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Phosphoinositide 3-Kinase (PI3K) p110α Catalytic Subunit in Adipose Tissue Regulates Systemic Metabolism

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Abstract of the Dissertation

Phosphoinositide 3-Kinase (PI3K) p110α Catalytic Subunit in Adipose Tissue Regulates Systemic Metabolism

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Adipose tissue is a highly insulin responsive organ that contributes to metabolic regulation. Insulin resistance in the adipose tissue affects systemic lipid and glucose homeostasis. Phosphoinositide 3-kinase (PI3K) mediates downstream insulin signaling in adipose tissue, but its physiological role *in vivo* remains unclear. Using Cre recombinase driven by the aP2 promoter, we created mice that lack the class 1A PI3K catalytic subunit p110α or p110β specifically in the white and brown adipose tissue. The loss of p110α, not p110β, resulted in increased adiposity, glucose intolerance and liver steatosis. Mice lacking p110α in adipose tissue exhibited a decrease in energy expenditure but no change in food intake or activity as compared to control animals. This low energy expenditure is a consequence of low cellular respiration in the brown adipocytes caused by a decrease in expression of key mitochondrial genes including uncoupling protein-1. We also found that increased bodyweight in male mice lacking adipose-p110α is preceded by a delay in

puberty onset and corresponds with elevated leptin gene expression in the white adipose tissue suggesting that changes occurring before obesity onset contribute to metabolic impairments with age. These results illustrate a critical role of adipose-p110α in the regulation of early juvenile development and adult energy expenditure through modulation of cellular respiration in the brown adipose tissue and suggest that compromised insulin signaling in adipose tissue might be involved in the onset of obesity.

This dissertation is dedicated to my father for inspiring my scientific curiosity, to my mother for encouraging it, and to my husband for enduring it.

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List of Abbreviations

ACC	Acetyl-CoA carboxylase
ArKO	Aromatase knockout mouse
ATGL	Adipose triglyceride lipase
BAT	Brown adipose tissue
C/EBPα	CCAAT-enhancer-binding-proteins
cAMP	Cyclic adenosine monophosphate
CRE	cAMP response element
CREB	cAMP response element-binding protein
DHEA	Dehydroepiandrosterone
E1	Estrone
E2	Estrodial
ER	Estrogen receptor
FAS	Fatty acid synthase
FASN	Fatty acid synthase
FATP	Fatty acid transport proteins
FFA	Free fatty acids
FIRKO	Fat-specific IR knockout mouse
GPCR	G-protein coupled receptors
GSK-3β	Glycogen synthase kinase
HFD	High fat diet
HSL	Hormone sensitive lipase
IR	Insulin receptor

IRS	Insulin receptor substrates
LPL	Lipoprotein lipase
MGL	Monoglyceride lipase
mTOR	Mammalian target of rapamycin
mTORC2	mTOR complex 2
PDK1	Phosphoinositide-dependent kinase-1
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1α
PI	Phosphatidylinositals
PI(3)P	Phosphatidylinositol 3-phosphate
$PI(3,4)P_2$	Phosphatidylinositol (3,4)-phosphate
$PI(3,4,5)P_3$	Phosphatidylinositol (3,4,5)-phosphate
PI3K	Phosphoinositide 3-Kinase
PKA	Protein kinase A
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response elemens
PTEN	Phosphatase and tensin homologue
SH2	Src homology domain
SHBG	Sex-hormone-binding globulin
SHIP2	SH2-containing inositol 5'-phosphatase-2
SREBP1c	sterol regulatory element binding proteins
UCP1	Uncoupling protein-1
Vps34	Vacuolar protein sorting-associated protein 34
WAT	White adipose tissue

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This dissertation would not have been possible without the culmination of hard work and effort given by many people and I owe them my gratitude.

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Chapter 1

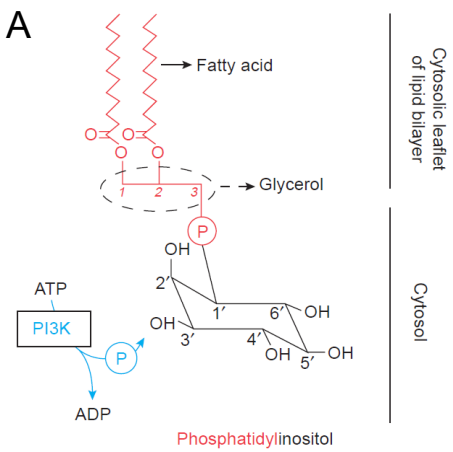
Introduction to PI3K and Adipose Tissue Physiology

1-1 Introduction to PI3Ks

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that phosphorylate the 3' hydroxyl position of the inositol ring in phosphatidylinositols (PIs) (Fig. 1-1A). These lipid products of PI3K are essential intracellular second messengers that embed their hydrophobic tail groups in a glycerol backbone into the plasma membrane. 3'-phosphorylated PIs are ubiquitously expressed in eukaryotic cells and promote signals relevant in a wide range of health and diseases. PI3K's are divided into three groups and classified based upon their PI substrate preference and their domain structures.

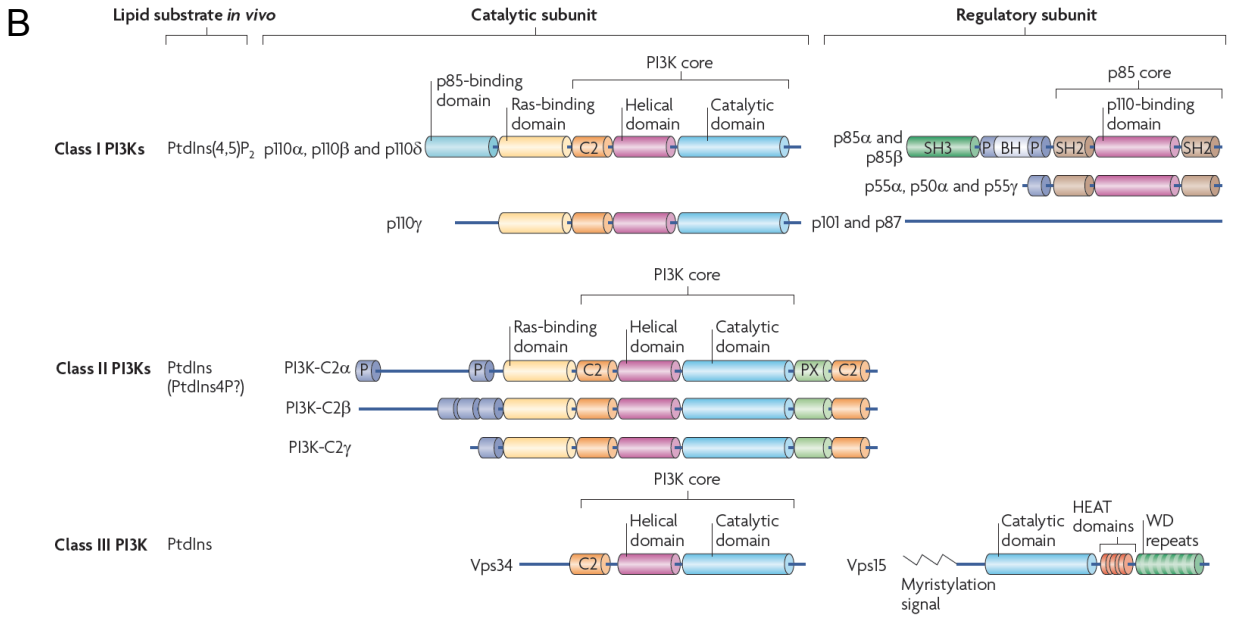
1-1.1 Class III PI3Ks

The only member of Class III PI3K is vacuolar protein sorting-associated protein 34 (Vps34). This kinase also is the only PI3K expressed in all eukaryotic cells [1]. Vps34 has a lipid substrate specificity for PI and which is used to create phosphatidylinositol 3-phosphate (PI(3)P). Vps34 dimerizes with Vps15, a protein kinase that acts as a regulatory subunit for Vps34 and this complex goes on to associate with other multiprotein complexes to regulate vesicle traffic within the cell including autophagy and endocytosis (Fig 1-1B) [1]. It is also suggested that Vps34 is required for full activation of mammalian target of rapamycin (mTOR) which regulates protein synthesis in response to nutrient availability [2].



Vanhaesebroeck 2000

FIG. 1-1. PI3K's are classified by their PI substrate preference and their domain structures. A: PI3K's phosphorylate PIs on their 3' hydroxyl position using the γ-phosphate of ATP. B: All PI3K subunits have a C2 domain, a helical and a catalytic domain. Class I PI3Ks use PI(4,5,)P_2 as their lipid substrate and complex with a regulatory subunit by binding SH2 domains with the exception of p101 and p87 subunits. Class II PI3Ks have two C2 domains located at both termini and lack regulatory subunits. They rely on PI(3)P and also PI as their lipid substrate. Class III PI3K consists of one catalytic member, Vps34, composed of only a C2, helical and catalytic domains that binds to one regulatory subunit, Vps 15. The catalytic domain of Vps15 is thought to be inactive. Class III PI3Ks use PI as a lipid substrate.



Vanhaesebroeck 2010

1-1.2 Class II PI3Ks

Class II PI3Ks are unique in comparison to the other PI3Ks in that they do not have a regulatory subunit. They express three isoforms of different catalytic domains located at their carboxyl terminus which provide the nomenclature for the three class II PI3Ks, PI3K-C2α, PI3K-C2β, and PI3K-C2γ (Fig 1-1B) [1]. Class II PI3Ks are broadly, but not ubiquitously, expressed in most tissues and are important in physiological cell processes including cell migration, glucose metabolism, exocytosis and apoptosis [3]. The main

substrate preference of class II PI3K is generally PI(3)P which is used to generate phosphatidylinositol (3, 4)-phosphate ($PI(3,4)P_2$). However, recent evidence suggests that they are also capable of generating PI(3)P from PI [4].

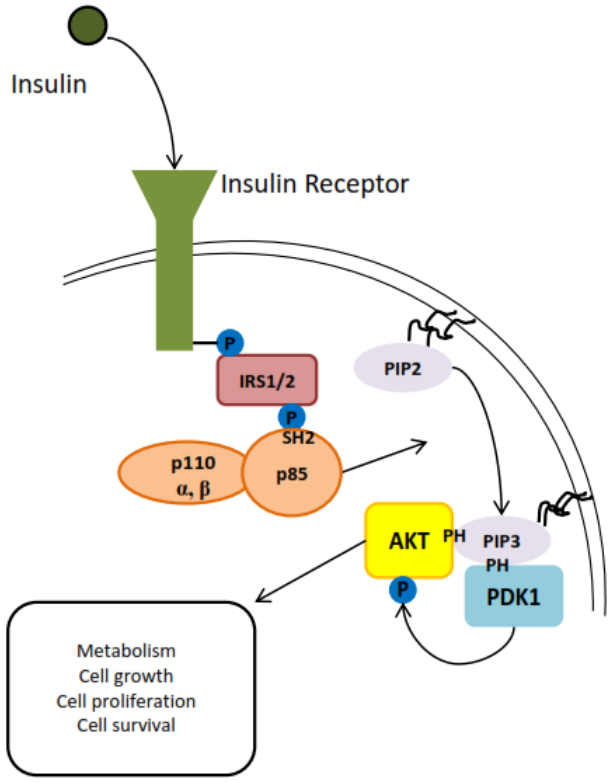
1-1.3 Class I PI3Ks

Class I PI3Ks, the best understood of the PI3Ks, are present in all mammalian cells [5]. They use the substrate $PI(4,5)P_2$ to generate phosphophatidylinositol (3,4,5)-phosphate ($PI(3,4,5)P_3$) [6]. Class I PI3Ks are further subdivided based upon their different regulatory domains (Fig 1-1B). Class IA PI3K's are distributed across most tissues. The class IA PI3K catalytic subunits, p110α, p110β and p110δ, are encoded by separate different genes and bind to a regulatory subunits, primarily p85α but also to p85β, and p55γ. The class IB p110γ associates with either p101 or p84 regulatory subunit and is expressed primarily in hematopoietic cell types [7]. Both class1A and 1B are regulated through stimulation of receptors on the cell surface. Class IA PI3K's are activated by receptor tyrosine kinases but p110β can also be activated by G-protein coupled receptors (GPCRs). Class IB PI3K is activated through G-protein coupled receptors (GPCRs). In addition, all class I PI3Ks can be activated by small G proteins through the Ras-Binding-Domain of p110 proteins (ref).

1-2 Class IA PI3K activation and signaling

Class IA p110α and p110β are activated in response to insulin stimulation of the insulin receptor (Fig 1-2). Insulin binds and stimulates the insulin receptor on the cell surface which increases its activity as a tyrosine kinase. The insulin receptor (IR) can then phosphorylate insulin receptor substrate proteins (IRS1/2). Phosphorylated IRS proteins recruit and activate PI3K at the plasma membrane by binding to the Src

FIG.1-2. PI3K signaling is regulated by insulin.



homology 2 (SH2) domain of p85. The p110α and p110β subunits are the primary insulin responsive catalytic isoforms of PI3K and will be further discussed in the following sections. The p110δ subunit of PI3K is not expressed in insulin responsive tissues. Once activated at the plasma membrane PI3K can phosphorylate $PI(3,4)P_2$ into $PI(3,4,5)P_3$ which is critical for normal cell physiology. This lipid product of PI3K activates downstream signaling components including phosphoinositide-dependent kinase-1 (PDK1) and, perhaps the most notable, Akt. Both PDK1 and Akt dock to $PI(3,4,5)P_3$ through their pleckstrin homology (PH) domain allowing for PDK1 to phosphorylate and activate Akt. Activated Akt phosphorylates many downstream targets including other kinases, signaling proteins or transcription factors and regulates many processes involved in cell metabolism, cell growth, cell proliferation and cell survival [8].

The actions of PI3K are opposed by phospholipid phosphatases which target PIs for dephosphorylation thereby attenuating PI3K signaling. Two major phospholipid phosphatases that target $PI(3,4,5)P_3$'s are phosphatase and tensin homologue (PTEN) and SH2-containing inositol 5'-phosphatase-2 (SHIP2). PTEN dephosphorylates PIs on the 3' hydroxyl position whereas SHIP2 targets the phosphorylated 5'-position [9]. Insulin

sensitivity can be regulated through these phosphatases. Whole-body deletion of PTEN in a mouse model improved insulin sensitivity and a knockout mouse model of SHIP2 provided protection against insulin resistance induced by obesity [10, 11].

$PI(3,4,5)P_3$ is a crucial intermediate in the insulin signaling pathway because it mediates insulin-dependent activation of PDK1 which activates Akt through phosphorylation of Akt at threonine 308 (T308) [12]. The T308 phosphorylation site is located in the activation loop of Akt and was shown to be required for catalytic activity of the enzyme. This same study also determined that T308 phosphorylation is important in reducing the binding affinity of Akt for associations at the cell membrane enabling the enzyme to mediate cytosolic signals [13]. However, full activation of Akt requires phosphorylation at a second site, serine 473 (S473), in addition to T308. S473 is located at the C-terminus and is phosphorylated by mTor Complex 2 (mTORC2), which is not directly regulated by PI3K signaling. Lack of phosphorylation at one site does not demolish phosphorylation at the other but in the absence of phosphorylated T308, Akt stays in the inactive state implicating a pivotal role of PI3K in regulating Akt activation [14].

1-3 Metabolic phenotypes associated with disruptions of Class IA PI3Ks

It is well understood that insulin regulates glucose and lipid homeostasis in most tissues. Insulin promotes glucose clearance from the bloodstream by increasing glucose uptake in the muscle and adipose tissue while inhibiting glucose production from the liver [15]. Insulin also stimulates fatty acid synthesis in the liver and adipose tissues through a process called lipogenesis or the conversion of acetyl-CoA conversion into fatty acids for assembly into triglycerides [15]. PI3K plays an important physiological role in insulin-

responsive tissues and PI3K signaling is impaired in states of insulin resistance. In almost all type 2 diabetic patients PI3K activity is decreased in the muscle, liver and adipose tissue [16].

Due to the importance of Class 1A PI3K in insulin signaling, intensive research has gone into understanding the role of *in vivo* PI3K activity in insulin-mediated metabolism (Table 1). The initial attempts to characterize the contributions of the PI3K insulin responsive catalytic isoforms, p110α and p110β, through a global homozygous deletion resulted in embryonic lethality for deletion of p110α and also p110β underscoring the absolute necessity for PI3K activity [17, 18]. Mice with whole-body heterozygous deletion of either p110α or p110β did survive but had no discernible phenotype. However, double heterozygous deletion of both p110α and p110β resulted in mild glucose intolerance and hyperinsulinemia in the fasted state indicating a requirement for maximal PI3K activity in normal glucose homeostasis [19]. Studies utilizing kinase-dead “knockin” mouse models suggest that PI3K activity is necessary to maintain insulin and glucose sensitivity. Full-body homozygous knockin of kinase-dead p110β results in mild insulin resistance and growth retardation [20]. The p110α-heterozygous kinase-dead knockin mouse produced a much more profound phenotype of glucose intolerance and hyperinsulinemia and also increased adiposity and reduced growth [21].

In an attempt to understand the precise contributions of PI3K in insulin signaling many groups have turned to mouse models of tissue specific knockout mouse models. Liver specific knockout models show that deletion of p110α affects glucose intolerance and impairs insulin signaling which may afford some protection against

Table 1: Metabolic phenotypes associated with PI3K catalytic isoform-specific disruptions

PI3K Catalytic Isoform	Affected Tissue	Metabolic Phenotype	Author
p110α	Global – homozygous deletion	Embryonic lethal	Bi et. al. 1999. J. Biol Chem
	Global – heterozygous knockout	No phenotype	Brachmann et.al. 2005 Mol Cell Bio
	Global – heterozygous kinase-dead knockin	-glucose intolerance -hyperinsulinemia -increased adiposity -growth retardation	Foukas et. al. 2006 Nature
	Liver	-glucose intolerance -insulin intolerant -mild obesity	Sopasakis et. al. 2010 Cell Metab
	Liver	protection against high-fat diet steatosis	Chattopadhyay et. al. 2011
	Muscle	-cardiac T tubule disruption -decreased muscle	Wu et. al. 2011 PLOS one
		-normal glucose tolerance -normal adiposity	Wu, Lin RZ Unpublished data
	Muscle	-impaired cardiac contractility	Lu et. al. 2009 Circulation
p110β	Global – homozygous deletion	Embryonic lethal	Bi et. al. 2002 Mamm Genome
	Global – heterozygous knockout	No phenotype	Brachmann et.al. 2005 Mol Cell Bio
	Global – homozygous kinase-dead knockin	-mild insulin resistance -growth retardation	Ciraolo et. al. 2008 Science
	Liver	kinase-independent impaired insulin/glucose sensitivity	Jia et. al. 2008 Nature
	Liver	hyperglycemia	Chattopadhyay et. al. 2011
	Muscle	No phenotype	Wu, Lin RZ Unpublished data

high-fat diet steatosis [22, 23]. p110β in the liver appears to be important in protection against hyperglycemia and is thought to regulate insulin sensitivity through a kinase-independent function of the p110β subunit [23, 24]. In the skeletal and cardiac muscle p110α knockout, the kinase was shown to be important for normal cardiac contractility

but this deletion did not affect glucose or lipid homeostasis [25, 26], Wu, Lin RZ unpublished data]. The current dogma is that the skeletal muscle is the insulin sensitive organ responsible for 70-90% of glucose disposal following carbohydrate loading [27]. As a result, skeletal muscle insulin resistance is highly indicated as a major contributor to disease states such as diabetes and metabolic syndrome [28]. Therefore it is surprising that attenuation of PI3K activity in the skeletal muscle of the muscle-specific p110α or p110β knockout mice does not result in any glucose or lipid phenotype (Table 1). This potentially suggests that muscle insulin resistance is a byproduct of disease onset in another organ system.

Missing from Table 1 is the adipose tissue which is also a highly insulin responsive organ. As will be discussed in the following sections the adipose tissue, which was once thought to be a metabolically inert fat storage depot, actually has the capability to be a potent regulator of global metabolism. However, the role of *in vivo* PI3K in the adipose tissue has remained unexplored. The primary goal of this dissertation is to provide evidence that adipose tissue PI3K is important not only for normal adipose tissue function but also for the regulation of systemic metabolic homeostasis.

1-4 Adipose tissues are metabolically important organs

Insulin resistance is known to play a role in the development of obesity and type 2 diabetes, yet little is known about the pathological mechanisms that lead to decreased insulin signaling. Adipose tissue is a key insulin-responsive tissue that contributes to the regulation of whole-body glucose and lipid homeostasis. PI3K is activated in response to insulin stimulation of adipocytes and may contribute to the regulation of metabolism by the adipose tissue.

Studies examining the role of PI3K in adipocytes relied on pharmacological inhibition of PI3K catalytic activity in isolated primary adipocytes or cell lines [29-32]. These studies suggest that PI3K mediates glucose and lipid homeostasis and insulin signaling in the adipocytes but were unable to describe the effect of PI3K on the adipose tissue as an active organ or on global metabolism. The role of specific PI3K isoforms in regulating adipose tissue functions that affect systemic glucose and lipid metabolism have not been studied.

The mechanisms surrounding the pathophysiology associated with the adipose tissue are a highly relevant research topic in today's population. Fueled by increased availability of food resources and increasingly sedentary lifestyles, obesity has grown to epidemic proportions. In 2005 over 1.6 billion adults were overweight and 400 million were obese [33]. This rise in obesity was predicted to surpass 700 million individuals in 2015 if no intervention occurred. However, in January of 2014 the Overseas Development Institute reported that this barrier had already been breached with 904 million people classified as obese. This equates to 1 in 3 people in the developing world with a BMI greater than 25 [34].

Obesity is a recognized as a medical condition and highly correlates with many other diseases including cardiovascular disease, insulin resistance, type 2 diabetes, and metabolic syndrome [35]. In addition adipose tissue accumulation around the visceral organs, commonly seen with abdominal obesity, is associated with higher mortality rates [36]. In the modern world environmental factors, such as increased availability of calorie dense foods, most likely are strong contributors to increased adiposity. However, researchers are beginning to recognize that obesity onset is likely a complex pathological

process that reflects both environmental and genetic interactions [35]. It was recently demonstrated that in longitudinal studies comparing mortality among obese populations to lean type 2 diabetic populations the mortality among the lean diabetics was higher than those of the obese non-diabetic cohorts [37]. This suggests that obesity alone cannot account for increased mortality rates among obese individuals and also suggests that impairment in insulin sensitivity can be extremely detrimental to long term survival. Adipocytes isolated from both lean type 2 diabetic individuals and obese individuals displayed similar levels of impaired insulin-stimulated PI3K activity and Akt activation [38]. It was further demonstrated that a genetic predisposition to diabetes is also associated with increased body weight and obesity suggesting that the mechanisms of pathophysiology between insulin signaling and adiposity are intertwined [39].

1-4.1 Adipose tissues are essential for energy homeostasis

High amounts of visceral adipose tissue can exert negative effects but in normal quantities the adipose tissue serves an important physiological role. The adipose tissue is the largest energy reserve in the body and is also the primary source of metabolic fuel. There are two types of adipose tissue, brown adipose tissue (BAT) and white adipose tissue (WAT). BAT is most prominently found in the interscapular region of infants [40]. BAT mass decreases substantially in adult humans but is still located in specific regions such as the paravertebral or supraclavicular regions [40, 41]. Brown adipocytes contain high levels of mitochondria and uncoupling protein-1 (UCP-1) that allow them to burn fat and produce heat instead of ATP. Thus, BAT plays a key role in non-shivering thermogenesis [42]. On the other hand, WAT is found in many depots throughout the body to store triglycerides. White adipocytes are larger than brown adipocytes because

they possess bigger unilocular lipid droplets whereas brown adipocytes have smaller multilocular lipid droplets [40].

There are two ways in which adipocytes accumulate triglycerides (Fig. 1-3). One way is through fatty acid uptake from the circulation [43]. After consumption of a meal, triglycerides are packaged in large lipoprotein particles called chylomicrons in the intestine and transported through the blood for uptake by the peripheral tissues [44]. Lipoprotein lipases secreted by adipocytes break down the triglycerides in the chylomicrons to produce free fatty acids (FFAs) and monoacylglycerol [45]. FFAs enter the adipocyte by either passive diffusion through the plasma membrane or by binding to fatty acid transport proteins (FATP) and CD36 at the cell surface [46-48]. Insulin stimulation increases translocation of FABP in 3T3-L1 adipocytes to the cell membrane and also increase CD36 translocation in the muscle [49, 50]. After FFAs have entered the adipocyte, they are converted in the endoplasmic reticulum back into triglycerides and stored in the lipid droplet until needed [51]

A second way in which adipocytes accumulate triglycerides is through *de novo* fatty acid synthesis or lipogenesis, a process by which lipids are generated from non-lipid precursors (Fig. 1-3). Lipogenesis contributes a minor portion of the fatty acids stored in the adipocytes but is a major source of lipid production in the liver during times of fasting to provide energy for the other organs. In *de novo* fatty acid synthesis, glucose enters the cells through the GLUT4 transporter in response to insulin stimulation [52]. Glucose is glycolytically converted into acetyl-CoA and then converted into malonyl-CoA by acetyl-CoA carboxylase (ACC), the committed step in lipogenesis [53]. Fatty acid synthase (FAS) then catalyzes a series of reactions using acetyl-CoA and malonyl-CoA to yield a fatty

acid. Fatty acyl-CoAs are then esterified onto glycerol 3-phosphate to form triglycerides which are stored until needed [51].

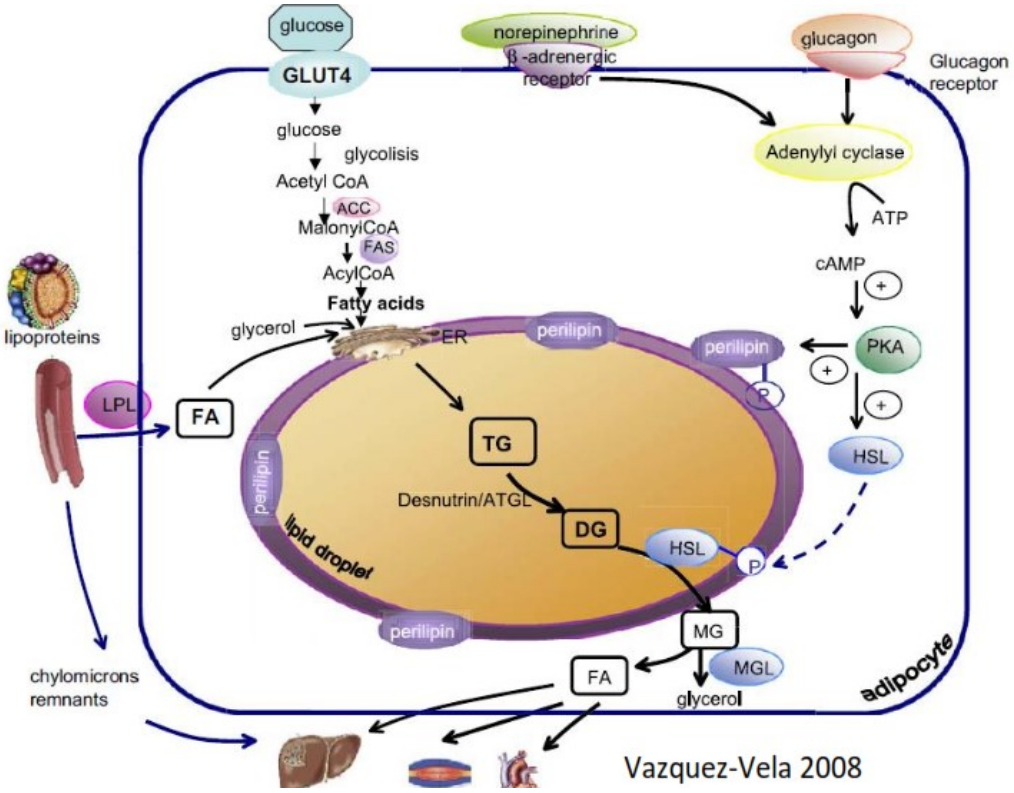


Fig. 1-3 Fatty acid flux through the adipocyte is maintained through fatty acid uptake, lipogenesis and lipolysis. Fatty acids from the diet are packaged into chylomicrons in the intestine and transported to the adipocyte where they are released into the cytoplasm by lipoprotein lipase. Lipogenesis involves the creation of fatty acids from acetyl-CoA, one of the end products of glycolysis. Fatty acids acquired through either uptake from the bloodstream or from lipogenesis are stored in the lipid droplet of the adipocyte as triglycerides until hormonal activation of adenylyl cyclase stimulates their release by lipolysis. The triglycerides are broken down by a series of lipases (ATGL, HSL and MGL) into free fatty acids and glycerol and released into the bloodstream for use as an energy substrate for other tissues.

