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titled

The Effect of FoxO1 on Glycemic Control and Skeletal Muscle Glucose Uptake and Lipid Metabolism

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in

Exercise Science

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An Abstract of

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Previous research has indicated a possible role for Forkhead Box protein O1 (FoxO1) in the pathogenesis of type 2 diabetes through its purported role in affecting whole body glycemic control and lipid metabolism. However, the literature indicating such a relationship is limited and has primarily relied on *in vitro* models. Thus, the purpose of the present study was to use *in vivo* and *ex vivo* methodology to examine the role of FoxO1 on whole body glycemic control, skeletal muscle glucose metabolism and lipid metabolism. Transgenic mice with skeletal muscle-specific overexpression of FoxO1 were subjected to intraperitoneal glucose tolerance testing (IPGTT) with concomitant plasma insulin analysis followed by *ex vivo* examination of skeletal muscle insulin stimulated glucose uptake, intramyocellular triglyceride (IMTG) storage and fatty acid oxidation. IPGTT and plasma insulin analysis revealed no differences between FoxO1 transgenic and wildtype (WT) mice. Likewise, basal level skeletal muscle protein expression levels of phosphoinositide kinase-3 (PI3K) and serine$^{473}$ phosphorylated Akt (pAkt$^{473}$) were not different between mouse strains. Additionally, FoxO1 did not exhibit
any impairment in insulin stimulated glucose uptake. However, IMTG and fatty acid oxidation capacity was significantly (p<0.05) reduced in FoxO1 transgenic mice. These findings indicate that \textit{in vivo} skeletal muscle FoxO1 overexpression does not impede whole body glycemic control or skeletal muscle insulin action. However, it does appear to reduce IMTG while impairing skeletal muscle lipid oxidation capacity.
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**List of Abbreviations**

Akt/PKB…………….Protein Kinase B
Apo………………….Apolipoprotein
ATGL……………….Adipose triglyceride lipase

IRS-1……………….Insulin receptor substrate-1
DAG………………..Diacylglycerol

FA………………….Fatty acid
FoxO1…………….Forkhead Box (O1)

G6P………………..Glucose-6-phosphatase
GLUT4…………….Glucose transporter protein-4

IMTG…………….Intramyocellular triglyceride
IPGTT……………..Intraperitoneal glucose tolerance test

JNK………………c-Jun kinase

LPL……………….Lipoprotein lipase

MTP……………….Microsomal triglyceride transfer protein
MuRF-1…………..Muscle ring finger protein-1

PEPCK…………….Phosphoenolpyruvate carboxylase kinase
PGC-1α…………..Proliferator-activated receptor gamma-coactivator 1-α
PKC……………….Protein Kinase C
PDK1/2…………..PI3K dependent kinase 1/2
PDK……………….Pyruvate dehydrogenase kinase
PDX1………………...Pancreatic homeobox factor-1
PI3K………………...Phosphoinositide kinase-3
PPAR………………...Peroxisome proliferator activated receptor

T2DM………………..Type 2 diabetes mellitus
Tg………………...Transgenic

VLDL………………..Very low density lipoprotein

WT………………...Wildtype
List of Symbols

*............................Statistical significance
Chapter 1

Introduction

Type 2 diabetes mellitus (T2DM) is a public health epidemic that threatens the economies of all nations. In 2012, the total cost for diabetes in the United States was $245 billion.\(^1\) According to the Centers for Disease Control (CDC), diabetes affects 25.8 million Americans with an estimated yearly increase of 1.9 million new cases.\(^2\) The global rise in T2DM mirrors that of obesity, exposing the danger of increasingly sedentary lifestyles and the overabundance of non-nutritive food. Additionally, the glucotoxicity associated with T2DM weakens both macro and microvasculature leaving those with T2DM two to four times more likely to die from heart disease, suffer a stroke or develop blindness, kidney disease or severe neuropathy.\(^2\)

T2DM is typically developed in adulthood and is characterized by insulin resistance in liver, adipose and skeletal muscle tissue.\(^3\) Consequently, pancreatic beta-cell compensation causes hypersecretion of insulin to maintain euglycemia. The disease progresses as various factors lead to beta-cell deterioration and severe hyperglycemia.\(^4,\,5\) The pathogenesis of T2DM is complex as dietary habits, aging, genetics and disease may cause insulin resistance. The metabolic triggers of insulin resistance bring about
excessive circulating glucose and fatty acids, which threaten normal intracellular insulin signaling and glucose oxidation.\textsuperscript{3, 6}

Recently, Forkhead Box O1 (FoxO1), a transcription factor and Forkhead Box family member has drawn scrutiny in relation to T2DM for its apparent role in antagonizing insulin action in the liver, adipose tissue, pancreatic beta-cells and skeletal muscle. In the liver, FoxO1 stimulates the production and secretion of glucose by increasing the expression of phosphoenolpyurate carboxylase kinase (PEPCK) and Glucose-6-Phosphatase (G6P), two critical gluconeogenic genes.\textsuperscript{7-9} Additionally, FoxO1 promotes hypertriglyceridemia by activating transcription of microsomal triglyceride transfer protein (MTP) and apolipoprotein CIII (apoCIII), two integral components in the assembly and release of hepatic very low-density lipoprotein (VLDL).\textsuperscript{10-12} FoxO1 exacerbates lipemia and systemic insulin resistance by mobilizing stored fatty acids through expression adipose triglyceride lipase (ATGL) while encouraging the deterioration of adipose tissue via repression of peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)).\textsuperscript{13-15} In the pancreas, FoxO1 stems the activity of pancreatic and duodenal homeobox factor-1 (Pdx1), diminishing the growth and proliferation of beta-cells required for compensatory hyper-insulin secretion in T2DM.\textsuperscript{16}

The effects of FoxO1 in the pathogenesis of T2DM is perhaps most profound in skeletal muscle which accounts for nearly eighty-percent of total body glucose disposal.\textsuperscript{17} Limited \textit{in-vivo} work suggests that skeletal muscle-specific overexpression of FoxO1 impairs glucose and insulin tolerance in mice.\textsuperscript{18} Various \textit{in-vitro} models have shown that FoxO1 inhibits skeletal muscle differentiation. This can occur through several pathways regulated by FoxO1 including upregulation of Notch and myostatin and proteasomal
degradation of mammalian target of rapamycin (mTOR).\textsuperscript{19-21} FoxO1 also mediates the process of skeletal muscle loss by activating the expression of atrogin-1/MAFbx and muscle ring finger-1 (MuRF-1), two integral effectors in ubiquitin proteasomal pathway-mediated skeletal muscle protein degradation.\textsuperscript{22-24}

Extensive study in skeletal muscle suggests that FoxO1 may also control the fuel switch from carbohydrate to fatty acids common in T2DM. Importantly, increased reliance on fatty acid oxidation increases the production of metabolites such as diacylglycerol (DAG). DAG acts through protein kinase C-θ (PKCθ) to inhibit insulin-stimulated activation of the phosphoinositide-3 kinase (PI3K)/Protein Kinase B (Akt) pathway.\textsuperscript{25} Similarly, increased fatty acid oxidation amplifies levels of acetyl-CoA which hinders glucose oxidation through the activation of pyruvate dehydrogenase kinases (PDKs).\textsuperscript{26}

In cultured muscle cells, FoxO1 has been shown to regulate the entry of fatty acids via direct transcriptional control over muscle lipoprotein lipase (LPL).\textsuperscript{27} Similar models have revealed that FoxO1 increases fatty acid uptake by regulating membrane distribution of fatty acid translocase, CD36.\textsuperscript{28} Further \textit{in-vitro} analysis has demonstrated that ectopic expression of FoxO1 increases oxidation in muscle cells.\textsuperscript{28} This appears to be accomplished by two main mechanisms. First, FoxO1 activation induces the expression of pyruvate dehydrogenase kinase-4 (PDK4), the major inhibiting force of skeletal muscle carbohydrate oxidation.\textsuperscript{28, 29} Second, FoxO1 promotes the oxidation of fatty acids over their synthesis by stimulating the expression of genes such as peroxisome proliferator-activated receptor gamma-coactivator 1-α (PGC1-α), acyl-CoA oxidase
(ACO) and peroxisome proliferator receptor-activated receptor-δ (PPARδ) while suppressing acetyl-CoA carboxylase (ACC)\textsuperscript{28,30}.

