s) underlying this alteration in skeletal muscle, liver, kidneys and heart in metabolic syndrome patients are presently unclear. Recent data have demonstrated that the p38 and extracellular-signal-regulated kinases (ERK 1/2) - mitogen-activated protein kinase (MAPK) proteins may be involved in the regulation of (Glucose transporter type 4) GLUT4 expression levels. Other data have suggested that miRNA may also play a role. **Purpose:** The purpose of this study is to compare the tissue content, phosphorylation of protein kinases (AKT), p38, c-JUN N-terminal kinase(JNK), ERK1/2, GLUT4 and transcriptional factor Myocyte enhancer factor-2 (MEF2) in the skeletal muscle, heart, liver and kidney of insulin resistant obese (fa/fa) and lean Zucker rats. **Methods:** Skeletal muscle, heart, liver and kidney were obtained from obese (n=6) and lean (n=6) Zucker rats. The amount and phosphorylation status of AKT, p38, JNK, ERK 1/2, GLUT4 and MEF2 were evaluated by immunoblotting. **Results:** Decreased GLUT4 expression levels were observed in the skeletal muscle, liver and heart of obese Zucker rats compared to lean zucker rats and are associated with alterations in the phosphorylation of MAPK family members. Levels of mir-1and 133a were altered in skeletal and cardiac muscles of obese Zucker rats when compared with their lean counterparts. **Conclusions:** These results suggest that MAPK members regulate GLUT4 expression differently in various insulin resistant tissues compared to lean counterparts and that these changes may play a key role in insulin resistance associated with glucose dysregulation.

Key words: Insulin resistance, Akt, P38, Erk1/2, JNK, MEF2, GLUT4

CHAPTER 1

Introduction

According to the World Health Organization diabetes fact sheet for January 2011 approximately 220 million people worldwide have diabetes and it is estimated that this will rise to 366 million by 2030. The Centers for Disease Control and Prevention has estimated that about 8.3 percent of the total United States populations are diabetic. Diabetes mellitus (DM) is a serious, life-threatening condition that is characterized by high levels of blood glucose resulting from defects in insulin production, insulin action, or both [1]. Individuals with type 2 diabetes typically exhibit characteristics of metabolic syndrome and insulin resistance characterized by hyperinsulinemia, hyperlipidemia and hyperglycemia [2]. The obese Zucker (fa/fa) rats are leptin deficient models that result in an early onset of type 2 diabetes and exhibit the characteristics of metabolic syndrome and are therefore thought to be an appropriate model for diabetes related studies.

Metabolic syndrome is associated with alterations in insulin signaling that can lead to insulin resistance and hyperglycemia [3]. Insulin resistance is the cause of type 2 diabetes thatis linked with several other factors and plays a key role in pathogenesis of metabolic syndrome [3]. A key regulator of insulin induced glucose uptake is the glucose transporter (GLUT4) [4]. It is thought that the GLUT4 is regulated, at least in part, by PI3K that, when phosphorylated causes recruitment of protein kinase B (PKB) and PDK1 [5]. There is overwhelming evidence to support the role of Akt/PKB in insulin stimulated glucose uptake [6-8]. Mitogen activated protein kinases are also thought to play a role in the GLUT4 expression and relocation. The stress-responsive kinase, p38 mitogen-activated protein kinase (p38-MAPK), ERK1/2 (extra

signal regulatory kinases) have been found to be involved in the regulation of GLUT4 expression by activating the transcription factor MEF2 that, in turn, activates the GLUT4 promoter [9]. Though it is well established that insulin resistance is associated with glucose dysregulation, whether differences exist between normal and insulin resistant tissues in the regulation of MAPK members and GLUT4 levels remains unclear. To our knowledge, the mechanism of insulin signaling in different sensitive tissues like skeletal muscle, liver, heart and kidney of normal and diabetic animal has not been investigated.

Purpose

Our long-term goal is to elucidate the organ specific differences in insulin induced signaling in lean and obese Zucker rats that could provide vital information in further understanding the molecular mechanisms and drug targets of insulin resistance. The objective of this study is to determine whether insulin resistance alters insulin related signaling mechanisms differently in skeletal muscle, liver, kidneys and heart. To achieve the objective we will compare the tissue content of phosphorylated levels of AKT, p38 mitogen activated protein kinases, JNK (c-Jun N-terminal kinases), ERK1/2 (extracellular regulatory kinases), GLUT4 (glucose transporter) and transcriptional factor Myocyte enhancer factor-2 (MEF2) in the skeletal muscle, heart, liver and kidney of Obese (fa/fa) and Lean Zucker rats. We will also investigate the role of different microrna in regulation of GLUT4 expression in type 2 diabetes that might be useful in designing therapeutic targets.

Specific Aims

Estimated global healthcare expenditures to treat and prevent diabetes and its complications are expected to total US Dollar (USD) 376 billion in 2010. By 2030, this number is projected to exceed some USD 490 billion (International diabetes federation, diabetes atlas). Diabetes is the sixth leading cause of death in the US. The economic burden posed by diabetes is daunting with the total cost of diabetes in the U.S. in 2007 at an estimated 174 billion and this cost is estimated to almost triple in the next 25 years (American Diabetes Association). As such, it is clear that the scientific community must continue to search for the most effective methods for the treatment and prevention of this disease. Recent data have suggested that the p38 and extracellular-signal-regulated kinases (ERK 1/2)-mitogen-activated protein kinase (MAPK) proteins may be involved in the regulation of (Glucose transporter type 4) GLUT4 expression levels. As of yet no concrete study has been done to indicate whether differences exist in insulin signaling in different insulin resistant tissues. The working hypothesis of this study is that differences exist between normal and diabetic skeletal muscle, heart, liver, kidney in the insulin signaling pathway. To test this hypothesis and accomplish the purpose of this study the following specific aims are proposed:

Specific Aim #1

To determine if the expression of total and phosphorylated levels of Akt, MAPK family, MEF2, GLUT4 pathway related proteins and mir-1,133a, 223 miRNA are altered in the skeletal and cardiac muscles of lean and obese Zucker rats with type 2 diabetes.

Hypothesis:

Type 2 diabetes will be associated with alterations in the expression of total and

Phosphorylated level of Akt, MAPK family, MEF2, GLUT4 pathway related proteins and mir-1,133a, 223 miRNA in the skeletal and cardiac muscles of lean and obese Zucker rats.

Specific Aim #2

To determine if the expression of total and phosphorylated levels of Akt, MAPK family, MEF2, GLUT4 pathway related proteins and mir-223 miRNA are altered in the liver of lean and obese Zucker rats with type 2 diabetes.

Hypothesis:

Type 2 diabetes will be associated with alterations in the expression of total and Phosphorylated levels of Akt, MAPK family, MEF2, GLUT4 pathway related proteins and mir-223 miRNA in the liver of lean and obese Zucker rats.

Specific Aim #3

To determine if the expression of total and phosphorylated level of Akt, MAPK family, MEF2, GLUT4 pathway proteins are altered in the kidney of lean and obese Zucker rats with type 2 diabetes.

Hypothesis:

Type 2 diabetes will be associated with alterations in the expression of total and Phosphorylated levels of Akt, MAPK family, MEF2, GLUT4 pathway proteins in the kidney of lean and obese Zucker rats

CHAPTER 2

Review of Literature

Introduction

A review of the literature pertaining to this study will be discussed here in this chapter in detail. The following topics that are aptly related will be addressed: 1) Type 1 and Type 2 diabetes, 2) models of metabolic syndrome, and 3) regulation of glucose uptake in different insulin sensitive tissues**.**

Type 1 and Type 2 diabetes

Diabetes mellitus (DM) is an emerging epidemic in United States and the world over that, if allowed to proceed unchecked, can progress into a deadly metabolic syndrome affecting almost all the vital organs in the body. Diabetes mellitus can be described as the uncontrolled regulation of circulating glucose levels that is oftentimes dues to a failure in insulin production or unresponsiveness of the tissues to insulin stimulation [10]. According to the World Health Organization, approximately 3.4 million people died from consequences of high blood sugar in 2004. It is currently estimated that 346 million people suffer from diabetes worldwide. Here in the United States, the American Diabetes Association has estimated that the total number of people with diabetes in 2011 will be approximately 25.8 million, or 8.3% of the total population. It is also estimated that the number of people exhibiting pre-diabetes in the United States will be 79 million by the end of 2011.