The triglycerides stored in lipid droplets are hydrolyzed by lipases to release FFAs in times of caloric need [54]. During periods of fasting, glucagon and catecholamines activate lipolysis in the adipocyte [51]. Glucagon is a hormone produced by the pancreas in response to low blood glucose levels and activates the glucagon receptor on the adipocyte membrane. Catecholamines, such as epinephrine, activate β-adrenergic receptors on the membrane of the adipocyte [43]. As shown in figure 1-3, activation of

both glucagon and beta-adrenergic receptors elevates the level of cyclic adenosine monophosphate (cAMP) in the cell and activates protein kinase A (PKA) [55]. PKA phosphorylates perilipin, which is localized on the membrane of the lipid droplet. Phosphorylation of perilipin causes CGI-58, normally bound to perilipin, to dissociate into the cytosol [55]. This release of CGI-58 from perilipin serves two roles. First, free perilipin can activate hormone sensitive lipase (HSL) at the membrane of the lipid droplet. Second, the newly released CGI-58 binds to another lipase, adipose triglyceride lipase (ATGL), in the cytosol, causing the enzyme to translocate to the lipid droplet where it becomes embedded in the membrane. The triglycerides stored in the lipid droplet are hydrolyzed in a sequential manner with each step releasing one fatty acid [56]. ATGL performs the initial cleavage converting the triglyceride into a diglyceride. HSL releases the second fatty acid creating a monoglyceride. At this point the monoglyceride is released from the lipid droplet out into the cytosol where the third and final lipase, monoglyceride lipase (MGL), performs the final cleavage, liberating the final fatty acid from the glycerol backbone [55]. Lipolysis is opposed by insulin in the adipocyte primarily through the actions of protein kinase B, also named Akt. Akt has many functions in the adipocyte including inhibition of PKA phosphorylation of HSL as well as activation of phosphodiesterases 3 and 4 which degrade cAMP [57-59]. Insulin thus serves as a regulator of lipid homeostasis in the adipose tissue through regulation of lipid storage and breakdown.

1-4.2 Brown adipose tissue is an important source of non-shivering thermogenesis

Normal human physiology is highly dependent on body temperature which is maintained in a narrow range around 37ºC. In order to prevent internal temperatures from

dropping in the presence of colder external temperatures muscles groups around the vital organs shiver to generate heat from the energy released in the breakdown of muscle ATP [60]. However this process is finite in its duration and is rate-limited by the availability of energy stores to the muscle. In the 1950's it was observed that rodents adapt to temperatures as cold as -10ºC and appear to thrive and even reproduce in conditions that originally seemed too harsh for small animals [61]. It was a very surprising discovery that after exposure to prolonged periods of cold, rodents stopped shivering yet still maintained an elevated metabolic rate that produced enough heat to maintain their body temperature [14, 62, 63]. Eventually it was determined that the source of this process, termed non-shivering thermogenesis, is the BAT.

Non-shivering thermogenesis is one of the primary functions of the BAT. The thermogenic capacity of the BAT is attributed mainly to the large quantities of uncoupling protein-1 (UCP-1) on the inner mitochondrial membrane which uncouples fatty acid oxidation from ATP synthesis allowing for energy from substrate oxidation to be dissipated as heat [64] (Fig 1-4A). This process is highly efficient. At its highest capacity, heat production by the BAT can reach 300W/kg meaning that while the brown adipose tissue accounts for only a small amount of body weight it can generate as much heat as the rest of the body [42].

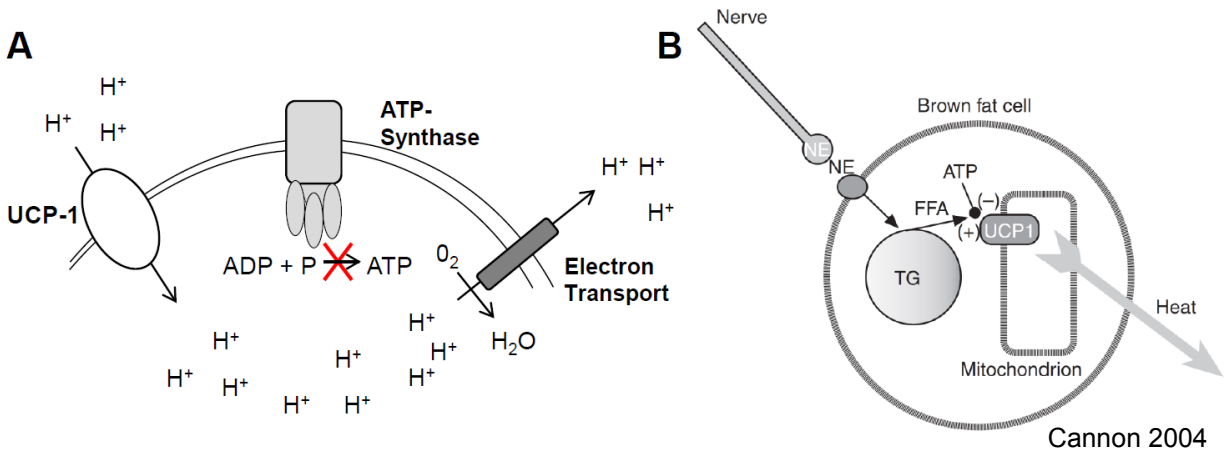


Fig. 1-4. Uncoupling protein-1 regulates heat production by transporting protons across the mitochondria. A. UCP-1 uncouples proton transport across the mitochondria membrane from ATP synthese for use in the electron transport chain. B. Sympathetic innervation releases free fatty acids (FFA) from the lipid droplet through lipolysis and activates UCP-1 allowing for efficient dissipation of energy as heat for non-shivering thermogenesis.

UCP-1 function is enhanced through adrenergic activation of the brown adipocyte. In response to cold, sympathetic innervations activates the adrenergic receptors on the adipocyte surface [65] (Fig 1-4B). Adrenergic activation releases FFAs from the lipid droplets of the brown adipocytes providing rapid energy substrates to facilitate the sudden increase in mitochondrial respiration. FFAs may also be required to bind directly to UCP-1 thereby inducing the conformational change allowing for proton transport across the mitochondrial membrane [42]. UCP-1 expression is a rate-limiting factor in adrenergic stimulation of thermogenesis. Brown adipocytes lacking UCP-1 do not show any increased in oxygen consumption after stimulation with norepinephrine indicating that BAT thermogenesis is blocked in the absence of UCP-1 and cellular respiration does not increase as seen in control brown adipocytes expressing UCP-1 (Fig 1-5).

UCP-1 expression is primarily controlled by transcription factors binding to multiple UCP-1 promoter regions. Among them are CRE promoter sites which are bound by nuclear cAMP response element-binding (CREB) after β-adrenergic

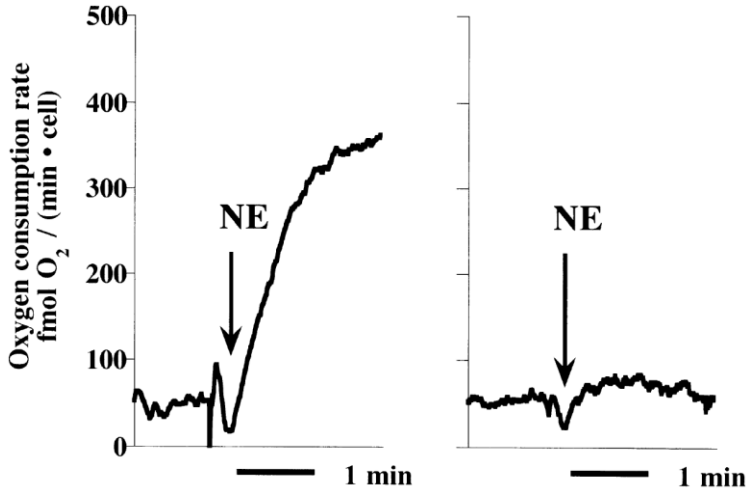


Fig. 1-5. Increased brown adipocyte respiration by non-shivering thermogenesis stimulated by adrenergic agonists is dependent on UCP-1 expression. Thermogenic response measured by oxygen output in isolated brown adipocytes in response to norepinephrine (NE) stimulation in wild type mice (left) and in mice lacking UCP-1 (right).

Adapted from Nedergaard 2000

stimulation of cAMP through PKA [66]. Another source multiple promoter regions is the distal complex enhancer region located upstream of UCP-1 [42]. Most notably is the PPAR response elements (PPREs) that bind peroxisome proliferator activated receptor gamma (PPARγ) [67, 68]. Treatment of brown adipocytes with PPARγ agonists increase the expression of UCP-1 indicating that this is a powerful mediator of the thermogenic response. PPARγ can also complex with other transcription factors in the nucleus that bind to UCP-1 promoter regions such as retinoid X receptor or thyroid response hormone which also have been shown to enhance UCP-1 expression [69]. The binding of PPARγ to other transcription factors is largely dependent on its interactions with peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) [70]. PGC-1α plays many roles in the cell most of them centering around the cellular respiration, mitochondrial biogenesis and gene expression [70]. PGC-1α gene expression is induced through β-adrenergic activation of PKA to phosphorylate CREB. The nuclear PGC-1α protein is activated through phosphorylation by p38 mitogen activating protein (p38 MAPK) where it can then increase nuclear transcription of mitochondrial-associated genes [70].

Insulin stimulation has been shown to increase UCP-1 expression in brown adipocytes cell lines [71]. Targets of insulin stimulation that increase UCP-1 expression include ccaat-enhancer-binding proteins (C/EBP) and indirectly sterol regulatory element-binding protein 1c (SREBP1c) [42, 71, 72]. SREBP1c binds to the E box promoter region of PGC-1α to increase its transcription and thereby indirectly regulate UCP-1 and mitochondrial gene expression. It is also possible that SREBP1c enhances the production of FFAs in the brown adipocytes which can increase UCP-1 activity [72].

1-4.3 The white adipose tissues are endocrine organs

Not only does WAT serve as an energy storehouse, but it also serves as an endocrine organ. The effects of this endocrine function are observed in patients with obesity or lipid deficiency. Obese individuals with increased visceral adipose depots often present with several aberrant metabolic symptoms such as increased insulin resistance, hyperglycemia and high levels of blood triglycerides [73]. Interestingly, patients who develop lipodystrophy as a result of HIV antiretroviral therapies also exhibit symptoms of metabolic syndrome despite degeneration of the visceral adipose tissue [74, 75]. Because both too much and too little adipose tissue can have harmful systemic metabolic effects, it is clear that the adipose tissue is involved in endocrine metabolic regulation.

Adipose tissue secretes adipocytokines that contribute to the regulation of systemic glucose and lipid metabolism [76]. Generally these adipokines are catergorized by their actions on glucose homeostasis. Anti-hyperglycemic adipokines include adiponectin and omentin, both of which increase insulin sensitivity and are reduced in obesity. On the other hand, some anti-hyperglycemic adipokines, including visfatin and

leptin, are secreted in proportion to adipose mass and increase glucose-stimulated insulin secretion from the pancreas [51, 77].

The best known anti-hyperglycemic adipose-derived hormone is leptin, which signals to the arcuate nucleus in the hypothalamus of the brain to signal satiety and decrease food intake. Leptin also increases insulin sensitivity in the liver and skeletal muscle and stimulates locomotor activity [78, 79]. Several mouse models have been used to study leptin deficiency, most notably the *ob/ob* and *db/db* mice developed at Jackson Laboratories. The *ob/ob* mouse has a mutation in its leptin gene rendering it unable to produce this adipokine. As a result the *ob/ob* mouse is hyperphagic and rapidly becomes morbidly obese. This *ob/ob* mouse is also hyperglycemic and insulin resistant with hyperlipidemia [80]. It is also known that leptin administration can reverse the hyperglycemia as well as increase overall locomotor activity [81, 82]. The *db/db* mouse has a mutation in the leptin receptor and presents with the same phenotype as the *ob/ob* mouse in addition to leptin resistance and hyperleptinemia. Taken together, this evidence indicates that adipose-derived leptin regulates glucose homeostasis and overall energy expenditure.

Estrogen is another hormone that is produced by adipocytes. Estrogen production is controlled by the activity of aromatase located in the endoplasmic reticulum which catalyzes the conversion of testosterone to estrogen [83]. Estrogen binds to estrogen receptors (ER) which are located throughout the body [84]. Once estrogen binds the ERs are activated and translocate into the nucleus to regulate gene transcription [83]. ERs can be negatively regulated by glycogen synthase kinase-3 (GSK-3β), a target of insulin stimulated Akt, which phosphorylates and inactivates ERs ability to act as a transcriptional

regulator [83]. Akt phosphorylation inhibits the activity of GSK-3β and prevents active GSK-3β from blocking the actions of ERs.

Despite estrogen's well known role in female reproduction, it also participates in the regulation of metabolic function in both males and females. In mouse models of estrogen deficiency using whole-body aromatase knockouts (ARKO) the male mice developed an array of metabolic dysfunctions including increased adiposity, metabolic syndrome, hyperleptinemia, hepatic steatosis and hypercholesterolemia [85, 86]. The male ARKO mice also had reduced food intake and decreased activity. Similar phenotypes were seen in estrogen receptor knockout mice, which exhibited increased visceral adiposity but no change in food intake. Further investigation revealed that site-specific deletion of the estrogen receptor in the hypothalamus resulted in obesity accompanied by an anabolic shift in energy expenditure caused by decreased activity and increased food intake [87].

1-4.4 PI3K mediates insulin signaling in adipose tissues

Class IA PI3Ks play a crucial role in insulin signaling yet little is known about the physiological roles of specific PI3K isoforms in mature adipocytes. Most evidence linking PI3K to the regulation of adipocyte metabolism relied on the use of PI3K inhibitors to examine the enzyme's role in insulin signaling. It was shown that insulin induces fatty acid uptake in 3T3-L1 adipocytes by promoting the translocation FATP1 and fatty acid uptake was reduced by the addition of wortmannin, a PI3K inhibitor [50]. Treatment of 3T3-L1 adipocytes with wortmannin also decreased insulin-stimulated glucose uptake through reduced translocation of the GLUT4 transporter and reduced the ability of insulin to suppress lipolysis [31, 88-90].

Use of isoform-specific PI3K inhibitors in 3T3-L1 adipocytes suggested that insulin signals primarily through the p110α catalytic subunit. After the administration of the p110α inhibitor PIK-75, insulin activation of Akt and GSK3β phosphorylation was dramatically reduced [30]. To further underscore the importance of p110α, mice heterozygous for whole body kinase-dead p110α developed severe metabolic defects including reduced growth, hyperinsulinemia, glucose intolerance, hyperphagia, leptin resistance, increased adiposity, and blunted insulin signaling in the muscle, liver and fat [21]. While the importance of p110α in maintaining normal metabolic function is obvious, the contribution of p110α in the adipose tissue to systemic glucose and lipid homeostasis has not been studied.

The focus of this dissertation is to evaluate the contributions of PI3K to the normal physiology of the white and brown adipose tissue as well as the ability of PI3K in the adipose tissue to influence systemic metabolism. We will utilize mouse models of adipose specific deletion of the PI3K catalytic subunits to study the requirements of PI3K in insulin signaling and how alterations in adipose PI3K affect whole body glucose and lipid homeostasis

Chapter 2

Adipose-Specific Ablation of PI3K increases adiposity and glucose intolerance

2-1 Introduction

Insulin resistance is known to play a role in the development of obesity and type 2 diabetes, yet little is known about the pathological mechanisms that lead to decreased insulin signaling. The leptin receptor mutant *db/db* mouse is a well-studied model of severe obesity, diabetes and dyslipidemia [91]. However, its relevance to the human disorder is limited since the vast majority of the obese patients do not have leptin receptor mutations [92].

IR knockout mice have provided unique insight into how organ-specific insulin resistance affects adiposity and glucose homeostasis. Muscle-specific IR knockout mice exhibited normal glucose tolerance associated with increased adiposity, while mice with liver-specific ablation of the IR were glucose intolerant and had normal fat mass [93, 94]. The fat-specific IR knockout mouse (FIRKO) exhibited reduced adiposity and normal glucose tolerance [95]. In fact, loss of IR in the fat tissue appears to be protective. The FIRKO mouse was protected from obesity and glucose intolerance despite hyperphagia as a result of gold thioglucose treatment to induce hypothalamic lesions. This protection afforded by loss of adipose IR seems to increase with age [96].

Class IA PI3Ks play a crucial role in insulin signaling. In fact, pharmacological inhibition of PI3K completely blocks the metabolic actions of insulin [97]. In addition to decreased insulin signaling with pharmacological inhibition of PI3K in adipocytes cell lines, mice expressing a kinase-dead mutant of p110α developed insulin resistance and glucose intolerance [21, 30]. PI3K is activated in response to stimulation by insulin and other hormones [98]. When stimulated with insulin, the insulin receptor phosphorylates insulin receptor substrate proteins (IRS) on tyrosine [8]. These IRS proteins bind to the

PI3K regulatory subunits to activate PI3K signaling. It has been demonstrated that PIP_3 activates downstream effectors such as the protein kinase Akt, which regulates a variety of cell responses including glucose uptake, lipolysis and adipokine secretion [31, 99, 100].

Little is known about the physiological roles of specific PI3K isoforms in mature adipocytes. It has been demonstrated that p110α is the primary insulin responsive catalytic subunit in the adipocyte [30]. However these studies were performed in 3T3-L1 fibroblasts which are not true adipocytes and do not provide information about the physiological role for p110α in a complete animal model. As previously described mice heterozygous for whole body kinase-dead p110α developed severe metabolic defects including blunted insulin signaling in the muscle, liver, and fat as well as adiposity and glucose intolerance [21]. The phenotypes seen in the kinase dead mouse are similar to those of insulin receptor knockouts specific to the muscle, liver or fat but do not provide insight into the tissue specific role of p110α. However, the FIRKO mouse model provides evidence that insulin signaling in the adipose tissue is very important in systemic metabolic homeostasis [95]. It has been further shown that PI3K activity is crucial to mediate the downstream effects of insulin stimulation in many tissues including the adipose tissue but the in vivo role of PI3K in the adipose tissue has not been examined. In order to investigate the specific role of PI3-Kinase in the adipose tissue we created we created mice that lack the class 1A PI3K catalytic subunit p110α or p110β specifically in the white and brown adipose tissue using Cre recombinase driven by the aP2 promoter.

2-2 Materials and Methods

Antibodies. The following antibodies were used: p110α, p110β, Akt phospho-T308, cytochrome c oxidase (COX) IV, and heat shock protein (HSP) 90 from Cell Signaling Technology; p85, Akt, PEPCK, insulin receptor substrate IRS-1, IRS-2 from Santa Cruz Biotechnology; Akt phospho-S473 from Epitomics; and β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Sigma-Aldrich.

Animals. $p110\alpha^{flox/flox}$ and $p110\beta^{flox/flox}$ mice (Lu 2009) were crossed with aP2-Cre transgenic mice (obtained from Bruce Spiegelman [101]) to produce experimental aP2-Cre/$p110\alpha^{flox/flox}$ and aP2-Cre/$p110\beta^{flox/flox}$ animals (called α-/- and β-/-, respectively) and control $p110\alpha^{flox/flox}$ or $p110\beta^{flox/flox}$ mice (called α+/+ and β+/+, respectively). These mouse strains are maintained in a mixed genetic background and the control animals for each study were littermates. Age-matched mice of both sexes were studied between 4 weeks and 4 months of age. The adipose tissue depots studied herein were perigonadal WAT and interscapular BAT. The mice were maintained under temperature- and humidity-controlled conditions with a 12-h light/12-h dark cycle and had free access to standard rodent chow and water. Body weights were measured weekly from 4 weeks to 4 months of age. At 4 months of age the mice were anesthetized with an intraperitoneal injection of ketamine/xylazine, and dual energy X-31ray absorptiometry (DEXA) scanning was performed with the Lunar PIXImus2 DEXA scanner (Faxitron X-ray Corporation, Wheeling, IL) to determine body composition. All animal studies performed in this investigation were approved by the University of Stony Brook Animal Care and Use Committee.

Histology. WAT and BAT were collected from 4-month-old male mice after whole body perfusion with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) administered through the inferior vena cava. The adipose tissues were collected and fixed overnight in 4% PFA. The adipose tissues and formalin-fixed liver were dehydrated and embedded in paraffin using the Leica ASP300S Tissue Processor (Leica Biosystems, Wetzlar, Germany). Sections (5 μm) were stained with hematoxylin and eosin (H&E). Frozen liver sections (8 μm) from 4-month-old male mice were stained with Oil Red O and counterstained with hematoxylin to visualize lipids. Sections were visualized with an Olympus BX43 microscope and Cell Sens Digital Imaging Software (Olympus).

Liver triglyceride content. Male mice (4 months old) were fasted for 6 h, and the livers were removed and frozen in liquid nitrogen. Triglycerides were extracted and quantified using the Serum Triglyceride Determination Kit (Sigma-Aldrich) as previously described [23].

Glucose tolerance test and serum chemistry. For glucose tolerance tests, 4-month-old male mice were fasted for 6 h. An intraperitoneal injection of glucose (2 g/kg body weight) was administered, and tail blood glucose levels were measured at various times using a OneTouch UltraMini glucose monitor (LifeScan, Milpitas, CA). Blood for serum chemistry was collected by a retro-orbital bleed under isoflurane anesthesia. Serum insulin was measured by an ELISA kit (Mercodia), as were serum leptin, adiponectin, fibroblast growth factor (FGF)-21 and resistin (R&D Systems). Serum triglycerides, free fatty acids and cholesterol were measured by Anilytics (Gaitherburg, MD).

Preparation of isolated adipocytes. White adipocytes were isolated according to Viswanadha and Londos [102]. In summary, WAT was collected from mice anesthetized with ketamine/xylazine. White adipocytes were isolated with 3 mg/mL type-1 collagenase (Worthington Biomedical Corp.) in Krebs Ringer Bicarbonate Solution HEPES buffer, pH 7.4 (supplemented with 3% fatty acid-free bovine serum albumin). Adipocytes were filtered through 297 μm polypropylene mesh, washed three times in Krebs Ringer Bicarbonate HEPES buffer, and collected for experiments. Brown adipocytes were isolated as previously described [103]. Briefly, BAT was collected from mice under ketamine/xylazine anesthesia. The BAT was subjected to an initial digestion with 1 mg/mL type-1 collagenase in Krebs Ringer Bicarbonate Solution HEPES buffer, pH 7.4 (supplemented with 4% fatty acid-free bovine albumin). The tissue was then minced, incubated again with collagenase, washed, and filtered through 297 μm mesh before the third and final incubation with collagenase. The isolated brown adipocytes were rinsed three times with Krebs Ringer Bicarbonate HEPES buffer and collected for experiments.

Human Perinephric Adipose Tissue. Human perinephric adipose tissue was a generous gift by Dr. Dennis Mynarcik and Stony Brook University Hospital as a byproduct of ongoing kidney transplants under IRB approval. Human perinephric adipose tissue was collected and stored at room temperature until use the and adipocytes isolated as described above.

PI3K activity assay. Pooled adipocytes isolated from WAT of 4-month-old male and female mice were treated with 100 nM bovine insulin (Sigma-Aldrich) for various times and then homogenized in general lysis buffer (10 mM sodium pyrophosphate, 50 mM HEPES, pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM sodium

orthovanadate, 1:1000 protease inhibitor cocktail (Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride). After centrifugation, aliquots of supernatant containing equal amounts of protein (measured by the Bio-Rad Protein Assay) were incubated overnight at 4°C with IRS-1 or IRS-2 antibody. IRS-associated PI3K was pulled down with protein A agarose, and PI3K activity was assayed in the immunoprecipitates as previously described [104]. For analysis of PI3K activity in isolated human perinephric adipose tissue, white adipocytes were isolated as previously described and stimulated with 100nM of insulin for 5 minutes and PI3K activity was measured as described above. Human adipocyte lysates were treated with 100nM of a p110α-specific inhibitor PIK-75 (Cayman Chemical, Ann Arbor MI) for 20 minutes prior to the addition of the substrate and reaction mix. For analysis of PI3K activity in BAT, mice fasted overnight were anesthetized with ketamine/xylazine, and the inferior vena cava was exposed by laparotomy through a midline incision. Human insulin (Novolin R, Novo Nordisk) at 1 U/kg body weight or an equal volume of saline was injected into the inferior vena cava. BAT was collected 15 min later and stored in liquid nitrogen. The tissue was homogenized in general lysis buffer, and PI3K activity was assayed as described above.

Western blotting. Tissues were collected and immediately frozen in liquid nitrogen. Tissue samples were homogenized in general lysis buffer, and adipose tissue homogenates were further sonicated for 10 min at 4°C. After centrifugation, aliquots of supernatant containing equal amounts of protein were subjected to SDS-PAGE and immunoblotting as previously described [105]. Signals were visualized using horseradish peroxidase-linked secondary antibodies and chemiluminescence reagents. Densitometry was performed using Image J (NIH) after imaging with film or using AlphaView version

3.4.0.0 software after imaging with the FluorChem E system (ProteinSimple, Santa Clara, CA).

Gene expression analysis. RNA was isolated from Liver, WAT and BAT using the RNeasy Lipid Tissue Kit (Qiagen), and cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad). WAT and BAT gene expression of *Lpl* (Lipoprotein lipase), *Slc27a1* (FATP1), *Slc27a1* (FATP4), *Cebpa (*C/EBPα*)*, *Srebf1* (SREBP1c) and *Actb* (β-actin) were analyzed with the SYBR Green PCR Kit from Qiagen. The primer sequences are listed below in table 2. Liver lipogenic and gluconeogenic gene expression gene expression was investigated using the following Taqman Gene Expression Assays (*Fasn (*Fatty acid synthase) Mm00662312_g1; *Srebf1* (SREBP1c) Mm00550338_m1; *Acaca* (Acetyl-Coenzyme A Carboxylase α) Mm01304285_m1; *Ppara (*PPARα) Mm00627559_m1; *Pparg* (PPARγ) Mm01184323_m1; *G6pc* (Glucose-6-phosphatase) Mm00839363_m1; *Pck1* (PEPCK1) Mm01247058_m1; Life Technologies) using *Actb* as a control. Relative gene expression was obtained using the delta Ct calculation.

Table 2
Primer sequences for WAT RT-PCR

Gene Name	Forward Primer	Reverse primer
Actb	TTCTTTGCAGCTCCTTCGTT	ATGGAGGGGAATACAGCCC
Cebpa	CCGGGAGAACTCTAACTC	GATGTAGGCGCTGATGT
Srebf1	CTGGATTTGGCCCGGGGAGATTC	TGGAGCAGGTGGCGATGAGGTTC
LpL	TCTGTACGGCACAGTGG	CCTCTCGATGACGAAGC
Slc27a1	GATGTGCTCTATGACTGCCTG	GTGTCGCTGCTCCACGTCG
Slc27a4	AGCAACTGTGACCTGGAGAG	CTTCCGAGCATCCAGATAGA

Glycerol measurements. Glycerol was measured in serum from overnight fasted α-/- and α+/+ control male mice at 30 days of age, prior to obesity onset, and 4 months of age, after obesity onset using the Free serum glycerol was measured with the Free Glycerol

Determination Kit purchased from Sigma (St. Louis, MO). Glycerol release from stimulated isolated white adipocytes was also measured with the free glycerol determination kit.

Statistics. Results are reported as means ± SEM. Significance was determined by Student's *t* test. Statistical significance was accepted when $P < 0.05$.

2-3 Results

2-3.1 Loss of adipose-specific p110α causes increased adiposity.

We created a mouse strain with adipose-specific deletion of p110α using the Cre-lox system. Western blotting confirmed reduced p110α protein expression in adipocytes isolated from WAT and BAT of α-/- mice as compared to α+/+ animals (Fig. 2-1A). By contrast, there was no difference in levels of the p110β catalytic and p85 regulatory PI3K subunits in WAT and BAT from mice of these genotypes (Fig. 2-1A). We also created mice with adipose-specific deletion of p110β. Western blotting confirmed the successful ablation of p110β in white and brown adipocytes of β-/- mice (Fig. 2-1B). No decrease in PI3K subunits was seen in the liver or skeletal muscle of α-/- or β-/- mice (Fig. 2-1).