Though comprehensive \textit{in-vitro} examination of FoxO1 in skeletal muscle metabolism suggests a potential role for FoxO1 in T2DM, it is imperative to corroborate these findings \textit{in-vivo}. Therefore, the objective of this project is to investigate the impact of FoxO1 on: 1) whole-body glycemic control, 2) skeletal muscle glucose metabolism and 3) skeletal muscle lipid metabolism. Our central hypotheses are that FoxO1 reduces whole-body glycemic control and skeletal muscle glucose uptake while increasing fatty acid oxidation and impairing intracellular insulin signaling.

**Specific Aim 1: Determine the effect of FoxO1 on whole-body glycemic control.** This aim will be achieved by administering intraperitoneal glucose tolerance tests (IPGTT) to both wild-type and mice with skeletal muscle-specific FoxO1 overexpression. Blood samples will be obtained at all time points during the IPGTT for insulin analysis. We hypothesize that FoxO1 transgenic mice will display impaired glucose and insulin tolerance.

**Specific Aim 2: Determine the effect of FoxO1 on skeletal muscle glucose metabolism.** The first part of this aim will be achieved by excising the gastrocnemius muscle from wildtype and FoxO1 transgenic mice to analyze protein expression levels of Phosphoinositide-3 kinase (PI3K) and Protein Kinase B (Akt), two key insulin signaling enzymes. The second part of the aim will be completed by harvesting the soleus muscle from a different set of mice for \textit{ex vivo} analysis of insulin stimulated glucose uptake. We hypothesize that both insulin signaling activity and insulin stimulated glucose uptake will be diminished in FoxO1 muscles.
Specific Aim 3: Determine the effect of FoxO1 on skeletal muscle lipid metabolism.

This aim will be achieved by applying an Oil Red O stain to sections of plantaris and soleus muscles to reveal intramyocellular triglyceride (IMTG) storage. Additionally, soleus muscles from a separate set of mice will be harvested and processed for \textit{ex vivo} fatty acid oxidation quantification. We hypothesize that in FoxO1 skeletal muscle, fatty acid oxidation and IMTG will be elevated.
Chapter 2

Literature Review

Diabetes mellitus (DM) is a metabolic disease that causes dysfunction to various organ systems as a result of chronic hyperglycemia. Chronically elevated plasma glucose produces advanced glycation endproducts (AGE) in microvasculature leading to diabetic neuropathy and retinopathy while AGE in macrovasculature leads to arterial disease. Nearly 80 years ago, Himsworth was among the first researchers to establish a distinction between “insulin sensitive” and “insulin insensitive” DM based upon presence of ketosis and an elevated insulin requirement during glucose challenge in the latter. Insulin insensitive DM, now termed type 2 diabetes mellitus (T2DM), is typically developed in adulthood and is characterized by insulin resistance in liver, adipose and skeletal muscle tissue. Consequently, pancreatic beta cell compensation causes hypersecretion of insulin to maintain euglycemia. The disease progresses as glucotoxicity lipotoxicity and chronic insulin hypersecretion eventually cause beta-cell deterioration and severe hyperglycemia. The pathogenesis of T2DM is complex as dietary habits, aging, genetics and disease may bring on insulin resistance. Equally complex are the metabolic triggers of insulin resistance, most important of which are reductions in cellular glucose uptake and oxidation with concomitant amplification of hepatic triglyceride secretion,
adipose tissue lipolysis, cellular fatty acid uptake and oxidation, and hepatic glucose production.\textsuperscript{11, 27, 28, 33-36}

**Insulin signal transduction**

Post-prandial euglycemia is achieved through a series of enzymatic reactions in insulin sensitive tissue. Insulin signal transduction begins with secretion of insulin from pancreatic beta cells which binds to the extracellular portion of the insulin receptor. Binding of insulin to the insulin receptor activates its kinase activity resulting in autophosphorylation of tyrosine sites on the intracellular domain.\textsuperscript{3} This enables insulin receptor substrate-1 (IRS-1), a scaffolding protein, to bind to intracellular tyrosine residues and become phosphorylated. Phosphoinositide-3 kinase (PI3K) then binds to phosphorylated tyrosine sites in the activated IRS-1 through its p85 regulatory subunit.\textsuperscript{37} This association activates PI3K allowing its p110 catalytic subunit to phosphorylate membrane-associated phosphatidylinositol 4, 5-bisphosphate (PIP2) which causes formation of phosphatidylinositol (3,4,5)-triphosphate (PIP3).\textsuperscript{3, 38} Protein kinase B (Akt) then translocates to the cell membrane and through its pleckstrin homology (PH) domain, binds to PIP3 which activates PI3K-dependent protein kinase 1 (PDK1/2).\textsuperscript{39, 40} PDK1/2 partially activates Akt by phosphorylating Thr308, one of two regulatory sites in Akt.\textsuperscript{40} Full activation of Akt results from phosphorylation of Ser473 by mammalian target of rapamycin 2 (riotor-mTOR2) complex.\textsuperscript{41, 42} Following activation, Akt phosphorylates AS160, a Rab GTPase-activating protein (GAP) protein.\textsuperscript{43} In the dephosphorylated state the GAP activity of AS160 maintains Rab in an inactive GDP-bound state, sustaining intracellular retention of glucose transporter-4 (GLUT4) vesicles.\textsuperscript{44} Akt phosphorylation
of AS160 diminishes its GAP activity, shifting Rab to an active GTP-bound state thereby facilitating GLUT4 vesicle movement toward, and docking and fusion with the plasma membrane (translocation). GLUT4 membrane translocation is further aided by protein 14-3-3 binding to phosphorylated AS160, triggering its dissociation from GLUT4 vesicles. The resulting increase in GLUT4 membrane association stimulates glucose uptake.

In relation to T2DM, a critical substrate for Akt mediated phosphorylation is Forkhead Box O1 (FoxO1), a member of the Forkhead Box super family of transcription factors. FOX proteins are named after the presence of a conserved 110 amino acid winged helix DNA binding domain referred to as the forkhead box. FoxO1 is ubiquitously expressed and its activity is regulated by phosphorylation, acetylation and ubiquitination. When dephosphorylated, FoxO1 translocates to the nucleus where it regulates transcription of a wide array of target genes involved in metabolism, cell cycle arrest, cell differentiation, oxidative stress response and muscle protein degradation.

Insulin stimulated Akt phosphorylation of FoxO1 causes its nuclear export and cytosolic sequestration via protein 14-3-3. Recent examination indicates that FoxO1 activation may encourage and perpetuate T2DM by antagonizing insulin action in the liver, adipose, pancreatic beta-cells and skeletal muscle.

**FoxO1 in the liver**

Hepatic glucose entry is insulin independent as it depends upon increased portal vein glucose concentration and subsequent translocation and membrane association of glucose transporter-2 (GLUT2), the major hepatic glucose transporter. In hepatic
tissue, insulin stimulation activates the PI3K/Akt pathway which acts to suppress hepatic gluconeogenesis, glycogenolysis and very low density lipoprotein (VLDL) secretion. Thus insulin resistance in hepatic tissue causes systemic post-absorptive hyperglycemia through excessive endogenous glucose production and induction of hypertriglyceridemia.

**Gluconeogenesis**

Increased hepatic glucose production is required during fasting or prolonged exercise in order to maintain energy homeostasis. However, fasting hepatic glycogenolysis and gluconeogenesis is significantly elevated in both mild and severe T2DM and obese human patients. This response holds true during hyperinsulinemic-euglycemic clamping. Hepatic gluconeogenesis converts three carbon, non-carbohydrate precursors such as glycerol, lactate and alanine to glucose. It is primarily regulated by the activity of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P), two rate limiting enzymes whose gene expression is inhibited by insulin. PEPCK catalyzes the conversion of citric acid intermediate oxoalacetate to phosphoenolpyruvate and carbon dioxide while G6P provides free glucose for entry into the bloodstream by dephosphorylating glucose -6-phosphate.

