MEMBRANE CHOLESTEROL BALANCE IN EXERCISE AND INSULIN RESISTANCE

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Dedication

I dedicate this thesis dissertation to my family. Thank you all for your help in big and small ways, and for putting up with the perpetual student. To my wife, your patience and support has overwhelmed and sustained me. To my son, you are the true motivation for my work and best reason to put it down every night. To my parents, without your support, guidance and occasional motivation; I would not be who I am today, nor who I will become. To my sister, brother, grandparents and in-laws; thank you for your encouragement and motivation.

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.

Abstract

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Study has shown that plasma membrane (PM) cholesterol and cortical filamentous actin (F-actin) influence skeletal muscle glucose transport. Of fundamental and clinical interest is whether diabetogenic insults promote membrane/cytoskeletal dysfunction amendable for therapy. As exposure to excess fatty acid (FA)s induce glucose intolerance by mechanisms imperfectly understood, we tested if PM cholesterol/F-actin changes could contribute to FA-induced glucose transporter GLUT4 dysregulation in skeletal muscle. High-fat fed, insulin-resistant animals displayed elevated levels of skeletal muscle PM cholesterol and a loss in cortical F-actin, compared to normal-chow fed animals. Consistent with a PM cholesterol component of glucose intolerance, human skeletal muscle biopsies revealed an inverse correlation between PM cholesterol and whole-body glucose disposal. Mechanistically, exposure of L6 myotubes to the saturated FA palmitate induced an increase in PM cholesterol that destabilized actin filaments and decreased insulin-stimulated PM GLUT4 and glucose transport, which could be reversed with cholesterol lowering. Next, study tested if the lipid-lowering action of the antidiabetic AMP-activated protein kinase

(AMPK) had a beneficial influence on PM cholesterol balance. Consistent with AMPK inhibition of 3-hydroxy-3-methylglutaryl CoA reductase, a rate-limiting enzyme of cholesterol synthesis, we found that AMPK activation promoted a significant reduction in PM cholesterol and amplified basal and insulin-stimulated GLUT4 translocation. A similar loss of PM cholesterol induced by β-cyclodextrin caused an analogous enhancement of GLUT4 regulation. Interestingly, PM cholesterol replenishment abrogated the AMPK effect on insulin, but not basal, regulation of GLUT4 translocation. Conversely, AMPK knockdown prevented the enhancement of both basal and insulin-stimulated GLUT4 translocation. As a whole these studies show PM cholesterol accrual and cortical F-actin loss uniformly in skeletal muscle from glucose-intolerant mice, swine, and humans. In vivo and in vitro dissection demonstrated this membrane/cytoskeletal derangement induces insulin resistance and is promoted by excess FAs. Parallel studies unveiled that the action of AMPK entailed lowering PM cholesterol that enhanced the regulation of GLUT4/glucose transport by insulin. In conclusion, these data are consistent with PM cholesterol regulation being an unappreciated aspect of AMPK signaling that benefits insulin-stimulated GLUT4 translocation during states of nutrient excess promoting PM cholesterol accrual.

Jeffrey S. Elmendorf, Ph.D., Chair

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Abbreviations

2-DG	2-deoxyglucose
ABC	ATP binding cassette transporter
ACAT	Acyl CoA cholesterol acyltransferase
ACC	Acetyl-CoA carboxylase
AMP	Adenosine monophosphate
AMPK	5'-AMP-activated protein kinase
APS	Adaptor protein containing PH and SH domains
Arp3	Actin related protein-3
AS160	Akt substrate of 160 kDa
ATM	Adipose tissue macrophage
ATP	Adenosine triphosphate

ATV	Atorvastatin
BMI	Body mass index
BSA	Bovine serum albumin
CaMKIV	Calmodulin-dependent protein kinase IV
CaMKK	Calmodulin-dependent protein kinase kinase
CAP	Cbl associated protein
CBS	Cytsathionine-β-synthase binding domain
Chol	Cholesterol
CoA	Coenzyme A
COPII	Coat protein complex II
CPT-1	Carnitine palmitoyltransferase I
CRP	C-reactive protein

CrPic	Chromium picolinate
DAG	Diacylglycerol
DMEM	Dulbecco’s modified Eagle’s medium
DNP	2,4-Dinitrophenol
ER	Endoplasmic reticulum
FA	Fatty acids
F-actin	Filamentous actin
FBS	Fetal bovine serum
FFA	Free FA
GAP	GTPase-activating protein
GEF	GLUT4 enhancer factor
GFAT	Glutamine:fructose-6-phosphate amidotransferase

GlcNAc	β-*N*-acetylglucosamine
GLUT	Glucose transporter
HBP	Hexosamine biosynthetic pathway
HDL	High density lipoprotein cholesterol
HM	Hydrophobic motif
HMG-CoA	3-hydroxymethyl-3-glutaryl coenzyme A
HMGR	HMG-CoA reductase
IKκB	The inhibitor of NF-κB kinase-β
IL-6	Interleukin-6
INSIG	Insulin-induced gene
IRAP	Insulin-responsive aminopeptidase
IRS	Insulin receptor substrate

JNK	Jun kinase
L6-GLUT4myc	L6 myotubes stably expressing GLUT4 that carries an exofacial myc-epitope tag
LDL	Low density lipoprotein
LXR	Liver X receptor
MEF-2	Myocyte enhancer factor-2
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NRF1	Nuclear respiratory factor 1
N-WASP	Neural Wiscott-Aldrich syndrome protein
OGA	O-linked-β-N-acetylglucosaminidase

O-GlcNAc	*O*-linked β-*N*-acetylglucosamine
OGT	*O*-linked-β-*N*-acetylglucosamine transferase
PAS	Phospho-Akt substrate
PBS	Phosphate buffered saline
PDK1	Phosphoinositol dependent kinase 1
PDK2	PDK 2/TORC2
PGC-1α	PPAR gamma, coactivator 1 alpha
PH	Pleckstrin homology
PI	Phosphoinositol
PI3K	Phosphatidylinositol-3-kinase
PIF	PRK2-interacting fragment
PIP2	Phosphatidylinositol 4,5 bisphosphate