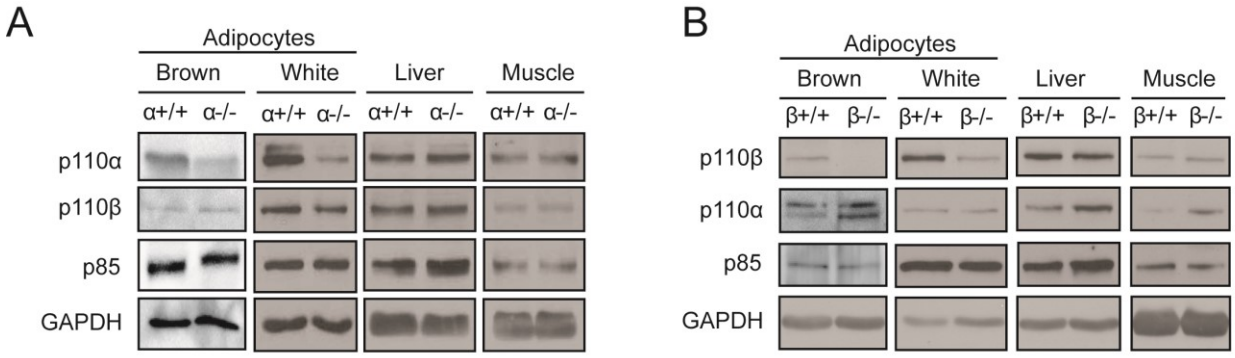


Fig. 2-1. Adipose tissue-specific loss of p110α and p110β proteins. Adipocytes were isolated from gonadal white or interscapular brown fat pads of adult p110α (A) and p110β (B) knockout mice and their controls. Isolated adipocytes from several animals of each genotype were pooled and subjected to western blotting using the indicated antibodies. Expression of PI3K subunits in the liver and gastrocnemius muscle of knockout and control mice is also shown. GAPDH serves as a loading control.

At birth, the α-/- mice appeared normal and were indistinguishable from their control littermates. However, starting at 6 weeks of age the male α-/- mice developed a progressive increase in body mass when compared to α+/+ controls (Fig. 2-2A). By 16 weeks of age male α-/- mice were 32% heavier than α+/+ mice when normalized to tibia

length (Fig. 2-2B). We assessed the body composition of 4-month-old male animals by DEXA scanning. The α-/- mice exhibited a 35% increase in fat mass when compared to the α+/+ controls, as well as a reduction in lean mass (Fig. 2-2C).

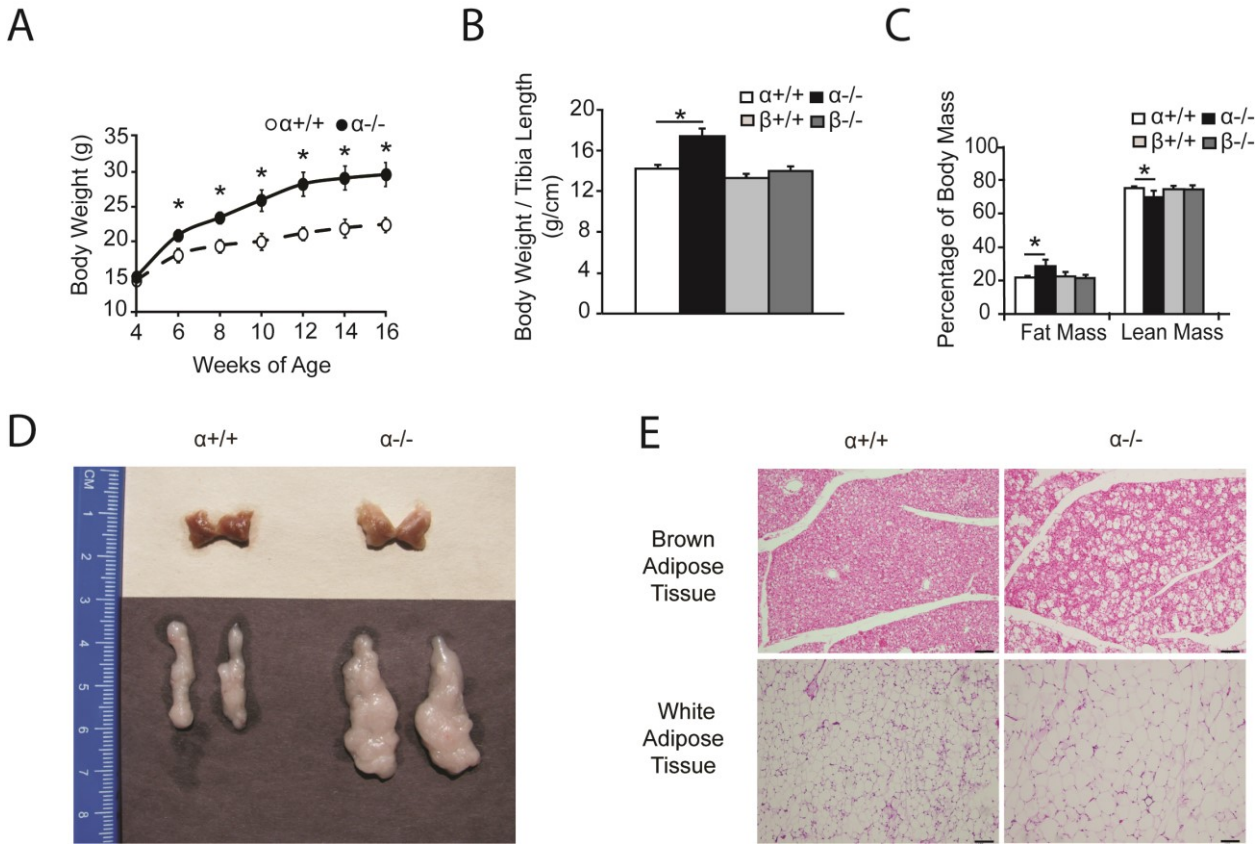


FIG. 2-2. Increased adiposity in male α-/- mice. A: Weight gain of α-/- and α+/+ mice (*n* = 4 per group). B: Body weights normalized to tibia length of 4-month-old mice (α, *n* = 8 per group; β+/+, *n* = 8; β-/-, *n* = 7). C: Fat mass and lean mass normalized to total body mass as measured by DEXA scan of 4-month-old mice (α, *n* = 11 per group; β, *n* = 6 per group). D: Interscapular brown (top) and epididymal white (bottom) fat pads of 4-month-old mice. E: Representative H&E-stained sections of epididymal white and interscapular brown fat pads from 4-month-old mice Scale bars are 100 μm.

Gross examination of the interscapular brown fat and epididymal white fat pads confirmed an increase in mass in both of these depots (Fig. 2-2D). The weight of the dissected epididymal adipose tissue from 4-month-old male α-/- mice was significantly increased over that of α+/+ mice when normalized to body weight (α+/+, 13.1 mg/g ± 1.5, *n* = 7; α-/-, 30.3 mg/g ± 3.2, *n* = 7; *P* = 0.0003). Histological examination of the fat pads

revealed hypertrophied white and brown adipocytes in the α-/- tissues (Fig. 2-2E). In contrast to the p110α knockout mice, 4-month-old β-/- mice showed no difference in body weight/tibia length, fat mass or lean mass as compared to the β+/+ controls (Fig. 2-2B and 2-2C).

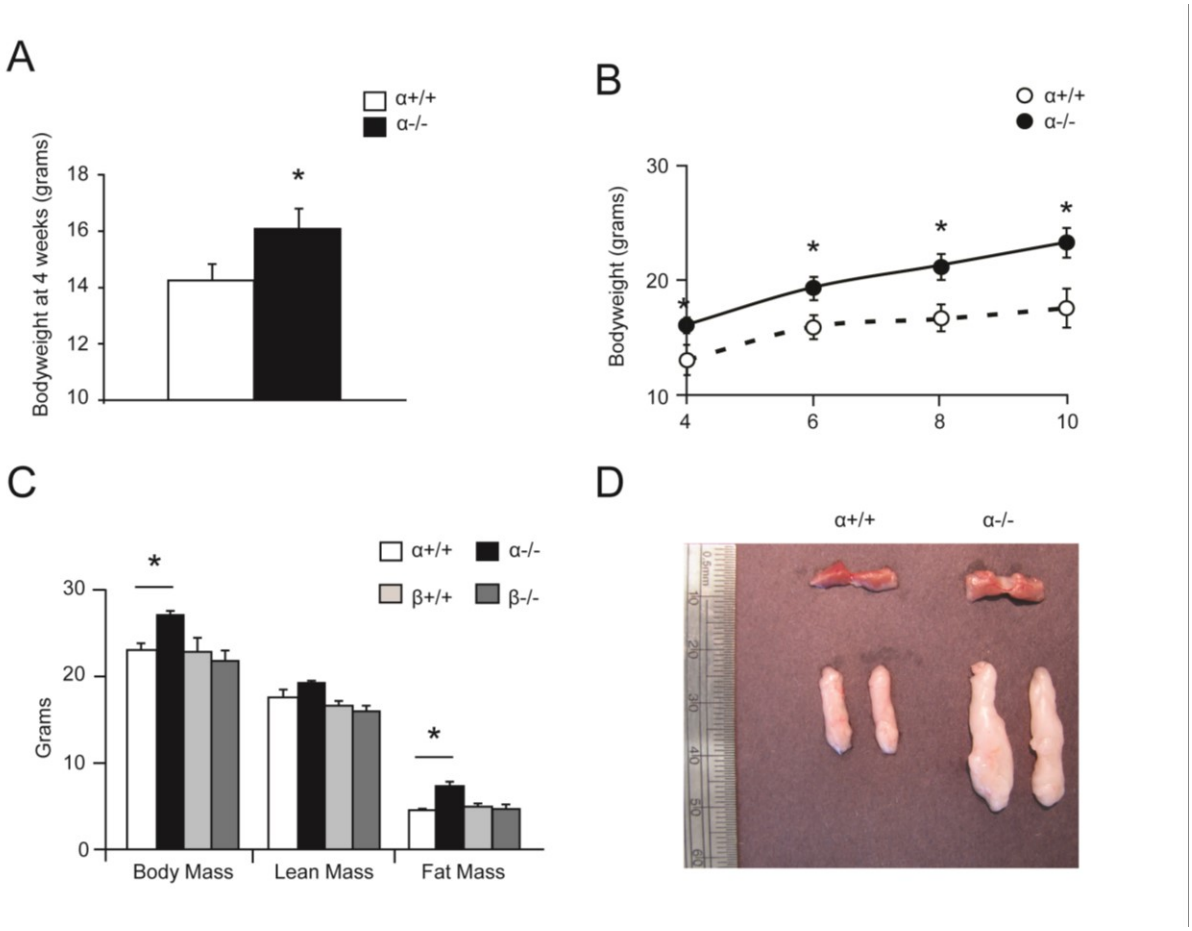


FIG. 2-3. Increased adiposity in female α-/- mice. Body weight is increased in 4 week old female α-/- mice (A) and continues with age (B) (α+/+ *n* = 5; α-/- *n* = 3). C: By 4 months the α-/- female mice have increased body mass as measured by DEXA scan while the female β-/- mice are unchanged from their β+/+ controls (*n* = 5 per group). D: Gross examination of the interscapular brown adipose tissue (top) and the gonadal white adipose tissue (bottom).

In contrast to the α-/- male mice the α-/- female mice displayed increased body weight compared to the female α+/+ controls starting at 4 weeks of age and continuing through 10 weeks of age (Fig. 2-3A, B). At 4 months of age the α-/- female mice have

increased body mass due to elevated fat mass with no change in lean mass in comparison to the female controls (Fig. 2-3C). We saw no change in the body mass or fat mass in the female β-/- mice (Fig. 2-3D).

2-3.2 α-/- mice have increased hepatic lipid deposition.

In addition to increased adiposity, 4-month-old α-/- mice exhibited an increase in liver weight to body weight ratio as compared to controls (Fig. 2-3A). This phenotype was not present in β-/- mice (Fig. 2-3A). To determine if the increased liver mass in α-/- mice was due to fat deposition, we measured liver triglyceride levels and found a significant increase in the lipid content of α-/- as compared to α+/+ tissue (Fig. 2-3B).

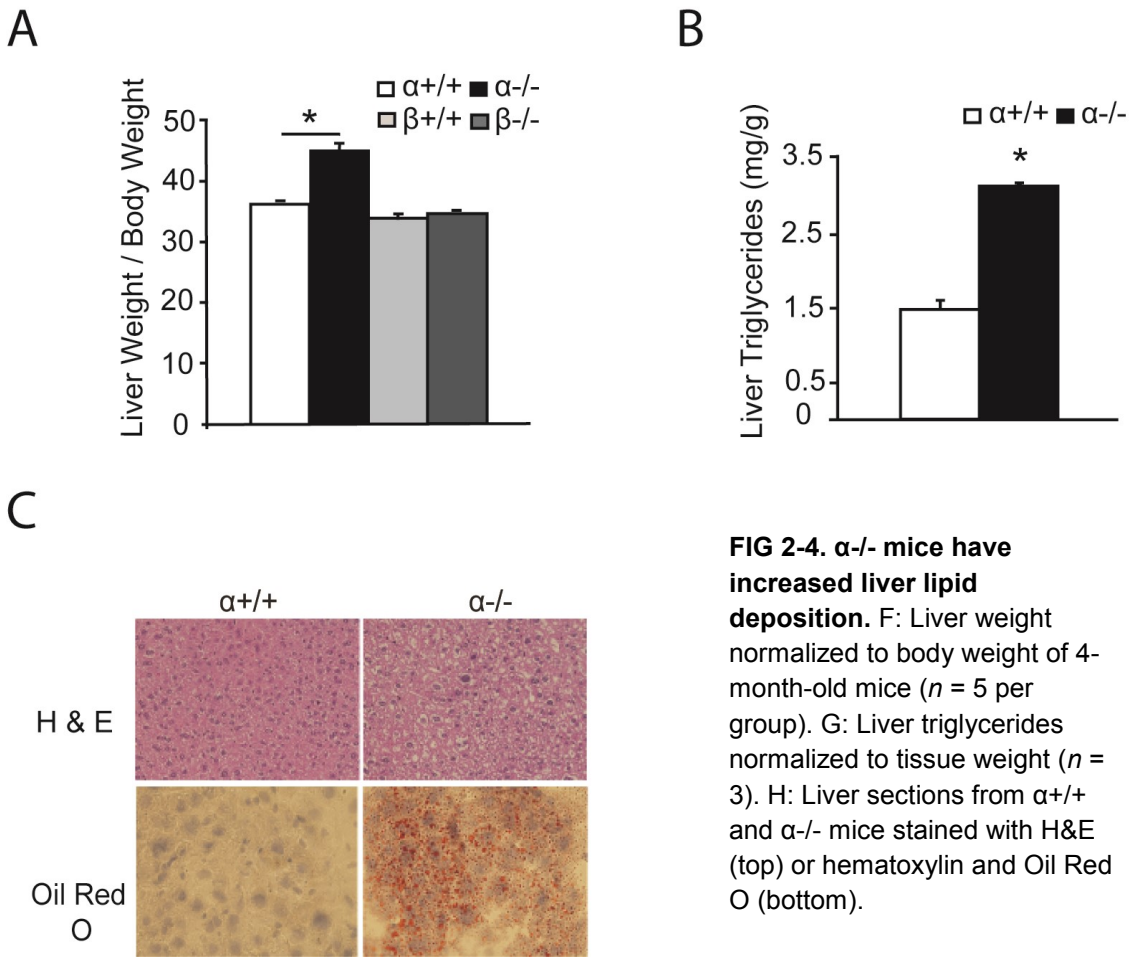


FIG 2-4. α-/- mice have increased liver lipid deposition. F: Liver weight normalized to body weight of 4-month-old mice (*n* = 5 per group). G: Liver triglycerides normalized to tissue weight (*n* = 3). H: Liver sections from α+/+ and α-/- mice stained with H&E (top) or hematoxylin and Oil Red O (bottom).

Increased accumulation of lipid in α-/- liver was also seen in sections stained with Oil Red O (Fig. 2-3C).

Increased lipid deposition in the liver of α-/- mice could be explained by increased lipogenesis. We examined the expression of lipogenic gene expression in the livers of 4 month old α-/- and controls during normal *ab lib* feeding and after fasting overnight (Table 3). Expression of the majority of lipogenic genes in the livers of α-/- mice are upregulated when compared to the α+/+ controls indicating that fatty acid synthesis is increased potentially accounting for increased hepatic triglyceride storage.

Table 3
Lipogenic gene expression in liver of α-/- and α+/+ mice

	Fold Change	
Gene Symbol	16 Hour Fast	*ad lib* Fed
Fasn	1.60	1.35
Srebf1	1.29	-1.10
Acaca	1.17	1
Ppara	1.28	1.15
Pparg	-1.72	2.30

Fold Change is expressed as $\frac{\alpha-/-}{\alpha++}$

We also looked at the levels of the gluconeogenic genes *G6pc* and *Pck1* both under fed and fasted conditions (Table 4). α-/- liver *Pck1* is increased primarily during fasting while *G6pc* is decreased in the α-/- liver during fasting but increased during *ad lib* feeding.

Table 4
Gluconeogenic gene expression in liver of α-/- and α+/+ mice

	Fold Change	
Gene Symbol	16 Hour Fast	*ad lib* Fed
G6pc	-1.34	1.55
Pck1	1.43	1.12

Fold Change is expressed as $\frac{\alpha-/-}{\alpha++}$

2-3.3 α-/- mice are glucose intolerant.

Increased adiposity in humans is often associated with insulin resistance and glucose intolerance. We performed glucose tolerance tests on 4-month-old male α-/- and α+/+ mice and found that the α-/- mice were relatively glucose intolerant despite having high fasted insulin levels (Fig. 2-5A and Table 5). By contrast, glucose tolerance and fasted insulin levels were essentially the same in β-/- and β+/+ mice (Fig. 2-5B and Table 5).

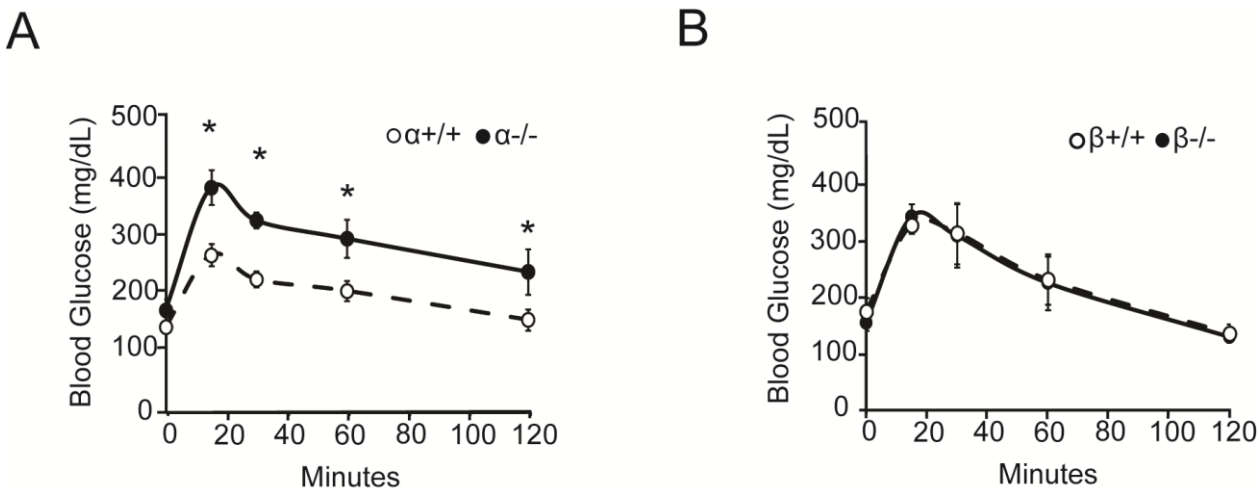


FIG 2-5: α-/- mice are glucose intolerant. A and B: Glucose tolerance tests on 4-month-old α (*n* = 5 per group) and β (*n* = 6 per group) mice. *Statistical significance at $P < 0.05$.

Similar to other mouse models of obesity [106], fasted α-/- mice had increased serum levels of triglycerides, cholesterol, and free fatty acids as compared to controls (Table 5). With *ad lib* feeding serum triglycerides normalized in the α-/- mice while serum cholesterol remained significantly elevated. Surprisingly serum free fatty acids fell to significance below that of the control α+/+ mouse during *ab lib* feeding. In addition, serum leptin was significantly increased in the α-/- mice, possibly reflective of higher fat mass (Table 5). Serum resistin levels were lower in α-/- mice than in the controls, potentially reflecting a compensatory response of the adipose tissue to hyperglycemia (Table 5). No change was observed in serum adiponectin or FGF-21 (Table 5).

Table 5

Blood chemistry of α+/+ and α-/- mice

	α+/+	α-/-
Resistin, Fed (ng/mL)	18.2 ± 2.5	10.7 ± 1.7 *
FGF-21, Fed (pg/mL)	110.2 ± 31.3	117.3 ± 19.9
Leptin, Fasted (ng/mL)	1.24 ± 0.35	10.17 ± 2.5 *
Adiponectin, Fasted (ng/mL)	3311 ± 857	3413 ± 1043
Glucose, Fasted for 6 h (mg/dL)	145.6 ± 7.9	174 ± 10.4
Insulin, Fasted (ng/mL)	0.21 ± 0.19	2.97 ± 0.86*
Glycerol, 30 days old, Fasted (μM)	81.5 ± 41.6	110.5 ± 73.1
Glycerol, 4 month old, Fasted (μM)	297.9 ± 56.6	125.6 ± 39.0 *
Triglycerides, Fasted (mg/dL)	52.4 ± 8.8	92 ± 9.8 *
Triglycerides, Fed (mg/mL)	179.7 ± 28.2	173 ± 15.7
Cholesterol, Fasted (mg/dL)	79.6 ± 2.0	119.8 ± 7.5 *
Cholesterol, Fed (mg/dL)	104 ± 10.3	162.7 ± 8.7*
Free Fatty Acids, Fasted (μmol/L)	507.8 ± 48.9	731 ± 52.2 *
Free Fatty Acids, Fed (μmol/L)	1543 ± 242.9	931.4 ± 60.9*

Data are means ± SE. Male mice 4 months of age (*n* = 5-9) were fasted for 16 h unless otherwise noted. * statistical significance at *P*< 0.05.

The serum adipokine levels measured in β+/+ and β-/- mice were statistically indistinguishable (Table 6).

Table 6

Blood chemistry of β+/+ and β-/- mice

	β+/+	β-/-
Leptin (ng/mL)	1.04 ± 0.32	1.18 ± 0.26
Adiponectin (ng/mL)	5583 ± 588	5931 ± 1018
Glucose, Fasted for 6 h (mg/dL)	177.6 ± 24.3	157.5 ± 13.8
Insulin (ng/mL)	0.06 ± 0.04	0.04 ± 0.02

Data are means ± SE. Male mice 4 months of age (*n* = 4-7) were fasted for 16 h unless otherwise noted. Statistical significance at *P*< 0.05.

2-3.4 Effect of p110α ablation on PI3K activity and insulin signaling.

Studies utilizing 3T3-L1 cells treated with PI3K inhibitors have suggested that p110α is the primary insulin-responsive PI3K in adipocytes [30]. To determine the effect of p110α ablation on insulin signaling in *bona fide* adipocytes, we isolated white adipocytes from perigonadal fat pads of α+/+ and α-/- mice, stimulated them with insulin *in vitro*, and measured the activation of PI3K and Akt. The absence of p110α resulted in a dramatic decrease in insulin-stimulated PI3K activity associated with IRS-1 (Fig. 2-6A) and IRS-2 (Fig. 2-6B). Insulin-induced phosphorylation of Akt T308 (a site that is controlled by PI3K via PDK1) was also attenuated in the α-/- adipocytes, especially after 30 min of insulin treatment (Fig. 2-6C). Phosphorylation of Akt S473 (a mTORC2 site) was decreased to a lesser extent in cells lacking p110α (Fig. 2-6C). These results confirm that p110α plays an important role in insulin-stimulated PI3K/Akt signaling in WAT.

In a second experiment, PI3K/Akt activation was evaluated in BAT that was harvested from mice injected with saline or insulin. Insulin strongly increased IRS-1–associated PI3K activity in BAT of α+/+ mice, but this response was blunted in p110α-null tissue (Fig. 2-6D). In contrast, insulin caused only a minimal increase in IRS-2–associated PI3K activity in α+/+ and α-/- animals (Fig. 2-6E). We also saw a marked suppression of Akt T308 phosphorylation in BAT of α-/- mice following insulin injection, whereas phosphorylation of Akt S473 was not affected (Fig. 2-6F). Interestingly, Akt T308 phosphorylation in the liver and both Akt T308 and S473 muscle tissue of α-/- mice was also reduced as compared to α+/+ animals in response to insulin (Fig. 2-5G).

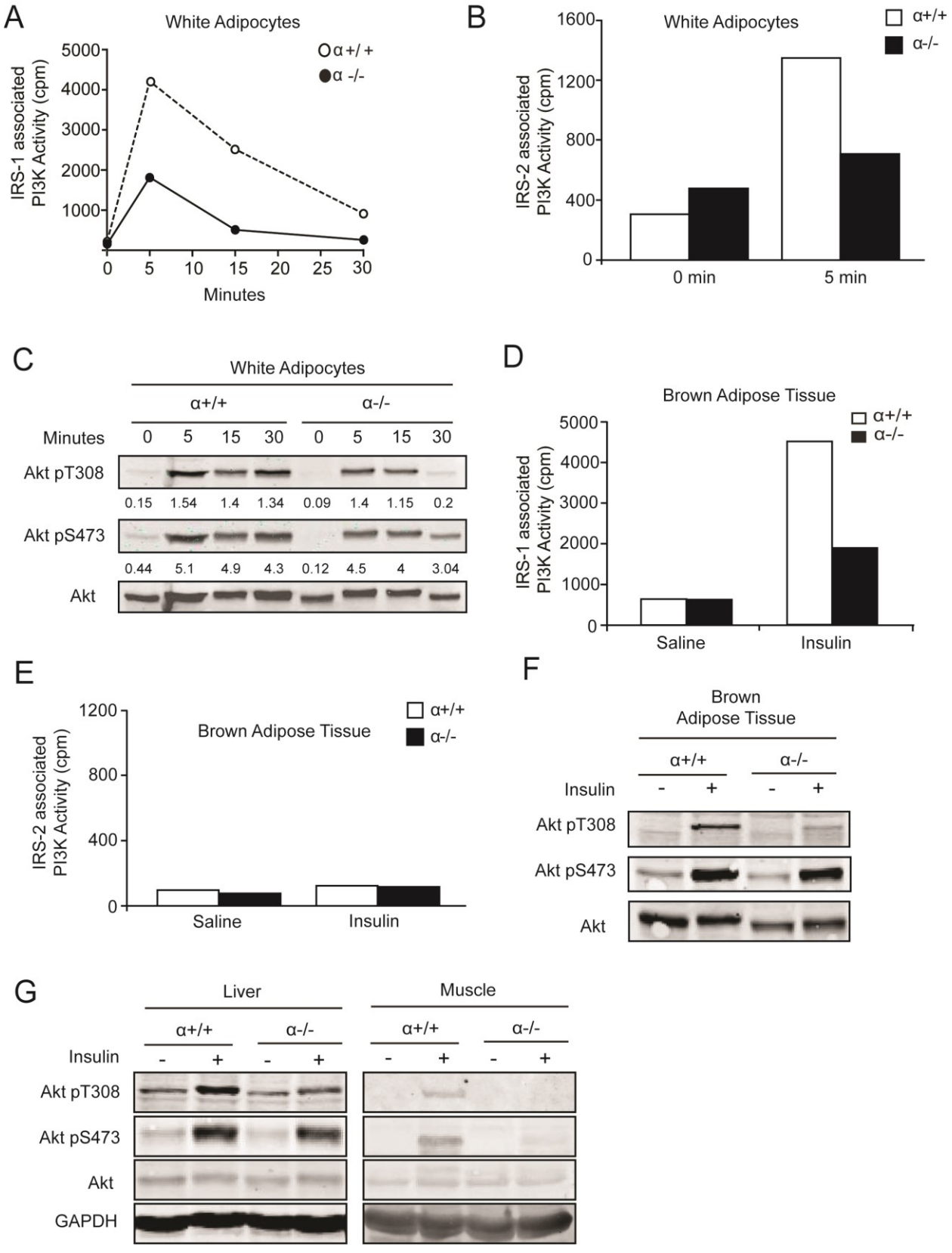

A
White Adipocytes
IRS-1 associated PI3K Activity (cpm)
5000
4000
3000
2000
1000
0
5
10
15
20
25
30
Minutes
α+/+
α -/-
B
White Adipocytes
IRS-2 associated PI3K Activity (cpm)
1600
1200
800
400
0
0 min
5 min
α+/+
α-/-
C
White Adipocytes
α+/+
α-/-
Minutes
0
5
15
30
0
5
15
30
Akt pT308
0.15
1.54
1.4
1.34
0.09
1.4
1.15
0.2
Akt pS473
0.44
5.1
4.9
4.3
0.12
4.5
4
3.04
Akt
D
Brown Adipose Tissue
IRS-1 associated PI3K Activity (cpm)
5000
4000
3000
2000
1000
0
Saline
Insulin
α+/+
α-/-
E
Brown Adipose Tissue
IRS-2 associated PI3K Activity (cpm)
1200
800
400
0
Saline
Insulin
α+/+
α-/-
F
Brown
Adipose Tissue
α+/+
α-/-
Insulin
-
+
-
+
Akt pT308
Akt pS473
Akt
G
Liver
Muscle
α+/+
α-/-
α+/+
α-/-
Insulin
-
+
-
+
-
+
-
+
Akt pT308
Akt pS473
Akt
GAPDH

FIG. 2-6. Reduced insulin activation of PI3K/Akt signaling in adipose tissue of α-/- mice. A, B: White adipocytes isolated from the perigonadal fat pads of 11 α-/- or 5 α+/+ mice were pooled and treated for the indicated times with 100 nM insulin. The cells were then used for (A) measurement of PI3K activity in IRS-1 immunoprecipitates, (B) measurement of PI3K activity in IRS-2 immunoprecipitates and (C) western blot analysis and densitometry of the bands normalized against total Akt to determine the activation of Akt. Mice were injected with saline or 1 U/kg insulin through the inferior vena cava. BAT, liver and muscle tissues were collected 15 min later (D,E,F,G). BAT PI3K activity in IRS-1 immunoprecipitates (D) and IRS-2 immunoprecipitates (E). Akt phosphorylation by western blotting after insulin IVC stimulation in BAT (F) and liver and muscle tissue (G).