Repression of insulin stimulated Akt activity in hepatocytes causes dephosphorylation, nuclear import and transcriptional activation of FoxO1. Reporter gene studies in HepG2 hepatoma cells reveal that in the absence of insulin FoxO1 is transcriptionally active in the nucleus, binding to the insulin response element (IRE) in target gene promoters. The IRE is found in the promoters of several genes inhibited by insulin such as PEPCK and G6P. With the addition of insulin, FoxO1 target gene
expression is inhibited due to Akt dependent phosphorylation and nuclear exclusion.\textsuperscript{56, 58} Transgenic mice with hepatic overexpression of FoxO1 have elevated fasting plasma glucose, impaired glucose tolerance and are hyperinsulinemic.\textsuperscript{60} Isolated hepatocytes overexpressing a mutant FoxO1 show increased expression of gluconeogenic genes.\textsuperscript{60} Further elucidating the role of FoxO1 in mediating insulin suppression of gluconeogenic genes, expression of a dominant negative FoxO1 in primary mouse hepatocytes was shown to inhibit dexamethasone/cyclic adenosine monophosphate (cAMP) induced PEPCK and G6P expression.\textsuperscript{8} Similarly, adenoviral delivery of dominant negative FoxO1 into wild-type mouse liver reduced PEPCK and G6P expression while injection of this dominant negative construct into diabetic \textit{db/db} mice was successful in lowering fasting plasma glucose levels.\textsuperscript{7} Likewise, pharmaceutical inhibition of FoxO1 in diabetic \textit{db/db} mice also reduced fasting plasma glucose levels through suppression of PEPCK and G6P gene expression.\textsuperscript{9} Optimized antisense oligonucleotides (ASO) that specifically inhibit FoxO1 expression were effective in reducing PEPCK and G6P expression in mouse hepatocytes. Additionally, ASO use in insulin resistant, obese mice reduced fasting plasma glucose and basal endogenous glucose production while improving glucose tolerance and both hepatic and peripheral insulin sensitivity.\textsuperscript{61} Consistent with the above findings, FoxO1 haploinsufficiency restores insulin sensitivity in part by diminishing hepatic PEPCK and G6P expression in insulin receptor haploinsufficient mice.\textsuperscript{16} Recently, Morinda citrifolia fruit juice was successful in slowing weight gain, decreasing fasting blood glucose and restoring glucose and insulin tolerance in high fat diet (HFD) fed mice. These improvements were associated with increased phosphorylation and nuclear exclusion of FoxO1 and hepatic suppression of PEPCK and G6P mRNA.\textsuperscript{62}
FoxO1 may also act in concert with peroxisome proliferator activated receptor gamma coactivator -1α (PGC-1α) to induce glucogenoginc gene expression. FoxO1 activates PGC-1α transcription by binding to three insulin response elements (IRE) in its promoter. Liver specific FoxO1 gene ablation in fasted mice induces hypoglycemia inhibits hepatic glucose production, and reduces PGC-1α gene expression. It seems that in the fasted state FoxO1 and PGC-1α coactivate PEPCK and G6P gene expression while in the fed state insulin induces Akt mediated phosphorylation and inactivation of both FoxO1 and PGC-1α.

Activation of pyruvate dehydrogenase complex (PDC) inhibits gluconeogenesis by the conversion of pyruvate to acetyl-Coa which promotes activation of the citric acid cycle and fatty synthesis. The major regulator of the PDC is pyruvate dehydrogenase kinase 4 (PDK4) which phosphorylates the E2 subunit of the PDC rendering it inactive. Inactive PDC allows pyruvate carboxylase to convert pyruvate to oxoalacetate, a known substrate for PEPCK. In HepG2 cells, FoxO1 activates transcription of PDK4 by binding to the IRE in its promoter. Further, mice with liver specific insulin receptor substrate (IRS-1) knockout show increased PDK4 and FoxO1 expression while adenoviral delivery of constitutively active FoxO1 in HepG2 cells induces PDK4 expression. However, fasting which normally activates FoxO1 does not induce PDK4 expression without peroxisome proliferator receptor –α (PPAR-α), suggesting a role for it as an important coactivator of PDK4.
Lipid metabolism

In the post absorptive state, the liver releases triglycerides into the blood in the form of very low density lipoproteins (VLDL) to maintain energy homeostasis in peripheral tissues. Triglyceride for hepatic VLDL formation is supplied by adipose tissue and is also synthesized in hepatocytes. VLDL assembly is heavily dependent upon free fatty acid (FFA) availability and occurs primarily through the coordinated actions of three proteins: microsomal triglyceride transfer protein (MTP), apolipoprotein B (apoB) and apolipoprotein C (apoCIII). MTP is a resident endoplasmic reticulum (ER) protein, which in a rate-limiting step, acts as a chaperone transferring lipid into nascent apoB molecules and forming the pre-VLDL particle. ApoB translation and translocation into the ER lumen depends upon increases in its substrate free fatty acid (FFA) availability and MTP activity. Newly lipidated apoB particles are then secreted out of the ER and into the Golgi where they acquire apoCIII, a lipoprotein which protects VLDL from membrane lipoprotein lipase action as it exits the cell.

Excessive postprandial secretion of VLDL and ensuing hypertriglyceridemia is widespread in T2DM. In response to a meal, increased insulin suppresses VLDL production preventing postprandial blood triglyceride excursion. Insulin exerts its inhibitory role in VLDL production by inhibiting adipose tissue hormone sensitive lipase, effectively decreasing substrate availability and apoB activity. Evidence also suggests that insulin may induce proteasomal degradation of apoB. Thus, hepatic insulin resistance promotes dyslipidemia by increasing free fatty acid (FFA) flux into the liver, increasing apoB activity and preventing VLDL assembly machinery from being degraded.
FoxO1 may mediate insulin action on VLDL production by inducing hepatic synthesis of lipid substrate and activating assembly machinery. Adenoviral mediated hepatic FoxO1 overexpression in mice results in impaired glucose tolerance and increased hepatic fat content. This is accounted for by increased expression of lipogenic genes such as peroxisome proliferator-activated receptor gamma coactivator-1beta (PGC-1β), fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC). In a similar model, constitutively active FoxO1 in mouse liver induces steatosis from increased triglyceride accumulation and decreased fatty acid oxidation. Pivotal work by Kamagate et. al., 2008 demonstrates that constitutively active FoxO1 in mice increases rates of hepatic VLDL production, serum apoB levels and hepatic MTP protein and gene (MTTP) expression. Additionally, FoxO1 transgenic mice displayed increased expression of hepatic lipogenic genes sterol response element binding protein-1c (SREBP-1c), FAS and ACC and reduced acetyl CoA oxidase-1 (ACOX-1) expression. Perhaps the most powerful finding was in work with cultured HepG2 cells which indicated that FoxO1 activates MTP transcription by binding to the IRE on its promoter. Taken together, these results indicate that FoxO1 increases hepatic lipid synthesis for VLDL assembly, not for oxidation. Further, FoxO1 also directly encourages VLDL assembly and secretion by increasing expression of MTP. Consistent with these findings, high fructose fed hamsters are rendered glucose intolerant, hyperinsulinemic, dyslipidemic, and display elevated SREBP-1c, MTP and nuclear FoxO1 protein expression. FoxO1 cDNA delivery into HepG2 cell reveals that FoxO1 also induces transcription of apoCIII by binding to the IRE in its promoter. Further, FoxO1 vector-treated mouse liver produces elevated plasma triglycerides, VLDL and apoCIII. Similarly, high fructose fed hamsters exhibit
amplified nuclear FoxO1 expression correlated with elevated apoCIII and impaired triglyceride metabolism.\textsuperscript{78}

**FoxO1 in adipose tissue**

Adipose tissue is integral in maintaining systemic energy homeostasis. In the face of energy deprivation, adipocytes can mobilize reserves of stored triglycerides which are used as energy substrates in peripheral tissue.\textsuperscript{79-81} Additionally, adipocytes secrete adipokines such as adiponectin, leptin, resistin, and tumor necrosis factor-α (TNF-α) which are essential in maintaining energy homeostasis.\textsuperscript{80, 82} Moreover, abnormality in adipose tissue structure and function is associated with disordered metabolic function.\textsuperscript{81, 83, 84} Indeed, insulin resistance in adipose tissue reduces adipocyte glucose uptake by inhibiting Akt mediated Glut4 membrane translocation.\textsuperscript{85, 86} Reduced insulin sensitivity in adipose tissue also promotes dyslipidemia through an increase in the ratio of lypolytic to lipogenic pathway activation.\textsuperscript{87} To this end, synthetic agonists of adipocyte nuclear receptor, peroxisome proliferator receptor-gamma (PPARγ), are commonly used in the treatment of T2DM.\textsuperscript{88}