Type 1 diabetes mellitus is characterized by failure of pancreatic islets of Langerhans to produce adequate amounts of insulin. Type 1 diabetes mellitus is an autoimmune mediated

disease that occurs in 1 of every 300 individuals in United States although it may sometimes be due to idiopathic nature [11]. Type I diabetes is commonly diagnosed in children and hence commonly referred to as juvenile onset diabetes or insulin dependent diabetes mellitus. This disorder represents about five to ten percent of all recorded diabetic cases. Currently there is no cure for type I diabetes [12].

Type 2 diabetes mellitus is characterized by insulin resistance where the responsiveness of the target tissues to circulating levels of insulin is decreased [13]. In Type 2 diabetes there is a gradual change from normal glucose tolerance to abnormal glucose tolerance. This shift in tolerance is characterized at first by an increase in postprandial glucose levels that gradually increases to hyperglycemia [14]. Previous studies have shown that genetics, obesity, physical inactivity and environmental factors pose as risk factors for development of Type 2 diabetes [15]. Recent studies have demonstrated that insulin resistance is associated with changes in GLUT4 expression and alterations in insulin receptor isoform expression and IGF-I receptor abundance [16, 17]. Other work has suggested that that impaired phosphorylation of IRS-1 receptor and reduced PI3Kinase activity may also play a role in mediating insulin resistance in skeletal muscle [18]. Whether a similar mechanism is present in other tissue types has, to our knowledge, not been investigated.

Summary

Type I diabetes mellitus is primarily due to an autoimmune disease in which the beta cells of the pancreas are destroyed leading to diminished insulin production. Type I diabetes comprises of about five to ten percent of all diabetic cases whereas Type II diabetes comprises about ninety to ninety five percent. Type II diabetes mellitus occurs primarily due to a failure of

cells to respond to insulin. Although the exact mechanism(s) are currently unclear, Type 2 appears to be associated with changes in receptor isoforms and the propagation of intracellular signaling.

Models of metabolic syndrome

A suitable experimental model is a prerequisite for the success of any scientific study. The selection of model depends primarily on the study and its potential applications. Research centered toward diabetes has widely used rodent models for study due to their short life span and the ability to control the external environment.

There are several strains of rodents for diabetic research such as Goto-Kakizaki rat, non-obese diabetic rats, Nagoya-Shibata-Yasuda mouse, Pound mouse, obese Zucker rats and others [19]. The selection of particular model among these is typically dependent upon the type of diabetes being investigated and the dependent variables that are being measured. For example the Non-Obese Diabetic (NOD/NHsd) rats serve as a model for research in Type 1 diabetes mellitus as they develop insulitis due to leukocytic infiltration of pancreatic islets [20]. In addition, the onset of diabetes in these rats is associated with glycosuria and non-fasting hyperglycemia. The Goto-Kakizaki (GK) rat is a non-obese Wistar sub strain which develops Type 2 diabetes and exhibits hyperinsulinemia, insulin resistance, neuropathy, retinopathy and nephropathy [21]. Similarly, the Nagoya-Shibata-Yasuda mouse develops spontaneous non-insulin-dependent diabetes. This model is particularly suitable for studying the genetics of diabetes [22]. In addition, this strain also exhibits some characteristics of diabetes seen in humans including impaired insulin secretion, visceral fat accumulation and the ability to induce development of this disease by dietary manipulation. Another model for type 2 diabetic is the Israel Sand rat or

Psammomys obesus, which belongs to the gerbil family [23]. These rats exhibit impaired insulin sensitivity accompanied by increased adiposity before the onset of insulin resistance in a manner that closely resembles human type 2 diabetes [23]. Another strain that has also been developed is the Pound mouse (C57BL/6NCrl-*Lepr*$^{db\text{-}db}$/Crl), which exhibits hyperinsulinemia, insulin resistance, dyslipidemia, metabolic syndrome, leptin deficiency and fatty liver disease [24]. This strain develops hyperinsulinemia by 8 weeks of age with insulin levels exceeding 300 ng / ml by 18 weeks.

Obese Zucker rat model

The most commonly and perhaps best suited model for type 2 diabetes study is the obese Zucker rat that was developed through selective breeding over many generations. These animals exhibit hyperinsulinemia, hyperphagia, hypercholesterolemia and hyperglycemia along with central obesity[25]. These animals are also considered to be excellent models for the study of metabolic syndrome as they also exhibit glucose intolerance, insulin resistance, increased oxidative stress in heart and liver, borderline hypertension, cardiac hypertrophy and increased mean arterial pressure [25-27]. Other characteristics include increased hepatic glycogen, impaired glycogen synthesis, steatosis and proteinuria, peripheral neuropathy and altered sympathetic and parasympathetic activity [28]. Taken together, these data suggest that the obese Zucker is highly suited for the study of Type 2 diabetes and metabolic syndrome.

Earlier studies on the hearts of obese Zucker rats demonstrated that the hyper-phosphorylation of IRS-1 may significantly contribute to pathogenesis of insulin resistance in the heart [29]. They also correlated the alterations in insulin-induced glucose transport activation and GLUT4 translocation to possible defects of the insulin signaling cascade [29]. Carvalho *et al.,*

demonstrated that insulin resistance in the adipocytes of obese Zucker rats is characterized by impaired glucose transporter 4 (GLUT4) translocation and diminished GLUT4 expression due to signaling defects associated with reductions in protein kinase B (AKT) and PI3K activation [30].

Summary

Millions of Americans have been diagnosed with type 2 diabetes, and many more are unaware that they are at high risk. The obese Zucker (fa/fa) rat appears to be an appropriate animal model for studying diabetes given its phenotypic properties and the similarities to that seen in humans. Few studies have investigated molecular mechanisms that are responsible for insulin resistant in the obese Zucker rat model. Elucidating the molecular events responsible for insulin resistance may be beneficial designing pharmacological interventions for the treatment of this disease.

Regulation of glucose uptake in different vital organs

A major metabolic defect associated with type 2 diabetes is the failure of tissues in the body to properly utilize glucose, thereby resulting in chronic hyperglycemia. Given that elevations in blood glucose levels can be toxic, it is of importance to understand the underlying molecular mechanisms that regulate insulin resistance. In the early 1980s, Dr. Cushman and colleagues proposed a novel mechanism in which vesicles containing glucose transporters could be mobilized to the plasma membrane by insulin stimulation , thereby effecting glucose transport into the cell [31]. Subsequent studies have largely confirmed this initial work and have begun to unravel the signaling mechanisms responsible for regulating glucose transporter movement to the cell membrane.

A key regulator of insulin-induced glucose uptake is the glucose transporter (GLUT4). GLUT4 is primarily expressed in skeletal muscle and adipocytes. Under conditions of low insulin, GLUT4 is sequestered in intracellular vesicles. Conversely, when insulin levels are increased, translocation of GLUT4 transporters occurs. This process appears to be initiated by insulin binding to the insulin receptor that then activates the receptor's tyrosine-kinase domain. The receptor, now activated, in turn phosphorylates the insulin receptor substrate-1 (IRS-1) that, in turn, binds the enzyme PI-3 kinase. PI-3 kinase then activates protein kinase B (AKT) through its phosphorylation. Activation of AKT through an as of yet underdetermined mechanism, then induces the movement of GLUT4 containing vesicles from the cytoplasm to the plasma membrane.

Intracellular signaling pathways that regulate GLUT4 activity and expression

The mitogen-activated protein kinases (MAPK) are serine/threonine-specific protein kinases that respond to extracellular stimuli. The MAPK proteins, when activated function to regulate various cellular activities including gene expression, differentiation, proliferation, and cell survival or apoptosis [32]. The p38-MAPK is a mammalian orthologue of the yeast Hog1p MAP kinase, which participates in a signaling cascade controlling cellular responses to cytokines and stress. There are four different isoforms of p38 MAPK: α, β, γ and δ. Previous studies have shown that hyperglycemia can activate p38 kinase in a protein kinase C (PKC) - δ isoform-dependent manner [33]. Michelle *et al.,* in 2003 proposed a model in which GLUT4 translocation by insulin was dependent upon both AKT/PKB and p38 MAPK activation [34]. Other data have shown that extremely elevated levels of glucose can also activate p38 kinase in a PKC-independent manner, most likely by inducing hyperosmolarity [33].

The extracellular signal regulated kinases (ERKs) are members of the MAPK family, which are involved in regulation of meiosis, mitosis, and in modulating gene expression. The ERK-MAPK proteins are activated by a variety of stimuli such as growth factors, cytokines, and carcinogens. Jing *et al*. showed that fatty acids can cause an increase in glucose uptake by activation of ERK1/2.