PIP3	Phosphatidylinositol 3,4,5 trisphosphate
PKC	Protein kinase C
PM	Plasma membrane
PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferator activated receptor
PTB	Phosphotyrosine binding
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RXR	Retinoic X receptor
SCAP	SREBP cleavage activating protein
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH	Src homology

siRNA	Small interfering RNA
SNARE	Soluble *N*-ethylmaleimide-sensitive fusion factor attachment receptor
SOCS	Suppressor of cytokine signaling
Sp1	Specificity protein 1
SRE	Sterol response element
SREBP	Sterol response elementSRE binding protein
STZ	Streptozotocin
T2D	Type 2 diabetes
TBC1D	Tre2/Bub2/Cdc16 domain family member
TBS	Tris buffered saline
TNFα	Tumor necrosis factor-alpha
TORC2	Transducer of regulated CREB activity 2

UDP	Uridine diphosphate
β-CD	Methyl-β-cyclodextrin

Chapter I

Introduction

Diabetes is a devastating and costly disease escalating in our country and throughout the developed world. This disease currently affects approximately 24 million individuals in the United States (8% of the population), with Type 2 diabetics accounting for the vast majority of those afflicted, over 90% [1]. In addition to those who are already afflicted, there are a staggering 57 million pre-diabetics who are likely to develop the disease, and the incidence is rising at a rate of 1.6 million people per year [1]. A well-recognized pathophysiological feature of type 2 diabetes (T2D), as well as pre-diabetes, insulin resistance appears to drive the progression of this disease and is highly correlated with cardiovascular risk factors which often account for the morbidity in these patients [2-5].

Although the exact mechanism/signal that elicits insulin-resistance is yet to be elucidated, nutritional excess and/or obesity are well-known factors which predispose individuals to develop insulin resistance and T2D. While the molecular links between obesity and insulin resistance are not well understood; increased levels of glucose, insulin, and free fatty acids (FFA)s have all been shown to be associated with a diminishment in insulin sensitivity, both *in vitro* and *in vivo* [6-12]. For example, high levels of glucose and lipids may prevent insulin's activation of key signaling intermediates [8, 13]. Importantly however, pathophysiologic nutrient toxicity appears to occur without altering insulin

signaling mechanisms, but rather via profound changes in plasma membrane (PM) lipids and cytoskeletal structure [11, 14, 15]. Interestingly, glucose toxicity has been implicated as the basis of both insulin- and lipid-induced insulin resistance [12, 16] providing a possible common mechanism, and therefore a common therapy, for multiple states of nutritional excess.

While the focus of my thesis work is dedicated to the study of PM and cytoskeletal dynamics and their influence on glucose transporter GLUT4 mediated glucose transport, this work builds upon the fundamental findings that have been established in the field. As such, the first four sections of this introduction will highlight insulin-regulated glucose homeostasis, cellular mechanisms of insulin action, insulin stimulated signal transduction, and obesity induced defects in insulin resistance; key findings that establish the framework for the study of glucose homeostasis and defects leading to insulin resistance.

A. Insulin-regulated glucose homeostasis

Insulin is a pancreatic hormone that regulates many cellular functions in a myriad of tissues throughout the body. A primary function of insulin entails the post-prandial regulation of glucose homeostasis. In the post-prandial state, elevated glucose levels stimulate the release of insulin from the β-cells of pancreatic islets. Once released into the blood stream, insulin acts on the liver, adipose tissue and skeletal muscle to clear circulating glucose and restore glucose homeostasis. At the liver, insulin binding inhibits hepatic glucose output from both glycogenolysis and gluconeogenesis. Conversely, in adipose and

striated muscle (skeletal and cardiac) tissues, insulin binding stimulates uptake/transport of glucose. The combined suppression of hepatic glucose production and export from the liver, and activation of glucose transport into fat and muscle by insulin are essential to the normal regulation of glucose homeostasis.

In adipose and striated muscle tissues, insulin mediated glucose uptake is contingent on the ability of insulin to stimulate the redistribution of the glucose transporter GLUT4 from an intracellular membrane compartment to the PM [17-19]. A failure in these tissues to respond to insulin stimulation (i.e. insulin resistance) is a central component of T2D, obesity, and the metabolic syndrome-X. This resistance initially leads to glucose intolerance, compensatory hyperinsulinemia, and dyslipidemia. However, as the resistance progresses, the β-cell expansion/compensation fails and thus, these cells can no longer secrete additional insulin and eventually decline in number. This loss of β-cells and the insulin hormone they produce results in frank T2D.

At the molecular level, insulin resistance has many facets and varies from tissue to tissue. In adipose tissue and skeletal muscle one of the key definitions of insulin resistance is a failure to recruit GLUT4 to the PM in response to insulin stimulation, while in the setting of normal GLUT4 protein expression. These findings argue the importance for elucidating the mechanisms of GLUT4 mediated glucose transport, in the hopes that one day it will be possible to treat insulin resistance at the molecular level. While a complete mechanism linking

insulin to GLUT4 translocation and glucose transport has yet to be elucidated, significant advances have been made over the last two decades in this regard.

The focus of this dissertation was to dissect mechanisms of glucose transport regulation in *skeletal muscle* and to define derangements that compromise this system. Therefore, the following sections/subsections will provide pertinent information on our current state of knowledge regarding regulated glucose transport and insulin resistance in skeletal muscle. It is important to note that skeletal muscle is by no means the only tissue involved in glucose homeostasis. As such, expanded information on hepatic and/or adipocyte insulin action can be found in several detailed reviews on these specific topics [20, 21].

B. Cellular mechanisms of insulin action in skeletal muscle

On the cellular level, insulin regulates many processes including glucose homeostasis. This whole body effect is a combination of both independent and interrelated mechanisms in several key systems including liver, skeletal muscle, and adipose tissues. In skeletal muscle and adipose tissues, insulin contributes to glucose homeostasis by stimulating the trafficking of GLUT4 to the PM, facilitating glucose transport. While together these tissues account for over 90% of the post-prandial glucose disposal, skeletal muscle is responsible for the vast majority [22]. In the absence of insulin, GLUT4 primarily resides in intracellular membrane pools. The binding of insulin hormone to its receptor stimulates a signaling cascade, concluding with the trafficking and subsequent incorporation

of GLUT4 containing vesicles into the PM. This exocytotic event is accompanied by a slowing of the endocytosis of GLUT4, leading to an accumulation of GLUT4 at the PM. Altogether these events dispose of excess blood-glucose, an event which is essential for maintaining normal glucose homeostasis.