This result suggests that ablation of p110α in the adipose tissue induces systemic insulin resistance.

We next examined white adipocytes from human perinephric adipose tissue to confirm the dominant role of p110α in insulin-stimulated PI3K activity. We isolated adipocytes from biopsies of human retroperitoneal fat pad and measured IRS-1 associated PI3K activity before and after insulin stimulation with and without 100 nM of the p110α specific inhibitor, PIK75 (Figure 2-7A). In samples from two separate patients PIK75 dramatically reduced PI3K activity in the presence of insulin supporting the results seen in the α-/- mouse white adipocytes. Quantification of the autoradiograph confirmed a decrease in insulin-stimulated PI3K activity in the presence of PIK-75 when compared to insulin-stimulation alone (Figure 2-7A *right)*. We then stimulated adipocytes isolated from a retroperitoneal adipose tissue biopsy from a third patient with 100 nM insulin in a time course and measured phosphorylation of Akt in the presence and absence of PIK75 (Figure 2-7B). Similar to the adipocytes of the α-/- mouse, treatment of human adipocytes with PIK75 attenuated phosphorylation of Akt primarily at the threonine 308 phosphorylation site. The studies shown here in the α-/- adipose tissue and in human adipose tissue demonstration that loss of p110α greatly reduces PI3K activity and subsequently decreases Akt phosphorylation.

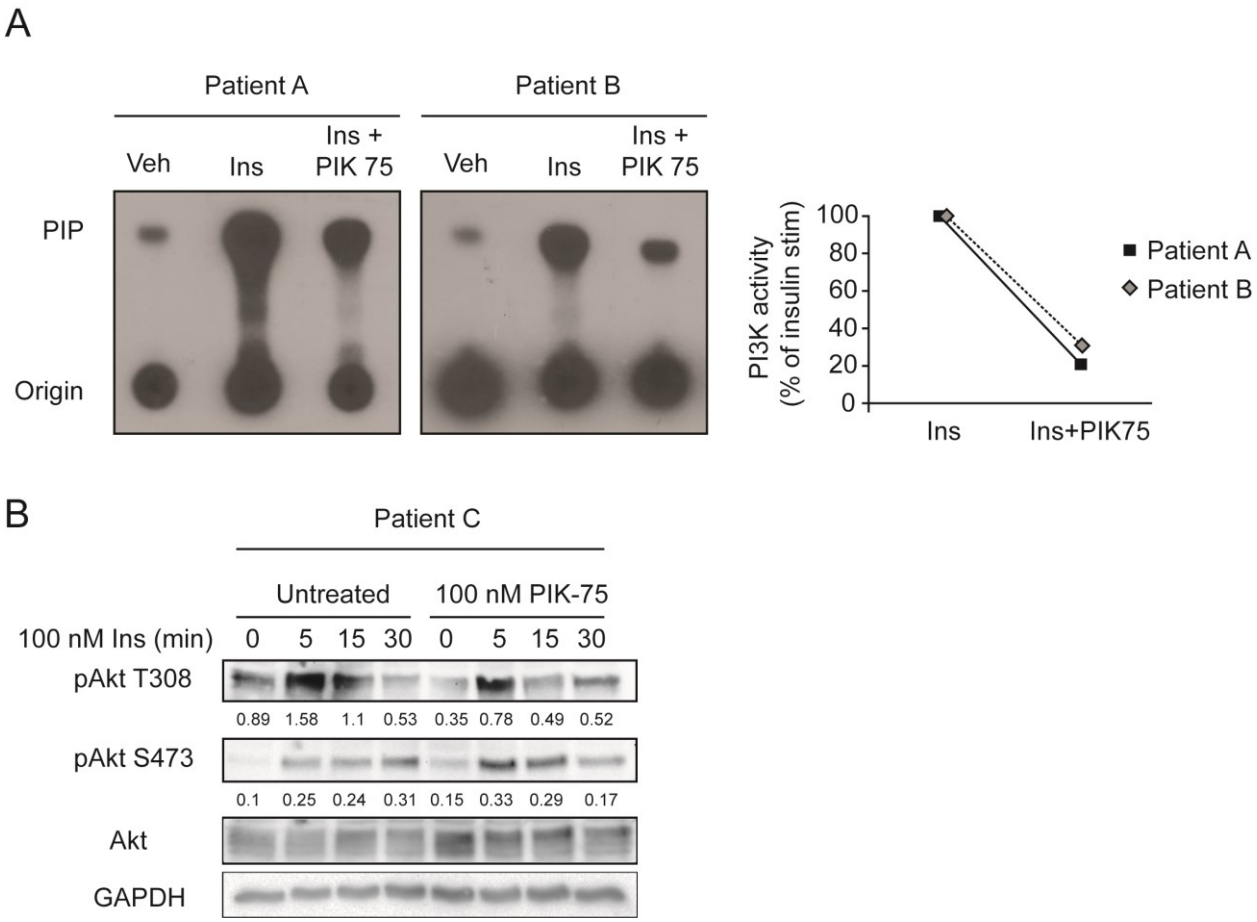


FIG 2-7: Inhibition of p110α reduces insulin activation of PI3K/Akt in human adipose tissue. A: Autoradiography of migration of phosphorylated phosphatidyinositol after immunoprecipatation of IRS-1 associated PI3K in isolated adipocytes from two patients with and without insulin stimulation after treatment with 100 nM PIK-75 (left) and quantified (right). B: Phosphorylated Akt in isolated adipocytes from a third patient stimulated in a time course with and without PIK75 pretreatment western was assessed with western blot and densitometry of the bands normalized against total Akt.

2-3.5 Lipid intake and lipolysis is unaffected in the α-/- white adipose tissue

It is possible that increased adiposity and altered serum profile of the α-/- mice is due to increased FFA uptake or lipogenesis in the white adipose tissue itself. To test this, we fasted α-/- and control mice overnight and measured expression of genes involved in lipid incorporation in the white adipose tissue. We saw no change in the expression of LPL or FATP1/4 under *ab lib* feeding (Figure 2-8A). We also saw no

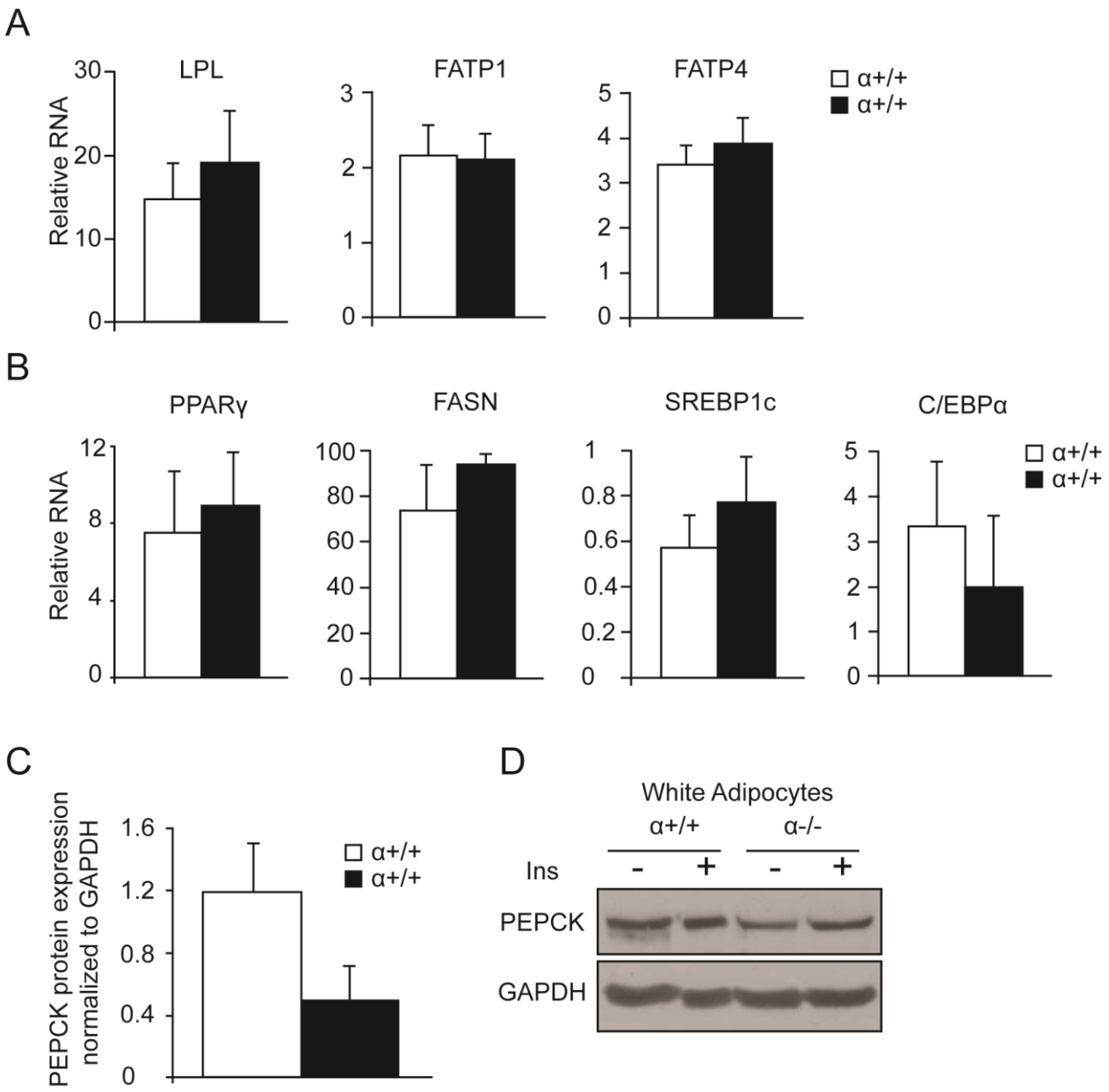


Fig 2-8: Lipid transporters and intracellular lipid regulating proteins are unchanged in the α-/- mice. A. Relative RNA expression of fatty acid transporters in the white adipose tissue of *ad lib* fed α-/- and α+/+ mice (*n* = 6 per group). B: Relative RNA expression of lipogenic genes in fasted α-/- mice and α+/+ controls at 4 month of age (*n* = 3,3 per group). Gene and protein expression of PEPCK in fed 4 month old mice measured with western blot with and without insulin stimulation (C) and with qPCR from *ad lib* fed mice (D) (*n* = 3 per group).

change in the expression levels of PPARγ, Fatty Acid Synthase, C/EBPα, or SREBP1c during fasting (Figure 2-8B). We also examined the protein expression of PEPCK.Over expression of PEPCK has been shown to increase adiposity in the white adipocyte by

promoting re-esterification of free fatty acids and subsequent storage into the adipocyte lipid droplet [107]. However, we did not see an increase in expression of PEPCK white adipose tissue of fed α-/- mice when compared to controls. Additionally, PEPCK appears to increase normally in response to insulin similar to the control adipocytes (Figure 2-8C and 2-8D). It is unlikely that the increased lipid deposition in the white adipocytes is caused by endogenous lipid storage mechanisms.

Another potential explanation for increased adiposity in the adipose tissue is decreased lipolysis, or breakdown of fatty acids from the glycerol head group of triglycerides for energy. In order to assess lipolysis in the α-/- mouse we measured serum glycerol release in response to an overnight fast in the obese 4 month old adults and also prior to the onset of obesity at 30 days of age. At 30 days there is no difference in the fasting glycerol levels of the α-/- mice from the control group. However a statistically significant decrease in α-/- serum glycerol is seen at 4 months of age (Table 5).

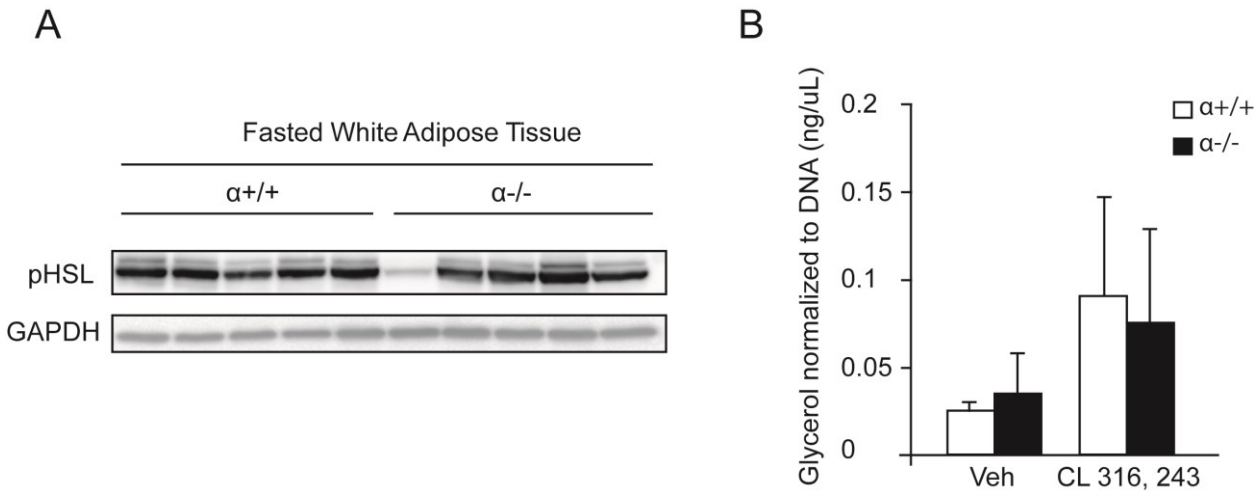


FIG 2-9: Lipolysis is unaffected in the α-/- white adipose tissue. A: Western blot of expression of phosphorylated hormone sensitive lipase (pHSL) in the white adipose tissue of mice fasted overnight. B: Glycerol release from pooled white adipocytes isolated from α-/- and controls with and without stimulation with β-adrenergic agonist (*n* = 3 per group).

We next looked at phosphorylation levels of hormone sensitive lipase (HSL), which is a rate limiting enzyme in lipolysis, in the white and brown adipose tissue. We saw no difference in HSL in either α-/- adipose tissue depot at 4 months of age (Figure 2-9A). Lipolysis is activated in response β-adrenergic stimulation. To measure the lipolytic response in white adipose tissue we isolated white adipocytes pooled from α-/- and control animals and measured glycerol release in response to stimulation with the β-adrenergic agonist, CL 316, 243 (Figure 2-9B). We saw no change in lipolysis with vehicle treatment or with β-adrenergic stimulation. We concluded that the decrease in fasting serum glycerol levels at 4 months of age are not due to alterations of white adipose tissue lipolysis.

2-4 Discussion

Skeletal muscle, liver and adipose tissue are major insulin-responsive tissues that control glucose and lipid homeostasis. Although the role of PI3K in the liver and muscle has been examined using genetically modified mice [22-24, 108], to our knowledge this is the first study that characterizes an adipose tissue-specific PI3K knockout mouse. We show that ablation of p110α in WAT and BAT causes obesity, fatty liver and glucose intolerance. In contrast to p110α, ablation of p110β in adipose tissue did not cause a gross metabolic phenotype.

We generated our p110α and p110β adipose-specific knockout mice using mice expressing cre recombinase under the control of the adipose-specific aP2 promoter produced and validated in the laboratory of Kleanthis Xanthopoulos in collaboration with Bruce Spiegelman. The researchers confirmed that detection of amplification of the cre segment using RT-PCR was limited to the white and brown adipose tissue only and was not present in the brain, liver heart or muscle [101]. In addition, the authors confirmed white and brown adipose-specificity when the aP2-Cre mice were crossed with mice expressing the reporter loxP-βgal and stained with X-Gal blue cytostaining. They saw no off-target expression in the heart, skeletal muscle or brain [101]. This is in sharp contrast to other lines of aP2-Cre mice that have been produced by independent laboratories. Unlike our mouse model, activity of cre in these other aP2-Cre mouse strains is not limited to the adipose tissue making them poor models for the study of adipose-specific gene effects [109, 110]. We purposefully chose to utilize the aP2-Cre mouse model created by the Xanthopoulos group because of its demonstrated specificity for targeting only the white and brown adipose tissue. It has been reported that aP2 is expressed in adipose

macrophages but its expression is dramatically reduced when compared to the levels of adipose tissue aP2 expression [109]. Therefore, it seems unlikely that PI3K deletion in macrophages contributes to the phenotypes associated with aP2-Cre knockouts.

In this current study we demonstrate that inhibition of p110α in the adipose tissues of the α-/- mice and in human adipocytes decreases PI3K activity and reduces phosphorylation of Akt at threonine 308. The mirrored effect of p110α inhibition between the human adipocytes and the α-/- mouse adipocytes supports our hypothesis that p110α is a potent catalytic driver of PI3K activity. While we successfully ablated p110α in the adipose tissue and reduced the levels of phosphorylated Akt (Fig. 2-1, Fig 2-6) we did not see decreased phosphorylated Akt to the levels of those observed by Knight [30]. However, these studies were performed in an adipocyte cell line and it is likely that in a complete physiological *in vivo* model loss of Akt activation by p110α is compensated for by other kinases, such as mTOR, through external stimuli to the adipocyte surface receptors by various growth factors.

Some of the phenotypes of our p110α and p110β knockout mice are similar to those of whole-body kinase-dead PI3K knockin mice [20, 21]. The global p110β kinase-dead mouse exhibited no change in adipose tissue mass and developed signs of mild insulin resistance with age, whereas global p110α kinase-dead heterozygous mice developed increased adiposity and glucose intolerance. The similarities between the global p110α kinase-dead mouse and our adipose-specific p110α-null mice suggest that the main site of action of p110α in regulating carbohydrate and fat metabolism might be the adipose tissue. However, whole-body kinase-dead p110α heterozygous mice were hyperphagic with high serum leptin levels, strongly suggesting leptin resistance [21].

Interestingly, our α-/- mice showed no decrease in food intake despite a ten-fold higher serum leptin level as compared to controls, indicating that lack of adipose-specific p110α might confer a loss of central leptin responsiveness. Recent studies have demonstrated that crosstalk between the insulin and leptin signaling pathways in the hypothalamus contributes to the regulation of energy homeostasis [111-113]. It was demonstrated by Qassab *et al.* that mice lacking p110β in the POMC neurons were unable to attenuate food intake despite high serum leptin due to decreased hypothalamic response to leptin and insulin [114]. It is possible that elevated glucose levels and hyperinsulinemia interfere with the ability of high serum leptin to control feeding behavior in our α-/- mice.

It is interesting that the FIRKO mouse and our α-/- animals, which were both produced to model insulin resistance in fat, display opposite phenotypes. FIRKO mice had low fat mass, normal or improved glucose tolerance, decreased serum triglycerides and fasted insulin, and normal serum free fatty acids and cholesterol as compared to controls [95]. In addition, UCP-1 expression was unchanged or increased in BAT of FIRKO mice [95]. Several explanations could account for the differences between these two models. First, distinct compensatory mechanisms might occur during development in each model. Second, the IR might activate targets other than p110α, so complete abrogation of IR signaling results in a gene expression pattern different from that caused by the loss of p110α alone. Third, p110α might be activated by multiple signaling inputs in BAT and not just IR signaling. Finally, it should be noted that both the IR and PI3K catalytic subunits have kinase-independent functions that are lost in knockout models but presumably still operate in insulin resistance [115, 116].

We showed that p110α protein expression is diminished only in the white and brown adipose tissue of the α-/- mouse and was not decreased in the liver and muscle tissue yet Akt phosphorylation is decreased in these peripheral insulin responsive tissues (Fig. 2-6). There are 3 Akt isoforms and it is known that Akt2 is the most abundant Akt isoform in the insulin responsive tissues and is also the most responsive to PI3K activation. It is not surprising then that the systemic phenotypes associated with adipose-specific loss of p110α would mirror that of the global Akt2 knockout mouse since the Akt2 isoform is highly expressed in the WAT, BAT, liver and muscle tissue [117]. Similar to our mouse model, the Akt2 knockout mouse is insulin resistant and glucose intolerant. The authors of this study found that systemic loss of Akt2 decreases insulin repression of hepatic glucose output while decreasing glucose uptake in the muscle [117]. Because of the strong association between liver and muscle Akt and glucose metabolism it is likely that this is the mechanism for glucose intolerance seen in our own α-/- mice.

It was also shown that Akt2 knockout mouse adipocytes have decreased glucose uptake and present with age-dependent lipoatrophy [117, 118]. This is in contrast to the obesity induced by loss of p110α in the adipose tissue. However, the adipose tissue also expresses Akt1 and to a lesser extent Akt3 [119]. While we did not examine the differences in Akt isoform expression in the α-/- adipose tissue, the discrepancies seen between the adipose tissue of the Akt2 knockout mouse and our mouse model likely arise from alterations in signals to Akt from loss of upstream activation by p110α. Any change induced by PI3K would affect all Akt isoforms whereas in Akt2 knockout mouse model, Akt1 and Akt3 are still activated.

In further support of a systemic phenotype as a result of adipose-p110α ablation, the α-/- mice have an extremely disrupted serum lipid profile (Table 5). It was curious that we saw an age dependent reduction in fasted glycerols levels in the α-/- serum yet the amount of fasting serum FFA remained high. This suggests that decreased lipolysis is not the driving force behind these low glycerol levels. The liver might be shuttling glycerol into triglyceride biosynthesis, which is also high in the α-/- mouse. This supports the notion that decreased activation of Akt increases hepatic glucose output by reducing the ability of the liver to respond to insulin. Another possibility is that the rate of glycerol conversion into glucose is higher in the α-/- animals through increased gluconeogenesis in an effort to cope with an apparent inability to utilize lipid or serum glucose for energy. However, this reduction in glycerol is not intrinsic to loss of adipose-specific p110α since it does not occur prior to the onset of obesity.

A defect in liver lipid metabolism is highly indicated in the α-/- mice. The trend of increased lipogenic gene expression in the knockout livers most likely contributes to the increased lipid deposition. In fact the knockout livers trend towards high lipogenic gene expression even in the fasting state indicating that they are always in an anabolic energy state. Liver steatosis is a common symptom in metabolic syndrome and usually develops in the presence of insulin resistance and the resulting compensatory hyperinsulinemia. It is well established that increased adipose tissue storage of lipids seen in obesity results in an increased flux of FFA to other tissues which compensate by increasing triglyceride storage [15]. The systemic effects seen in the α-/- mouse are most likely a secondary response to obesity and insulin resistance induced in the mouse as a result of insulin resistance in the α-/- adipose tissue.

Accumulated visceral adipose tissue produces and secretes a number of adipokines which can dramatically affect the function of the internal organs. The α-/- mouse has low serum resistin and extremely high serum leptin (Table 5). Many tissues express leptin receptors including the liver. Under normal conditions leptin reduces the lipid content in hepatocytes but in patients with non-alcholic fatty liver disease leptin levels are elevated and directly correlate with the severity of the disease [120]. The changes in liver function of the α-/- mice may be contributed to by some degree of leptin resistance and/or by some other hormone affected by loss adipose insulin resistance.

It is clear that by 4 months of age that the loss of p110α in the adipose tissue results in disruption of systemic glucose and lipid homeostasis. Given what we already know about loss of Akt activation in the insulin responsive organs as discussed above, the glucose intolerance in the α-/- mouse is not unexpected. We were surprised to see the extreme changes in systemic lipid metabolism especially since the knockout of the insulin receptor in the fat was protective. However, we detected no change in the lipogenic gene expression in the WAT and also no change in WAT lipolysis. Therefore, the gonadal white adipose tissue alone cannot be directly tied to the changes seen in systemic lipid regulation of the α-/- mouse. We did see changes in serum hormones and adipokines in the α-/- mouse and it may be that these errant signals are ‘talking’ with the other tissues in a way that promotes both lipid storage and lipid release into the circulation. The obesity-related phenotypes discussed in this chapter appear to be a reactionary consequence to adipose p110α ablation and not the primary cause of the metabolic breakdown. It is possible that other adipose tissue depots are regulating systemic metabolism and the brown adipose tissue depot will be discussed in chapter 3.

Chapter 3

Adipose-specific p110α regulates respiration in the brown adipose tissue

3-1 Introduction

Brown adipose tissue (BAT) is responsible for the production of heat by non-shivering thermogenesis and is typically activated in response to cold exposure. Once thought to be present only in infants, recent studies have shown that BAT is also present and highly active in adults [121]. Stimulation of the sympathetic nervous system activates β_3 adrenergic receptors in BAT to increase cAMP production and upregulate expression of UCP-1 [42]. UCP-1 is crucial for BAT thermogenesis and serves to uncouple heat generation from the respiratory chain, allowing for efficient substrate oxidation and low ATP synthesis [64]. UCP-1 is required for non-shivering thermogenesis, as evidenced by the inability of the UCP-1 knockout mouse to adapt to cold temperatures [122]. The UCP-1 knockout mouse became obese when housed at thermo-neutrality (the temperature at which shivering is no longer required to produce heat), indicating that UCP-1 can regulate adiposity [123].

The effect of BAT activation dramatically affects metabolic parameters. Mouse models show that activation of BAT ameliorates the negative effects of a high fat diet and normalizes symptoms of metabolic syndrome through improved glucose uptake and increased rates triglyceride clearance while BAT ablation induces obesity [124, 125]. In lean human subjects cold-induced BAT activation increases energy expenditure and improves glucose tolerance but this response is blunted in obese individuals [121, 126-129]. However, a recent study by Vosselman *et al* demonstrated that BAT activation in human subjects is not necessarily dependent on activation of the β-adrenergic pathway [130]. This study revealed that direct treatment with an adrenergic agonist did increase energy expenditure and increased glucose uptake in human subjects through the

upregulation of the thermogenic program in the skeletal muscle while the BAT remained unactivated [130]. This suggests that BAT activation is not exclusively controlled by the β-adrenergic pathway and may require another stimulus.

Insulin has been shown to affect UCP-1 expression in the BAT. Studies have shown that fasting decreases the thermogenic capacity in an effort to preserve the energy stores during times of caloric need [131]. This attenuation in thermogenesis is marked by decreased expression of UCP-1 and other thermogenic proteins in the brown adipose tissue while refeeding mice after fasting restores the thermogenic capacity of the BAT [131, 132]. It was later shown that this thermogenic restoration in brown adipocytes can be mimicked by insulin treatment providing a potential role for insulin in BAT activation [29, 71]. However, the molecular mechanisms underlying insulin regulation of thermogenesis in the BAT has not been well examined. In the present study, we show that adipose specific loss of p110α-PI3K catalytic subunit results in obesity and glucose intolerance due to loss of BAT respiration and decreased UCP-1 gene expression.

3-2 Materials and Methods

Antibodies. The following antibodies were used: cytochrome c oxidase (COX) IV, and heat shock protein (HSP) 90 from Cell Signaling Technology; Translocase of outer mitochondrial membrane 40 (TOM40) from Santa Cruz Biotechnology; UCP-1 from Abcam; and β-actin and from Sigma-Aldrich

Western blotting. Tissues were collected and immediately frozen in liquid nitrogen. Tissue samples were homogenized in general lysis buffer, and adipose tissue homogenates were further sonicated for 10 min at 4°C. After centrifugation, aliquots of supernatant containing equal amounts of protein were subjected to SDS-PAGE and immunoblotting as previously described [105]. Signals were visualized using horseradish peroxidase-linked secondary antibodies and chemiluminescence reagents. Densitometry was performed using Image J (NIH) after imaging with film or using AlphaView version 3.4.0.0 software after imaging with the FluorChem E system (ProteinSimple, Santa Clara, CA).