**FoxO1 and adipocyte differentiation**

Terminal differentiation of adipocytes is initiated by the expression of adipogenic transcription factors such as CCAAT/enhancer-binding protein (C/EBPβ) and C/EBPδ.\textsuperscript{89} This is followed by induction of PPARγ, a nuclear receptor and ‘master regulator of adipogenesis’ responsible for coordinating the expression of many genes in charge of adipocyte maturation.\textsuperscript{90} PPARγ expression is partly dependent upon transactivation by
E2F transcriptions factors during G1 phase of the cell cycle. E2F activation is contingent upon its detachment from pocket protein family members, retinoblastoma (Rb), p130, and p107. Cyclin dependent kinase (cdk) phosphorylation of E2F stimulates this detachment. FoxO1 increases expression of p130 and cyclin dependent kinase inhibitors (CDKI), p21 and p27, effectively halting cell cycle progression and inhibiting E2F mediated expression of PPARγ. Furthermore, yeast two-hybrid screening shows that FoxO1 interacts with PPARγ and antagonizes its transactivation capability by interfering with its DNA binding activity. Moreover, study in primary rat adipocytes, illustrates that FoxO1 binds to the PPARγ promoter, repressing its activity.

FoxO1 acetylation status can affect its activity. Acetylation of FoxO1 enhances its insulin mediated phosphorylation and nuclear exclusion. In response to oxidative stress FoxO1 is phosphorylated by c-jun N-terminal kinase (JNK) causing its nuclear import. Here, FoxO1 recruits NAD-dependent deacetylase, silent information regulator (Sir2) mammalian ortholog, sirtuin 1 (Sirt1) which in turn deacetylates FoxO1 promoting its nuclear inclusion and transcriptional activity. Interestingly, in white adipocytes, addition of Sirt1 blocks adipocyte differentiation through repression of PPARγ. In visceral human adipocytes, addition of resveratrol, a Sirt1 agonist up regulates FoxO1 gene expression while down regulating PPARγ.
FoxO1 and adipocyte function

Obesity and diabetes in humans and mice is associated with whole-body oxidative stress and reduced anti-oxidative capacity, particularly in adipose tissue.\textsuperscript{98, 99} Treatment of 3T3-L1 adipocytes with FFA decreases FoxO1 protein expression dose dependently while increasing reactive oxygen species (ROS) production and pro-inflammatory adipokine gene expression. Additionally, db/db mice treated with Sirt1 agonist, resveratrol, exhibit lower adipocyte ROS production and a partial restoration to the pro-inflammatory adipokine expression pattern.\textsuperscript{100} These results indicate that interaction between FoxO1 and Sirt1 may play a role in protecting adipocytes from diabetes and obesity–induced dysfunction.

Apart from protecting adipocytes from diabetes and obesity-induced oxidative stress, the FoxO1-Sirt1 pathway may oppose insulin-mediated inhibition of adipose tissue lipolysis. Upregulation of Sirt1 in differentiated adipocytes causes lipolysis and FFA mobilization.\textsuperscript{96} In 3T3-L1 adipocytes, FoxO1 drives gene transcription of adipose triglyceride lipase (ATGL) by binding to its promoter.\textsuperscript{13} Recently, it was shown that in cultured adipocytes, knockdown of Sirt1 with small hairpin RNA decreases isoproterenol-stimulated lipolysis with an associated suppression of ATGL gene transcription.\textsuperscript{14} This suggests that a lack of Sirt1 mediated FoxO1 deacetylation may act to repress transcription of ATGL.\textsuperscript{14}
**FoxO1 in pancreatic beta-cells**

Pancreatic beta-cells are located in endocrine compartments of the pancreas known as islets of Langerhans. Beta-cells bare a central role in maintaining glucose homeostasis by producing and secreting insulin in response to circulating glucose and other hormonal factors.\(^\text{101}\) Though beta-cells rely on insulin independent glucose uptake via glucose transporter 2 (Glut2), insulin stimulation in beta cells is crucial for proper growth and function.\(^\text{50}\) Additionally, insulin regulates the beta-cell adaptive response to increased insulin demand brought on by peripheral insulin resistance.\(^\text{5, 102}\) Thus, hyperinsulinemia is a compensatory response orchestrated by the beta-cell and must occur to avoid severe hyperglycemia.

**FoxO1 and beta-cell compensation**

In the face of escalating insulin demand, heightened insulin secretion is modulated by increases in beta-cell mass and proliferation. This requires both activation of the insulin/PI3K/Akt pathway and FoxO1 nuclear exclusion.\(^\text{36, 101}\) Several factors enhance insulin signal transduction including insulin, glucose, glucagon like peptide-1 (GLP-1), and glucose-dependent insulinoctropic polypeptide (GIP).\(^\text{101}\) Ensuing Akt activation results in phosphorylation and FoxO1 nuclear export whereas downregulation of Akt promotes its activation and nuclear translocation. Consistent with this paradigm, haploinsufficiency of FoxO1 restores beta-cell proliferation in mice with beta–cell specific deletion of Irs2 and PDK.\(^\text{103, 104}\) Similarly, constitutively active FoxO1 attenuates
increased in beta-cell mass in insulin resistant mice expressing the insulin receptor solely in the liver, beta-cell and brain.\textsuperscript{105} In a different transgenic model using mice with pancreas-restricted IGFII production, the same group showed that beta-cell-specific FoxO1 overexpression blocks beta-cell proliferation.\textsuperscript{105} Likewise, transgenic mice with hepatic and pancreatic FoxO1 overexpression display reductions in compensatory beta-cell growth and insulin secretion.\textsuperscript{16} Zealand obese (NZO) mice exhibit marked hyperglycemia and concomitant dephosphorylation of FoxO1 and beta-cell apoptosis.\textsuperscript{106} Fructose treatment induces proliferation and hyper-insulin secretion in INS-1 cultured beta-cells. Administration of the flavonoid, quercitin, was successful in reversing proliferation and hyper-insulin secretion in fructose-treated INS-1 beta-cells by increasing nuclear FoxO1 content.\textsuperscript{107}

FoxO1 impedes beta-cell growth and proliferation by downregulating pancreatic duodenal and homeobox factor-1 (Pdx1). Pdx1 is a key transcription factor that oversees the development and function of beta-cells. Ablation of Pdx1 in mice is enough to induce diabetes.\textsuperscript{104} Conversely, beta-cell-specific overexpression of Pdx1 alone can rescue beta-cell mass and function in the diabetic Irs2 knockout mouse.\textsuperscript{108} FoxO1 and Pdx1 exhibit mutually exclusive patterns of nuclear inclusion.\textsuperscript{103} Likewise, GLP-1 mediated PI3K/Akt activation induces FoxO1 nuclear expulsion while increasing Pdx1 expression.\textsuperscript{109-112} It appears that FoxO1 controls Pdx1 expression by two mechanisms. First, FoxO1 may affect Pdx1 nuclear localization.\textsuperscript{113} Second, Foxo1 may act as a repressor of Foxa2-dependent expression from the Pdx1 promoter.\textsuperscript{103}
FoxO1 and beta-cell function

Chronic exposure to excessive glucose or lipids increases the production of ROS in beta-cells.\textsuperscript{5, 114-116} Increased ROS in beta-cells activates JNK which disrupts glucose-stimulated insulin secretion and lead to apoptosis.\textsuperscript{114, 117} As previously stated, JNK phosphorylates FoxO1 causing its nuclear translocation.\textsuperscript{48} In HIT-T15 beta-cells, induction of oxidative stress causes the activation of JNK along with nuclear localization of FoxO1 and nuclear exclusion of Pdx1.\textsuperscript{113} Those events were reversed following JNK suppression. Following oxidative stress induced nuclear import, FoxO1 is acetylated by p300/CREB binding protein (CBP) and then deacetylated by Sirt1. The latter action helps maintain nuclear localization of FoxO1 thereby increasing its transcriptional activity.\textsuperscript{48} Interestingly, two key FoxO1 gene targets are insulin (Ins2) transcription factors, NeuroD and MafA.\textsuperscript{118} This underscores the complex role that FoxO1 plays in the beta-cell; though it may repress its growth and proliferation, it also helps the beta-cell maintain activity in times of metabolic stress.