To better detail these signaling events, and the subsequent glucose transport, the following subsections will provide in-depth analysis of the known effectors of insulin-stimulated glucose transport and defects that result in insulin resistance and glucose dysregulation.

B.1. Insulin stimulated signal transduction

The precise mechanism of action by which insulin stimulates the translocation and fusion of GLUT4 vesicles into the PM is a highly coordinated and regulated assemblage of signaling networks initiated by the binding of insulin to its receptor. The insulin receptor is a member of the protein tyrosine kinase (PTK) family of proteins. It is a transmembrane protein comprised of two extracellular α-subunits and two β-subunits that consist of the transmembrane and intracellular tyrosine kinase domains. The receptor subunits are connected by disulphide bonds that link the α-subunits to each other, as well as to the β-subunits. The signaling cascade is initiated by the binding of insulin to the α-subunit of the insulin receptor. This binding causes a conformational change in the receptor, allowing for the auto-phosphorylation of tyrosine residues on the β-subunit [23, 24]. In the auto-phosphorylated state, the insulin receptor kinase is more catalytically active, and rapidly phosphorylates tyrosine residues of the insulin

receptor substrates (IRS) as well as additional scaffolding proteins (i.e. Grb2 and Shc) [25]. The IRS proteins are a class of six cytosolic proteins (IRS1-6) characterized by the presence a phospho-tyrosine binding (PTB) and Pleckstrin homology (PH) domains. These domains allow the IRS proteins to bind to the phospho-tyrosine residues of the β-subunit, leading to subsequent tyrosine phosphorylation of the IRS proteins by the receptor kinase. Mechanistic studies in transgenic and knockout mice, as well as small interfering RNA (siRNA) knockdown studies in L6 myotubes, suggest that it is the IRS1 isoform that is responsible for the propagation of this signaling cascade in the context of insulin stimulated glucose uptake [26-28]. This initial phase in the signaling cascade is also subject to negative feedback. The phosphatase activity of protein tyrosine phosphatase 1B (PTP1B) and the ubiquitin ligase activity of suppressor of cytokine signaling 3 (SOCS3) have both been shown to inactivate the insulin receptor and its substrates [25].

Following the receptor-mediated tyrosine phosphorylation, IRS1 is converted into a suitable docking site for effector proteins containing Src 2 homology (SH2) domains, which associate with the phosphorylated tyrosine residues. The first IRS1 associated SH2 protein to be identified was Class 1A phosphatidylinoisitol-3-kinase (PI3K) [29]. PI3K exists as a dimer consisting of a 110-kDa protein (p110) with catalytic activity and an 85-kDa (p85) regulatory subunit, which stabilizes and conformationally inhibits the catalytic activity of p110. The regulatory subunit contains two SH2 domains which both associate with the phosphorylated tyrosine residues of IRS1 [30]. In the absence of IRS1

phospho-tyrosine residues, the p85 subunit inhibits activity of the p110 subunit via binding to the p110 subunit by means of a p110 binding domain contained near the C-terminus of regulatory subunit. Upon stimulation and subsequent phosphorylation of IRS tyrosine residues, SH2 domains of the p85 subunit bind to the phospho-tyrosine residues, relieving the p85-mediated inhibition of the p110 catalytic subunit [31]. In addition to exposing the catalytic site, the N-terminal Ras binding domain is also uncovered; bringing the kinase into close proximity with the PM. Now catalytically active, the p110 subunit acts on its lipid substrate, phosphatidylinositol 4,5 bisphosphate (PIP_2), phosphorylating the 3 position of the inositol ring to generate phosphatidylinositol 3,4,5 trisphosphate (PIP_3) [32].

The accumulation of PM PIP_3 is an essential node in the insulin regulation of GLUT4 translocation, as evidenced by the ablation of GLUT4 and glucose uptake in the presence of the PI3K inhibitor wortmannin [33]. The generation of this membrane lipid provides for docking and activation of downstream effector proteins containing PH domains. Important among these effector proteins are Akt (also referred to as protein kinase B, PKB) and phosphoinositide-dependent-kinase-1 (PDK1). Both of these proteins are recruited, via their PH domains, to the PM in response to the accumulation of PIP_3 [34]. The activity of the Ser/Thr kinase Akt is tightly regulated and highly dependent upon cellular location. The activation of Akt results from the recruitment of this kinase from cytosolic pools, where it is inactive, to the PM. Once at the PM Akt is phosphorylated on Ser^{473} of its hydrophobic motif (HM) by PDK2 (reviewed in the following reference [35]). This recently identified enzyme is comprised of the mammalian target of rapamycin

(mTOR) in a complex with its regulatory protein, rictor [36, 37], collectively known as TORC2. The phosphorylated HM of Akt stabilizes and activates PDK1 via its PRK2-interacting fragment (PIF)-pocket, which then phosphorylates Akt on Thr^{308}. Following Thr^{308} phosphorylation, the HM of Akt now prefers association with its own PIF-pocket, resulting in dissociation from PDK1 and maximal activation of Akt. This activation results in the regulation of many cellular processes including those of glucose and lipid metabolism (i.e., regulation of glycogen synthase kinase and fatty acid synthase). While three isoforms of Akt have been identified (Akt1-3) in mammalian cells; transgenic animal, knockout mouse, and siRNA knockdown studies have suggested that it is the Akt-2 isoform that is specifically responsible for the transmission of insulin stimulation in glucose transport [38-41].