Indirect calorimetry. Metabolic gas exchange, food intake and ambulatory activity were measured using the Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH). Four-month-old male mice were individually housed for 96 h in calorimetry cages equipped with activity monitors and with free access to food and water. Data from the final 24 h were analyzed. Energy expenditure (EE) was calculated without normalization to body weight as per Cannon and Nedergaard using the following equation: $EE = \left(3.815 + 1.232 * (\frac{VCO_2}{VO_2})\right) * VO_2$, where VCO_2 is the volume of CO_2 produced and VO_2 is the volume of O_2 consumed [65].

Cell respiration. Brown adipocytes were isolated as described above, except that the cells were isolated in Hank's Balanced Salt Solution. Brown adipocytes from 4 mice were pooled for each experiment and resuspended in Gey's Balanced Salt Solution (Sigma-Aldrich). The cell suspension was bubbled with 5% CO_2 in air at 37°C for 15 min. The adipocytes were added to a sealed chamber with a Clark-type electrode in a final volume of 2 mL. Oxygen tension was monitored with a Yellow Springs oxymeter and recorded as a function of time with a Zip-Konnen chart recorder as previously described [133]. After baseline measurements were obtained, additional measurements were made after addition of 10 μM CL 316,243 (Sigma-Aldrich). Respiration rate was normalized to the protein expression of COX IV in the pooled adipocytes, as determined by densitometry of Western blots.

Mitochondrial DNA quantification. Total DNA was isolated from the BAT of male mice at 16-22 weeks of age. Two ng of total DNA was used to assess mtDNA normalized to nuclear DNA quantified by RT-PCR. The primer sequences are listed in the table below.

Table 7
Primer sequences for mitochondrial and genomic DNA quantification

Gene Name	Forward Primer	Reverse Primer
mt-Co2	CCGTGGGGAATGTATGAGCA	CTTCGCAGGAAGGGGATGTT
Actb	ATGCCCTGAGGCTCTTTTCC	TGCTAGGAGCCAGAGCAGTA

Transmission electron microscopy. Tissues were isolated after whole body perfusion (as described above) with 2% PFA and 2% glutaraldehyde in PBS. Postfixation with OsO_4 and embedding were performed by the Central Microscopy Imaging Center (Stony Brook University, Stony Brook, NY). Images were captured with a FEI BioTwinG^2 Transmission Electron Microscope equipped with an AMT digital camera.

Gene expression analysis. RNA was isolated from BAT using the RNeasy Lipid Tissue Kit (Qiagen), and cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad). Gene expression of *Ucp1*, *Ppargc1α* (PGC-1α), *mt-Nd1* (NADH dehydrogenase 1), *Cebpa (*C/EBPα*)*, *Srebf1* (SREBP1c), *mt-COX1* (Cytochrome *C* oxidase subunit 1) and *mt-Cytb* (Cytochrome *B*) and *Actb* (β-Actin) was analyzed with the SYBR Green PCR Kit from Qiagen. The primer sequences used are listed below in Table 8. *Pparg* (PPARγ) gene expression was investigated using a Taqman Gene Expression Assay (Mm01184323_m1; Life Technologies) using *Actb* as a control. Pooled BAT from several male mice was used to examine changes in gene expression using the Mouse Mitochondrial Energy Metabolism RT^2 Profiler PCR array (Qiagen). Relative gene expression was obtained using the delta Ct calculation.

Table 8
Primer sequences for BAT qRT-PCR

Gene Name	Forward	Reverse
Ucp1	TCCTAGGGACCATCACCACC	GCAGGCAGACCGCTGTACA
Ppargc1α	CATTTGATGCACTGACAGATGGA	CCGTCAGGCATGGAGGAA
Actb	TTCTTTGCAGCTCCTTCGTT	ATGGAGGGGAATACAGCCC
Cebpa	CCGGGAGAACTCTAACTC	GATGTAGGCGCTGATGT
Srebf1	CTGGATTTGGCCCGGGGAGATTC	TGGAGCAGGTGGCGATGAGGTTC
mt-Nd1	AATCGCCATAGCCTTCCTAACAT	GGCGTCTGCAAATGGTTGTAA
mt-COX1	CCCAATCTCTACCAGCATC	GGCTCATAGTATAGCTGGAG
mt-Cytb	TATTCCTTCATGTCGGACGA	AAATGCTGTGGCTATGACTG

Histology. WAT and BAT were collected from 4-month-old male mice after whole body perfusion with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) administered through the inferior vena cava. The adipose tissues were collected and fixed overnight in 4% PFA. The adipose tissues were dehydrated and embedded in paraffin using the Leica ASP300S Tissue Processor (Leica Biosystems, Wetzlar, Germany).

Sections (5 μm) were stained with hematoxylin and eosin (H&E). Sections were visualized with an Olympus BX43 microscope and Cell Sens Digital Imaging Software (Olympus).

Body temperature measurements and Cold Tolerance Test. Body temperatures were taken rectally at room. Additionally 4 month old α-/- mice and controls were subjected to a 12 hour cold tolerance test at 4ºC and temperature sampled every two hours. After 12 hours exposure to 4ºC brown adipose tissue was collected and analyzed for either protein expression or mice perfused and tissues collected for sectioning and histochemical analysis as described above.

COXIV enzyme activity assay. Cytochrome C Oxidase activity was assessed in BAT lysates of *ad lib* fed α-/- and controls with the Complex IV Rodent Enzyme Activity MIctoplate Assay Kit purchased from Mitosciences (Abcam, MA).

Statistics. Results are reported as means $\pm$ SEM. Significance was determined by Student's *t* test, one-way ANOVA or Mann-Whitney Rank Sum (SPSS, IBM, Chicago, IL). Statistical significance was accepted when $P < 0.05$.

3-3 Results

These studies used the same adipose-specific knockout mouse models for p110α (α-/-) that were used in Chapter 2.

3-3.1 α-/- mice have decreased energy expenditure.

Obesity is a result of increased caloric consumption or decreased energy expenditure. α-/- mice consumed the same amount of food as α+/+ controls over a 24-h period (Fig. 3-1A). While overall the α-/- mice did differ from the controls in their total food intake. We did detect a slight increase temporal feeding at the onset of the dark period or night (Fig. 3-1B). α-/- mice also exhibited the same ambulatory activity as compared to their lean controls (Fig. 3-1C). No change in overall food intake or activity suggested that there must be a difference in the ability of α-/- mice to utilize energy. To test this possibility, we measured energy expenditure using indirect calorimetry. α-/- mice had no change in carbon dioxide production but did exhibit decreased oxygen consumption (Fig. 3-1D, 3-1E) and reduced energy expenditure (Fig. 3-1F) when compared to their α+/+ controls. In other words, the α-/- mice have a positive energy balance that contributes to their increase in adiposity. A decrease in oxygen consumption is reflected by an increase in the respiratory exchange ratio (RER), which is calculated as the ratio of carbon dioxide produced to the amount of oxygen consumed and indicates which fuel is being metabolized to produce energy [134]. The RER of α-/- mice was significantly higher than that of α+/+ animals during parts of the night and day (Fig. 3-1G), signifying a decrease in usage of fat as an energy source that may further contribute to the obese phenotype.

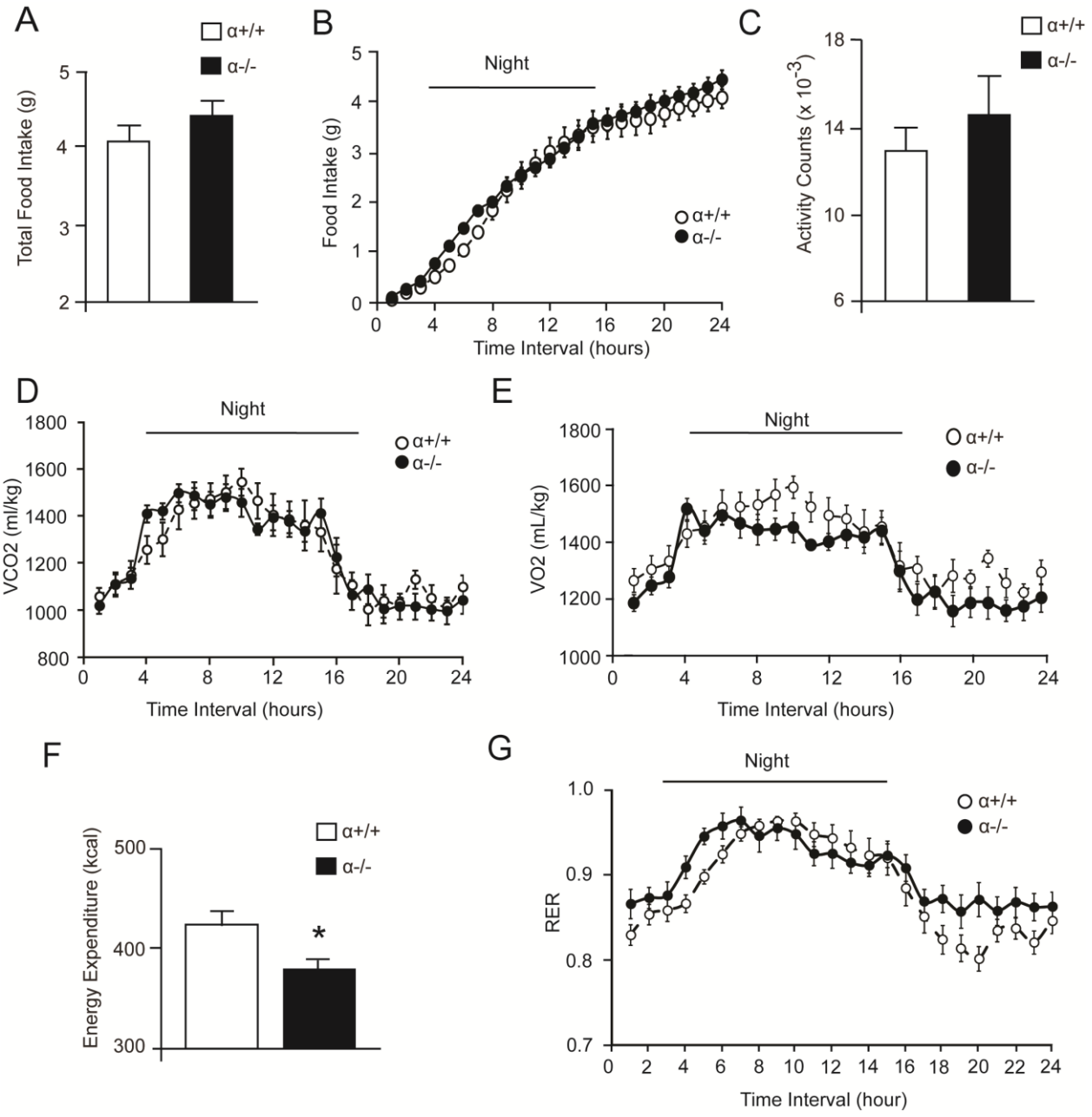


Figure 3-1. Decreased energy expenditure in α-/- mice. Whole body metabolic measurements of 4-month-old male α+/+ (*n* = 12) and α-/- (*n* = 10) mice were collected over a 24-h period using the Comprehensive Lab Animal Monitoring System. Total food intake (A) and cumulative food take during 24 hour period (B). C: Ambulatory activity. C: Volume of carbon dioxide (CO2) produced normalized to body weight compared to control animals. D: Volume of oxygen consumed (VO2) normalized to body weight was significantly decreased in the α-/- mice compared to controls throughout the 24-h period. F: Energy expenditure calculated from the VO2 before normalization to body weight. G: RER measured as the ratio of the volume of CO_2 produced and the volume of O_2 consumed was significantly higher in the α-/- mice during the day. *Statistical significance determined by Student's *t*-test (Food intake, Activity and Energy Expenditure) $P < 0.05$, or by one-way ANOVA (CO2, VO2 and RER analysis) $P < 0.05$

3-3.2 Reduced respiration in the BAT of α-/- mice.

BAT is a major regulator of energy expenditure and can greatly affect adiposity. We therefore tested whether BAT metabolism is dysregulated in α-/- mice. Brown adipocytes were isolated from adult α-/- mice and age-matched controls, and cell respiration was measured before and after addition of the β_3-adrenergic agonist CL 316,243. α-/- brown adipocytes had a significantly lower basal respiration rate than α+/+ cells, and a significantly lower respiratory response after the administration of the β_3-adrenergic agonist (Fig. 3-2A, left). However, the fold change of respiration in response to CL 316,243 was unchanged between the α-/- and α+/+ adipocytes (α+/+, 1.97 $\pm$ 0.49; α-/-, 1.88 $\pm$ 0.21) suggesting that the β_3-adrenergic response is intact but overall respiration is impaired. We normalized the respiration rate to protein expression of COX IV (Fig. 3-2A, right). Normalization of the OCR data to total DNA content did not change the results (data not shown). Further examination of the α-/- BAT revealed that cytochrome c oxidase activity was decreased when compared to control BAT (Fig. 3-2B). Gene expression analysis of the α-/- BAT confirmed significantly decreased gene expression of cytochrome c oxidase subunit 1 (*mt-Co1*) and cytochrome b (*mt-Cytb)* but no change in expression of NADH dehydrogenase subunit 1 (*mt-Nd1*) when compared to the BAT of the control mice (Fig. 3-2C).

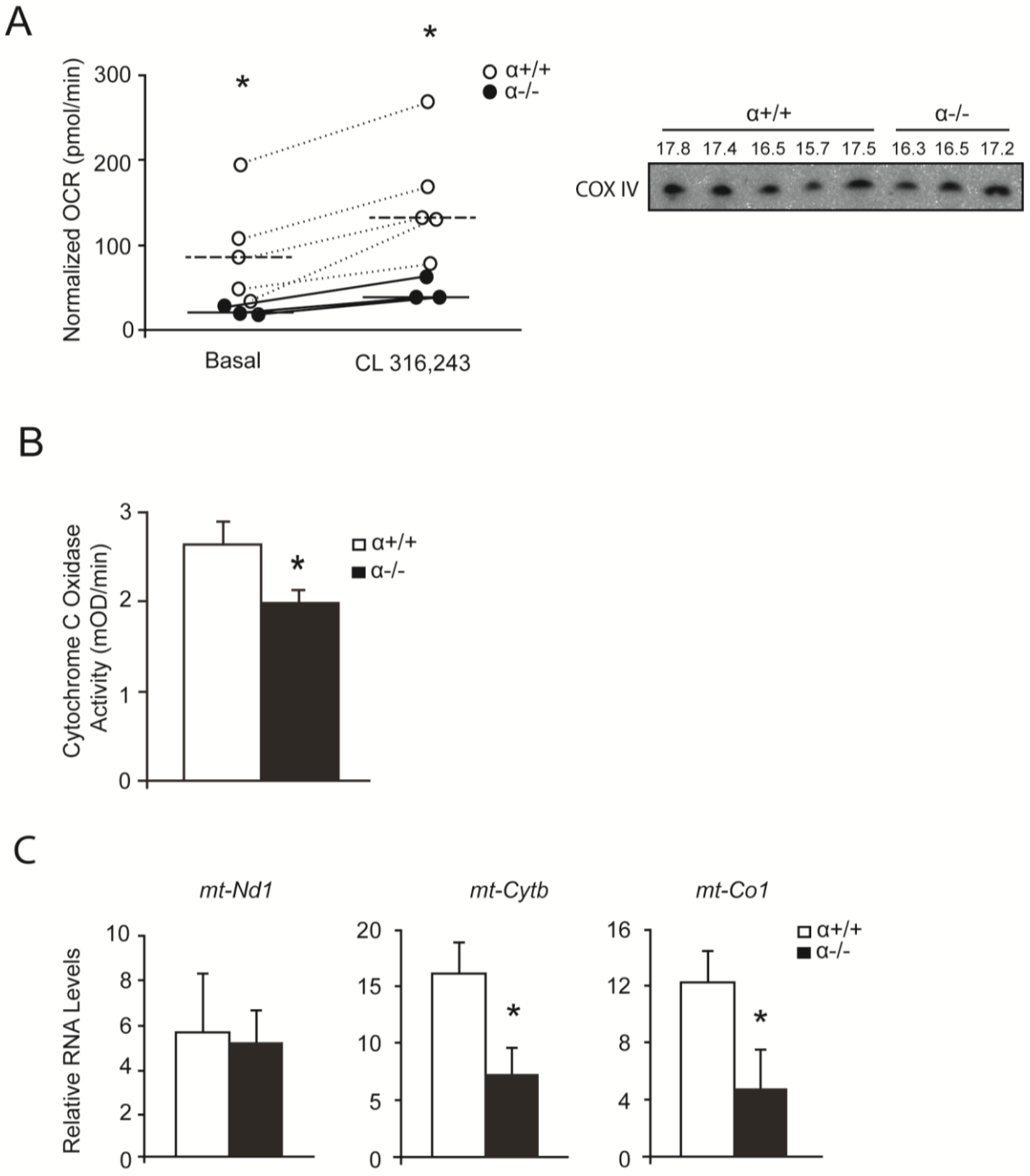


Figure 3-2. Impaired respiration in α-/- brown adipocytes. A: Oxygen consumption rate (OCR) in isolated brown adipocytes before and after stimulation with CL 316,243. Values were normalized to the expression of COX IV protein (right). *Significance was determined by Mann-Whitney Rank Sum test ($P < 0.05$). B: Cytochrome c oxidase activity in the brown adipose tissue of α-/- and α+/+ mice ($n = 5$ per group). C: BAT mitochondrial RNA expression (*mt-Nd1, mt-Cytb, mt*-Co10 normalized to Actb (n = 6 per group). *Statistical significance was determined by Student's *t*-test $P < 0.05$

Reduced respiration in the α-/- BAT could be caused by a decrease in the amount of mitochondria in the α-/- BAT. However, we did not detect a decrease in protein expression of the mitochondrial markers COX IV or TOM40 in α-/- BAT as compared to the control (Fig. 3-3A). The relative amount of mitochondrial DNA was unchanged, indicating that mitochondrial number is unaffected in the α-/- BAT (Fig. 3-3B). Electron microscopy revealed no obvious difference in the morphology of mitochondria from α-/- and α+/+ BAT (Fig. 3-3C). Taken together, these results indicate that the lower respiration in the α-/- BAT is contributed to by decreased cytochrome c oxidase activity but neither result can be explained by lower numbers of mitochondria.

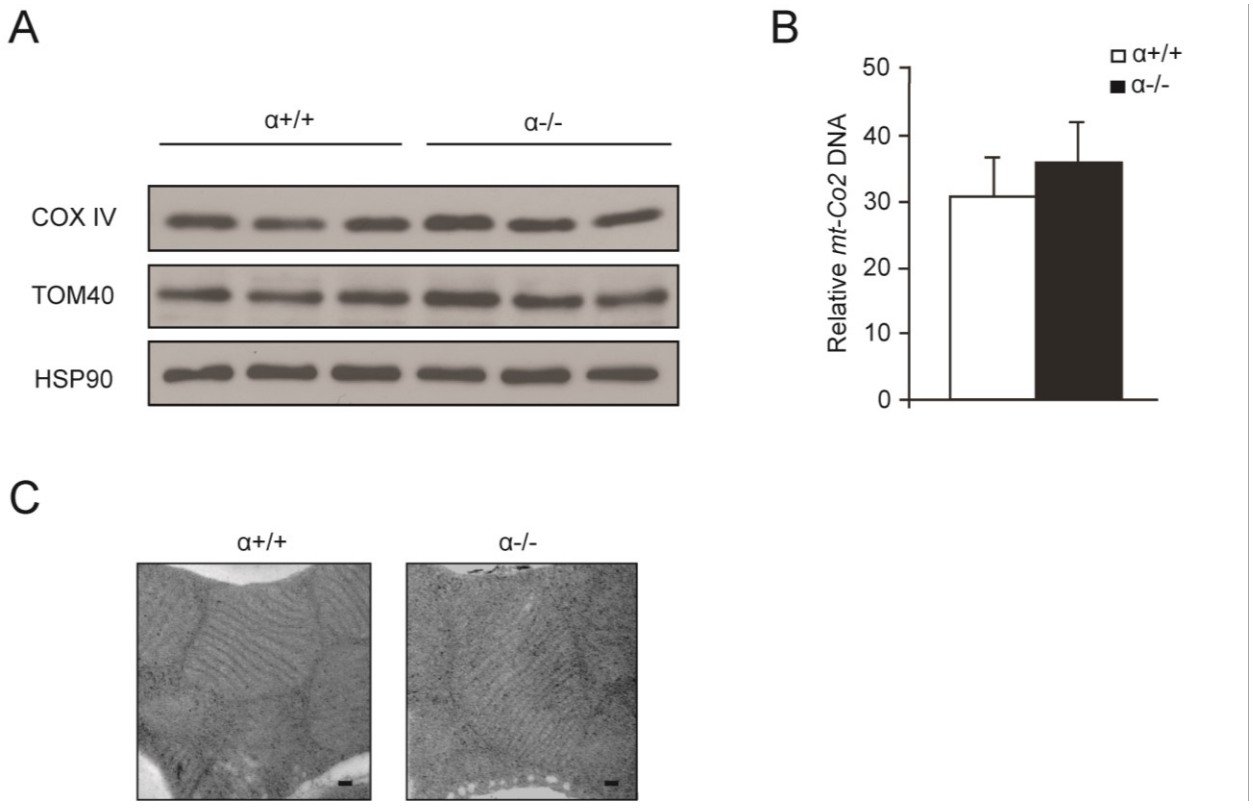


FIG. 3-3. Mitochondrial number is unchanged in α-/- brown adipose tissue. A: Western blot of mitochondrial proteins in BAT from 3 mice of each genotype. HSP90 serves as a loading control. B: DNA was isolated from BAT, and quantitative PCR was used to determine the amount of a mitochondrial gene (*mt-Co2*) relative to a nuclear gene (*Actb*; *n* = 4 per group). C: Transmission electron microscopy of mitochondria in BAT. Scale bars are 500 nm.

To further explore how ablation of p110α in the BAT changes mitochondrial efficiency, we used a quantitative real-time PCR array to profile key genes involved in mitochondrial energy metabolism. The array identified many genes that were downregulated in the α-/- BAT as compared to the control (Fig. 3-4A and Table 9). The genes with the largest decrease were *Ucp-1* and two subunits of the cytochrome c complex, *Cox6b2* and *Cox8c* (Table 9). Because of the essential role of UCP-1 in the regulation of BAT thermogenesis and its ability to affect mitochondrial respiration, we analyzed the expression of UCP-1 by qRT-PCR and western blotting. Both of these methods confirmed a significant decrease in UCP-1 expression in the BAT of α-/- mice as compared to the control (Fig. 3-4B and 3-4C). Further analysis of the α-/- BAT also revealed a decrease in gene expression of peroxisome proliferative activated receptor-γ (*Pparg*) and peroxisome proliferative activated receptor-γ coactivator 1-α (*Ppargc1a*), both of which are involved in activating the thermogenic program (Fig. 3-4B). These results suggest that reduced respiration of α-/- BAT is caused by a decrease in expression of genes important for mitochondrial function.

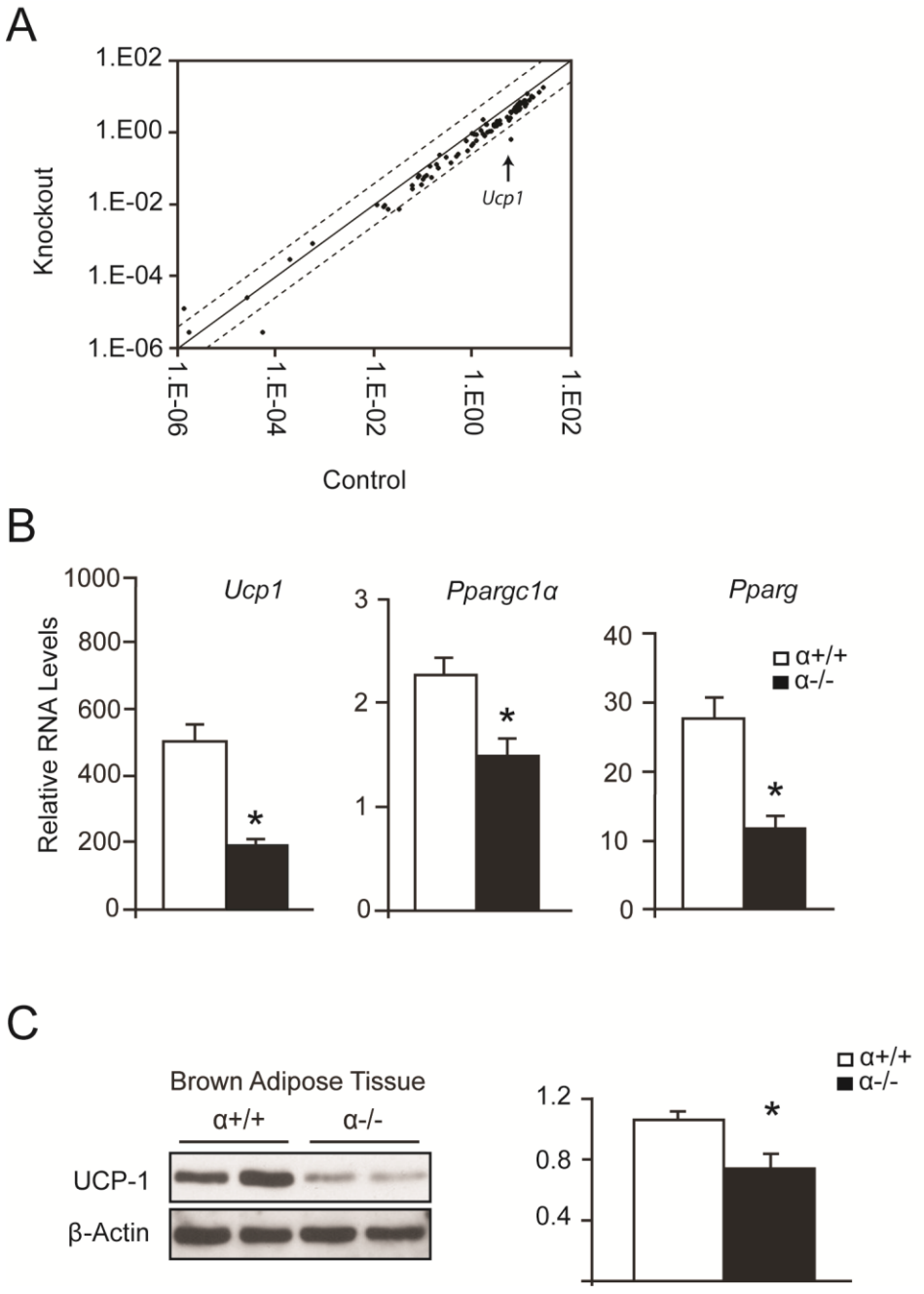


Figure 3-4 Altered gene expression in BAT of α-/- mice. A: Microarray analysis of mitochondrial-specific and associated gene expression in pooled brown adipocytes of α+/+ and α-/- mice (3 per group). The dashed lines represent 4-fold change. B: mRNA expression of thermogenic genes (*Ucp1*, *Ppargc1α, Pparg*) normalized to *Actb* (*n* = 6 per group). C: Western blot (left) showing decreased expression of UCP-1 in the brown adipose tissue of α+/+ and α-/- mice. Western blots were quantified (right) by normalizing UCP-1 to the loading control (*n* = 5 per group). *Statistical significance determined by Student's *t*-test, $P < 0.05$.