In addition to the PI-3K, Akt and the MAPK proteins, the myocyte enhancer factor-2 transcription factor may also play a role in regulating glucose uptake through its ability to regulate GLTU-4 protein expression. The myocyte enhancer factor-2 (MEF2) proteins are a family of transcription factors, which control gene expression and regulate cellular differentiation and development during the embryonic development. In adults, MEF2 is involved in regulation of various stress responses. Thus far, four isoforms of MEF2 have been delineated: MEF2A, MEF2B, MEF2C and MEF2D. It has been shown that p38 MAPK can phosphorylates MEF2, ATF-2, and MAX transcription factors under different conditions. Previous studies have shown that MEF2 protein regulates cardiac hypertrophy and tissue remodeling in skeletal muscle in response to stress [35, 36] . Mora *et al*., demonstrated that cardiac and skeletal muscle expression of GLUT4 is dependent upon a MEF2A-MEF2D heterodimer [37]. Recently, Christophe *et al*., showed that activation of p38 MAPK caused an increase in the expression of GLUT4 in response to IGF-1 in cardiac myocyte. In addition, this group also showed that MEF2 was the main transcription factor transducing the effect of p38 MAPK activation on the GLUT4 promoter [9]. Whether the mechanisms regulating the expression and activation of GLUT4 expression differ in different organs is not clear. Elucidating the molecular events responsible for alterations in GLUT4 expression in different organs of no diabetic and diabetic animals may be

beneficial for the development of new pharmacological interventions to treatment this Type 2 diabetes.

MicroRNAs

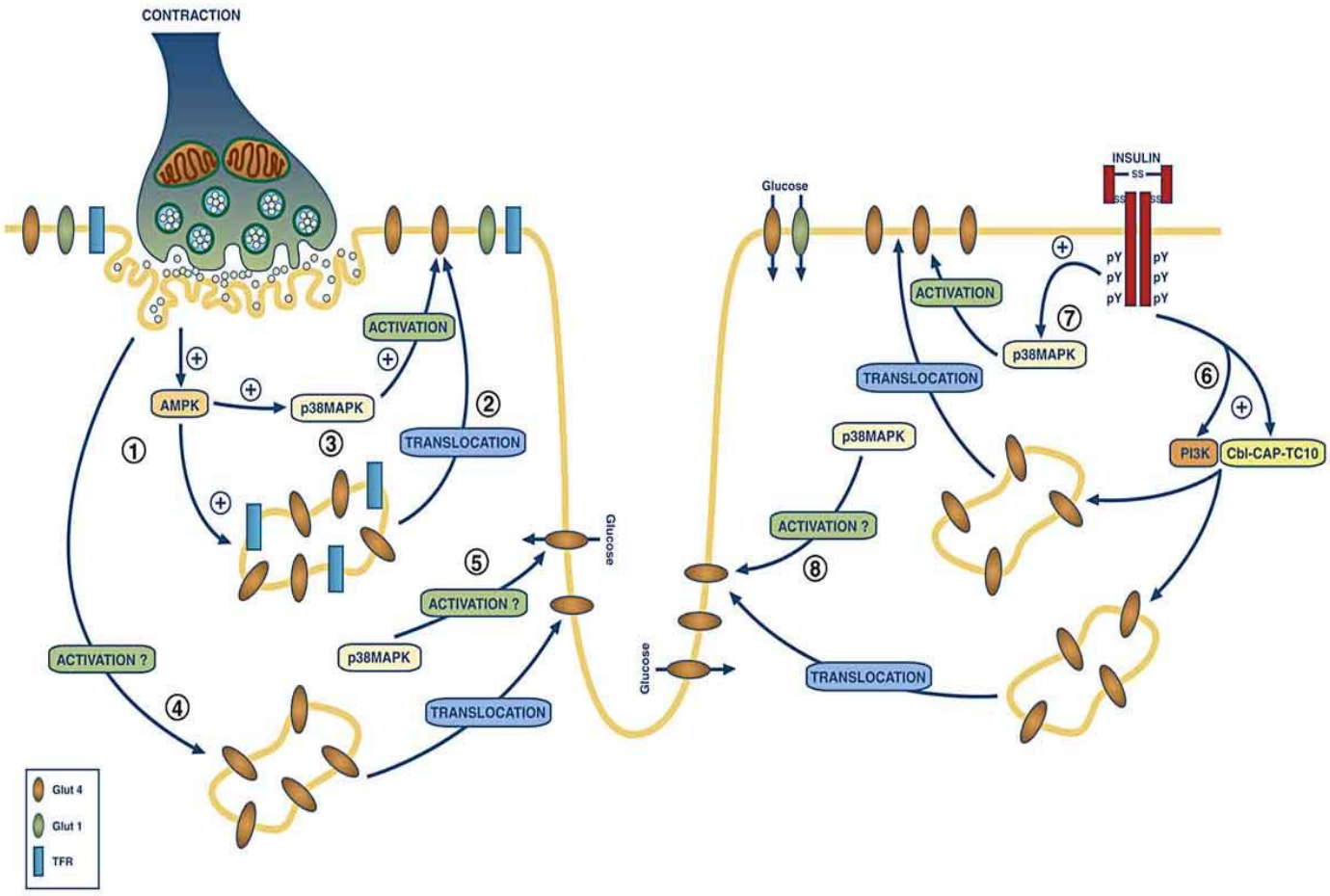
MicroRNAs are short ribonucleic acid molecules that act as post-transcriptional regulators by binding to complementary sequences on target messenger RNA transcripts. This binding, in turn, results in translational repression or target degradation. Elia *et al*., showed that miR-1 controls the expression of insulin-like growth factor-1 and IGF-1 receptor protein levels by translation in a reciprocal relationship [38]. Fred *et al*., showed that high glucose suppresses human Islet insulin biosynthesis by inducing miR-133a [39]. Recently Lu *et al*., provided interesting evidence with regards to regulation of glucose uptake by mir-223. They found that over expression of miR-223 resulted in an increase in expression of glucose transporter4 (Glut4) protein without effecting PI3Kinase or AMP Kinase activity [40]. Whether similar findings are observed *in vivo* is currently unclear.

Summary

Type 2 diabetes is characterized by unresponsiveness of the cells to normal levels of circulating insulin. GLUT4 is a primary glucose transporter protein for skeletal and cardiac muscle. Under elevated glucose conditions, GLUT4 is translocated to plasma membrane where it is responsible for up taking glucose into the cell. The regulation of GLUT4 function and expression appears to be mediated by the activity of the PI3K, Akt and MAPK signaling systems. The MEF2A transcription factor is activated by the MAPK pathways by high glucose

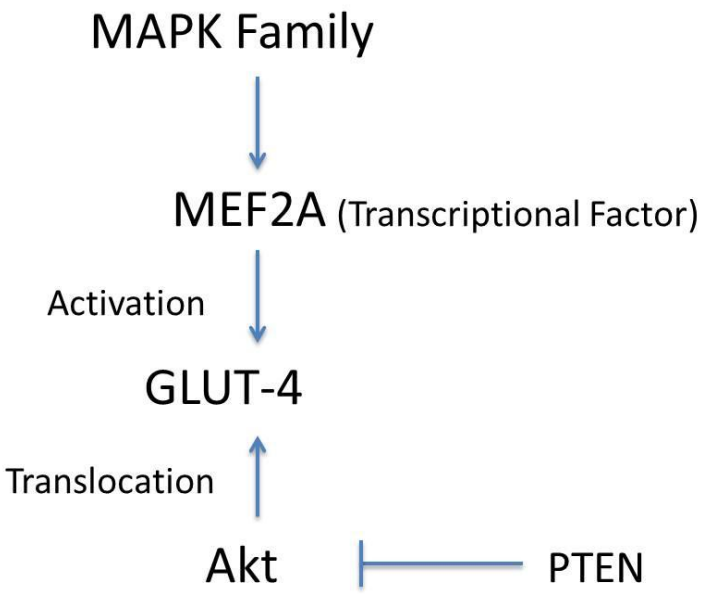
levels and may participate in the regulation of GLUT4 expression levels. Recent data suggest that miRNA may also play a role in regulating GLUT4 expression. It is currently unclear if the regulation of GLUT4 translocation and expression is regulated similarly in different tissue types.