While the pivotal role of Akt in insulin-regulated glucose transport has been investigated for some time, the identification of the Akt substrate of 160 kDa (AS160) in 2002 by Lienhard and colleagues defined a new distal point in the canonical insulin signaling pathway. AS160, also known as Tre2/Bub2/Cdc16 domain family member 4 (TBC1D4), and its closest relative TBC1D1 [42]; were found to contain a Rab-GTPase-activating protein (GAP) domain at the C-terminus, suggesting a role in the regulation of vesicular trafficking [43]. Mutation of the Akt target phosphorylation sites (Ser^{318}, Ser^{588}, Thr^{642}, and Ser^{751}) to alanine reduced insulin stimulated GLUT4 translocation [44]. This study suggested that AS160 was responsible for retention of intracellular GLUT4 vesicles in the absence of insulin stimulation and when phosphorylated, the inhibition was

released allowing for trafficking of these vesicles to the PM. Furthermore, it was shown that an intact GAP domain was necessary to maintain the reduction in insulin-stimulated GLUT4 translocation [44]. Additional studies utilizing siRNA knockdown of AS160 confirmed the role of AS160 in GLUT4 vesicular retention [45]. As virtually all vesicle trafficking systems are regulated by small GTP-binding proteins [46], (such as Rabs), it is likely that a target of AS160/TBC1D1 specifically regulates the translocation of GLUT4-containing vesicles. To identify the target/s of AS160/TBC1D1 multiple groups have utilized immunoprecipitation of GLUT4 vesicles from 3T3-L1 adipocytes followed by mass spectrometry. These studies have identified Rab10, Rab11 and Rab14 as targets, with Rab10 being the most likely candidate [47, 48]. siRNA knockdown studies further confirmed the role Rab10 as an AS160 target and effector of GLUT4 translocation [49, 50]. In muscle cell lines the target of TBC1D1 is less well elucidated; however, emerging data suggest that the Rab8a and the Rab11 effector Rip11 may be the AS160/TBC1D1 target responsible for GLUT4 translocation [51, 52].

While the canonical insulin signaling network described above appears to be a dominant regulator; there are at least two other pathways of interest with regards to insulin-mediated regulation of GLUT4. The first of these pathways is referred to as the atypical protein kinase C (aPKC) cascade. In addition to activating Akt, insulin-stimulated PDK1 is known to activate the aPKC family members PKCλ and PKCζ [53]. Once activated by PDK1 these Ser/Thr kinases stimulate GLUT4 translocation by promoting the association of Rab4, the microtubule motor protein KIF3, and the microtubules of the cytoskeleton [54]. In

addition to promoting translocation of GLUT4 from cytosolic retention pools to the PM, aPKCs promote fusion of GLUT4-containing vesicles with the PM through the phosphorylation of VAMP2 [55]. This action results in the association of this SNARE protein with Munc18c, leading to dissociation of the syntaxin4/Munc18c complex and subsequent fusion of the GLUT4 containing vesicles with the PM [56]. In addition to the aPKC cascade, recent studies suggest that tyrosine phosphorylation of Cbl is also important for insulin regulated GLUT4 translocation [57, 58]. In the presence of insulin stimulation Cbl and the adaptor protein Cbl associated protein (CAP) are recruited to the insulin receptor kinase by the adaptor protein containing PH and SH domains (APS) [59]. Once phosphorylated, Cbl recruits the adaptor protein CrkII and the guanyl exchange factor protein C3G to lipid rafts [60]. This clustering of effector and adaptor proteins results in the activation of the guanosine triphosphate-binding protein TC10 [61]. Activated TC10 has been documented to regulate actin dynamics and phosphoinositides in 3T3-L1 adipocytes. Actin regulatory targets of TC10 include neural Wiskott-Aldrich syndrome protein (N-WASP) [62], the actin related protein-3 (Arp3) [62], and the exocyst protein complex [63]. While N-WASP and Arp3 regulate actin polymerization [62], the exocyst protein complex is thought to influence docking/tethering of the GLUT4 containing vesicles at the PM [63]. Unfortunately dominant negative TC10 mutant studies in myoblasts and myotubes do not induce defects in insulin stimulated GLUT4 translocation [64], making the relevance of this exciting actin regulatory pathway somewhat questionable, especially in the context of my thesis studies. As detailed above and shown schematically in Fig.

1, insulin-stimulated glucose uptake is complex and highly regulated. However, a multitude of pathologies have been documented to disrupt this regulation and lead to insulin resistance in humans. Among the various resistance inducing insults, the largest population of defects are associated with obesity [65, 66]. As such the following subsection will highlight known and postulated defects in insulin regulation that have been associated with obesity and over-nutrition.

B.2. Obesity induced defects in insulin resistance

In the context of modern lifestyle, with abundant nutrient supply and reduced physical activity, it is of interest if excess FAs could decrease skeletal muscle insulin responsiveness. In human subjects insulin resistance is highly associated with obesity [65, 66], increased circulation of FAs, and accumulation of lipids in muscle and fat cells [67, 68]. Given that the etiologies of obesity-associated insulin resistance are complex and likely involve an imperfectly understood interplay of many factors, several well supported mechanisms have been described as the basis of fatty acid-mediated regulation of insulin sensitivity [69].

Numerous groups in the field have put forth the hypothesis that fatty acids and their metabolites are directly responsible for the insulin resistant state. The first mention of FAs in the context of glucose metabolism defects was made by Randel *et. al.* This hypothesis suggested that FAs competed with glucose for the same oxidative pathway, thus causing impaired glucose metabolism [70]. Although this early hypothesis was promising, recent ^{13}C and ^{31}P NMR spectroscopy analysis of insulin resistant subjects has suggested that it is glucose uptake [71]

and muscle glycogen synthesis [72], rather than glucose catabolism that is impaired [73]. Further supporting a role for defects in glucose uptake, it was shown that lipid infusion impaired tyrosine phosphorylation of IRS and was associated with activation of PKCθ. Additionally, this Ser/Thr kinase is known to be expressed at greater levels in high fat fed rats [74]. From these observations, Shulman and colleagues have suggested that serine phosphorylation of IRS is the primary mechanism for FA-induced insulin resistance. In this model FAs and their metabolic intermediates (i.e. acyl-Co enzyme As [CoA]s, ceramides, and diacylglycerides (DAGs)) act as signaling molecules. In conditions that elevate these signaling lipids kinases such as PKCθ, Jun kinase (JNK), and the inhibitor of nuclear factor-κB (NF- κB) kinase-β (IKKβ) are activated and can phosphorylate serine residues of IRS, thus causing defects in the tyrosine phosphorylation of IRS and impeding canonical insulin signaling [71, 75].