Table 9

Mitochondrial-associated gene expression in BAT of α-/- and α+/+ mice

Gene Symbol	Fold Change	Gene Symbol	Fold Change	Gene Symbol	Fold Change
Cox8c	-18.08	*Cox8a*	-1.91	*Ndufa6*	-1.54
Ucp1	-9.11	*Sdhd*	-1.88	*Cox6a1*	-1.51
Cox6b2	-4.14	*Cox5a*	-1.85	*Cox6c*	-1.49
Cox6a2	-2.50	*Sdhb*	-1.83	*Ndufb5*	-1.48
Lhpp	-2.49	*Ndufa11*	-1.82	*Ndufs6*	-1.47
Oxa1l	-2.47	*Ndufv2*	-1.80	*Ndufa8*	-1.47
Ppa1	-2.46	*Ndufs2*	-1.77	*Ndufa3*	-1.42
Atp5c1	-2.46	*Ndufb8*	-1.76	*Cox4i1*	-1.41
Ppa2	-2.42	*Atp5b*	-1.75	*Ndufs5*	-1.36
Atp5o	-2.25	*Sdhc*	-1.75	*Ndufb2*	-1.34
Ndufs3	-2.15	*Ndufab1*	-1.74	*Uqcr11*	-1.32
Atp5g2	-2.14	*Ndufa5*	-1.73	*Atp6v0a2*	-1.29
Ndufb10	-2.13	*Ndufv1*	-1.73	*Ndufc1*	-1.28
Cox7b	-2.12	*Atp6v1c2*	-1.72	*Atp5g3*	-1.26
Cox11	-2.12	*Ndufs4*	-1.72	*Bcs1l*	-1.23
Ndufs7	-2.11	*Atp5j2*	-1.71	*Ndufb7*	-1.23
Uqcrh	-2.07	*Atp5f1*	-1.69	*Atp5g1*	-1.13
Atp5d	-2.04	*Cox5b*	-1.67	*Atp5h*	-1.12
Ndufb4	-2.04	*Sdha*	-1.67	*Atp4b*	-1.11
Uqcrc2	-2.03	*Ndufa4*	-1.67	*Uqcrc1*	-1.07
Ndufb6	-2.00	*Ndufb9*	-1.65	*Uqcrq*	-1.05
Ndufc2	-2.00	*Cox4i2*	-1.65	*Atp4a*	1.05
Ndufa2	-1.98	*Ndufb3*	-1.61	*Uqcrfs1*	1.16
Ndufs1	-1.96	*Ndufa7*	-1.61	*Atp5j*	1.45
Ndufs8	-1.96	*Ndufa1*	-1.61	*Atp6v0d2*	1.56
Cox7a2l	-1.93	*Ndufa10*	-1.57	*Cox7a2*	1.63
Atp5a1	-1.92	*Cox6b1*	-1.56	*Atp6v1g3*	1.75
Cyc1	-1.91	*Ndufv3*	-1.55	*Atp6v1e2*	10.14

Fold Change is expressed as $\frac{\alpha-/-}{\alpha+/+}$

3-3.3 α-/- BAT is less sensitive to insulin induced upregulation of UCP-1

The α-/- mice have high serum insulin and leptin and both hormones have been previously reported to affect UCP-1 expression [71, 135]. We therefore wanted to determine if this repression of UCP-1 and adipocyte respiration is contributed to by reduced sensitivity of the α-/- BAT to either leptin or insulin. We injected leptin into the inferior vena cava of the α-/- mice and controls and stimulated for 15 minutes before collecting the BAT. We compared the protein expression of UCP-1 after leptin stimulation to that after insulin IVC treatment and saline treatment (Fig. 3-5A). Basal expression of UCP-1 levels were decreased in the α-/- BAT compared to the control BAT after saline treatment and after insulin stimulation. Treatment with leptin normalized UCP-1 levels in the α-/- mouse suggesting that extremely high levels of leptin might be able to overcome UCP-1 insulin resistance in the BAT after loss of p110α. The levels used in the IVC injections are well above physiological levels normally seen in mouse models. We therefore looked at levels of brown adipose tissue UCP-1 in response to fasting and also after mice were subjected to an overnight fast followed by 2 hours of *ad lib* feeding to produce physiological insulin stimulation. As expected fasting drastically reduced UCP-1 expression in both the α+/+ and α-/- brown adipose tissue (Fig. 3-5B). After the re-feeding period the UCP-1 expression levels increased in the control mice but did not normalize in all α-/- BAT samples (Fig. 3-6B). We were able to see increased UCP-1 expression with treatment of supraphysiological leptin but not with insulin stimulation. We next examined two targets of insulin, C/EBPα and SREBP1c, that have been reported to mediate insulin upregulation of UCP-1 of thermogenic genes including Ucp-1 and PGC-1α [71, 72].

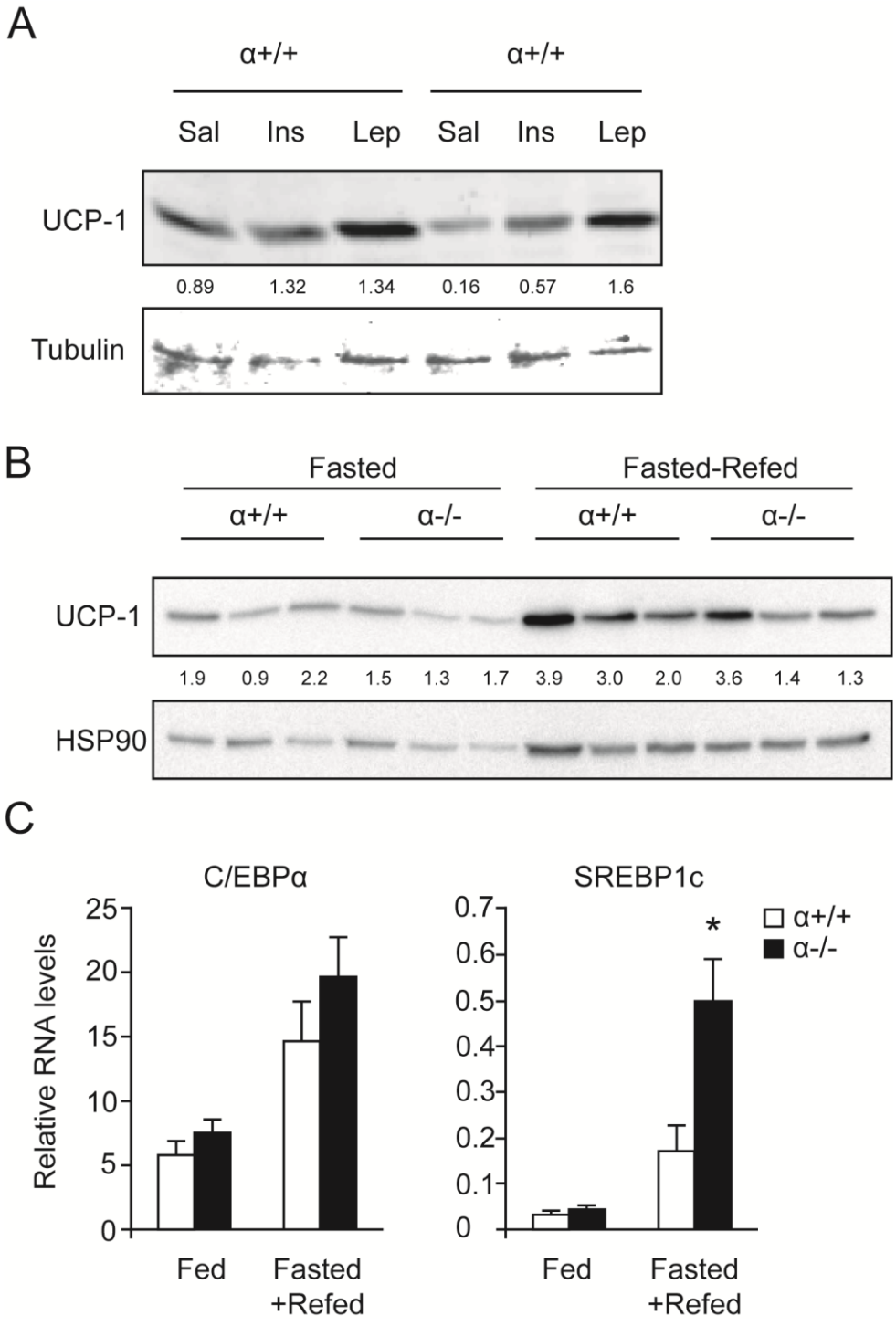


Figure 3-5.Expression of UCP-1 in the α-/- BAT in response to insulin. A: 8 week old α-/- mice and controls were injected with either saline, 1U/kg Insulin, or 25ug Leptin for 15 minutes through the inferior vena cava and tissues collected and examined for UCP-1. B: UCP-1 protein expression in the α-/- mice and controls after an overnight fast followed by two hours of *ad lib* refeeding. C: Relative RNA expression of C/EBP (left, n = 3,3) an SREBP1c (right, n = 5,5) after an overnight fast and after *ad lib* refeeding normalized to β-Acting levels. *Statistical significance determined by Student's t-test, $P < 0.05$.

We examined the relative RNA abundance of these two transcription factors in the α+/+ and α-/- brown adipose tissue during normal *ad lib* feeding and after an overnight fast followed by a 2 hour re-feeding period (Fig. 3-5C). We did not see a decrease in either transcription factor gene expression and actually SREBP1c protein expression is significantly increased in the α-/- brown adipose tissue after re-feeding. The inability of insulin to upregulate UCP-1 in the BAT of the α-/- is not due to decreased expression of either C/EBPα or SREBP1c and must be facilitated through other mediator proteins.

3-3.4 α-/- BAT is responsive to cold stimulus

UCP-1 is required for thermogenesis in order to protect against cold stress [136]. To measure if loss of UCP-1 is directly responsible for decreased respiration in the α-/- brown adipose tissue as a result of impaired thermogenesis we performed a cold tolerance test on the α-/- mice. We housed mice at either room temperature or 4ºC for 12 hours before collecting the brown adipose tissue for analysis. The α-/- mice maintained the same body temperature as their α+/+ controls at room temperature and also throughout a 12 hour cold exposure (Figure 3-6A and 3-6B). Histological examination of the brown adipose tissue revealed that after 12 hour cold exposure the α+/+ adipocytes stain strongly when compared to tissues collected at room temperature reflecting the expected upregulation of mitochondrial activation and protein synthesis characteristic of thermogenic activation and decreased lipid droplet size due to the extraction of fatty acid from the lipid droplet to be used for β-oxidation. The staining was performed simultaneously for all sections and the brown adipose tissue of the α-/- mice showed denser staining after exposure to 4ºC when compared to the α-/- mice at room temperature. Additionally the lipid droplets of the α-/- mice

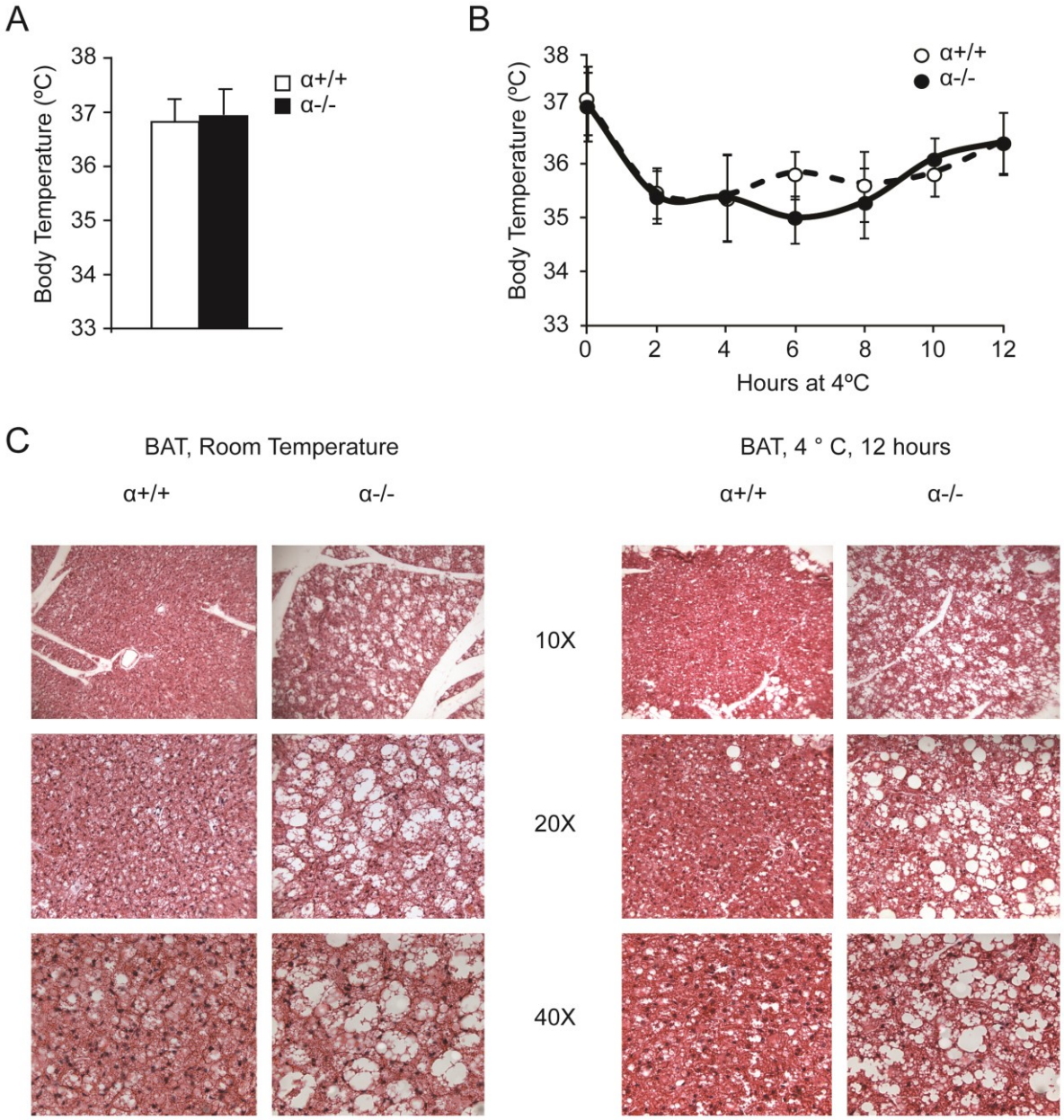


Figure 3-6 α-/- mice are tolerant to cold exposure A:Body temperature of α-/- and control animals kept at room temperature (α+/+ *n* = ,α-/- *n* =) B: α-/- mice were able to maintain body temperature when compared to controls during 12 hour cold tolerance test at 4ºC (α+/+ *n* = ,α-/- *n* =). H&E staining of brown adipose tissue at in the control α+/+ mice and α-/- mice at room temperature (C) and at the end 12 hour 4º exposure (D).

appeared to decrease slightly in at 4ºC indicating that the knockout mice can activate thermogenic resources in response to acute cold stress.

We next examined the protein expression in the brown adipose tissue of the α-/- mice after exposure to cold. As expected the protein expression of UCP-1 increased in response to cold in the α+/+ brown adipose tissue (Fig. 3-6D). However, while UCP-1 protein levels did increase in the α-/- mice with cold compared to room temperature UCP-1, the overall expression of UCP-1 in the BAT of the α-/- was still lower compared to that of the cold α+/+ control BAT.

3-3.5 α-/- have no change in UCP-1 protein expression prior to obesity onset.

We wanted to examine UCP-1 expression prior to obesity onset in the α-/- mice when compared to their controls. We collected BAT from mice between 3-4 weeks of age and measured UCP-1 expression with western blot (Fig. 3-7A). We did not see any obvious changes in UCP-1 protein between the α-/- and α+/+ control mice. We observed a slight decrease in the gene expression of UCP-1 and PGC-1α but no statistically significant changes in the BAT of the young α-/- mice (Fig. 3-7B).

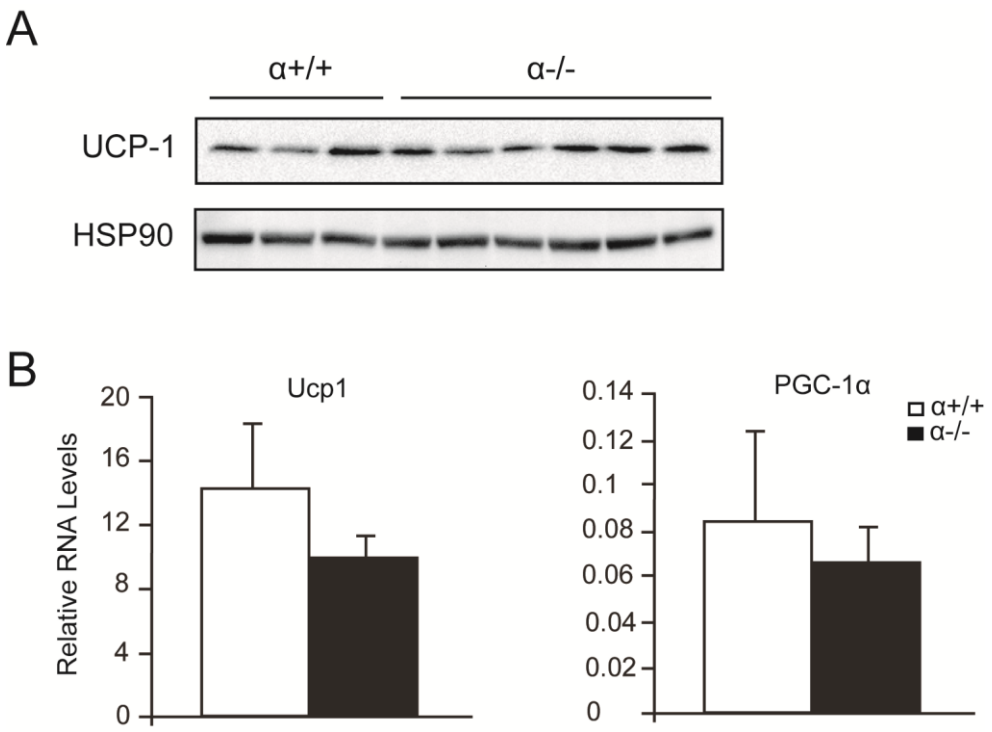


Figure 3-7: UCP-1 expression prior to obesity onset in young α-/- mice. A. Western blot of UCP-1 protein expression in fed mice between 3-4 weeks of age. B. Gene expression of UCP-1 and PGC-1α normalized to β-Actin in 3-4 week old α-/- mice (α+/+ *n* = 3 ; α-/- *n* = 6)

3-4 Discussion

As presented in chapter 2, the α-/- mouse is obese and glucose intolerant. Our data suggest that the α-/- phenotypes arise at least in part from low mitochondrial-related gene expression including low UCP-1 and impaired respiration in the BAT. It is our belief that the reduced respiration in the BAT is the main contributor to the observed decrease in energy expenditure of α-/- animals. Since activity and food intake are the same in α-/- and control mice, this creates a positive energy balance permissive for increased lipid storage in the α-/- mice which ultimately results in obesity.

The phenotypes seen in our α-/- mice are similar to those seen in a mouse model of transgenic toxigene BAT ablation. Mice lacking BAT developed obesity, hyperglycemia, hyperinsulinemia, hypertriglyceridemia and hypercholesterolemia [125]. Like our α-/- mice, increased adiposity of young BAT-deficient mice was linked to a decrease in oxygen consumption with no change in food intake. However, by 8 weeks of age BAT-ablated animals developed hyperphagia that further contributed to their obesity [125]. Our 4-month-old α-/- animals did not display hyperphagia even though they had high serum leptin levels, suggesting that they have leptin resistance.

The respiration defect in BAT from the UCP-1 knockout mouse was substantially different from that of α-/- mice [137]. Brown adipocytes from our α-/- mice showed decreased respiration at basal conditions and in response to acute adrenergic-stimulation. By contrast, the basal respiration rate was not affected in brown adipocytes from UCP-1 knockout mice, but the acute adrenergic response was greatly reduced [137]. We believe that the changes seen in the α-/- BAT cannot be fully accounted for by decreased UCP-1 expression. The α-/- BAT also had decreased expression of the majority of

mitochondrial energy metabolism genes, including a strong reduction in mitochondrial and nuclear transcribed cytochrome c oxidase subunits corresponding to decreased cytochrome c oxidase activity. Cytochrome c oxidase is an important regulatory enzyme in the electron transport chain and reduced cytochrome c oxidase activity is commonly seen in cases of mitochondrial dysfunction [138]. Reduced gene expression of the transcription regulators PGC-1α and PPARγ might contribute to suppression of the mitochondrial metabolic program. PGC-1α is known to interact with PPARγ and to regulate mitochondrial respiration through changes in gene expression [70]. While the majority of the genes in the array were down-regulated in α-/- BAT, we also observed a 10-fold increase in expression of *Atp6v1e2* (ATPase lysosomal V1 subunit E2). Whether increased expression of this lysosomal ATPase contributes to the reduction in cellular respiration is unclear.

This study provides evidence that BAT PI3k-p110α regulates basal and insulin stimulated UCP-1 expression. Previous studies have demonstrated that leptin resistance in the adipose tissue reduces UCP-1 expression and overall energy expenditure [139]. However, treatment of the α-/ mice with insulin did not restore UCP-1 to the level of the control animals while supraphysiological concentrations of leptin was able to induce upregulation of UCP-1 indicating that loss of UCP-1 expression in the α-/- adipose tissue is a direct consequence of attenuated insulin signaling while leptin signaling remains intact (Fig. 3-5A). It also suggests that leptin and insulin signaling do not necessarily converge in the BAT, as they do in other tissues, in the upregulation of UCP-1 [106, 113, 140]. It should be noted that both leptin and insulin are likely increased during refeeding following an overnight fast yet the more-normal physiological levels of both hormones

induced in α-/- mice are not enough to restore UCP-1 expression (Fig. 3-5B). We did observe that the insulin (and to a smaller extent leptin activated) transcription factors known to increase UCP-1 expression in the BAT were not decreased in the insulin resistance α-/- BAT (Fig. 3-5C). This implies that p110α might regulate UCP-1 expression through other transcription factors.

We were surprised to see that the α-/- mice were tolerant to acute cold exposure despite reduced UCP-1 (Fig. 3-6A). However in acute cold exposure the animals retain their ability to protect their body temperature by skeletal muscle shivering and it is likely that the α-/- animals are heavily relying on this mechanism. It is possible to remove this protection by storing the animals at their thermoneutral temperature before cold exposure so the ability to shiver is reduced and the differences. We do see that the α-/- animals are unable to activate UCP-1 to the levels of the α+/+ control during acute cold exposure indicating that while the α-/- animals are cold tolerant they cannot utilize UCP-1 for heat generation (Fig. 3-6D).

It is well established that insulin resistance strongly correlates with impaired mitochondrial function. Patients with type 2 diabetes or morbid obesity exhibited a decrease in mitochondrial respiration and mitochondrial gene expression [141-144]. Adipocytes from type 2 diabetic *db/db* mice showed depressed cell respiration stemming from a reduction in mitochondrial proteins and low UCP-1 expression [145, 146]. Experiments on immortalized brown adipocytes showed that long term insulin treatment upregulated UCP-1 via a pathway that required IRS-1 signaling to PI3K and Akt [71]. Furthermore, UCP-1 expression in IRS-1–null adipocytes was increased in the absence of insulin upon retroviral expression of PPARγ. It is possible that low PPARγ and reduced

insulin activation of PI3K associated with IRS-1 in α-/- BAT are responsible for low expression of UCP-1 and other genes involved in mitochondrial energy metabolism. PI3K may also positively regulate cellular respiration in a manner independent of changes in gene expression through Akt-dependent phosphorylation of mitochondrial proteins [147], and these events are expected to be suppressed in α-/- BAT.

Insulin acts as an anabolic hormone and, as discussed in the above paragraph, is required for mitochondrial protein and gene expression but also increases the mitochondrial oxidative capacity as well as ATP production [148, 149]. In the muscle, liver, and adipose tissues insulin is found to upregulate the expression of genes and proteins important in cell development as well as glucose and lipid metabolism [150-152]. Most of these insulin-regulated products are found to be controlled through Akt activation [153]. Akt is a powerful effecter of cellular function. Alterations in its activity have been documented to produce profound affects in cell homeostasis [153]. In the α-/- brown adipocytes we find that the expression of not just one gene but many genes are affected by reduced PI3K/Akt signaling. Since Akt itself has many downstream targets and has been documented to regulate general cell metabolism we did not identify one direct mechanism of PI3K in the regulation of BAT respiration but rather identified many gene targets that are important mitochondrial function.

The presence of such a wide scope of BAT transcriptional repression supports the hypothesis that the PI3K/Akt signaling pathway regulates a program of nuclear receptors and/or transcription factors which mediate these observed changes in respiratory genes. For example, a recent study demonstrated that the nuclear receptor Re-verbα acts as a repressor on UCP-1 expression and reduces the ability of the animals to regulate body

temperature in the response to long-term cold exposure after being maintained at thermoneutrality [154]. The study showed that Re-verbα binds to a proximal promoter region of UCP-1 and inhibits UCP-1 expression. This is relevant to our study of the α-/- mice because re-verbα is a circadian protein involved in the maintenance of the circadian clock of all cells including the adipocyte [155]. The adipose circadian clock primes the cells for lipid disposal and lipid release to correspond to meals and periods of fasting. It has been proven that disruption of the adipose circadian rhythm and clock proteins results in the development of adipose insulin resistance and increased lipid deposition [156]. Activation of Re-verbα is regulated through phosphorylation by GSK-3β which is a known target of insulin-stimulated Akt [157]. Activated GSK-3β phosphorylates and stabilizes re-verbα which then suppresses UCP-1 expression [157]. Phosphorylation of GSK-3β by Akt inhibits its activity and thus insulin stimulation restores UCP-1 expression.

The aforementioned studies clearly show a direct path between re-verbα and UCP-1 but is just one signaling pathway known to be affected by insulin. The advances made in next generation sequencing and the ability to provide insight about the genome, transcriptome, epigenome and many other –ome's of any species frees researchers from the limitations of searching only for direct single molecule to molecule interactions to explain system-wide phenotypic changes. In fact, in the liver re-verbα regulates gene transcription at the cistrome, the *in vivo* genome-wide location of transcription factors and histone modifications, to coordinate broad metabolic gene expression [158-160]. It is likely that re-verbα is capable of acting in a similar manner in the adipocytes affecting the expression not just one gene but many.

Of course, re-verbα is just one example of potential mechanisms for Akt regulation of a genome wide metabolic program in the brown adipose tissue. The Insulin/PI3K/Akt pathway regulates many transcription factors and nuclear receptors that have been shown to affect brown adipocyte metabolism. FOXO1 is a target of Akt and has been shown to inhibit transcription of many brown adipocyte nuclear mitochondrial genes including the majority of genes involved in the mitochondrial electron transport chain as well as UCP-1 [161]. Other factors cited in the literature that are insulin responsive and effectors of cellular respiration are PPARγ, C/EBPα, SREBP1c, NRF-1, and PGC-1β, to name a few [29, 158, 162-165]. There are no shortages of potential targets to affect cellular respiration in the brown adipose tissue.

It is important to note that suppression of BAT UCP-1 by PI3K in the α-/- mice appears to be age dependent Fig (3-7). While we showed that UCP-1 expression is decreased in the adult α-/- mice when compared to the controls we did not observe any changes in UCP-1 protein or gene expression in the young adolescent α-/- mice. It is possible that a mechanism exists to protect the pubescent animals from potentially harmful changes in nutritional and energy status and this protection is lost with age. This possibility will be explored in chapter 4.

Our results show that reduced p110α signaling in adipocytes causes profound effects on basal BAT respiration and produces a whole body phenotype similar to that seen in metabolic syndrome. A better understanding of how p110α regulates BAT function may provide insight into the pathogenesis and treatment of this disorder.

Chapter 4

Loss of p110α in the adipose tissue delays male puberty onset

4-1 Introduction

In both male and females the adipose tissue plays an important role in the hypothalamic-pituitary-gonadal axis (HPG axis). The HPG axis is a classic endocrine axis that mediates hormonal stimulation on peripheral organs via hypothalamic and pituitary drives. These hypothalamic drives are inhibited by a feedback loop that aids in the ultradian rhythms of many hormonal responses (Fig. 4-1) [166].

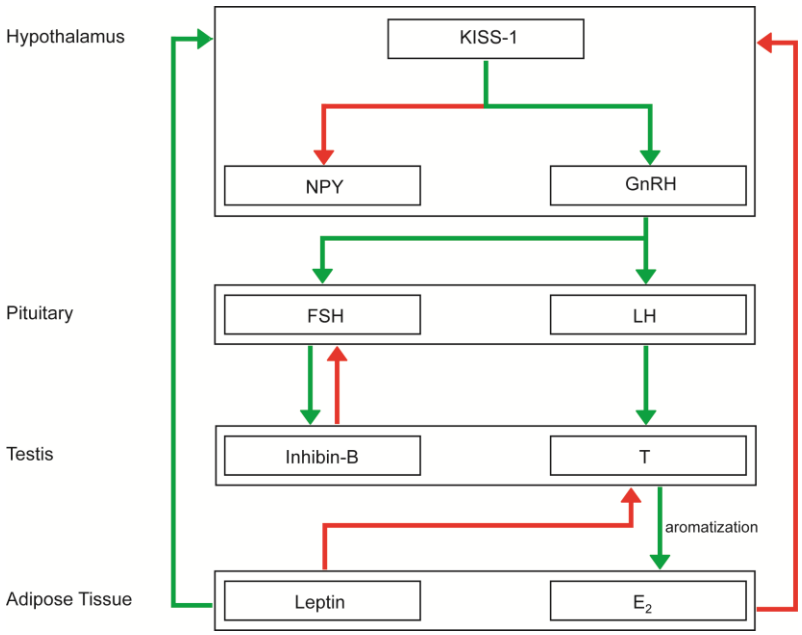


Fig. 4-1. Male Hypothalamic-pituitary-gonadal axis interacts with the adipose tissue. The adipose tissue provides a feedback loop to the hypothalamus through leptin secretion and estrodiol synthesis. The green lines represent stimulatory pathways while the red lines represent inhibitory pathways. E_2: estrodiol; FSH: Follicular stimulating hormone; GnrH: gonaotrophin-releasing hormone; KISS-1: kisspeptin neuron; LH: Luteinising hormone; NPY: neuropeptide Y; T: testosterone

Modified from Michalakis 2013

While the fluctuations of and sex hormones differ between sexes the basic principles guiding the HPG axis remain the same. Stimulation of the hypothalamus signals the production and release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior portion of the pituitary gland. In turn FSH and LH signal directly to the gonads to produce either testosterone in males or estrogen in females. The adipose tissue mediates part of the regulatory negative feedback through the synthesis of estrogen from testosterone which is inhibitory on the hypothalamic production of GnRH.