Figure 1. Pictorial representation of activation and translocation of GLUT4 via MAPK family



Front Biosci. 2003 Sep 1;8:d1072-84

Figure 2. Schematic representation of MAPK mediated GLUT4 regulation



CHAPTER 3

Altered regulation of Mitogen-activated protein kinase (MAPK) mediated GLUT4 expression in skeletal muscle, liver, heart and kidney of Obese Zucker rat

ABSTRACT

Background: Metabolic syndrome is characterized by insulin resistance and hyperglycemia. The molecular mechanism(s) underlying this alteration in skeletal muscle, liver, kidneys and heart in metabolic syndrome patients are presently unclear. Recent data have demonstrated that the p38 and extracellular-signal-regulated kinases (ERK 1/2) - mitogen-activated protein kinase (MAPK) proteins may be involved in the regulation of (Glucose transporter type 4) GLUT4 expression levels. Other data have suggested that miRNA may also play a role. **Purpose:** The purpose of this study is to compare the tissue content, phosphorylation of protein kinases (AKT), p38, c-JUN N-terminal kinase(JNK), ERK1/2, GLUT4 and transcriptional factor Myocyte enhancer factor-2 (MEF2) in the skeletal muscle, heart, liver and kidney of insulin resistant obese (fa/fa) and lean Zucker rats. **Methods:** Skeletal muscle, heart, liver and kidney were obtained from obese (n=6) and lean (n=6) Zucker rats. The amount and phosphorylation status of AKT, p38, JNK, ERK 1/2, GLUT4 and MEF2 were evaluated by immunoblotting. **Results:** Decreased GLUT4 expression levels were observed in the skeletal muscle, liver and heart of obese Zucker rats compared to lean zucker rats and are associated with alterations in the phosphorylation of MAPK family members. Levels of mir-1and 133a were altered in skeletal and cardiac muscles of obese zucker rats when compared with their lean counterparts. **Conclusions:** These results suggest that MAPK members regulate GLUT4 expression differently in various insulin resistant tissues compared to lean counterparts and that these changes may play a key role in insulin resistance associated with glucose dysregulation.

Key words: Insulin resistance, Akt, P38, Erk1/2, JNK, MEF2, GLUT4

Introduction

The Centers for Disease Control and Prevention estimates that nearly 26 million Americans or about 8% of the total population are diabetic [10]. Diabetes remains the sixth leading cause of death in the United States and costs $174 billion annually, including $116 billion in direct medical expenses. Perhaps most worrisome is the fact that an additional 35% of all adults are considered pre-diabetic.

Type 2 diabetes accounts for about 90-95% of all diabetes cases and is thought to be caused, at least in part, by insulin resistance [3]. The regulation of glucose uptake into the cell in response to elevations in extracellular insulin is not fully understood. A key regulator of insulin-induced glucose uptake is the glucose transporter (GLUT4) [4]. GLUT4 redistributes from the intracellular membrane pool to plasma membrane upon insulin stimulation [41]. Activation of insulin receptor by insulin is thought to stimulate the PI3-kinase pathway. The PI3-kinase, in turn, recruits the serine/threonine protein kinases protein kinase B (PKB or Akt) and PDK1 [5], which results in the stimulation of glucose uptake [6-8]. In addition to Akt, the mitogen activated protein kinases (MAPK) are also thought to play a role in the GLUT4 expression and relocation. The p38-MAPK has been found to be involved in the regulation of GLUT4 expression by activating the transcription factor MEF2, which functions to activate the GLUT4 promoter [9]. Besides p38-MAPK, regulation of glucose uptake has also been found to be influenced by the extracellular-regulated kinase 1/2 (ERK1/2)–MAPK as the phosphorylation of this molecule is thought to be involved in regulating insulin receptor signaling and GLUT4 translocation [42-44]. Conversely, the activation of c-Jun N-terminal Kinase (JNK) has been found to be impair the insulin-signaling pathway by serine phosphorylation of IRS proteins [45]. How insulin

regulated signaling and GLUT4 expression is regulated in different tissues is to our knowledge, not been investigated.

The purpose of this study was to determine whether insulin resistance alters insulin related signaling mechanisms differently in skeletal muscle, liver, kidney and heart. To accomplish this goal, we examined the regulation of Akt, MAPK proteins, MEF2A and GLUT4 in normal and insulin resistant obese Zucker rats. Taken together, our findings suggest that insulin related signaling appears to be regulated differently in different insulin sensitive tissues.

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 Lean Zucker (LZ, *n* =6) and Obese Zucker (OZ, *n* =6) rats were obtained from the Charles River Laboratories. All animals were 12 weeks of age at completion of this study. Rats were housed two per cage in an American Association for the Accreditation of Laboratory Animal Care approved vivarium. Housing conditions consisted of a 12:12-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.

Tissue collection

Rats were anaesthetized with a ketamine-xylazine (4:1) cocktail (50 mg/kg i/p) and supplemented as necessary for reflexive response before tissue collections. The plantaris muscles, liver, kidney, heart were collected, trimmed of excess connective tissue, weighed and flash frozen in liquid nitrogen. Samples were stored at -80 °C until further analysis.

Tissue homogenization and determination of protein concentration

Muscles, liver, kidney, heart samples were homogenized in Pierce Tissue Protein Extraction Reagent (10 ml/g tissue; Rockford) that contained protease inhibitors (P8340; Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitors (P5726; Sigma-Aldrich). 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). Homogenate samples were boiled in a Laemmli 2X sample buffer (Sigma-Aldrich) for 5 min.

SDS-PAGE and immunoblotting

Total protein from each sample (45µg) was separated on a 10% PAGEr Gold Precast gel (Lonza, Rockland, ME) and then transferred to a nitrocellulose membrane. Visual verification of transfer and equal protein loading among lanes were accomplished by Ponceau S staining of the membranes. For immunodetection, membranes were blocked for 1 h at room temperature in blocking buffer [5% nonfat dry milk in 20 mMTris-base, 150mM NaCl, and 0.05% Tween 20 (TBS-T), pH 7.6], serially washed in TBS-T at room temperature, and then probed with antibodies for the detection of Akt (#9272), phospho-Akt (Ser473) (# 9271), PTEN (# 9552),

phospho-PTEN (Ser380/Thr382/383) (#9549), p38 MAPK (#9212), phospho p38 MAPK (# 9211), P44/42 MAPK (ERK1/2) (#9102), phospho p44/42 MAPK (Thr202/Tyr204) (#9101), SAPK/JNK (#9252), phospho SAPK/JNK (Thr183/Tyr185), MEF2A (#9736), GLUT4 (#2299) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#2118) (Cell Signaling Technology ,Beverly, MA). Membranes were incubated overnight at 4°C in primary antibody buffer (5% BSA in TBS-T, pH 7.6. Primary antibody diluted 1:1,000) followed by washing in TBS-T (5 min each), and incubation with horseradish peroxidase conjugated secondary antibody [anti-rabbit (# 7074) or anti-mouse (#7076); Cell Signaling Technology, Danvers, MA] in blocking buffer for 1 h. After removal of the secondary antibody, membranes were washed (3x5 min) in TBS-T, and protein bands were visualized on reaction with ECL reagent (Amersham ECL Western Blotting reagent RPN 2106; GE Healthcare Bio-Sciences, Piscataway, NJ). Target protein levels were quantified by Alpha Ease FC image analysis software (Alpha Innotech, San Leandro, CA) and normalized to GAPDH.

RNA extraction and miRNA analysis

Skeletal muscle, heart, kidney and liver of lean and obese Zucker rats are homogenized Individually, on ice in a TRI reagent (Ambion, Austin, TX) at a ratio of 1 mL of TRI reagent Per 100 mg of tissue. The homogenate was incubated for 5 minutes on ice to allow nucleoprotein complexes to completely dissociate, and centrifuged at 4°C for approximately for 15 mins at 12,000 x g. The supernatant was separated from the pellet and transferred to a 2 ml RNAse-free tube. A 200-µl aliquot of chloroform (Sigma-Aldrich, Inc., St. Louis, MO) was added to the supernatant. The solution was mixed and incubated at room temperature for 10 mins, followed by centrifugation at 4°C for 15 mins at 12,000 x g. The aqueous phase of the solution was

collected and transferred to another tube. A 500- µl aliquot of isopropanol (Sigma-Aldrich, Inc., St. Louis, MO) was added to the aqueous phase, followed by thorough mixing. The resultant solution was incubated at room temperature for 10 mins, followed by centrifugation at 4 °C for 10 minutes at 12,000 x g. The supernatant was then carefully removed without disturbing the pellet. The RNA pellet was washed free of salts by centrifugation in 75% ethanol at 7500 x g for 5 minutes. The ethanol wash was carefully removed without disturbing the pellet, and the residual ethanol was evaporated at room temperature by speed vacuuming for 1 minute. The RNA pellet was dissolved in 50 µl of RNAse free water and quantified at 260 nm using a NanoVue UV-Vis Spectrophotometer (GE Healthcare, Piscataway, NJ). The integrity of the RNA from each sample was determined through use of RNA 6000 Nano Kit and an Agilent 2100 Bio analyzer (both from Agilent Technologies, Santa Clara, CA). Two micrograms of RNA were reverse transcribed (RT) using the QuantiMiR RT kit (System Bioscience) according to standard methods (Systems Bioscience). Control RT reactions were done in which the RT enzyme was omitted. The control RT reactions were PCR amplified to ensure that DNA did not contaminate the RNA. Cyber green-based real-time qPCR was performed by using a 7500 Real-Time PCR system (Applied Bio systems, Foster City, CA) and gene-specific primers for the miRNA of interest designed according to the guidelines outlined in the QuantiMiR RT kit (System Bioscience). Melt analysis was used after each PCR run to ensure amplification of only a single product. All samples were normalized to an internal control housekeeping gene (U1 small nuclear RNA (snRNA)). Relative fold changes in miRNA within each group and basal differences between groups were determined from the Ct values after normalization to their respective housekeeping genes using the $2^{\Delta ct}$ method.