Based on the observation that saturated FAs clearly decrease insulin sensitivity, while unsaturated FAs exert a weaker effect [76]; an alternative, yet related, hypothesis for FA-induced insulin signaling defects has been suggested by Summers and colleagues. This work was bolstered by the observations that palmitate, the most prevalent saturated FA in circulation and muscle [77], stimulates *de novo* synthesis of ceramide. This common sphingolipid dramatically inhibits insulin signaling at the level of Akt phosphorylation [10, 13, 78]. Additionally, ceramide content is negatively correlated with insulin sensitivity in humans [79], and when cultured myotubes and adipocytes are treated with ceramide analogues, they display diabetic-like defects in insulin-stimulated

glycogen synthesis and glucose uptake [10, 13]. Mechanistic studies in cultured C2C12 myotubes and human myotubes have revealed that blocking ceramide accumulation using fumonisisn B1, cycloserine, or myriosine (inhibitiors of ceramide synthesis), prevented the palmitate induced defects in Akt signaling [10, 80-82]. Furthermore, depletion of cellular ceramide pools via overexpression of acid ceramidase recapitulates the resistance to palmitate insult [83]. The mechanism by which ceramides inhibit insulin signaling appears to be multifaceted including activation of protein phosphatase 2A (PP2A), inhibition of Akt translocation to the PM, and activation of the Ser/Thr kinases JNK and IKK. One of the earliest identified targets for ceramide induced insulin resistance was PP2A. This phosphatase is known to dephosphorylate Akt, thus blunting the insulin signaling and inducing resistance [84, 85]. Ceramides have also been suggested to inhibit Akt translocation to the PM, preventing its phosphorylation and subsequent activation. This inhibition has been attributed to PKCζ-mediated phosphorylation of Ser^{34} on the PH domain of Akt, which acts to prevent binding of Akt with PIP_3 [86]. Further confirming these findings, the negative effects of ceramide were reversed in the presence of PKCζ inhibitors or expression of a dominant negative PKC construct [80]. A final mechanism by which ceramides may induce insulin resistance is through the activation of Ser/Thr kinases JNK and IKK by facilitating the inflammatory cytokine tumor necrosis factor alpha (TNFα) [87-89].

The hypothesis that the inflammatory state associated with obesity may induce insulin resistance is not isolated to ceramide associated defects. It has long been appreciated that the chronic, systemic inflammation that is associated

with obesity-related insulin resistance may in fact have a causal role in its development [90-92]. This systemic inflammation is characterized by an infiltration of the adipose by macrophages (adipose tissue macrophages [ATM]) [93, 94]. Thus in the expanding fat-mass an activation of the ATMs stimulates the production and accumulation of inflammatory cytokines associated with insulin resistance such as tumor necrosis factor-alpha (TNFα), C-reactive protein (CRP), and interluekin-6 (IL-6). The accumulation of these inflammatory molecules is likely to cause the activation of JNK seen in skeletal muscle, leading to serine phosphorylation of IRS and insulin resistance [71, 75]. Additionally, cytokine-mediated insulin resistance is also associated with activation of the SOCS proteins [95, 96]. These proteins induce resistance by decreasing IRS tyrosine phosphorylation or targeting the IRS proteins for proteasomal degradation [97, 98]. Dysfunctions in mitochondria processes and the production of reactive oxygen species (ROS)/oxidative stress are also of considerable interest in having a hand in compromising insulin action (these specific topics are reviewed in the following references [99, 100]). Although it is possible that one of these mechanisms dominates, a consensus in the field is that these mechanisms are interdependent, and it is likely that their dynamic interplay underlies the pathophysiology of insulin resistance [69].

Although the mechanisms of regulation and resistance covered in the previous two subsections focused primarily on signaling events/defects, a growing hypothesis in the field is centered on regulation of GLUT4 translocation by PM and cytoskeletal dynamics. As this is also of great interest to our group,

and the work described in this text, the following subsection will highlight cytoskeletal and PM regulation of GLUT4.

B.3 Cytoskeletal and plasma membrane regulation of GLUT4

While the primary mode of cellular glucose transport is regulated by the insulin and its signaling pathway, a growing body of literature has established a role for cytoskeletal and PM dynamics in the regulation of glucose transport. The cortical actin cytoskeleton is a highly dynamic meshwork located immediately beneath the PM and shown to play an important role in insulin-stimulated GLUT4 translocation and glucose transport in skeletal muscle and adipose tissue [62, 101-105]. Furthermore, pharmacological disruption of the cortical actin cytoskeleton with latrunculin B [106-108] cytochalasin D [103], or botulinum toxin C2 [109] inhibits insulin-stimulated GLUT4 translocation, adding additional evidence to the role of actin in insulin-stimulated GLUT4 translocation [110]. A well documented effect of insulin stimulation on the actin cytoskeleton in cultured myotubes and adipocytes is membrane ruffling [111, 112]. This dynamic reorganization of the actin cytoskeleton has been observed as early as 20 seconds after insulin stimulation and may regulate vesicle accumulation at these membrane sites [113]. A role for the regulation of GLUT4 translocation by the actin cytoskeleton is further evidenced by the formation of actin comet-tails during insulin-stimulated translocation in both adipocytes and muscle cells [107, 114, 115]. A possible mechanism by which GLUT4 containing vesicles interact with the actin cytoskeleton is through the insulin-responsive aminopeptidase (IRAP) protease.

This constitutive member of the GLUT4 vesicles [116-118] contains an amino terminal domain that may regulate actin comet tails [119] necessary for translocation [43, 114].

The proximity of the cortical actin cytoskeleton to the PM may be important to its regulation as several components of the PM are known to regulate its remodeling [102, 111]. *In vitro* analyses suggest that this may be mediated through proteins that regulate cytoskeletal architecture including the Rho GTPase cdc42 [120], the neural Wiskott-Aldrich syndrome protein (N-WASP) [121], and the actin capping/severing protein gelsolin [110].