Additionally, the production of leptin can directly inhibit sex steroid production from the gonads of both males and females [166].

The normal function of the HPG axis is dependent on normal energy balance and obesity is clearly not a state of normal energy homeostasis. It is known that obesity is strongly associated with changes in sex steroid hormone concentrations and results in reproductive dysfunction [166]. In females, incidences of menstrual irregularities are increased with waist circumference and insulin resistance and BMI are negatively correlated with serum levels of FSH and LH in female patients [167, 168]. It is also observed that early menarche occurs at a higher frequency in obese young pubescent females with decreased insulin sensitivity. Subsequent menstrual irregularities continue with age in half of these obese adolescent girls [169, 170].

It is becoming increasingly evident that low male serum testosterone is associated with obesity and metabolic dysfunction. Serum testosterone is negatively correlated with BMI and is significantly reduced in men with metabolic syndrome [171, 172]. Additionally, low serum testosterone strongly correlates with fasting glucose over 110 mg/dL and triglycerides over 150 mg/dl [173]. As previously mentioned, estrogens can be synthesized from testosterone in the adipose tissue. This conversion of testosterone into estrogens, specifically estrone (E1) and the more potent estrodial (E2) was found to attribute to low serum testosterone in morbidly obese men [173].

It is now known that the beneficial effects of estrogens in males on the reproductive axis are far greater than originally understood. Male estrogens are required for cardiovascular protection and normal bone development as well as healthy lipido and sexual function in addition to providing anti-inflammatory protection in the periphery and

CNS [174-176]. However, these beneficial effects of estrogens depend upon tight regulation of intracellular and circulating estrogens within a strict physiological range. Recent research shows that there is an drop in estrogen levels in both middle-aged women and men that corresponds to increased adiposity while regulation of lean mass and muscle strength in males is attributed to by testosterones [176].

The adipose tissue is a critical mediator of the reproductive axis primarily through the production of adipokines including leptin. Leptin strongly influences the HPG pathway. Leptin is synthesized in the white adipose tissue and is secreted in direct proportion to fat mass. The classic site of leptin action is on the hypothalamus to signal satiety in response to food intake and reduce consumption in order to maintain energy balance. Conversely, starvation and fasting reduces leptin concentrations which promotes the desire to eat [177]. The higher the fat mass in the individual the more leptin is secreted. There is a propensity in obese individuals with chronically high leptin expression to develop leptin resistance which promotes chronic overfeeding further increasing leptin production and secretion by the adipose tissue [178].

Elevated leptin receptors are ubiquitously expressed on tissues throughout the body including the liver, adipose tissue, and also the ovaries and testes [179]. High leptin concentrations in obese females inhibit the production of E2 by the ovaries in response to FSH and inhibit steroidal precursor production resulting in irregular cycles and infertility [180]. In obese males high leptin from elevated fat mass reduces serum testosterone by inhibiting testosterone production in the leydig cells of the testes [181].

Leptin is thought to be a required permissive factor in puberty onset. A study examining the effects of leptin receptor mutations in severe early-onset obesity

discovered that, while rare, complete loss-of-function mutations do occur in the leptin receptors and results in hyperphagia and obesity, as well as hypogonadism and loss of the pubertal growth spurt [92]. The same effect is seen in cases of human leptin deficiency where loss of leptin signaling inhibits pubertal development and promotes obesity and induces hyperphagia [182]. In this case, leptin replacement permits the progression of pubertal development in addition to reducing body mass and decreasing food intake. The permissive role of leptin in puberty onset is further supported by mouse models of leptin deficiency. The male *ob/ob* mice have attenuated testosterone, low gonadotropin concentrations, hypertrophied testes, infertility and delayed puberty onset [183]. Exogenous administration of leptin to the *ob/ob* mice induces puberty through normalization of serum gonadotrophins and subsequent gonadal maturation which ultimately restores fertility [111, 183].

While these models of leptin deficiency do provide convincing evidence for the role of leptin and obesity in the regulation of reproductive development, they illustrate rare conditions that affect a small percentage of the population [92]. Yet, reproductive impairments coinciding with high serum leptin are often seen in obese patients that do not have any changes in leptin receptor function or expression. High leptin in obese young females typically accompanies premature puberty onset and conversely excessive leanness delays puberty onset whereas the opposite effect is observed in young males with obesity correlating with delayed puberty onset [184-186]. This is perhaps indicative that elevations in endogenous leptin caused by obesity exerts gross peripheral effects disrupting the HPG axis and normal reproductive function. We showed in chapter three that the obese male p110α-null mice have elevated serum leptin. Our aim was to

determine if this high serum leptin seen in the α-/- mice was a direct effect of obesity and if these changes in leptin correspond to alterations in puberty development.

4-2 Materials and Methods

Serum hormone measurements. Serum samples from fed male α-/- and control mice were collected from cardiac puncture in animals anesthetized with an intraperitoneal injection of ketamine/xylazine at 30 days of age, 8 weeks, and 8 months of age between the hours of 9:00AM to 11:00AM and free serum testosterone was measured by an ELISA kit (IBL-international). Serum leptin from overnight fasted male mice at 30 days of age were collected during the hours of 9:00-11:00AM and quantified with an ELISA (R&D Systems).

Characterization of male and female puberty onset. Mice were weaned at 21 days of age and maintained in same-sex housing. From 21 days of age until puberty onset the ano-genital distance was measured in both male and female α-/- and control mice. In male mice balanopreputial separation was used as determinant of puberty onset. Full separation was determined when the prepuce could be fully retracted. In the female α-/- and control mice puberty onset was marked on the day of vaginal opening.

Gene expression analysis. RNA was isolated from WAT using the RNeasy Lipid Tissue Kit (Qiagen), and cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad). WAT gene expression of *Leptin, Cebpa*, *Srebf1* and *Actb* were analyzed with the SYBR Green PCR Kit from Qiagen. The primer sequences are below listed in table 10**.**

Table 10
Primer sequences for RT-PCR

Gene Name	Forward Primer	Reverse primer
Lep	TGACACCAAAACCCTCATCA	AGCCCAGGAATGAAGTCCA
Cebpa	CCGGGAGAACTCTAACTC	GATGTAGGCGCTGATGT
Srebf1	CTGGATTTGGCCCGGGGAGATTC	TGGAGCAGGTGGCGATGAGGTTC
Actb	TTCTTTGCAGCTCCTTCGTT	ATGGAGGGGAATACAGCCC

Statistics. Results are reported as means ± SEM. Significance was determined by Student's *t* test, one-way ANOVA or Mann-Whitney Rank Sum (SPSS, IBM, Chicago, IL). Statistical significance was accepted when $P < 0.05$.

4-3 Results

These studies used the same adipose-specific knockout mouse models for p110α (α-/-) that were used in Chapter 2 and 3.

4-3.1 Puberty onset and pubertal delayed growth in the male α-/- mice

Obesity correlates strongly with alterations in puberty onset [185, 187]. To asses potential changes in puberty development caused by loss of adipose-specific p110α in both male and female mice we recorded the age of puberty onset as the day of balanopreputial separation in males, the age at which the prepuce can be retracted, or the day of vaginal opening for female mice. In addition we also recorded the anogenital distance from weaning beginning at 21 days until puberty onset. We did not see any difference in puberty development between female α-/- and α+/+ controls either in the age of vaginal opening or in anogenital growth (Fig. 4-2A, B). We next examined the young male mice. As expected the male α+/+ controls displayed a larger anogential distance than female mice (Fig. 4-2B, D). We also observed that the α+/+ control mice achieved balanopreputial separation around 30 days of age but a separation in the α-/- mice is delayed until 33 days of age (Figure 4-2B). This delay corresponds with a significant lag in anogenital growth, determined by one way ANOVA, starting at 23 days of age and continues through puberty onset (Fig. 4-2C). It is possible that a changes in body weight are affecting pubertal development in the α-/- mice. However we saw no changes in bodyweight between the male α-/- and control mice at 30 days of age (Fig. 4-2E). We did find that α-/- mice have a decrease in testes weight at 30 days of age (Fig. 4-2F). Since the testes are the primary site of testosterone production in male animals loss of testicular function in the α-/- mice might explain the delay in puberty onset.

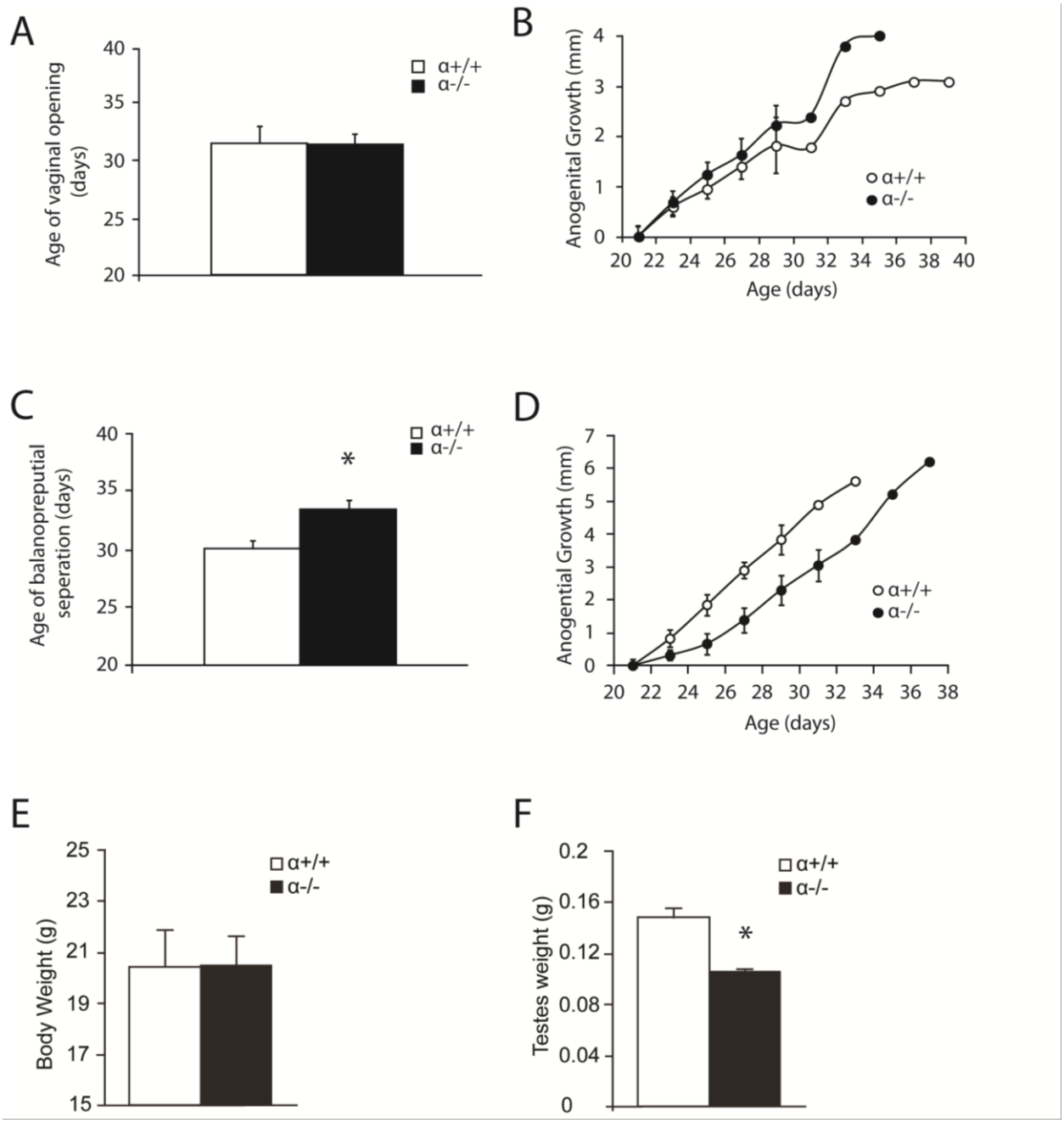


Fig. 4-2. Onset of puberty is delayed in α-/- males but not α-/- females. Female puberty onset was marked as day of vaginal opening (A) and anogenital distance was measured from weaning until vaginal opening (B) (α+/+ *n* = 9 , α-/- *n* = 6) for both female measurements).Male puberty onset was determined as the day of balanopreputial separation (C; α+/+ *n* = 12 , α-/- *n* = 9) and anogenital distance measured from weaning until preputial separation (D; α+/+ *n* = 12, α-/- *n* = 7). Bodyweight (E) and testes (F) weight measured from *ab lib* fed 30 day old male α-/- mice and controls (α+/+ *n* = 4; α-/- *n* = 5), * Statistical Significance determined by Students t-test (Balanoseperation, Testes weight) $P < 0.05$ or by one-way ANOVA (Anogenital growth) $P < 0.05$

4-3.2 Free serum testosterone is not decreased in male α-/- mice.

Puberty onset can be delayed by low serum testosterone in male patients [188]. To find out if this correlation exists in the α-/- mice we measured free serum testosterone, unbound and therefore biologically active testosterone, levels in male α+/+ and α-/- mice with age (Fig. 4-3). Surprisingly, we did not see a decrease in free serum testosterone in the α-/- mice when compared to the control animals. In fact, at 2 months of age we noted a statistically significant increase in the α-/- free testosterone levels. We concluded that low serum testosterone is not the cause of delayed puberty onset in the young male α-/- mice.

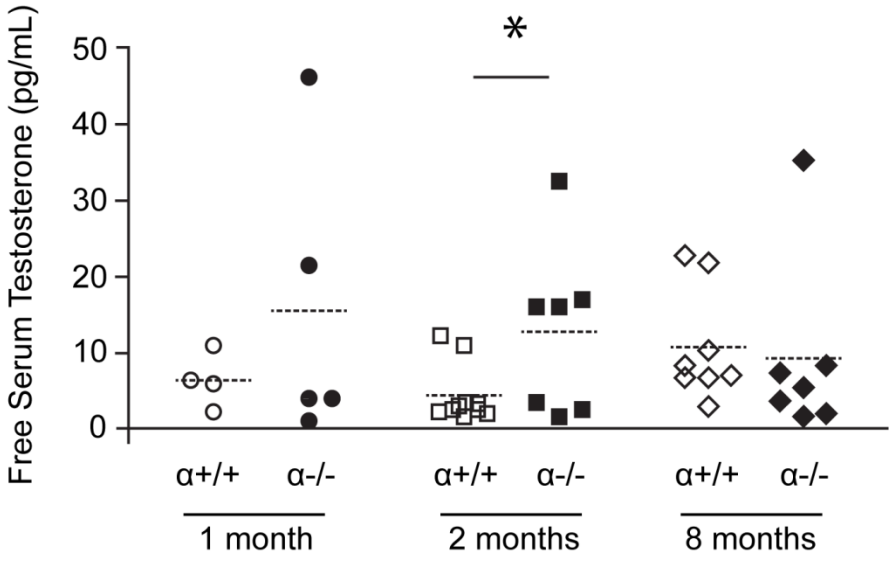


Fig. 4-3. Free serum testosterone in male α-/- mice. Serum was collected from *ad lib* fed male α-/- mice and controls and free serum testosterone measured by ELISA. Samples were collected at 30 days (α+/+ *n* = 4; α-/- *n* = 5), 2 months (α+/+ *n* = 10; α-/- *n* = 7), and 8 months of age (α+/+ *n* = 8; α-/- *n* = 7). *Statistical significance determined by Student's t-test $P < 0.05$

4-3.3 Fasting serum leptin levels do not correlate with body weight in male α-/- mice.

Leptin is considered to be a 'permissive' factor for puberty onset and is capable of affecting reproductive signals directly through the HPG axis [166]. Under normal conditions, leptin is synthesized in direct proportion to fat mass. We already showed that fasting serum leptin is high in the obese 4 month old α-/- mice and we confirmed an increase in fasting leptin levels in the pre-obese 30 day old α-/- mice (Table 11).

Table 11
Fasting serum leptin of male α-/- and α+/+ mice

	α+/+	α-/-
Leptin, Fasted (ng/mL), 4 months old	1.24 ± 0.35 (*n* = 7)	10.17 ± 2.5 * (*n* = 7)
Leptin, Fasted (ng/mL), 30 days old	1.03 ± 0.02 (*n* = 4)	1.25 ± 0.5 * (*n* = 5)

Data are means ± SE. Mice were fasted for 16 h * Statistical significance at $P < 0.05$.

We wanted to determine if the high serum leptin seen in the adult α-/- mice can be accounted for by the observed increase in body weight due to increased adiposity and if this elevation in serum leptin exists in the young α-/- mice prior to obesity onset. At 4 months of age, fasting serum leptin does correspond to increases in body weight in both the α-/- and α+/+ control mice yet normalization of leptin to bodyweight fails to normalize the α-/- serum levels to that of the control mice (Fig. 4-4A,B). In the 30 day α+/+ mice fasting leptin also increase with bodyweight but the leptin levels of the α-/- mice are above higher than control mice at similar bodyweight (Fig. 4-4C). This equates to a significant increase in serum leptin of the α-/- mice over the controls when normalized to bodyweight (Fig. 4-4D).

Quantification of leptin mRNA in the WAT of the overnight fasted 30 day old mice confirmed a dramatic increase in leptin gene expression in the α-/- mice (Fig. 4-4E).

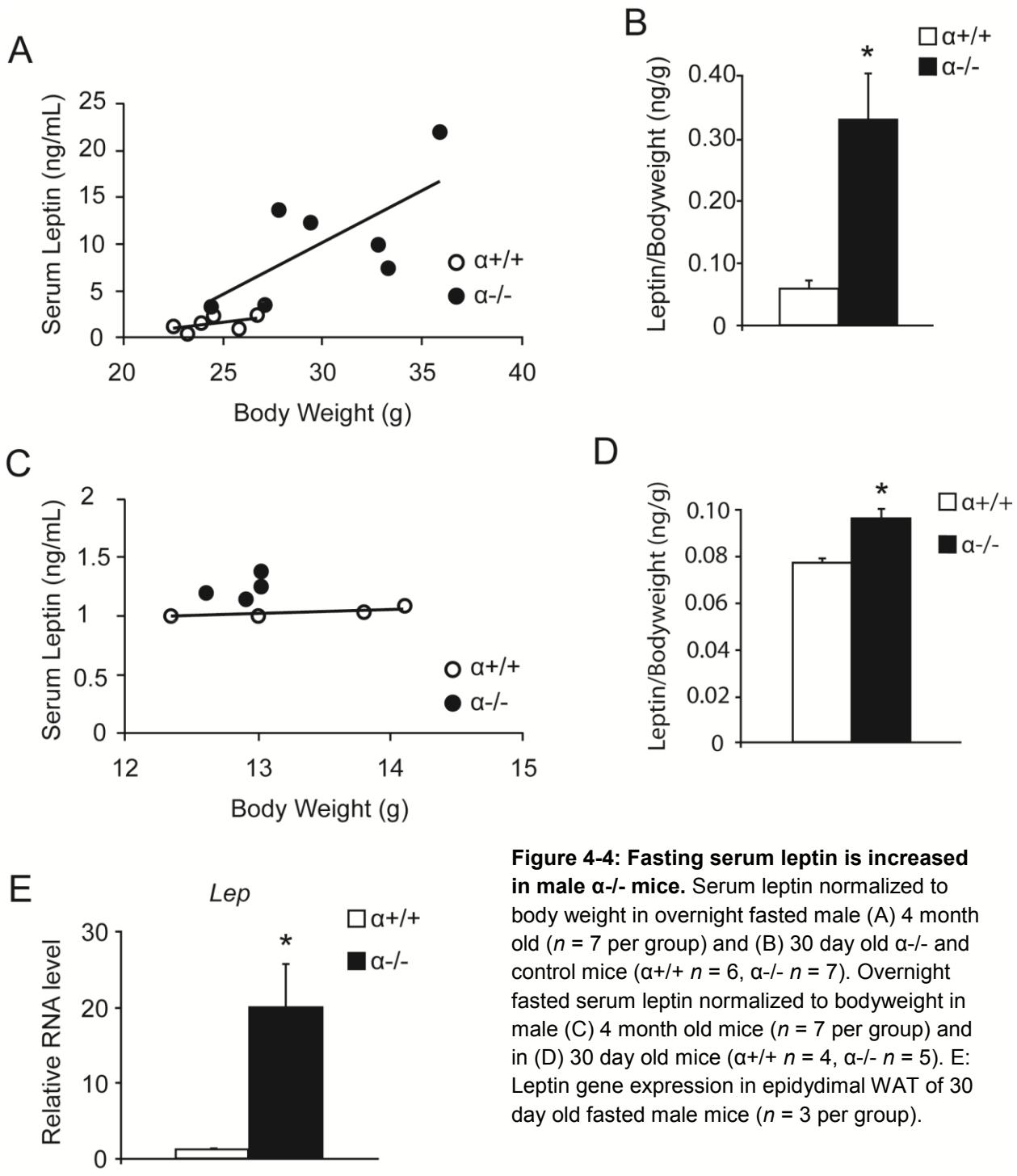


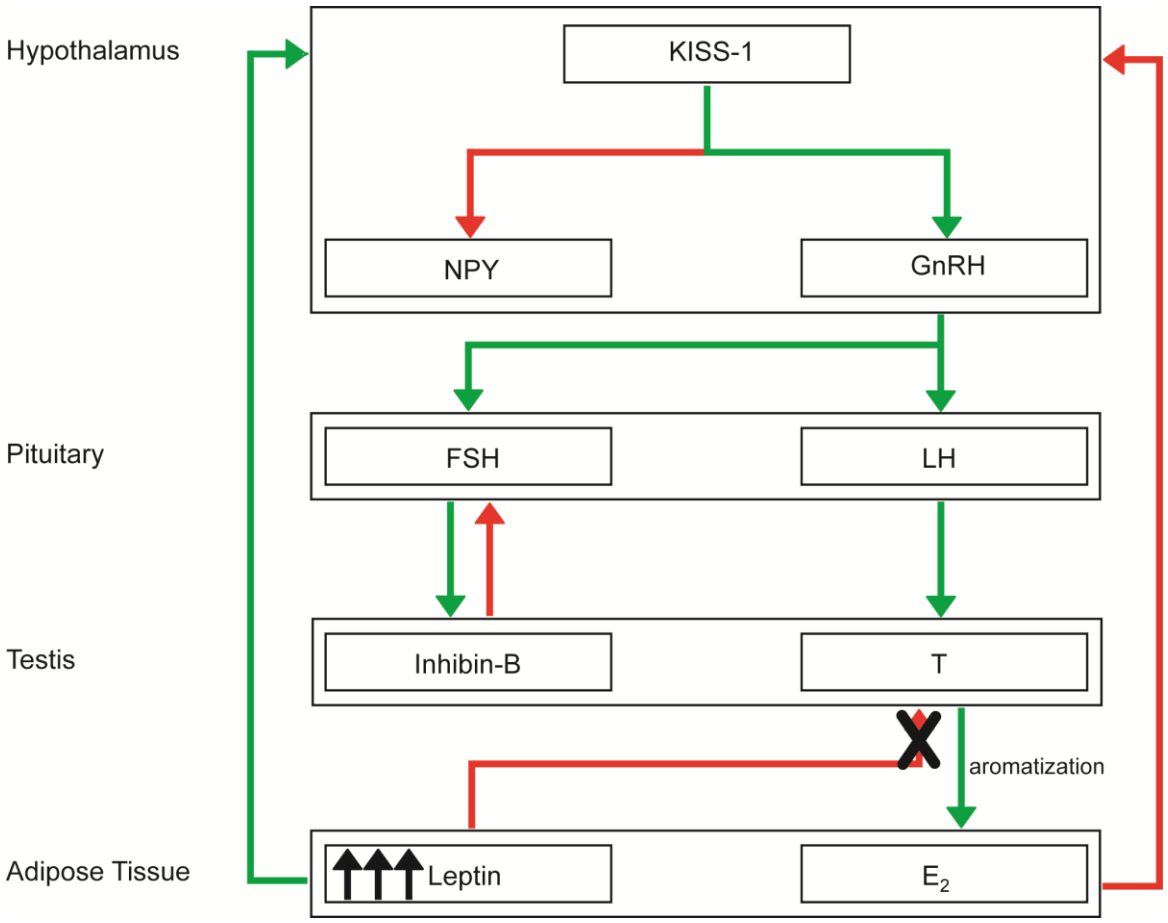
Figure 4-4: Fasting serum leptin is increased in male α-/- mice. Serum leptin normalized to body weight in overnight fasted male (A) 4 month old (*n* = 7 per group) and (B) 30 day old α-/- and control mice (α+/+ *n* = 6, α-/- *n* = 7). Overnight fasted serum leptin normalized to bodyweight in male (C) 4 month old mice (*n* = 7 per group) and in (D) 30 day old mice (α+/+ *n* = 4, α-/- *n* = 5). E: Leptin gene expression in epididymal WAT of 30 day old fasted male mice (*n* = 3 per group).

Studies have shown that activation of the insulin signaling pathway can increase leptin production and secretion by the adipose tissues (Wrann 2012). In the fasted state the obese 4 month old α-/- mice have elevated insulin in addition to high leptin (Table 5). It is

possible that high serum insulin seen with fasting in the α-/- mice activates signaling cascades independent of PI3K-p110α to activate insulin-responsive transcription factors. To investigate the link between loss of adipose p110α and increased leptin transcription we examined the expression of insulin-regulated transcription factors known to stimulate leptin transcription in the white adipose tissue of overnight fasted male mice 30 days of age. We did not see any changes in the levels of C/EBPα (α+/+ 0.22 $\pm$ 0.03; α-/- 0.26 $\pm$ 0.08; *n* = 3 per group) or SREBP1c (α+/+ 0.39 $\pm$ 0.1; α-/- 0.37 $\pm$ 0.2; *n* = 3 per group) possibly suggesting that loss of p110α in the adipose tissue allows for insulin-independent leptin synthesis.

4-4 Discussion

The work presented here demonstrates a link between adipose tissue PI3K and the mechanisms that regulate male puberty onset. Thus far, the evidence indicates that high leptin levels occurring prior to the onset of obesity in the male α-/- mice might be interfering with the ability of the HPG axis to regulate puberty (Fig. 4-5).



Adapted from Michalakis 2013

Fig. 4-5. Working model of HPG-puberty delay in the male α-/- mice. Normal free serum testosterone in the pubescent α-/- mice suggest that elevated serum leptin is not inhibiting testosterone production by the testes.

Because we see male-specific delayed puberty onset in the α-/- mice while the female α-/- mice have normal pubertal development it is likely that the mechanism of action is

related to testosterone. We confirmed that testosterone levels are not changed in the prepubescent α-/- mice from that of the controls indicating that leptin is not inhibiting testosterone production by the testes (Fig. 4-5). Future experiments should focus on dissecting out the point of dysfunction along the HPG axis.

As discussed in the introduction, testosterone is converted into estrogen by aromatase in the white adipose tissue [83]. It is possible that even though the α-/- mice express normal concentrations of serum testosterone, the conversion rate into estrogens is increased potentially providing a negative feedback to the puberty stimulatory signals by hypothalamus (Fig. 4-5). To confirm if the action of aromatase is altered in the male α-/- mice, aromatase activity should be measured at the time of puberty onset in the 30 day old male mice. Additionally measurement of serum estrogens in the young male mice should be taken as another determinant of aromatase activity. Serum estrogens can should be measured in age-matched α-/- and controls prior to puberty onset and in sexually mature adult mice at 4 months of age. As previously mentioned testosterones are the precursor for estrogens and this conversion is mediated by the aromatase enzyme. While estrogen deficiency in males is not thought to be associated with changes in pubertal development, it has been suggested that lack of estrogen signaling in males can be compensated for by increased conversion of testosterone into estrogens [189, 190]. Potentially, elevated aromatase activity in the white adipose tissue resulting from p110α ablation could cause increased turnover of serum testosterone into estrogens.