Statistical analysis

Results are presented as means ± SE. Data were analyzed using the Sigma Stat 3.5 statistical program. The effects of insulin resistance on protein phosphorylation were analyzed using a t- test. Differences were considered Significant at $P < 0.05$.

Results

Insulin resistance decreases muscle and liver GLUT4 levels.

GLUT4 levels were 16-, 33- and 45% lower in the obese Zucker plantaris, liver and heart, respectively (P<0.05). Conversely, GLUT4 expression was 51% higher in the obese Zucker kidney (P<0.05) (Figure 1).

Insulin resistance decreases the amount of Akt phosphorylation in skeletal muscle and liver.

Compared to lean animals, the ratio of phosphorylated (Ser 473) to total Akt was 32% and 27% lower in the skeletal muscle and liver of the obese Zucker rats (P<0.05) (Figure 2). Conversely, the ratio of p-Akt to total Akt was 12 and 11% higher in the obese Zucker heart and kidney (P<0.05). Compared to that observed in the lean animals, the ratio of phosphorylated (Ser380/Thr382) to total PTEN were 19- and 10% higher in the plantaris and liver, respectively (P<0.05) (Figure 4). Compared to that seen in the lean animals, the ratio of p-PTEN to PTEN was 8- and 24% lower in the obese Zucker rat heart and kidney (P<0.05).

Insulin resistance affects the phosphorylation of MAPK proteins

Compared to that observed in the lean Zucker animals, the ratio of phosphorylated p38 (Thr180/Tyr 182) 21-, 122- and 19% higher in the obese Zucker skeletal muscle, liver and kidney, respectively ($P<0.05$) (Figure 3).

Compared to that seen in the lean animals, the ratio of phosphorylated ERK1/2 (Thr202/Tyr204) to total ERK1/2 levels were higher in skeletal muscle (46%) but 19- and 39% lower in the liver and heart of the obese Zucker rats ($P<0.05$) (Figure 5). Similar to what we observed for ERK1/2, the ratio of phosphorylated (Thr183/Tyr185) to total JNK was 29% higher in skeletal muscle (29%) and 19% lower in the hearts of the obese Zucker rats compared with their lean counterparts ($P<0.05$) (Figure 6). MEF2A protein levels were 17- and 19% lower in the muscle and hearts of obese Zucker rats ($P<0.05$) (Figure 7).

Insulin resistance is associated with alterations in miRNA expression

The relative expression of mir-1 was 63% lower in the hearts of the obese Zucker rat compared to that observed in the control animal ($P<0.05$) (figure 8) Conversely, mir-1 expression was 218% higher in the insulin resistant muscle. The expression of mir-133a was lower in obese Zucker skeletal muscle (96%) ($P<0.05$) but 120% higher in the obese Zucker heart (120%) (Figure 9). Compared to lean animals, the relative expression of mir-223 was 88- and 93% lower in the skeletal and hearts of the obese Zucker animals ($P<0.05$). Conversely, mir-223 expression was 250% higher in the liver of obese compared to lean Zucker animals ($P<0.05$) (Figure 10).

Discussion

Insulin resistance is thought to be one of the primary causes of Type 2 diabetes [1]. Liver, skeletal muscle and fat are important insulin sensitive tissues that play a primary role in the regulation of blood glucose levels. Impaired insulin sensitivity can affect cellular metabolism and lead to an increased reliance on fatty acids for the production of energy. This response, while first adaptive, can led to increased secretion of inflammatory cytokines and a further decrease in the insulin sensitivity of other tissues [49, 50].

The primary finding of this investigation was that the mechanism of insulin resistance appears to be regulated differently in different tissue types. Specifically, we found that the amount of GLUT4 appeared to be lower in the muscle, heart and liver but not in the kidney of the obese Zucker rats (Figure 1). These changes in GLUT4 expression were, in turn, associated with alterations in Akt, MAPK, and MEF2 expression and activation that appeared to differ between tissue types.

Insulin-stimulated translocation of GLUT4 to the cell membrane has been shown to be a key regulator of glucose uptake [46]. King et al demonstrated that insulin resistance in gastrocnemius muscle of obese rats is characterized by an inability to activate Glut4 translocation following insulin stimulation [47]. Similar findings have been reported by Wai et al in hearts of obese Zucker rats who showed that the cardiac expression of GLUT4 in the plasma membrane was depressed in the fatty Zucker rat in both the basal and insulin stimulated states [48]. The mechanism(s) responsible for the diminished activation of GLUT4 following insulin stimulation are not clear however recent data has demonstrated that the activation of protein kinase B (Akt) and p38-MAPK may play a pivotal role [34].

Herein we demonstrate that insulin resistance is characterized by decreases in the amount of phosphorylated (activated) Akt and increases in the amount of phosphorylated p38-MAPK in the skeletal muscle and liver of the obese Zucker rat (Figures 2 and 3). Shao and coworkers suggested that that decreased insulin-stimulated Akt kinase activity but not MAPK is an important component underlying impaired GLUT4 translocation and insulin resistance in skeletal muscle of db/db mice [49]. Niu and colleagues observed that GLUT4 activity is regulated by insulin in skeletal muscle cells during their maturation into myotubes and that this process is related to the ability of insulin to stimulate p38 MAPK [50]. Subsequently Christophe et al found that p38 is a strong inducer of GLUT4 expression in cardiac myocyte [9]. Taken together, these findings suggest that the regulation of GLUT4 by p38 MAPK appears to differ across various tissues and perhaps at different time points within the same tissue.

PTEN is a dual specificity phosphatase protein involved in tumor suppression and is a negative regulator of the Akt signaling pathway. Previous studies have shown that muscle specific deletion of PTEN resulted in enhanced insulin stimulated glucose uptake and Akt phosphorylation in soleus but not in the extensor digitorum longus muscle, suggesting that the activity of PTEN in glucose regulation is restricted to certain tissues or perhaps dependent on other factors [51]. Shen and colleagues recently demonstrated that resistin mediated PTEN expression is regulated by p38 MAPK signaling, which may activate target transcription factor ATF-2 in human endothelial cells [52]. Herein, we observed that the ratio of phosphorylated to total PTEN are increased in the obese Zucker skeletal muscle and liver but decreased in obese hearts and kidneys (Figure 4). Whether these findings are directly related to the elevations we see if p38 MAPK in the obese Zucker heart and liver is currently unclear.

Besides p38 MAPK, it is also thought that ERK1/2 and JNK MAPK may also play a role in the development of insulin resistance. Jiro and coworkers demonstrated that JNK1 results in substantial protection from obesity-induced insulin resistance [53]. Other work has shown that the activation (phosphorylation) of JNK-MAPK inhibits insulin signaling in the rat soleus muscle by enhancing the phosphorylation of IRS1 at Ser 307 [54]. Bouzakri et al demonstrated that ERK1/2 is phosphorylated to a greater extent in skeletal muscle cells in type 2 diabetic patients and that this finding is correlated with phosphorylation of IRS-1 at Ser 636 [55] .Consistent with this finding, we observed an elevation in the amount of phosphorylated ERK1/2, JNK -MAPK in the skeletal muscle of the obese Zucker rats (Figure 5, 6).

Myocyte enhancer factor 2A is a member of MEF2 family transcription factors which act as muscle-specific DNA binding proteins. Work by Martin et al., in 1998 showed that MEF2 binding activity is necessary for GLUT4 regulation in skeletal muscle and adipocytes [56]. Around this same time Zhao and colleagues found that p38 MAPK regulates MEF2A activity by phosphorylating the MEF2A molecule at threonine residues 312 and 319 [57]. More recently, work by Morgia and co-workers demonstrated that regulation of GLUT 4 enhancer is dependent on MEF2 activity [58]. Consistent with the work of Martin, and our findings of decreased GLUT4 expression in the skeletal muscle and hearts of the obese Zucker rat, we also demonstrate that the expression of MEF2A was lower in the diabetic heart and skeletal muscle.