Recent investigations of hyperinsulinemia-induced insulin resistance have identified a therapeutically targetable lipid-based mechanism for impaired GLUT4 translocation. This mechanism entailed an increase in PM cholesterol that weakened cortical filamentous actin (F-actin) structure important for GLUT4 regulation [122-126]. A role of cholesterol in the pathogenesis of cardiovascular disease is well recognized and an appreciation for this lipid in other abnormalities such as neurodegenerative disorders and glucose intolerance is emerging [127-129]. In direct support of a regulatory role of cholesterol, decreases in membrane fluidity dampen insulin action [130] and pathological increases in membrane cholesterol due to disease states impair insulin receptor activation [131]. A report from Younsi *et al*. [132] recently found that erythrocyte membranes from insulin-resistant subjects had significantly higher cholesterol content than erythrocyte membranes from insulin-sensitive individuals. It has been shown by several groups that hydrolysis of sphingomyelin by sphingomyelinase activates GLUT4

translocation and glucose transport [8, 124, 133]. Further findings from our group have demonstrated that this insulin independent effect on GLUT4 translocation was associated with a loss of PM cholesterol [124]. Additional observations which have confirmed this initial finding involve depletion of membrane cholesterol and enhancement of insulin and GLUT4 action by methyl-β-cyclodextrin (βCD), [124] nystatin, and filipin treatments [124] and more recently with chromium picolinate (CrPic) [125, 134]. While these findings suggest that excess membrane cholesterol may play a role in cellular insulin resistance, the hypothesis that membrane cholesterol accrual, and its reciprocal regulation of cortical F-actin, may be induced by the diabetic milieu has yet to be determined and as such is a primary focus of my work presented in Chapter II A and B.

In light of the defects observed in T2D and obesity, intense research is focused on those endogenous systems and pharmaceutical therapies that may correct or slow the progression of insulin resistance. One of the more promising of these therapies, the activation of the 5'-AMP Dependent Protein Kinase (AMPK), is the focus of studies described in Chapter II C. As such the structure/function, targets, and regulation of insulin response/sensitivity by this kinase will be highlighted in the following section and subsections.

C. 5'-AMP Dependent Protein Kinase

It is well appreciated that physical exercise positively modulates glucose homeostasis in healthy individuals, as well as in individuals with T2D [135]. This modulation is based on the fact that muscle contraction is a potent stimulus of

glucose transport activity [136, 137], as well as an enhancer of insulin sensitivity in skeletal muscle [138-140], which is the primary tissue responsible for whole body glucose disposal [22]. This exciting characteristic of exercise, in the context of insulin resistance, may be central to explain the phenomenon that regular exercise can prevent or delay the onset of T2D [141, 142]. Although the exact mechanism/signal that elicits the glucose response has yet to be elucidated, several effectors such as; calcium [143, 144], nitric oxide (NO) [145, 146], bradykinin [147] and AMPK [148, 149] have also been implicated in contraction/exercise-stimulated glucose transport. Among these possible mechanisms, a central, though not exclusive, role for the energy sensing kinase AMPK has been established. In addition to being activated during contraction/exercise, AMPK has also been proposed as the mediator of multiple antidiabetic therapies such as; metformin [150, 151], the plant sterol β-sitosterol [152], polyphenolic compounds such as resveratrol [153-156] and epigallocatechin gallate [157, 158], berberine [159], and bitter melon [160]. Furthermore, AMPK has been identified as a nexus for endogenous insulin-sensitizing adipokines and cytokines such as adiponectin [161, 162], leptin [162-164], and IL6 [165-167]. Together these findings establish AMPK as a candidate of great interest for insulin resistance and T2D therapy.

C.1. AMPK Structure and Regulation

Often referred to as a “fuel gauge” for cellular energy regulation; AMPK functions to maintain cellular energy homeostasis [168, 169]. This heterotrimeric Ser/Thr protein kinase is found in a multitude of tissues [170] and is conserved from

yeast to human [171]. The kinase is composed of three subunits the catalytic α, and regulatory β and γ [172]. There are two isoforms of the α subunit. α1 containing complexes are ubiquitously expressed and α2 containing complexes, which are more highly expressed in the heart, liver, and skeletal muscle [173, 174]. Cellular location and target specificity of the two isoforms is varied [175, 176], hinting that these isoforms may have different roles in maintaining cellular homeostasis. In addition to stabilizing the kinase complex, the β and γ subunits also contribute regulatory roles to the kinase activity. Specifically, the β subunit has been shown to contain a glycogen binding domain, and more importantly this domain is associated with inhibition of the kinase when glycogen is bound [177, 178]. Furthermore, the γ subunit contains multiple cytsathionine-β-synthase binding domains (CBS), important for the binding of adenosine containing molecules [179], and specifically 5'-adenosine monophosphate (AMP) which activates the kinase in an allosteric manner [180].

Many of the activators of AMPK in skeletal muscle are cellular stressors that lead to the depletion of high-energy molecules such as 5'-adenosine triphosphate (ATP), phosphocreatine, and glycogen. This leads to the accumulation of AMP, which binds to the CBS domain of the γ subunit [180] and leads to a subtle increase in kinase activity. In addition to the allosteric activation, this binding causes a conformational change that makes the α subunit a more favorable target for its upstream kinases on Thr^{172} of the activation loop [181-183]. Recent work has elucidated two potential kinases for residue Thr^{172} of AMPKα, LKB1 [184-186] and the β isoform of calmodulin-dependent protein kinase kinase

(CaMKK-β) [187, 188]. This phosphorylation has been documented to be essential [182, 189, 190] and highly associated with the level of kinase activity [190].

C.2. AMPK Targets

Once activated AMPK induces metabolic changes both acutely, due to direct regulation through phosphorylation of targets, and chronically, through regulation of gene expression [191-193], to restore intracellular energy homeostasis. Many of these metabolic responses are similar to the adaptive processes induced by endurance training such as increased uptake and metabolism, especially oxidation, of glucose and fatty acids. Additionally, AMPK activation has been documented to regulate the accumulation of those enzymes central to the catabolism of these energy sources, including those of the mitochondria [191-197]. Acutely, AMPK activation functions to restore energy homeostasis by inhibiting energy consuming (anabolic) pathways, while stimulating catabolic pathways which increase cellular energy status (specifically ATP) [168, 169]. Well documented targets include phosphorylation of acetyl-CoA carboxylase (ACC) on Ser^{79} and 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) on Ser^{872}. The phosphorylation of ACC stimulates a switch from synthesis to oxidation in fatty acid metabolism via inhibition of the activity of this enzyme. This phosphorylation-mediated inhibiton results in a decreased level of its product, malonyl-CoA [198-200]. Additionally, AMPK has been shown to activate malonyl-CoA decarboxylase, further depleting malonyl-CoA levels [201]. The decrease in this key intermediate of FA synthesis relieves the inhibition of carnitine palmitoyltransferase I (CPT-1),

allowing for increased transport of fatty acids into the mitochondria for oxidation [202-204] while concomitantly decreasing substrate for fatty acid esterification. Similarly AMPK directly phosphorylates and inhibits HMGR resulting in decreased lipid biosynthesis, and specifically that of cholesterol [205-207]. As this interaction is of key importance to my thesis work, this regulation will be detailed in a subsequent section of this chapter.