FoxO1 in skeletal muscle

Skeletal muscle is the largest organ in the human body, accounting for 40\% of its total mass. Its primary role is movement of the body though it plays an integral role in governing energy metabolism.\textsuperscript{119} Skeletal muscle contributes to more than 30\% of resting metabolic rate and 80\% of whole body glucose uptake.\textsuperscript{17} Under resting conditions, skeletal muscle glucose uptake is insulin-dependent, therefore maintaining its mass and
function is critical for sustaining normal glycemic control. In fact, progressive loss of muscle mass and function is associated with the onset of the metabolic syndrome and T2DM.\textsuperscript{120} \textsuperscript{121-123} Accordingly, resistance training aimed at increasing muscle mass is now considered an efficacious treatment for increasing whole body insulin sensitivity.\textsuperscript{121, 124, 125}

\textit{FoxO1 and skeletal muscle myogenesis}

While most peptide growth factors promote myoblast proliferation and inhibit differentiation, insulin and insulin-like growth factor-1 (IGF-1) stimulate both.\textsuperscript{126-128} This occurs through activation of the PI3K-Akt pathway and involves several downstream effectors such as p70S6 kinase, mammalian target of rapamycin (mTOR1), and FoxO1.\textsuperscript{128-130} Expression of a constitutively active form of Foxo1 inhibits differentiation in C2C12 muscle cells while also blocking myotube differentiation induced by constitutively active Akt. Conversely, a dominant negative mutant FoxO1 partially rescues wortmannin-mediated inhibition of C2C12 differentiation. Likewise, siRNA-mediated knockdown of FoxO1 increases myoblast differentiation.\textsuperscript{131} Similarly, C2C12 cells continue to differentiate with reduced FoxO1 activity. At the same time, reduced differentiation of C2C12 muscle cells was attributed to FoxO1 mediated upregulation of Notch, a known inhibitor of myoblast differentiation.\textsuperscript{19} In the same study, muscle-specific FoxO1 ablation increased formation of fast-twitch muscle fibers at the expense of slow-twitch fibers. Furthermore, inducible activation of a FoxO1 mutant blocks C2C12 differentiation by inducing proteasomal degradation of mTOR and associated components, raptor, tuberous sclerosis complex 2 (TSC2), and S6 kinase 1.\textsuperscript{21} Additionally, FoxO1 may inhibit myoblast differentiation by inducing expression of
myostatin, an inhibitor of muscle cell differentiation. In contrast to the aforementioned studies, FoxO1 is shown to be sequestered in the cytoplasm of proliferating myoblasts where upon serum withdrawal it translocates to the nucleus and binds to DNA. Further, while phosphorylation of nuclear FoxO1 blocks myocyte fusion, expression of a dominant negative FoxO1 lacking the transactivation domain also prevents fusion activity. These conflicting results may be attributable to temporal patterns of FoxO1 expression during differentiation.

*FoxO1 and skeletal muscle loss*

Skeletal muscle loss, also known as atrophy, is a debilitating response brought on by fasting, aging, immobilization, cancer, renal failure, sepsis and diabetes. Muscle atrophy reflects dysregulation to the balance of intracellular protein synthesis and protein degradation, favoring the latter. During fasting or disease, a majority of amino acids from degraded proteins are mobilized for energy production through hepatic gluconeogenesis while some can be oxidized in the muscle. Global muscle atrophy is primarily induced by ubiquitin proteasomal pathway (UPP) mediated protein degradation. Moreover, the UPP mediates proteolysis of insulin signaling machinery. The UPP is an energy dependent process coordinated by three enzymes, ubiquitin-activating enzymes (E1) ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3) which target and mark various proteins for proteasomal degradation. There are two muscle-specific ubiquitin E3 ligases: atrogin-1 (MAFbx) and muscle ring finger-1 (MuRF-1).
Induction of atrogin-1 and MuRF-1 precedes muscle atrophy due to starvation while knockout of either gene attenuates atrophy triggered by denervation.\textsuperscript{139, 141}

The activity of FoxO1 is crucial in conditions that promote skeletal muscle atrophy. Fasting and glucocorticoid treatment, two conditions that promote muscle atrophy, upregulate FoxO1 gene expression.\textsuperscript{67, 142} Additionally, streptozitocin-induced diabetes leads to muscle mass loss and increased FoxO1 levels. However, elevated FoxO1 expression is abrogated by insulin treatment.\textsuperscript{142} Further, in glucocorticoid treated C2C12 myotubes, FoxO1 increases activity of the MuRF-1 promoter through direct binding.\textsuperscript{143} In mouse muscle atrophying due to starvation, siRNA knockdown of FoxO1 decreases atrogin-1 expression.\textsuperscript{24} In the same study, \textit{in-vitro} and \textit{in vivo} expression of a dominant negative form of FoxO1 reduces muscle atrophy subsequent to decreased levels of atrogin-1.\textsuperscript{24} In transgenic models, skeletal muscle-specific overexpression of FoxO1 induces muscular atrophy as well as upregulation of atrogin-1 and MuRF-1.\textsuperscript{22, 23} FoxO1 mediated regulation of atrogenes depends upon downregulation of the insulin/IGF-1/PI3K/Akt pathway.\textsuperscript{24, 144} IGF-1 injected into mouse muscle prevented the induction of atrogin-1 and MuRF-1 and associated muscle atrophy due to denervation.\textsuperscript{144} Additionally, constitutively active FoxO1 prevents Akt-mediated inhibition of MuRF-1 and atrogin-1/MAFbx.\textsuperscript{144}

Of particular interest is the involvement of FoxO1 mediated proteolysis in insulin resistant states. Transgenic mice with skeletal muscle-specific FoxO1 overexpression exhibit reductions in both skeletal muscle mass and muscle fiber cross-sectional area concurrent with impaired glucose tolerance and insulin resistance.\textsuperscript{22} Microarray analysis of muscle from fasted, cancer-stricken, uremic and diabetic rats reveals significant
upregulation of many UPP related genes including those encoding proteasomal subunits 19s and 20s to specific polyubiquitins and ubiquitin ligases such as FoxO1 target, atrogin-1/MAFbx. This was accompanied by downregulation of pyruvate dehydrogenase complex component genes which are imperative in regulating glucose metabolism.\textsuperscript{26}

Consistent with these findings, transgenic mice with skeletal muscle specific MuRF1 overexpression display fasting hyperinsulinemia and decreased protein levels of pyruvate dehydrogenase (PDH). In addition, MuRF1 was shown to interact with PDH, pyruvate dehydrogenase kinase 2 (PDK2), and pyruvate dehydrogenase kinase 4 (PDK4).\textsuperscript{145} PDH is a key mitochondrial enzyme regulating glycolytic flux and PDK4 inhibits its action in skeletal muscle through phosphorylation of its E1-alpha subunit.\textsuperscript{146} Tying together the above findings, diabetic \textit{db/db} mice exhibit reduced skeletal muscle mass, elevated rates of muscle protein degradation, and amplified UPP activity. Furthermore, reduced PI3K and Akt activity was associated with upregulation of atrogin-1/MAFbx and Murf-1 protein expression and reduced phosphorylated and inactive FoxO1.\textsuperscript{147}

\textit{FoxO1 and skeletal muscle metabolism}

Reduced systemic insulin sensitivity shifts energy substrate utilization in skeletal muscle from carbohydrate to free fatty acids (FFA).\textsuperscript{28} Under these circumstances, two processes stimulated by FoxO1, adipose tissue lipolysis and hepatic VLDL output, are responsible for supplying FFA to skeletal muscle.\textsuperscript{10, 13} Skeletal muscle lipoprotein lipase (LPL) hydrolyzes plasma triglyceride to glycerol and fatty acids in preparation for skeletal muscle uptake. Skeletal muscle-specific deletion of LPL in mice improves
muscle insulin sensitivity through increased Akt activation.\textsuperscript{148} Not surprisingly, in C2C12 cells, FoxO1 overexpression induces expression of LPL.\textsuperscript{27}