In addition to the potential role that different protein signaling pathways may play in the regulation of GLUT4 emerging evidence also suggests that miRNA may also be involved. MiRNA are short RNA molecules which act as post-transcriptional regulators by binding to complementary mRNA sequence. Recent studies have shown that mir-133a and 223 regulate the expression of Glut4 in cardiac myocytes [40]. Other work has shown that mir-133a expression is

elevated in the diabetic rabbit heart [59]. In the present study we found that the expression of mir-133a is higher while mir-223 expression is lower in the insulin resistant heart. Iai et al conducted an extensive study and showed that mir-133a is lowered in skeletal muscle biopsies of diabetic humans [60]. We demonstrate a similar finding here where we found that the expression of mir-133a, and mir-223 was lower in obese skeletal muscle compared to lean skeletal muscle [60]. Whether these changes in miRNA expression are directly related to the changes in GLUT4 we observe in the obese Zucker skeletal muscle is currently unclear.

Taken together, our data suggests that GLUT4 regulation appears to be regulated by different mechanisms in different tissues. In skeletal muscle, diminished GLUT4 levels appear to be correlated with decreases in Akt activation, MEF2A and mir-223 expression. In the liver, GLUT4 appears to be regulated, at least in part, through diminished Akt and ERK1/2 MAPK activation while in the heart, GLUT4 regulation is tied to decreased ERK1/2 MAPK, JNK-MAPK, and MEF2A and mir-223 expression. Given these data, it is likely that future studies perhaps looking at other molecules are likely needed to further understand the regulation of GLUT4 expression in insulin sensitive tissues.

CHAPTER 4

Conclusions

Type 2 diabetes is characterized by insulin resistance which leads to hyperglycemia. According to the survey conducted by NIH about 72,000 people died in United States in 2007 alone due to the effects of diabetes. The main intent of the current study was to investigate how insulin resistance affected the expression and activation of signaling molecules thought to play key roles in the insulin- stimulated glucose uptake. The following conclusions were drawn from the present study:

1. The skeletal muscle, heart and liver of the obese Zucker rat's exhibits decreased GLUT4 protein expression.
2. In skeletal muscle, diminished GLUT4 levels appear to be correlated with decreases in Akt activation, MEF2A and mir-223expression.
3. In the liver, GLUT4 appears to be regulated, at least in part, through diminished Akt and ERK1/2 MAPK activation.
4. In the heart, GLUT4 regulation is tied to decreased ERK1/2 MAPK, JNK-MAPK, MEF2A and mir-223 expression.
5. The kidneys of obese Zucker rat exhibit increases in GLUT4 expression which may be related to increased p38 MAPK activation.

Future Directions

Future directions based on this study should focus on examining other molecules thought to be involved in the regulation of insulin sensitivity or other *in vitro* models which may permit the testing of how changes in gene expression may directly affect GLUT4 protein expression. Other work could also examine the effects of the p38 inhibitor SB-681323 on MEF2 phosphorylation and how this might affect GLUT mRNA levels.

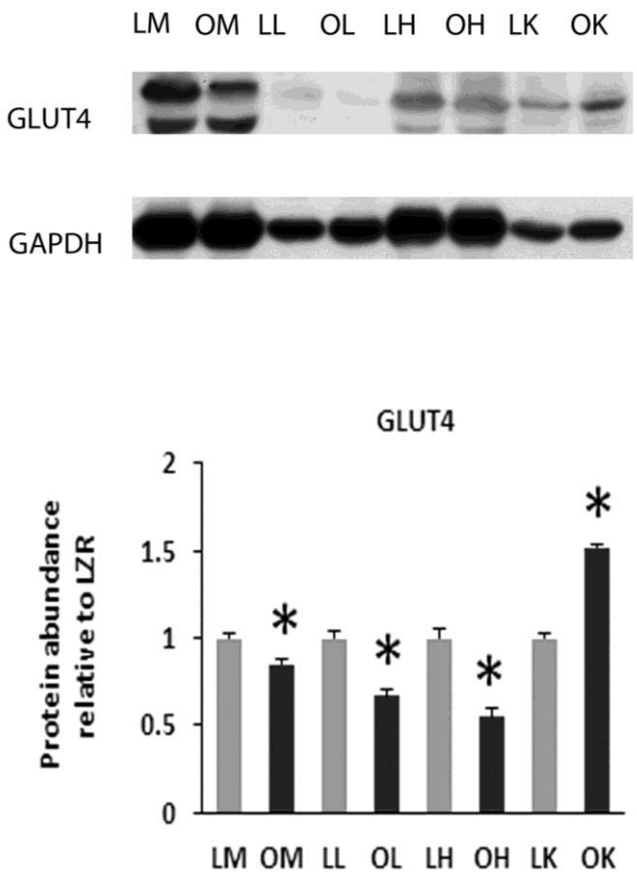


Figure 3. Total levels of GLUT4 are altered in diabetic rats

Total levels of GLUT4 in different tissues of lean and obese Zucker male rats at 12weeks of age. Results are expressed as arbitrary units for comparison.GAPDH was used as an internal control. An asterisk (*) indicates significant differences in obese Zucker rats from the lean Zucker age matched controls ($P< 0.05$). Abbreviations: LM, lean skeletal muscle; OM, obese skeletal muscle; LL, lean liver; OL, obese liver; LH, lean heart; OH, obese heart; LK, lean kidney; OK, obese kidney.

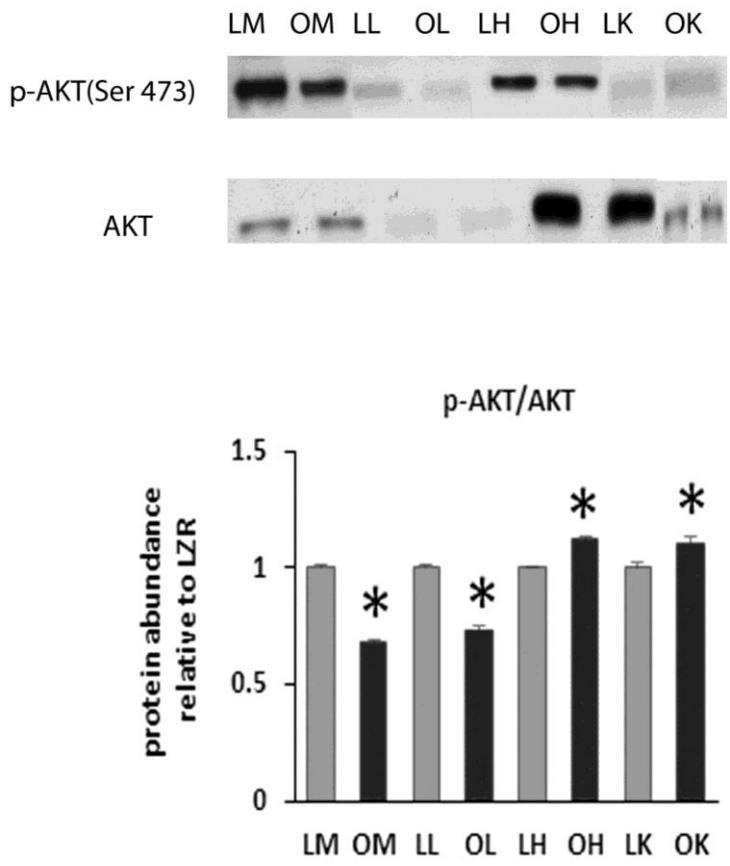


Figure 4. Phosphorylated to total levels of Akt are altered in diabetic rats.

Phosphorylated to total levels of Akt in different tissues of lean and obese Zucker male rats at 12 weeks of age. Results are expressed as arbitrary units for comparison. An asterisk (*) indicates significant differences in obese Zucker rats from the lean Zucker age matched controls ($P< 0.05$). Abbreviations: LM, lean skeletal muscle; OM, obese skeletal muscle; LL, lean liver; OL, obese liver; LH, lean heart; OH, obese heart; LK, lean kidney; OK, obese kidney.