There is cross-talk between the insulin and estrogen signaling pathways that possibly converge on PI3K. In fact, it has been demonstrated that PI3K is required to activate estrogen receptor α (ERα) and can do so in the absence of estrogen itself [191].

In models of breast cancer it has been shown that PI3K can phosphorylate the cytosolic estrogen receptor thereby activating its function as a nuclear receptor to modulate gene transcription [192]. It is also known that PI3K can be activated down-stream of estrogen and both estrogen and ERα can activate Akt independently of PI3K signaling in breast cancer cells lines [193, 194]. However, the direct interactions between estrogen signaling and PI3K activity in the adipose tissue are not known.

Increased conversion of testostosterone into estrogen is thought to be affected by a drop in sex hormone-binding globulin (SHBG) which decreases with obesity [195]. SHBG is mainly produced by the liver and specifically binds to circulating E2 and testosterone in the bloodstream. It should be noted that its affinity for testosterone is twice that of E2 [196]. Unbound testosterone and E2 is considered “free” and biologically active since unbound hormones enter cells and activate androgen or estrogen receptors, respectively. As SHBG levels fall a larger amount of testosterone is converted into E2 by the adipose tissue [197]. Low SHBG concentrations are associated with an increased risk of type 2 diabetes and, in some cases, SHBG may be a better of indicator metabolic syndrome than low testosterone [198-200]. Furthermore, current research suggests that SHBG might influence the insulin signaling pathway and may potentially be an important player in insulin resistance. SHBG levels decrease with insulin resistance and hyperinsulinemia after the onset of obesity but SHBG also decreases with elevated glycated hemoglobin (A1c) in patients without diabetes which suggests a relationship between SHBG and glucose homeostasis [201, 202].

However, whether SHBG is a cause or effect of insulin resistance has yet to be determined. It is entirely possible that in the α-/- mouse which is obese and, insulin

resistant and glucose intolerant the levels of SHBG are altered. In order to test this hypothesis, serum samples from mice 30 days of age, prior to the onset of obesity, and at 8 weeks of age, post-obesity onset, should be collected and levels of SHBG quantified. Glucose tolerance and serum insulin levels should also be measured at 30 days of age to examine the relationship between SHBG and insulin resistance in the young mice prior to the onset of obesity and during the time of puberty onset. In addition, we see evidence of disruption of the normal liver function of the α-/- mice with high cholesterol and serum triglycerides (Fig. 2-4, Table 5). Changes in serum SHBG may be an extension of this systemic effect resulting from adipose p110α deletion.

The introduction discusses the importance of leptin signaling in the normal progression of puberty. The male α-/- mice displayed elevated serum leptin that did not correlate with their bodyweights as it did in the control animals (Fig. 4-4). This elevation in serum leptin occurs during pubertal development and corresponds to a delay in puberty onset in the α-/- mice (Fig. 4-2). As discussed in previous chapters the FIRKO mouse, with adipose-specific deletion of the insulin receptor, displayed a lean protective phenotype that resisted glucose intolerance [95]. However, while the FIRKO mouse did not have a statistically significant elevation in serum leptin, the FIRKO leptin levels in mice 2 months old was elevated when normalized to their bodyweight and thus was inappropriately elevated in the knockout mice [95]. Unfortunately the authors did not examine puberty onset in the young FIRKO mice. Even so the FIRKO study and our own α-/- mice support that attenuated activation of the insulin receptor-PI3K pathway increases leptin production by the adipose tissue.

In contrast to the FIRKO and α-/- mice who have high leptin under attenuated insulin signaling in the adipose tissue, insulin stimulation has been shown to increase leptin expression in both primary white and brown adipocytes possibly through upregulation of the insulin responsive transcription factors C/EBPα and SREBP1c [203-206]. We found that neither gene is changed in the fasting adipose tissue of the young 30 day old α-/- mouse with high serum leptin in the white adipose tissue suggesting that in a physiological *in vivo* model insulin-regulation of leptin expression is more complicated than in primary wild-type cells. One possibility is that the *in vivo* insulin-dependent regulation of leptin depends on the protein expression of C/EBPα and SREBP1c and also on the nuclear localization of these factors and not soley on gene expression. Unfortunately the antibodies available to us for western blotting of C/EBPα and SREBP1c in the white and brown adipose tissues produced several nonspecific bands and we were unable to analyze protein expression or localization. Further efforts should be taken to identify antibodies that recognize C/EBPα and SREBP1c proteins in primary adipocyte in order to dissect out the contribution of these factors to increase in leptin expression in the adipose tissue of the α-/- mice.

It is commonly stated that leptin is a 'permissive' hormone in terms of reproductive development and is required for normal pubertal development but its presence alone is not sufficient to cue puberty onset [207]. A study demonstrated that acute *in vitro* leptin administration to the adult rat testis inhibits both basal and stimulated testosterone production independent of the nutritional status of the animal from which the tissues were collected [208]. However, the authors did not see the same effect in tissues collected from prepubertal animals and suggested the presence of a protective mechanism during

puberty that prevented leptin inhibition of testosterone production during sexual development. This protective mechanism might be maintaining the testosterone production in the prepuberal α-/- mice in the presence of high serum leptin but cannot control the effects of serum leptin.

A hypothesis to delayed puberty in the male α-/- mice is that the high serum leptin in the α-/- mice exerts central effects and affect production and release of other sex hormones such as FSH and LH. Leptin is a very potent hormone to the hypothalamus; however, it does not appear that leptin alone can mediate changes in puberty through the hypothalamus. As previously mentioned the HPG axis is sensitive to regulation by nutrient availability but it is likely that this is a cooperative effort by many metabolic signals including leptin and insulin [140]. The α-/- mice have elevated insulin levels with many other changes in metabolic factors which may affect hypothalamic function (Table 5). The α-/- animals also do not show a change in food intake despite high serum leptin which is already suggestive a certain degree of hypothalamic leptin resistance indicating that the hypothalamic response is already altered from that of wild-type mice (Fig 3-1A,B). If hypothalamic leptin and insulin signaling were impaired then this could result in a drop in levels of GnRH, FSH and LH since they are driven directly through the HPG (Fig 4-5).

This model of adipose-specific p110α ablation will undoubtedly prove beneficial in the study of puberty onset and its regulation. The sexual dimorphism between male and female puberty onset in the α-/- mice is quite remarkable and will provide unique insight into the regulatory factors that contribute to male specific reproductive development. So far the majority of the literature on pubertal development has focused on the female aspect and the data on male puberty is sparse. Little attention has been afforded to the

male development, in part, due to lack of available relevant models. The α-/- mice provides evidence that adipose tissue insulin resistance delays puberty onset in male mice. Since this delay occurs before the onset of obesity in males and also since the female α-/- mice display no change in puberty onset but still develop obesity it would seem that this is an independent phenotype from the errant lipid regulation discussed in chapter 2. Future experiments should focus on identifying the point of action of adipose p110α on leptin regulation and how this affects the HPG axis.

Chapter 5

General Discussion and Future Work

The research presented in this dissertation characterizes a unique mouse model of adipose specific deletion of p110α, the primary insulin responsive PI3K catalytic subunit, and the effects of p110α ablation on the white and brown adipose tissue as well as the systemic effects induced by disruption of normal adipose tissue function. We discovered that loss of p110α in the adipose tissues induces obesity and glucose intolerance contributed to, at least in part, by reduced respiration in the brown adipose tissue. We also found that in male mice without adipose-p110α obesity is preceded by a delay in puberty onset while in females there are no discernible changes related to puberty despite prepubertal obesity onset. While the connections between obesity onset and delayed puberty in the α-/- male mice remain unclear, it provides the opportunity for unique insight into the overlap between pubertal development and adiposity.

To fully appreciate the α-/- mouse model a critical examination over the lifespan of the organism is necessary. The majority of the research presented in this thesis was acquired from adolescent animals around 8 weeks of age to adult animals at 4 months of age and provides a relatively complete characterization of the metabolic phenotypes in adult α-/- mice. However, current research indicates that obesity and glucose intolerance during childhood may compromise normal glucose and lipid metabolism later in life [209]. Future research should continue the work performed in chapter 4 and continue to investigate the contribution of adipose p110α to puberty development in the young α-/- mice to understand and unite the changes seen in juvenile development to the mechanisms of obesity observed in the α-/- adult animals. In human, the incidence of diabetes and insulin resistance increases with age. It would also be interesting to conduct an aging study the α-/- mouse model and to determine if the metabolic phenotype worsen

with age or not, and how this abnormality affects life span. Somewhat surprisingly, it is well established that downregulation of PI3K and its downstream signaling molecules increases longevity in many animals.

5-1 Puberty progression and insulin resistance

The differences seen in the time of obesity and puberty onset between the male and female α-/- mice suggests a sexual dimorphic response to the loss of adipose p110α. This mouse model presents an obese female mouse that undergoes normal pubertal development but an obese male mouse that has delayed puberty. This is an extremely useful and rare model of metabolism affecting male puberty and should aid in future studies concerning the mechanisms affecting male puberty development.

It is important to note that the timing of obesity onset is different between the α-/- male and the α-/- female. In the α-/- females an increase in bodyweight is seen prior to the onset of puberty which develops normally when compared to the control animals (Fig. 2-3, 4-2). It has been reported that obesity in young female patients induces earlier menarche and puberty onset [186]. But, curiously, obesity in the female α-/- mice does not appear to affect the timing of puberty onset. In contrast, the male α-/- mice do not show any difference in weight compared to controls until after puberty onset which occurs around 34 days of age (Fig 4-2) and an increase in body weight recorded around 42 days of age (Fig 2-2). It is suggestive that the necessary hormonal signals for puberty progression already present in the female α-/- mice are triggered post-puberty in the α-/- males and serve to increase lipid deposition in both sexes.

We examined the effects of adipose-specific p110α ablation in male and female mice as early as 4 weeks of age. However, in the case of the α-/- female mice it is apparent that this age might be too late to examine the onset of obesity because the divergence in the female body weights has taken place before we starting collecting data. Examination of both male and female animals at this time will shed light upon the factors contributing to obesity including the degree of both whole-body insulin resistance and insulin resistance in the adipose tissue itself. The aP2 promoter is expressed embryonically so targeting of p110α-loxp sites by Cre recombinase should have already occurred by birth. We would expect some degree of insulin resistance by the adipose tissue very early in the α-/- mice but it is possible that the loss of p110α in the adipose tissue can be compensated for early on in postnatal development by other PI3K subunits.

Much research is focused on the adverse effects of reduced insulin sensitivity but it appears that insulin resistance is integral part in prepubertal development. A 9-year longitudinal study following healthy children from 5-14 years of age documented a naturally occurring rise of age-dependent insulin resistance [210]. The authors found that insulin resistance begins well before the initial rise in LH associated with puberty initiation and before the appearance of any endocrine or anatomical indicators of puberty. While healthy prepubescent female children have a more significant increase in insulin resistance when compared to male children of the same age both genders experience a rise in insulin resistance around 7 years of age. This rise occurs around the same time as adrenarche or the development of the adrenal zona reticularis of the adrenal cortex. Adrenarche occurs independently of the HPG axis and raises the levels of dehydroepiandrosterone (DHEA), the primary precursor to natural estrogens, and to a

lesser extent androgens such as testosterone [211]. It is unclear if adrenarche is directly affected by insulin resistance or if the coincidence of these events is incidental. The authors postulate that childhood insulin resistance might serve to increase DHEA production by the adrenal cortex to promote fat accumulation necessary to meet the increased nutritional demands required for growth and development through puberty [210].

As previously noted, we expect that the α-/- mice have adipose tissue-specific insulin resistant from birth. It is possible that the early onset of insulin resistance in the α-/- mice might cue chronic lipid accumulation in the adipose tissue beginning at a very early age and continue through adulthood. The gender divide of the α-/- mice in terms of puberty onset might be a result of errant gender-specific sex hormone production by the adrenal gland in response to premature insulin resistance. Both genders are expected to have insulin resistance but a difference in puberty onset induced by elevated estrogens in the female α-/- mice may be buffered by the inherent requirements for higher levels of estrogens in female control mice than in male mice. Male mice may have a higher sensitivity to changes in estrogens which is one possible contribution towards delayed puberty onset in the α-/- male mice.

5-2 Serum estrogens affect insulin sensitivity and metabolism

Estrogens are traditionally thought to be important in the development of female sex characteristics and for development and maintenance of reproductive capacity. Serum E2 levels and insulin sensitivity decline with age in postmenopausal women [83]. It has been reported that in female patients, age-induced insulin resistance is corrected with E2 replacement [212, 213]. During the reproductive age the ovaries are the primary

source of estrogen production. A link between ovarian function and glucose homeostasis in female rodents exists as insulin tolerance fluctuates in concordance with the estrous cycle [214]. It was shown that ovariectomy in female mice increases white adipose tissue while impairing glucose intolerance and increasing insulin resistance and these diabetic characteristics are reversed with estrogen replacement [215, 216].

It is not until recently that scientists have realized the full extent of estrogen signaling in males. It has been recently confirmed that in both men and women estrogens decrease with age and this promotes adipose tissue depositions and increased cholesterol [176]. Originally thought to be a result of diminishing testosterone levels, increases in male adiposity with age is thought to be prompted by a decline in E2 whereas low testosterone correlates with reduced lean mass [176]. As previously indicated, estrogen synthesis in the adipose tissues of both males and females is tightly regulated by the enzyme aromatase. Reductions in aromatase activity, and thereby estrogen, is known to be a contributing factor to obesity and metabolic syndrome [217, 218]. Aromatase deficiency in males causes decreased bone growth and osteopenia along with high insulin, cholesterol, and triglycerides [219]. Therefore, it would be interesting to examine the hormonal profile and adiposity phenotype of old (>12 month of age) α-/- mice and their controls.

To examine the direct contribution of estrogen signaling in metabolism researchers have generated knockout mouse models of estrogen deficiencies that closely mirror the human condition. These mouse models share many of the same phenotypes as our own α-/- mice. Both the estrogen receptor knockout mice (ERKO) and the aromatase receptor knockout (ArKO) mice develop increased adiposity, hyperleptinemia, hyperinsulinemia,

insulin resistance and glucose intolerance which are characteristics seen in our adult α-/- mice [220-223]. In further support of the similarities towards the α-/- mice, ArKO mice have high serum testosterone in addition to elevated cholesterol, triglycerides in the presence of a fatty liver [221]. The ArKO mice also have decreased lean mass which we saw in 4 month old α-/- male mice (Fig. 2-2) [86].

The changes in adiposity in the ERKO mice are contributed to by increased lipid deposition in the adipocytes resulting in hypertrophied adipocytes [222]. It was found that adiposity is likely a result of reduced energy expenditure with no change in food intake much like the phenotype in the α-/- mice. The ERKO mouse is representative of whole body estrogen receptor signaling and therefore illustrates the importance of estrogen signaling in systemic metabolism. Adipose specific deletion of the estrogen receptor results in hypertrophied adipocytes in males and females, as in the whole body deletion, but only female mice have increased body weight contributed to by increased fat weight [224]. The male adipose specific ER knockout mice are glucose intolerant but normal in body weight and adiposity [224]. Based on the broad spectrum of glucose and lipid phenotypes associated with deletion of ER which we also see with p110α deletion it seems likely that the α-/- mice could have developed some impairment in estrogen signaling that might be contributing to the observed increased in α-/- adiposity and glucose intolerance.

The role of estrogen signaling in the BAT is less well understood but BAT function does seem to be affected by changes in sex hormones. The ERKO mouse had no change in BAT weight but the researchers did not examine any functional changes [224]. In vitro studies have found that treatment of brown adipocytes with sex hormones does affect cell

metabolism and mitochondrial function. Treatment of a brown adipocyte cell line with E2 and testosterone reduced the adrenergic response and inhibited mitochondrial gene expression [225, 226]. Another group showed E2 treatment of brown adipocyte cell lines activated the PI3K/Akt pathway through inhibition of PTEN which coincided with a reduction in mitochondrial-associated gene expression [226]. Administration of testosterone did not appear to affect PI3K but did inhibit transcription of PGC-1α and UCP-1 indicating that sex hormones can directly affect the thermogenic capabilities of BAT [226]. In a human fetal BAT cell line ERα was demonstrated to localize in the mitochondria and colocalize with PPARγ and PGC-1α possibly indicating a potential mechanism for estrogen to modulate mitochondrial activation and biogenesis [227]. We clearly showed that loss of p110α in the brown adipose tissue impaired cellular respiration and reduced mitochondrial gene expression. It is possible that the changes in mitochondrial function are indicative of alterations of sex steroid signaling in the α-/- mice and are contributing to changes in puberty onset as well as post-puberty obesity onset. As discussed in chapter 4, PI3K is thought to interact with ERα to stimulate its actions as a nuclear receptor and alterations in the activity of PI3K could easily change the role of ERα.

5-3 A comparison between high fat diet model of obesity and α-/- mice

In an era where much of the world is afflicted with constant exposure to high fat calorie dense food availability it is very relevant to discuss the impact of high fat diets and the effect on insulin signaling. Mouse models of a high-fat diet (HFD) give us the ability to tightly control calorie intake and examine the physiological consequences that would otherwise be unavailable in human patients. The systemic phenotypes exhibited by the

adult α-/- mice also closely mirror that of mice kept on a high-fat diet. Mice that were kept on HFD starting at 4 weeks of age gained approximately 5 grams by 4 months over control mice kept on normal chow which is a similar body weight increase seen in our α-/- mice [228]. HFD mice are also glucose intolerant and insulin resistant characterized by peripheral insulin resistance and high fasting serum insulin [146]. Additionally, mice on HFD develop elevated plasma leptin within one week of diet initiation and this increase remains constant for the duration of the diet [229]. Prolonged fasting for 48 hours did not resolve HFD leptin levels to that of the mice on the control diet. It is also relevant to our study of the α-/- mice to note that the plasma leptin concentrations seen in HFD strongly correlate with serum insulin levels under both fasting and *ad lib* fed conditions but are independent of body weight which is very similar to what we observe in young α-/- mice prior to onset of obesity [229]. The remarkable aspect of our mouse model is that they are kept on normal chow from birth but still develop many of the phenotypes seen with HFD treatment. It would also be interesting to investigate how the α-/- mice respond to HFD, i.e. would the diet accentuate adipose deposition and worsen the metabolic abnormality in these animals.

Our research confirms a link between adipose tissue insulin sensitivity and mitochondrial function. This is in support of previous studies utilizing HFD mice and the hyperphagic *db/db* mouse model. Both *db/db* mice and HFD mice have suppressed mitochondrial biogenesis and mitochondrial function in the adipose tissues and are insulin resistant in the adipose tissues [146]. Expression of mitochondrial genes involved in oxidative phosphorylation, the TCA cycle, and fatty acid oxidation were decreased in these two mouse models in addition to reduced UCP-1 expression. Treatment with

Rosiglitazone, a known insulin sensitizer and PPARγ agonist of the thiazolidinedione drug class, restored mitochondrial function in these animals to that of the normal chow fed controls [204]. This relationship between insulin sensitivity and adipose mitochondrial function was further proved in human patients with type 2 diabetes resulting in less mitochondria, reduced PGC-1α and low citrate synthase (an enzyme important in the TCA cycle) activity [141]. Expression of PGC-1α and mitochondrial biogenesis was increased in the type 2 adipocytes with Piglitazone, another thiazolidinedione drug. We see both decreased PPARγ and UCP-1 in the less metabolically active α-/- brown adipose tissue. The low expression of mitochondria-associated genes resulting in low cellular respiration is seen in HFD mice, leptin deficient animals and with the loss of adipose-p110α is highly suggestive of the importance of insulin signaling in maintaining normal adipose tissue metabolism.

5-4 Regulation of leptin production by insulin

While the defects seen in the brown adipose tissue respiration are no doubt contributing to increased adiposity and liver lipid deposition it may not be the only cause. UCP-1 levels are not reduced prior to obesity onset (Fig. 3-7) and their reduction might be a side effect of age-related metabolic dysfunction from loss of adipose-p110α. It is possible that mechanisms other than impaired brown adipocyte respiration are responsible for the obese phenotypes of the α-/- mice. In light of the elevation of leptin that appears to be independent of body weight occurring in a mouse model of insulin resistance, the dynamic interplay between leptin and insulin must be taken into consideration. Leptin is thought to be a permissive factor for puberty onset and also displays a positive correlation with elevated insulin levels in adults [230]. This positive

correlation between leptin and insulin holds true for both prepubescent and pubescent children [207]. In studies of rodent models, hyperinsulinemia has also been implicated as a cause of high serum leptin indicated by increased leptin production in isolated rodent adipocytes treated with insulin [231-234]. In contrast, insulin deficiency in rats induced by streptozotocin treatment suppressed adipose tissue leptin mRNA abundance and this expression was restored by insulin injections [203].

We examined the expression of two transcription factors targeted by insulin, C/EBPα and SREBP1c, both known to upregulate leptin expression but did not detect any changes [205, 206]. However, as discussed in chapter 3, there are many targets of the Insulin/PI3K/Akt pathway which may affect leptin production. It is possible that p110α serves as a gate-keeper for leptin production and aids in the regulation of leptin secretion into the serum. In the absence of p110α there is no inhibition on the intracellular factors that would increase leptin synthesis and the leptin levels elevate. A better experimental approach to determine how insulin signaling in the adipocyte is regulating leptin production is to perform genome wide sequencing from the adipose tissue of the α-/- mice and their controls. Examination of the expression of genomic irregularities in the α-/- mice when compared to the controls will help to direct research into the signaling pathway that connects insulin signaling to leptin production.

5-5 Effects of leptin and insulin resistance in the peripheral tissues

High serum leptin in the presence of high insulin is capable of inducing systemic changes. It is well understood that insulin and leptin signaling overlap in major lipid and glucose regulating tissues including the liver, muscle and β-cells of the pancreas [235]. Leptin activation normally exerts anti-diabetic effects in the skeletal muscle and the liver.

In skeletal muscles, leptin induces glucose uptake and fatty acid oxidation, while in the liver, leptin serves to suppress glucose production as well as induce fatty acid oxidation [236, 237]. Hyperleptinemia has been implicated in the development of non aclhocolic fatty liver disease and correlates strongly with elevated serum lipids, glucose and insulin [238]. A recent study demonstrated that PI3K and leptin signaling are required for reduction of glucose as an anti-diabetic effect of duodenal-jejunal bypass surgery effectively improving diabetic phenotypes before weight loss [239]. The signaling pathways are thought to overlap through the IRS proteins which can bind to leptin activated Janus kinase 2 (JAK2) and then the resulting IRS-JAK2 complex can bind to the regulatory subunit of PI3K.

Our data indicates that, in addition to insulin resistance, the α-/- mice have a certain degree of leptin resistance demonstrated by their normal food intake despite almost a 10-fold increase in serum leptin (Fig 3-1, Table 5). If this is true, it would account for several of the glucose and lipid phenotypes seen in the α-/- mouse. The majority of the current research on the effect of attenuated leptin signaling on the peripheral tissues is performed with the leptin deficient *ob/ob* mouse model. With the exception of hyperphagia these animals share many similarities to our own α-/- mice including hyperinsulinemia, hyperglycemia and increased liver lipid deposition [80]. These symptoms caused by loss of leptin signaling are reversed with leptin administration and are not thought to be solely a result of increased food intake since these mice with impaired leptin signaling kept on food restriction do not resolve these phenotypes [240, 241]. Additionally, under normal conditions leptin has been found to inhibit insulin secretion by the pancreatic β-cells and

the treatment of the *ob/ob* mice with leptin reduces insulin secretion thereby correcting hyperinsulinemia [242].

The α-/- mice have high leptin and insulin but are insulin resistant as illustrated by the lack of peripheral phosphorylated Akt (Fig. 2-6) and potentially are leptin resistant in other tissues as well. Leptin resistance in the liver and muscle of the α-/- mice might be contributing to the increases in serum glucose, insulin and hepatic steatosis seen in the α-/- mice. The suggestion of systemic leptin and insulin resistance in support of the α-/- mice must be examined in the young mice before obesity onset. Continuous high serum leptin and high serum insulin from a very early age in the α-/- mice could maximally stimulate the signaling pathways in the peripheral tissues and attenuate their effectiveness.

5-6 Central leptin resistance and puberty onset

Central leptin resistance is highly suggestive in the α-/- mice. It is remarkable that in the presence of almost a 10-fold increase in serum leptin the obese animals show no change in food intake from their controls (Table 5, Fig. 3-1). We documented an increase in serum leptin at 30 days of age in the α-/- animals that persists until 4 months of age. It is possible for chronically elevated leptin to result in central leptin resistance [243]. Leptin is not thought to induce puberty onset but rather allows for puberty to happen. A theory of the role of leptin in puberty is that an ample supply of leptin signals to the CNS that nutrition stores are good enough to meet the high energy demands of pubertal development [187].

As discussed in chapter 4 leptin signals to the hypothalamus to activte GnRH neurons and increase GnRH pulse frequency. Leptin also directly stimulates LH and FSH.

In obese male patients with elevated serum leptin, the amplitudes of LH pulses are unchanged but the frequency is reduced [166]. Puberty onset may occur in the α-/- males once the levels of LH and FSH are attained with lower burst frequency accounting for the delay. LH and FSH levels should be tracked in the young male α-/- mice from weaning through puberty onset to determine how the hypothalamus is being regulated. Because leptin is only permissive during puberty onset there may be other hormonal signals from the adipose tissue, for examples serum estrogens, that are delaying male α-/- puberty despite high serum leptin. This places further importance on complete characterization of the adipose tissue and its genomic expression in young male α-/- mice and controls with techniques such as RNA-seq to examine changes in gene exression profiles related to leptin synthesis and puberty development.

5-7 Leptin resistance and energy expenditure

In the leptin deficient *ob/ob* mice leptin treatment increased energy expenditure through increased sympathetic signals to the brown adipose tissue [244].Chronic leptin administration to wild type rats increased oxygen consumption through upregulation of UCP-1 in the BAT [139]. This effect of leptin was ameliorated by surgically denervation of the BAT [245]. It was further shown by this group that direct administration of leptin into the CNS increased BAT UCP-1 and this effect was diminished with hypothalamic leptin resistance [243].

As previously discussed, it is very possible that the α-/- mice potentially have central leptin resistance. We showed that acute administration of leptin directly to the BAT increased the expression of UCP-1 demonstrating that the BAT itself is still leptin responsive (Fig. 3-5). The possibility of central leptin resistance in the hypothalamic

regions of the α-/- mice must be examined. Under artificial conditions the α-/- BAT is capable of responding to leptin but the literature strongly indicates that *in vivo* regulation of UCP-1in the BAT is under control of the CNS. The α-/- mice already live with chronically high leptin which does not produce anorexic effects. It must be considered that leptin resistance in the hypothalamus might be reducing BAT respiration by decreasing CNS signals to the adipose tissue itself. We should that the α-/- adipose tissue is responsive to β-adrenergic stimulation but if, *in vivo,* the tissue doesn't receive the stimulatory input due to central resistance then the intact tissues will also be affected. In future studies, attention should be given to the effect of leptin as it relates to the control of BAT cellular respiration and systemic energy expenditure in the α-/- mice.

General Conclusion

The data presented in this dissertation provides evidence describing the importance of PI3K signaling in adipocytes and the ability of adipose insulin signaling to impact the rest of the body. Most research on adipose PI3K has used models of cell culture and thus has not been able to recognize the systemic impact of adipose PI3K activity on the entire organism. The previous studies have also failed to recognize the extent of the widespread reduction in genes involved in respiration and energy expenditure of the brown adipose tissue. While these cell lines do provide strong evidence in support of the dominance of p110α in insulin signaling, they do little to describe the impact of p110α in a physiological model. We have demonstrated that p110α in the adipose tissue is critical in maintaining lipid and glucose stores and loss of adipose p110α reduces PI3K activity and causes cellular and global metabolic dysfunction.

Obesity is increasing across the world in epidemic proportions yet a unifying cause for obesity onset still eludes the medical community. The emergence of increased availability of high-fat calorie-dense food is an attractive explanation for increased adiposity but adiposity alone does not necessarily account for the development of diabetes and metabolic syndromes [246]. The research presented in this dissertation suggests that obesity and glucose intolerance stems from insulin resistance caused by loss of adipose tissue PI3K activity. My thesis supports the benefits of regulating insulin resistance as a measure against obesity and specifically points to the use of therapeutic agents targeting PI3K activation in adipocytes in the prevention and treatment of metabolic disorders.

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