Subsequent to hydrolysis, fatty acids are taken into the muscle cell via CD36, a plasma membrane fatty acid translocase.\textsuperscript{28} Bastie et al., 2005\textsuperscript{28} utilized an inducibly active form of FoxO1 to study its effects on CD36 expression and fatty acid oxidation. The inducibly active construct is generated by fusing FoxO1 to the ligand binding domain of the estrogen receptor allowing for activation by Tamoxifen (TAM), an estrogen receptor agonist. Additionally, FoxO1 undergoes site-directed mutagenesis, substituting Threonine 24, Serine 256, and Serine 319 to Alanine rendering it impervious to Akt-mediated phosphorylation and inactivation. In C2C12 myotubes, FoxO1 activation via TAM altered the subcellular dispersal of CD36 as membrane expression was elevated without alteration in total expression. Additionally, activated FoxO1 amplified fatty acid uptake and oxidation.\textsuperscript{28} Furthermore, in C2C12 cells, increased fatty acid flux or forced CD36 expression upregulates FoxO1 expression and activity while CD36 knockdown reverses this affect.\textsuperscript{149}

In addition to supplying FFA and inducing their uptake, FoxO1 promotes the oxidation of FFA over carbohydrates by several mechanisms. In C2C12 myotubes, activated FoxO1 increases expression of acyl-CoA oxidase (ACO) and peroxisome proliferator receptor-delta (PPARδ), two genes associated with increased fatty acid oxidation. In the same study, FoxO1 suppressed gene expression of acetyl-CoA carboxylase (ACC) an enzyme that promotes fatty acid formation rather than oxidation through formation of malonyl-CoA.\textsuperscript{28} FoxO1 may also regulate the expression of adiponectin receptor-1 (AdipoR1), ligation of which transduces a signal promoting fatty
acid oxidation. Insulin, which stimulates PI3K/Akt activity in cultured myocytes decreases AdipoR1 and FoxO1 expression while acute aerobic exercise increases both AdipoR1 and FoxO1 gene expression.

As mentioned above, PPARδ is an important regulator of skeletal muscle fatty acid oxidation. Two major PPARδ target genes are PDK4, which inhibits glucose oxidation and carnitine palmitoyltransferase-1α (CPT-1α) which mediates the transfer of long chain fatty acyl-CoAs into the mitochondria for beta oxidation. In C2C12 cells, fatty acids directly activate PPARδ which in turn binds to the FoxO1 promoter increasing its activity. Following PPARδ mediated activation, FoxO1 induces LPL expression and increases membrane distribution of CD36. Additionally, FoxO1 promotes fatty acid oxidation by regulating the expression of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), a co-activator of PPARδ. In human skeletal myotubes, insulin induces Akt mediated FoxO1 phosphorylation and nuclear exclusion along with repression of PGC-1α mRNA. In contrast, in insulin resistant myotubes where insulin is unable to phosphorylate Akt and FoxO1, palmitate treatment augments both nuclear FoxO1 protein levels and PGC-1α mRNA.

The pyruvate dehydrogenase complex (PDC) is a critical mitochondrial enzyme complex that governs the rate of carbohydrate oxidation in skeletal muscle. In response to insulin or a meal, the PDC is relatively active, manufacturing acetyl-CoA, a substrate for both fatty acid synthesis and energy production by the citric acid cycle. Starvation or insulin resistance inhibits PDC action and subsequently, glucose oxidation. PDC activity is regulated by a variety of factors, the most important of which are competing pyruvate dehydrogenase kinase (PDK) and phosphatase (PDP) reactions. Phosphorylation
of the E1-α subunit of PDC by PDK is inhibitory while de-phosphorylation by PDP is stimulatory. Of the four PDK isoenzymes (PDK1, 2, 3 and 4) PDK4 is the most abundant in skeletal muscle and thus has the most profound effect on PDC activity.\textsuperscript{26, 146}

FoxO1 appears to regulate skeletal muscle PDK4 in a variety of contexts. In the presence of glucocorticoids, FoxO1 co-activates transcription PDK4 by binding to three insulin response elements on its promoter.\textsuperscript{29} However, insulin-mediated Akt activation eliminates FoxO1 from the PDK4 promoter despite the presence of glucocorticoids. Interestingly, inducible activation of FoxO1 in C2C12 cells upregulates PDK4 gene expression without obstruction to glucose uptake or oxidation.\textsuperscript{28} However, this study did not address factors that may counter the inhibitory effect PDK4 such as PDP activity or pyruvate content. Rats with lipopolysacharride induced endotoxaemia display elevated levels of nuclear FoxO1 concurrent with increased PDK4 protein expression and decreased PDC activity.\textsuperscript{156} Additionally, a high-fat diet amplifies resting human skeletal muscle PDK4, FOXO1, and peroxisome proliferator-activated receptor-α (PPARα) mRNA and reduces PDC activation.\textsuperscript{146} Similarly, fatty acid treatment induces PPARδ/β dependent FoxO1 and PDK4 expression in C2C12 cells.\textsuperscript{149} This emphasizes an important sequence of events whereby fatty acid flux activates PPARδ/β which in turn binds to and activates the promoters of both FoxO1 and PDK4.\textsuperscript{149, 157} This perpetuates fatty acid entry and oxidation via CD36, LPL and PGC1-α while impeding glucose oxidation.
Chapter 3

Experimental Methods

*Animals*- Three-month-old male and female wild-type C57BL/6 (WT) and skeletal muscle-specific FoxO1 overexpressing mice (FoxO1\(^{+/−}\); C57BL/6 background) used in the experiments were obtained from an established breeding colony at The University of Toledo. FoxO1 overexpression is driven within skeletal muscle of transgenic mice via a skeletal muscle α-actin promoter, as previously described.\(^1\) All procedures were performed in accordance with University of Toledo Institutional Animal Care and Use Committee guidelines. All animals were housed in clear polycarbonate cages, exposed to a 12:12 hour light-dark cycle (lights on at 0800 hours), and provided a standard rodent diet and water ad libitum. For all experiments, Line A2 FoxO1\(^{+/−}\) mice were used, as these mice possess the highest FoxO1 transgene copy number incorporation and, therefore, express the highest amount of the FoxO1 protein.\(^1\)

*Intraperitoneal glucose tolerance test*- The IPGTT assessed whole-body glucose tolerance and insulin response following glucose challenge. Following an overnight semi-fast (16-18 hours), mice (5 mice/group) were anesthetized via intraperitoneal (IP) injection of pentobarbital (3-5 mg/100g body weight (BW)). Glucose was administered
via IP injection of (2g/kg BW) and blood samples were obtained from the retro-orbital sinus plexus at 0 (before glucose injection), 5, 10, 30, 60, and 120 minutes after glucose injection. Blood glucose concentrations were measured using an ultrasensitive glucometer (AlphaTrack; Abbott; North Chicago, IL). For insulin analysis, blood samples were obtained from the retro-orbital sinus plexus using capillary tubes (~50ul/time point). Samples were centrifuged and plasma was extracted and stored at -80° for analysis of insulin.

Muscle glucose transport- Mice (5 mice/group) were anesthetized using pentobarbital (3-5 mg/100 g BW) after overnight semi-fast. Soleus muscles from the hind limbs of each mouse were surgically excised and incubated for 30 minutes at 30°C in micro centrifuge tubes containing 0.5 ml of oxygenated Krebs-Henseleit Buffer (KHB) supplemented with 0.1% bovine serum albumin and either 0 or 1200 pmol/L insulin. All muscles were blotted and incubated for 20 minutes in media containing KHB, 2-deoxy-D-[\textsuperscript{1,2-3}H]glucose (1mmol/L), [U-\textsuperscript{14}C] mannitol (39 mmol/L), and 0.1% bovine serum albumin. Muscles were blotted and frozen in liquid nitrogen and stored at 80°C. All tubes were gassed continuously with 95% O2-5% CO2 throughout the experiment. Frozen muscles processing consisted of trimming tendons and boiling for 60 minutes in 1ml of water. Extracts were vortexed and centrifuged for 10 minutes at 1,000g. Duplicate 200-ul aliquots of the muscle extract supernatant and incubation medium were counted for intracellular 2-deoxy-D-[\textsuperscript{1,2-3}H]glucose accumulation in nmol/g wet muscle/20 min.
**Basal level signaling**- To assess inherent insulin signaling expression in skeletal muscle, gastrocnemius muscles will be harvested from non-insulin stimulated, semi-fasted mice followed by western blot analysis of homogenates containing the aforementioned muscle. Muscles samples will be homogenized in RIPA buffer (10 mM Tris-HCL, 1mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) containing HALT™ protease inhibitor single-use cocktail supplemented with 10 mM Na3VO4 and spun at 14,000 x g for 15 min at 4°C. Muscle homogenates (50 µg) will be solubilized in Laemmeli sample buffer and boiled for 5 min, resolved by SDS-PAGE on 7.5% tricine gels, transferred to a polyvinyl difluoride membrane (PVDF-FL; Millipore; Billerica, MA) via wet-transfer (IRS-1; Hoefer TE-22; 400mA constant for 2 hrs; PI3K; Hoefer TE-22; 200mA constant for 1 hr; Akt Hoefer TE-22; 200mA constant for 30 minutes) blocked in 5% non-fat dry milk in TBS for 1 hr at room temperature, and immunoblotted overnight at 4°C with PI3K(p85), phospho-Akt (Ser473) antibodies (1:500; Cell Signaling, Beverley, MA). Equal protein loading was verified using GAPDH expression (1:5000; Cell Signaling, Beverley, MA). After a 1 hr incubation with an infrared-conjugated Alexa Fluor 680 secondary antibody (1:5000; Molecular Probes, Carlsbad, CA) at room temperature, the immunoreactive proteins will be observed via infrared detection (Odyssey Imaging System, LI-COR BioSciences, Lincoln, NE) and quantified by densitometry.