C.3. AMPK Regulation of Glucose Transport

As previously mentioned, AMPK activation has been documented to positively regulate transcription of genes essential for glucose transport/oxidation such as: GLUT4 [192, 208-210], citrate synthase, succinate dehydrogenase, cytochrome C [194], and Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) [211-213]. The increased expression of GLUT4 has been mechanistically traced to activation of the key transcription factors myocyte enhancer factor-2 (MEF-2), GLUT4 enhancer factor (GEF) [214], and nuclear respiratory factor 1 (NRF1) [193, 215]. In addition to expanding the long-term capacity for glucose transport/oxidation, AMPK activation has also been documented to increase glucose transport acutely in skeletal muscle [216 209, 217] and cultured myotubes such as: L6 [218-220], C2C12 [221], and H-2K^{b} [222, 223]. Furthermore this transport has been shown to be mediated by GLUT4 accumulation at the PM [224]. In addition to the insulin-mimetic actions of AMPK, activation of this kinase is also known to increase insulin sensitivity/response [225-227]. While the precise mechanism(s) for both the insulin-mimetic and insulin-

sensitizing actions of AMPK are not well understood, several hypotheses have been put forward. Most well received among these possible explanations is the phosphorylation of AS160/TBC1D4 or its homolog TBC1D1 by AMPK. This distal member of the canonical insulin signaling pathway is phosphorylated in response to both insulin and AMPK activation [228-230], and has been directly tied to regulation of GLUT4 trafficking. Furthermore, mutation of the punitive phosphorylation residues on this Rab-GAP prevents AMPK-mediated accumulation of GLUT4 at the PM [231]. Alternatively, the role of AMPK as a regulator of cellular cholesterol homeostasis, as well as previous work from our group highlighting a role for PM cholesterol in the regulation of GLUT4 trafficking, may suggest that at least a portion of the AMPK-mediated accumulation of GLUT4 at the PM is regulated in a PM cholesterol-dependent manner and as such is a primary focus of my work presented in Chapter IIC.

With the hypothesis that AMPK may be regulating the *cellular* cholesterol distribution, it is important to understand the normal production, trafficking, and efflux of this essential membrane component. The following section and subsections will outline cellular cholesterol homeostasis in the context of its synthesis, efflux, and defects that occur in states of insulin resistance.

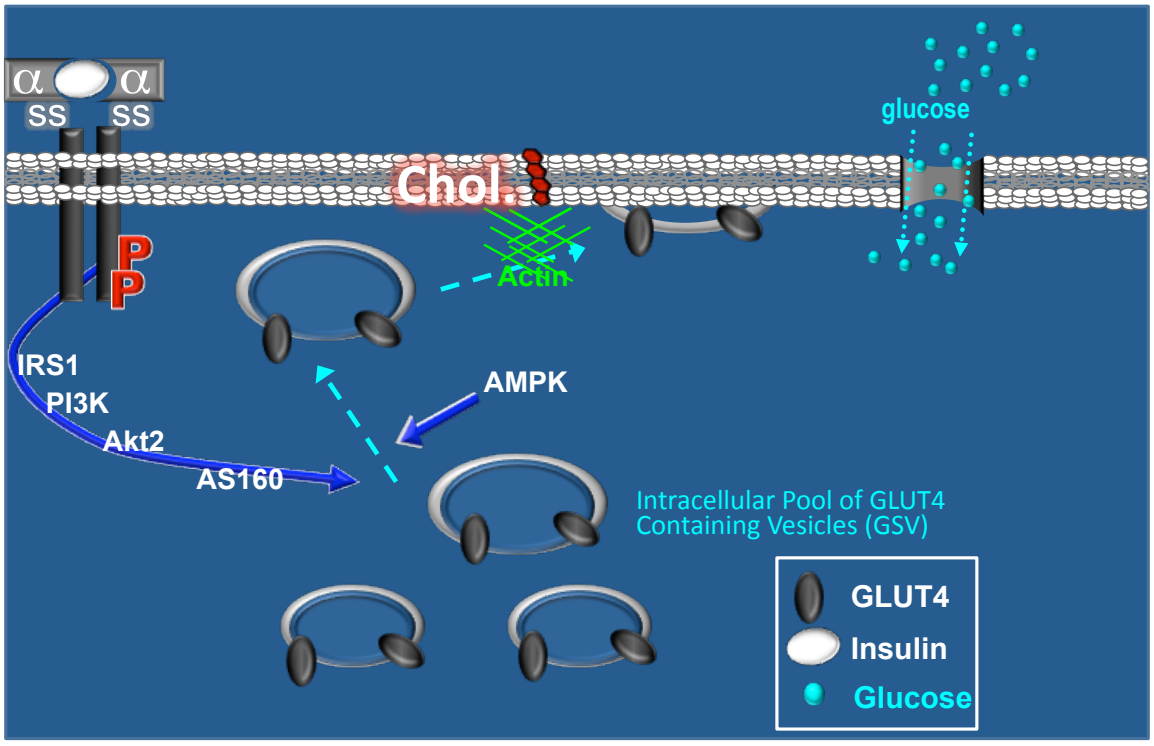

Fig. 1 Insulin mediated regulation of glucose transport in skeletal muscle. Schematic of several cellular components necessary for regulated GLUT4 trafficking in response to insulin or AMPK stimulation. These events lead to the insertion of GLUT4 transporters into the PM and subsequent glucose transport. Detailed discussion of each component shown above can be found in the previous sections.