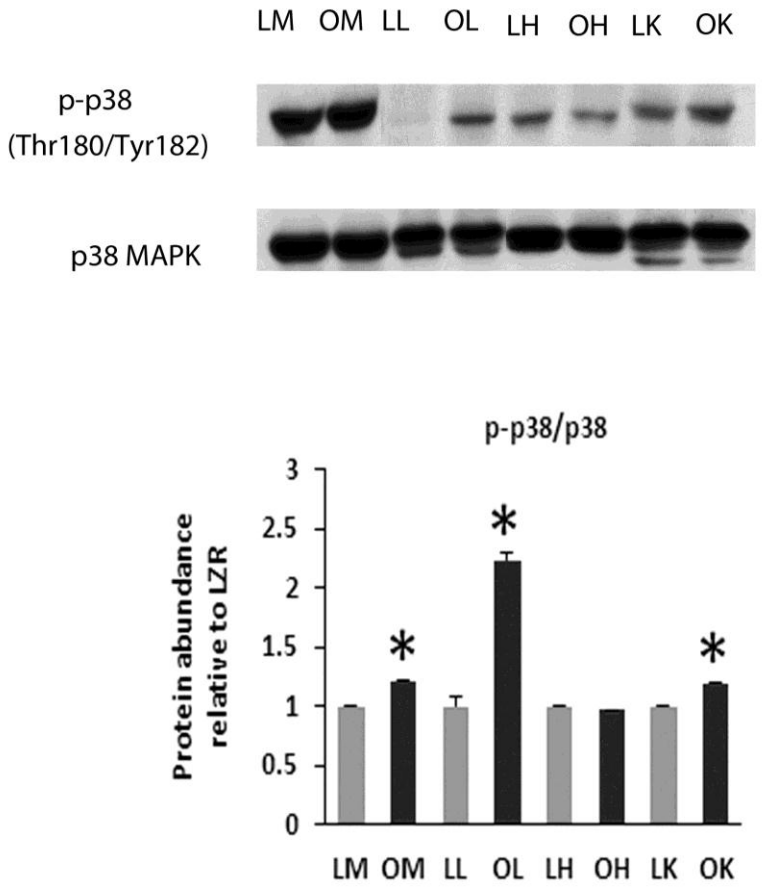


Figure 5. Phosphorylated to total levels of p38MAPK are altered in diabetic rats.

Phosphorylated to total levels s of p38MAPK in different tissues of lean and obese Zucker male rats at 12 weeks of age. Results are expressed as arbitrary units for comparison. An asterisk (*) indicates significant differences in obese Zucker rats from the lean Zucker age matched controls ($P< 0.05$). Abbreviations: LM, lean skeletal muscle; OM, obese skeletal muscle; LL, lean liver; OL, obese liver; LH, lean heart; OH, obese heart; LK, lean kidney; OK, obese kidney.

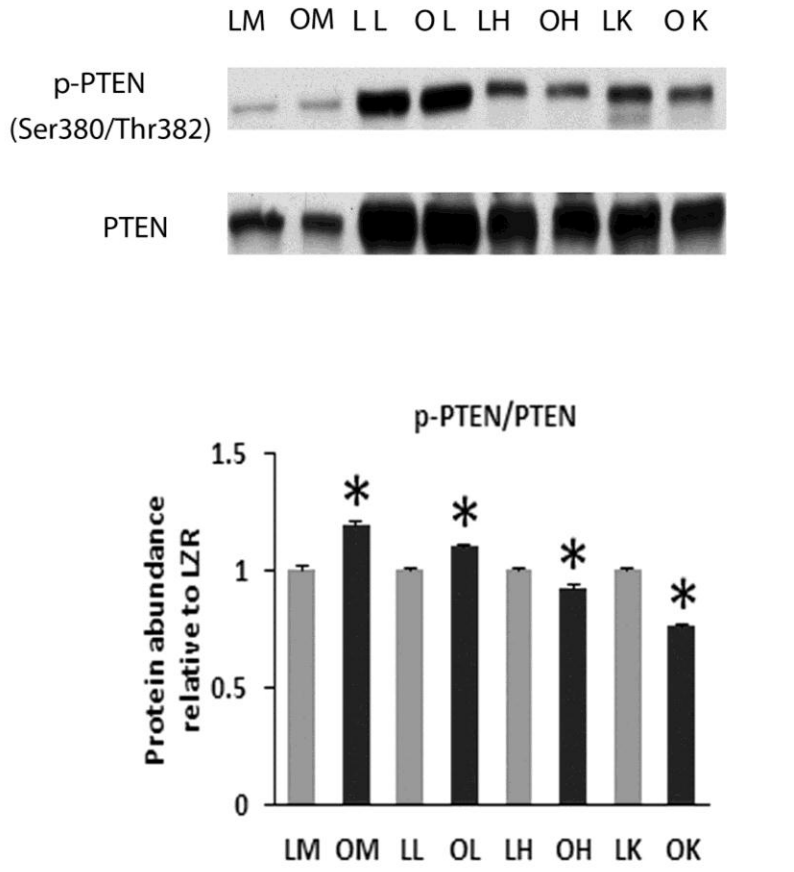


Figure 6. Phosphorylated to total levels of PTEN are altered in diabetic rats.

Phosphorylated to total levels of PTEN in different tissues of lean and obese Zucker male rats at 12 weeks of age. Results are expressed as arbitrary units for comparison. An asterisk (*) indicates significant differences in obese Zucker rats from the lean Zucker age matched controls ($P< 0.05$). Abbreviations: LM, lean skeletal muscle; OM, obese skeletal muscle; LL, lean liver; OL, obese liver; LH, lean heart; OH, obese heart; LK, lean kidney; OK, obese kidney.

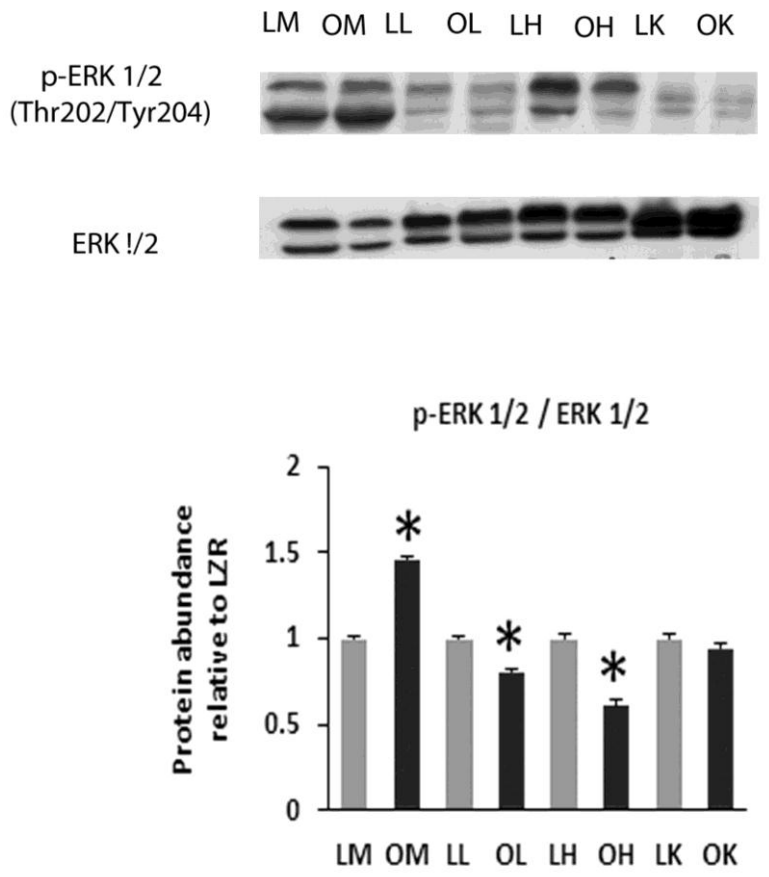


Figure 7. Phosphorylated to total levels of ERK1/2 are altered in diabetic rats.

Phosphorylated to total levels of ERK1/2 in different tissues of lean and obese Zucker male rats at 12 weeks of age. Results are expressed as arbitrary units for comparison. An asterisk (*) indicates significant differences in obese Zucker rats from the lean Zucker age matched controls ($P< 0.05$). Abbreviations: LM, lean skeletal muscle; OM, obese skeletal muscle; LL, lean liver; OL, obese liver; LH, lean heart; OH, obese heart; LK, lean kidney; OK, obese kidney.