**Muscle palmitate oxidation**- Mice (5 mice/group) were semi-fasted overnight and anesthetized before the removal of soleus, gastrocnemius and quadriceps muscles. The muscles were weighed and homogenized at 20-fold dilution in buffer (10 mmol/L Tris,
pH 7.2, 300 mmol/L sucrose, 2 mmol/L ethylenediaminetetraacetic acid [EDTA]). Homogenate (1 mL) was injected via syringe into a sealed beaker at 30°C to initiate the reaction in incubation buffer (0.2 mmol/L of [1-14C]palmitate at 0.5 uCi/mL, 100 mmol/L sucrose, 10 mmol/L Tris-HCl, 5 mmol/L potassium phosphate, 80 mmol/L potassium chloride, 1 mmol/L magnesium chloride, 2 mmol/L L-carnitine, 0.1 mmol/L malic acid, 2 mmol/L adenosine triphosphate, 0.05 mmol/L Coenzyme A, 1 mmol/L dithiothreitol, 0.2 mmol/L EDTA, and 0.5% bovine serum albumin, pH 7.4). After 45 minutes, the reaction was terminated with glacial acetic acid. Completely oxidized CO$_2$ will be trapped in a well suspended above the medium filled with benzothoniumhydroxide. Trapped CO$_2$ radioactivity as measured by liquid scintillation in CytoCint (MP Biomedicals, Solon, OH).

Intramyocellular Triglyceride Storage- Plantaris and soleus muscles from 6 mice (3 mice/group) were harvested, submerged into liquid freezing media and frozen dry ice chilled-isopentane in preparation for cryosectioning. Oil Red O was applied to 10µm muscle belly sections and stained triglyceride was analyzed by brightfield microscopy. Stain intensity was quantified by Image Pro Plus software.
Insulin- Insulin was analyzed from the plasma fraction using an ultra-sensitive mouse insulin ELISA Kit (Crystal Chem., Downers Grove, IL) from the remaining blood collected in EDTA-coated tubes and kept in ice.

Data analysis- Two-way mixed factor ANOVAs (Mouse strain [WT, FoxO1] x Time [0, 5, 10, 30, 60, 120mins]) were used to compare blood glucose and insulin levels. One-way ANOVAs were used to compare differences in glucose and insulin area under the curve (AUC) as well as muscle glucose uptake and palmitate oxidation. Student’s t-tests were used to compare differences in signaling activity and triglyceride storage. Data was analyzed with SPSS version 19.0 statistical software (SPSS Inc. Chicago, IL) using an alpha level of p<0.05 to assess significance in all experiments.
Chapter 4

Results

FoxO1 and whole body glycemic control

To gain insight into whether FoxO1 influences whole body glucose metabolism, we subjected both wild type and FoxO1 transgenic mice to intraperitoneal glucose tolerance testing (IPGTT) while simultaneously obtaining blood for plasma insulin analysis. Blood glucose concentration was not significantly different between mouse strains throughout all time points during the IPGTT (Fig. 1A). Additionally, there was no significant difference in total glucose response (AUC) during the glucose tolerance test (Fig. 1B). Although total insulin response (AUC) was not significantly different between mouse strains Fig 2B), plasma insulin levels were significantly (p<0.05) reduced in FoxO1 transgenic mice at the 120-minute time point while the 60-minute time point trended towards significance (p=0.67) (Fig. 2A). These results demonstrate that FoxO1 does not impair whole body glucose metabolism and may in fact improve insulin sensitivity.
**FoxO1 and skeletal muscle glucose metabolism**

To determine if FoxO1 alters the inherent ability of skeletal muscle to assemble the machinery required for effective insulin signaling, we measured protein expression levels of PI3K and phosphorylated Akt (p-Akt\textsuperscript{ser473}) in non-insulin treated skeletal muscle harvested from semi-fasted mice. Neither PI3K nor p-Akt\textsuperscript{ser473} protein expression levels differed between FoxO1 and wild type mice (Fig. 3). Following this, we applied an *ex vivo* method wherein insulin stimulated glucose uptake was assessed in isolated gastrocnemius muscle. Glucose uptake was not significantly different between mouse strains in response to insulin treatment (Fig. 4). Taken together, these results demonstrate that FoxO1 does not interfere with either the inherent assembly of insulin signaling machinery or insulin action in skeletal muscle.

**FoxO1 and skeletal muscle lipid metabolism**

Type 2 diabetes is often characterized by alterations in normal skeletal muscle lipid metabolism. Therefore, to better understand how FoxO1 impacts type 2 diabetes we sought to study its effects on skeletal muscle lipid metabolism. This was carried out by first quantifying intramyocellular triglyceride (IMTG) storage in skeletal muscle from FoxO1 and wild type mice. IMTG storage was significantly (p<0.05) reduced in FoxO1 transgenic mice (Fig. 5). Second, to better understand how intramyocellular lipid is used in FoxO1 transgenic mice, we assessed palmitate oxidation in skeletal muscle harvested and processed from both strains of mice. Palmitate oxidation was significantly (p<0.05) decreased in FoxO1 transgenic mice (Fig. 6). These data suggest that FoxO1 mediated reduction in IMTG may also limit mitochondrial capacity for fatty acid oxidation.
**Fig. 1.** Whole body glucose tolerance in FoxO1 transgenic (Tg) and wild type (WT) mice. A, dextrose (D50) was administered via intraperitoneal injection (2g/kg BW) and blood samples were obtained from the retro-orbital sinus plexus at 0 (before glucose injection), 5, 10, 30, 60, and 120 minutes after dextrose injection. B, total glucose load expressed as area under the curve. Values are expressed as mean ± S.E. (n=5).
Fig. 2. Whole body insulin sensitivity in FoxO1 transgenic (Tg) and wild type (WT) mice. A, Whole blood obtained at each time point during the GTT was centrifuged for plasma collection. Plasma insulin was quantified by ELISA. B, Total insulin response expressed as area under the curve. Values are expressed as mean ± S.E. (n=5). Asterisk indicates significant difference: *, p<0.05 within 120 minute time point versus WT.
**Fig. 3.** Basal level insulin signaling activity in skeletal muscle of FoxO1 transgenic (Tg) and wild type (WT) mice. A, phosphoinositide-3 kinase (PI3-k) protein expression levels in gastrocnemius muscle. B, phosphorylated (Ser\textsuperscript{473}) Akt protein expression levels in gastrocnemius muscle. Values are expressed as mean ± S.E. (n=4).
**Fig. 4.** Basal level and insulin stimulated glucose uptake in isolated FoxO1 transgenic (Tg) and wild type (WT) skeletal muscle. Insulin stimulated muscle specific glucose uptake was assessed by enrichment of \[^{3}H\]-2-deoxy-glucose in the gastrocnemius. Values expressed as mean ± S.E. (n=5). *, (p<0.05) significant main effect for treatment.
Fig. 5. Intramyocellular triglyceride storage. Panel A, WT soleus; B, FoxO1 soleus; C, WT plantaris; D, FoxO1 plantaris; E, Soleus stain quantification; F, Plantaris stain quantification in arbitrary units (a.u.). Values expressed as mean ± S.E. (n=3). *, p<0.05 versus WT.
Fig. 6. Skeletal muscle fatty acid oxidation in FoxO1 transgenic (Tg) and wild type (WT) mice. Fatty acid oxidation was determined by production of $^{14}$CO$_2$ from 14C-palmitate in gastrocnemius, plantaris, soleus and quadriceps homogenate. Values expressed as mean ± S.E. ($n$=5). *, p<0.05 versus WT.
Chapter 5

Discussion

To examine the role that FoxO1 has in the pathophysiology of type 2 diabetes, we utilized transgenic mice overexpressing FoxO1 predominantly in skeletal muscle. This provides a robust and physiologically relevant means of studying glucose metabolism as skeletal muscle is responsible for up to eighty-percent of whole body glucose disposal. Our findings indicate that FoxO1 overexpression does not induce type 2 diabetes, as FoxO1 transgenic mice possess normal whole body glucose tolerance and insulin sensitivity. We show that this is likely due to the fact that FoxO1 transgenic mice exhibit normal insulin stimulated glucose uptake in skeletal muscle. Furthermore, a potential antidiabetic role for FoxO1 is evidenced through suppressed intramyocellular triglyceride (IMTG) storage in FoxO1 transgenic mice. Finally we demonstrate that FoxO1 transgenic mice exhibit a reduced capacity for fatty acid oxidation, an effect likely born out of reduced IMTG storage.

The incidence of FoxO1-mediated alteration to glucose metabolism was established by first studying the effect it has on whole body glycemic control. This was accomplished by subjecting wild type and FoxO1 transgenic mice to intraperitoneal glucose tolerance testing (IPGTT) with concomitant plasma insulin analysis. Our data demonstrate that whole body glycemic control is not impaired in FoxO1 transgenic mice.
as glucose tolerance was comparable to wild type while plasma insulin levels throughout
the last sixty minutes of the IPGTT were in fact reduced. These findings refute a previous
report indicating that in vivo skeletal muscle specific FoxO1 overexpression blights
whole body glycemic control.18 However, recent reports confirm that the surgical
methods we used to assess whole body glycemic control more effectively minimize
endocrine and metabolic interference.159-161 Therefore, we offer the first evidence
indicating that chronic overexpression of FoxO1 in skeletal muscle does not impair whole
body glucose metabolism.

To elucidate how FoxO1 affects glucose metabolism at the tissue level, we followed
up our whole body analysis with an investigation of the FoxO1 transgene target, skeletal
muscle. Insulin stimulation of the PI3K/Akt signaling pathway represents the dominant
means by which glucose is transported into the muscle cell.162, 163 In the present study, we
found that without insulin stimulation, PI3K and phosphorylated Akt protein expression
levels were unaltered in skeletal muscle of FoxO1 transgenic mice. This indicates that
skeletal muscle FoxO1 overexpression does not affect the inherent ability of the muscle
cell to generate and activate the necessary machinery for effective insulin signaling.
More importantly, our ex vivo analysis demonstrates that insulin stimulated glucose
uptake is not diminished in skeletal muscle of FoxO1 transgenic mice, a finding that has
been previously supported.28 Thus, skeletal muscle FoxO1 overexpression likely does not
inhibit insulin action in skeletal muscle. These results corroborate our whole body data,
which confirms that the normal whole body glucose tolerance observed in FoxO1
transgenic mice is a function of effective glucose uptake in skeletal muscle. Moreover,
our whole body and skeletal muscle-specific analyses reveal that in vivo skeletal muscle-
specific FoxO1 overexpression does not inhibit glucose metabolism and more importantly does not induce type 2 diabetes.

As skeletal muscle represents the predominant source of glucose uptake, it is not surprising that shifts in intramyocellular lipid metabolism are tightly associated with type 2 diabetes.\textsuperscript{35, 158, 164-168} Additionally, it has been documented that FoxO1 may play a role concerning myocellular fatty acid uptake, storage and oxidation.\textsuperscript{28, 146} Consequently, our final objective in scrutinizing the impact of FoxO1 on type 2 diabetes was to assess intramyocellular lipid metabolism in FoxO1 transgenic mice. Interestingly, our histochemical results suggest that intramyocellular triglyceride (IMTG) storage is significantly reduced in FoxO1 transgenic mice. This finding appears to support a potential antidiabetic role for FoxO1 as the typical diabetic myocellular phenotype is towards increased IMTG.\textsuperscript{158, 165} As IMTG is largely a function of cellular fatty acid uptake, it seems that the cellular machinery required for this task, such as fatty acid translocase CD36 and muscle LPL may be downregulated in FoxO1 transgenic mice.\textsuperscript{158} Though these findings conflict with a previous analysis suggesting that FoxO1 overexpression leads to increased IMTG, the present study used intact whole muscle as opposed to cultured myoblasts. Whereas cultured myoblasts are immature muscle cells, whole muscle analysis provides the benefit of obtaining \textit{in vivo} data generated from intact, mature cells with fully developed metabolic pathways.

In light of our IMTG data, we sought to elucidate precisely how intramyocellular lipid is used in FoxO1 transgenic mice. Generally, fatty acids have many fates upon myocellular uptake including esterification into IMTG, direct agonist activation of PPAR\(\delta/\beta\), conversion to signaling active intermediates such as diacylglycerol and
ceramide and oxidation for energy production. Since type 2 diabetes is highly characterized by changes to mitochondrial function and fatty acid oxidation capacity our final objective was to assess the effect of FoxO1 on fatty acid oxidation. This was attained by measuring radiolabeled (14C) CO2 produced by ex vivo skeletal muscle palmitate oxidation. We found that fatty acid oxidation was markedly reduced in FoxO1 transgenic mice, perhaps suggesting mitochondrial dysfunction. These findings are substantiated by previous work indicating that expression of several structural and functional mitochondrial genes is suppressed in FoxO1 transgenic mice. Additionally, the authors noted an apparent fiber type alteration induced through FoxO1 overexpression which produced skeletal muscle with a paler appearance. This phenotypic shift is likely indicative of reduced oxidative capacity. Though reduced fatty acid oxidation and mitochondrial dysfunction are prevalent in type 2 diabetes, it is likely that our data reflect a novel scenario in which the reduced IMTG storage observed in FoxO1 transgenic mice has led to a reduction in IMTG turnover and subsequently, the mitochondrial machinery required for fatty acid oxidation. This in turn may inhibit mitochondrial fatty acid entry, beta-oxidation, and/or tricarboxylic acid (TCA) cycle flux.

In conclusion, our IPGTT and concomitant plasma insulin data show that FoxO1 does not impair whole body glucose tolerance and may in fact improve insulin sensitivity. In relation to this, our ex vivo analysis of insulin stimulated glucose uptake illustrates that FoxO1 does not interfere with insulin action in skeletal muscle. This is a key finding in that skeletal muscle serves as the primary reservoir for glucose disposal. We also provide histochemical analysis of skeletal muscle that reveals a marked suppression of IMTG in FoxO1 transgenic mice. Our ex vivo fatty acid oxidation studies demonstrate
that lipid oxidation is significantly reduced in FoxO1 transgenic mice. We propose that this observed mitochondrial dysfunction in FoxO1 transgenic mice might be associated with the absence of IMTG pools. Moreover, this study lays the groundwork for a potentially intriguing role for FoxO1 in the pathogenesis of type 2 diabetes. We provide evidence suggesting that the FoxO1 pathway in skeletal muscle is a potentially attractive pharmaceutical target especially for those type 2 diabetes patients suffering from abnormally elevated IMTG. The benefit of such a therapy is that it may reduce IMTG without affecting whole body or skeletal muscle glucose metabolism. However, our IMTG and fatty acid oxidation data necessitates further scrutiny of the impact of such therapy on skeletal muscle fatty acid uptake and mitochondrial function. Specifically, future investigation should utilize in vivo methods to resolve the effect of skeletal muscle FoxO1 overexpression on the activity levels of fatty acid translocase CD36 and muscle LPL as well as mitochondrial enzymes responsible for fatty acid entry and oxidation such as carnitine palmitoyltransferase (CPT1) and citrate synthase respectively.
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