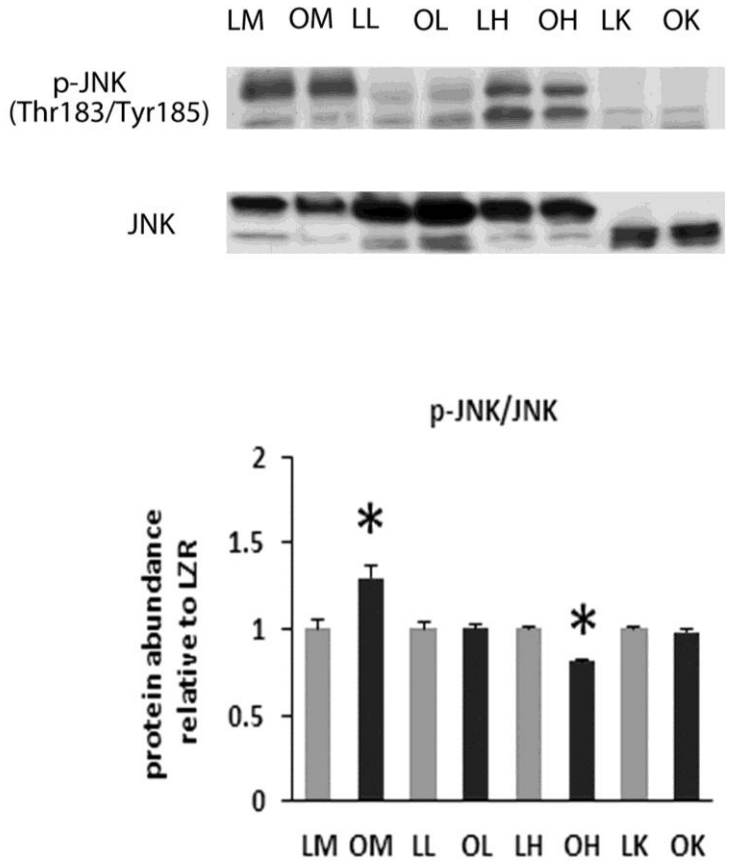


Figure 8. Phosphorylated to total levels of JNK are altered in diabetic rats.

Phosphorylated to total levels of JNK in different tissues of lean and obese Zucker male rats at 12 weeks of age. Results are expressed as arbitrary units for comparison. An asterisk (*) indicates significant differences in obese Zucker rats from the lean Zucker age matched controls ($P < 0.05$). Abbreviations: LM, lean skeletal muscle; OM, obese skeletal muscle; LL, lean liver; OL, obese liver; LH, lean heart; OH, obese heart; LK, lean kidney; OK, obese kidney.

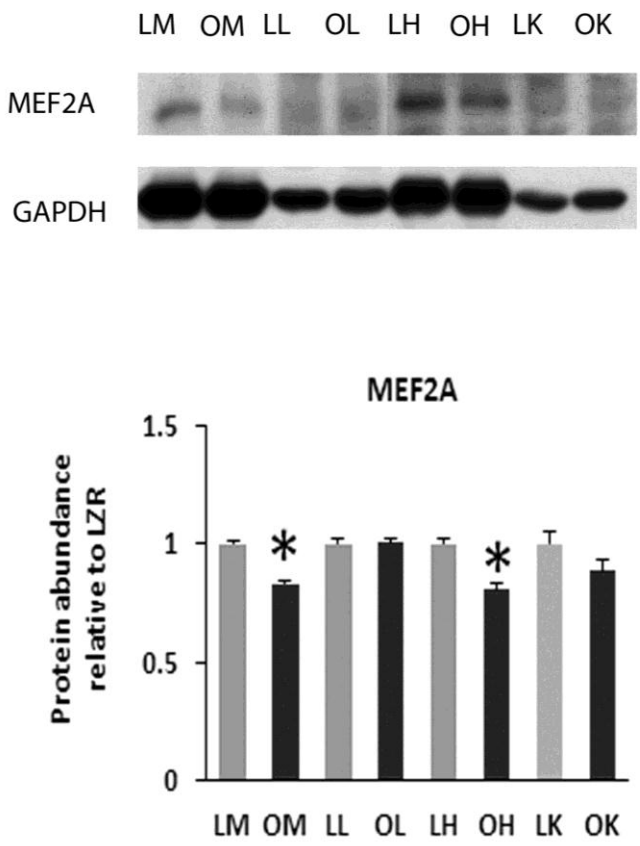


Figure 9. Total levels of MEF2A are altered in diabetic rats.

Total levels of MEF2A in different tissues of lean and obese Zucker male rats at 12weeks of age. Results are expressed as arbitrary units for comparison. GAPDH was used as an internal control. An asterisk (*) indicates significant differences in obese Zucker rats from the lean Zucker age matched controls ($P< 0.05$).

Abbreviations: LM, lean skeletal muscle; OM, obese skeletal muscle; LL, lean liver; OL, obese liver; LH, lean heart; OH, obese heart; LK, lean kidney; OK, obese kidney.

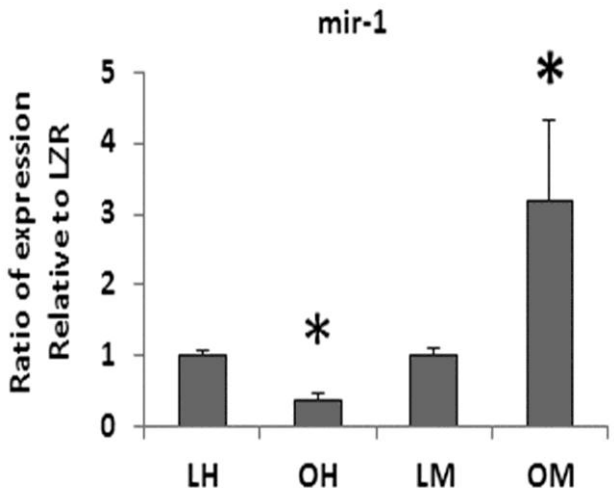


Figure 10. The expression of mir-1 miRNA is altered in diabetic rats.

Ratio of expression mir-1 miRNA in different tissues of lean and obese Zucker male rats at 12weeks of age. An asterisk (*) indicates significant differences in obese Zucker rats from the lean Zucker age matched controls ($P< 0.05$). Abbreviations: LH, lean heart; OH, obese heart; LM, lean skeletal muscle; OM, obese skeletal muscle.

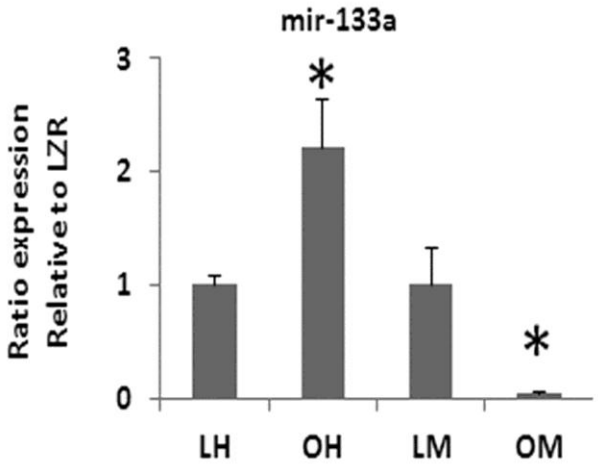


Figure 11. The expression of mir-133a miRNA is altered in diabetic rats.

Ratio of expression of mir-133a miRNA in different tissues of lean and obese Zucker male rats at 12weeks of age. An asterisk (*) indicates significant differences in obese Zucker rats from the lean Zucker age matched controls ($P< 0.05$). Abbreviations: LH, lean heart; OH, obese heart; LM, lean skeletal muscle; OM, obese skeletal muscle.

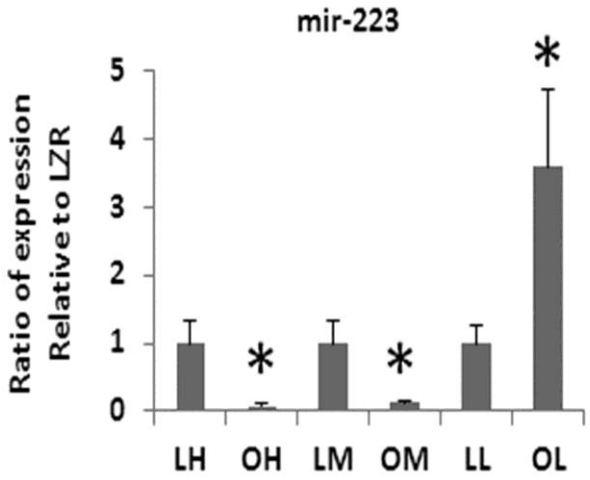


Figure 12. The expression of mir-233 miRNA is altered in diabetic rats.

Ratio of expression of mir-233 miRNA in different tissues of lean and obese Zucker male rats at 12weeks of age. An asterisk (*) indicates significant differences in obese Zucker rats from the lean Zucker age matched controls ($P< 0.05$). Abbreviations: LH, lean heart; OH, obese heart; LM, lean skeletal muscle; OM, obese skeletal muscle; LL, lean liver; OL, obese liver.

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