D. Intracellular Cholesterol Homeostasis

Cellular membranes are comprised of cholesterol and phospholipids, at a ratio slightly less than 1:1 [232]. Both lipid types are essential for the regulation of both membrane structure and function [233, 234]. Cholesterol organization within membranes has been documented to be complex and dependent upon the composition of the membrane as well as the concentration of cholesterol [235, 236]. Furthermore, it has been demonstrated that cholesterol displays preferential interaction with specific membrane lipids and proteins to form microdomains [237, 238] termed lipid rafts. These rafts have been documented to be essential to cell function, influencing signaling, adhesion, and motility [239]. This differential distribution is not restricted within the PM but is also found between membranes of the cell. The vast majority of cellular cholesterol, 65-90%, is found in the PM [232] where it constitutes 35-45% of all PM lipids [240]. Cholesterol is also distributed in membranes of the endocytic pathway and endoplasmic reticulum (ER), (10 and 0.5% of total cholesterol, respectively) [241-244]. Although extracellular cholesterol is delivered via low density lipoprotein (LDL), it can be synthesized *de novo* by all nucleated cells. This synthesis utilizes acetyl CoA through a pathway involving the conversion of mevalonate to lanosterol, which is then further modified by several enzymes to form cholesterol [245].

D.1 Regulation of Cholesterol Synthesis and Efflux

While it is an essential membrane lipid, cholesterol can also be extremely detrimental to cellular function. Because cells accumulate cholesterol from both internal (synthesis) and external (LDL) sources, the overall balance must be

tightly regulated. As such, mammalian cells have developed an exquisite regulatory system for the regulation of cholesterol. This regulation is primarily achieved through feedback control of HMGR protein levels, the rate-limiting enzyme of cholesterol synthesis [246]. The regulation of HMGR protein levels are maintained through the actions of specific membrane bound proteins of the ER including; the sterol response element binding protein (SREBP), SREBP cleavage-activating protein (SCAP), and insulin-induced protein (INSIG) 1&2 [247]. A schematic representation of this important system is shown in Fig 2. As only a small portion of the total cellular cholesterol (0.5%) is contained in the ER membrane [243, 244], and therefore in contact with the major regulatory mechanism, constant and rapid exchange with the PM is necessary to monitor the largest pool of cellular cholesterol [248, 249]. This trafficking ensures that rather subtle changes in PM cholesterol levels stimulate rapid [244] changes in ER cholesterol regulatory proteins. Specifically, in the presence of sufficient sterol levels (>5% of total ER lipids) [250], HMGR and SCAP both bind to INSIG. Once bound to the INSIG protein HMGR is ubiquitinated and degraded, while SCAP and the bound SREBP are retained in the ER [251, 252]. As SREBPs are transcription factors that activate transcription of all required cholesterogenic genes [253, 254], sequestration of these proteins prevents further sterol synthesis and maintains appropriate membrane structure/function. When sterol levels drop below 5% of total ER lipids, HMGR and SCAP no longer bind INSIGs [250]. This release from INSIG prevents the degradation of HMGR while facilitating the trafficking of SCAP/SREBP from the ER to the Golgi complex via coat protein complex (COP)

II coated vesicles [255, 256]. Once in the Golgi apparatus, SREPBs are proteolytically cleaved from the immature 125 kD form to produce the mature and active 68 kD transcription factor that translocates to the nucleus [247]. This proteolytic processing is mediated by Site-1 [257, 258] and Site-2 proteases [259], two Golgi-resident, membrane-bound proteases. The mature SREBPs bind to the sterol response element (SRE) and activate transcription of all necessary genes for sterol synthesis [253, 254], including up to a 200-fold increase in HMGR [260, 261].

Several different isoforms of SREBPs (SREBP-1a, -1c, and 2) are expressed in mammals and each regulates a slightly different set of lipogenic genes [262]. SREBP-1c is encoded from the SREBP-1 gene that also encodes an almost identical protein designated SREBP-1a [262]. Interestingly, expression of SREBP-1 is enhanced by insulin in liver, fat, and skeletal muscle [263-266]. Similarly, levels of SREBP-1 are increased in the presence of hyperinsulinemia [267-269]. SREBP-1 plays an active role in regulating the transcription of genes involved in fatty acid synthesis and, albeit to a lesser extent, those involved in cholesterol synthesis [253]. The other SREBP isoform (SREBP-2), is relatively specific to cholesterol synthesis, is derived from a different gene and expression of this isoform is controlled by cellular sterol concentration [270].

In addition to the regulation of cholesterol via expression, enzyme activity levels can be modulated by phosphorylation [271]. It has long been documented that HMGR activity is increased in the presence of insulin and repressed by glucagon treatment [271]. Interestingly, the antidiabetic AMPK (described previously) has been shown to phosphorylate and regulate HMGR in response to

decreased cellular energy status [272, 273]. Furthermore HMGR is regulated by the cholesterol synthetic pathway. In a classic example of feedback inhibition, lanosterol, a downstream intermediate of cholesterol synthesis, can stimulate proteasomal degradation of HMGR [274].

In addition to HMGR regulation, cholesterol levels are decreased through increased Acyl-coenzyme A:cholesterol acyltransferase (ACAT) mediated esterification and storage [275] or trafficking events such as ATP-binding cassette transport proteins (ABCA1 & ABCG1) mediated efflux [276, 277]. The efflux of cellular cholesterol is facilitated by the actions of the nuclear factor, liver X receptor (LXR), which binds in heterodimers with the retinoic-X-receptor (RXR[278]). Activation of LXR is mediated by the binding of this nuclear factor with its ligands. Though not completely elucidated, the likely endogenous ligands for LXR are oxysterols. Several candidates have been suggested including intermediates from steroid hormone synthesis or from cholesterol synthesis shunt pathways such as 27-hydroxycholesterol [279] or 24,25-epoxycholesterol [280], respectively. Upon the binding of ligand to LXR, the nuclear factor is able to bind target promoters such as ABCA1 [281], inducing gene transcription and increasing cellular cholesterol efflux.

As detailed above, the regulation of cholesterol is tightly maintained in mammalian cells via production and efflux; however, this homeostatic regulation can be disturbed. The following subsection will describe the dysregulation of cholesterol homeostasis during insulin resistance

D.2 Dysregulation during Insulin Resistance

The extremely sensitive systems of regulation described in the previous section ensure that cellular cholesterol levels are constantly monitored, and presumably held in check. However, disease states are often characterized by a disruption of cellular homeostasis. Specifically, insulin resistance and the metabolic syndrome-X are closely associated with dyslipidemia including elevated LDL and decreased high density lipoprotein (HDL) levels, suggesting a dysrugulation in cholesterol homeostasis driven by the insulin resistant state [282]. As previously mentioned, it is of interest to note that expression of SREBP-1 is enhanced by insulin in skeletal muscle, liver, and fat [263-266]. Additionally, SREBP activity has been documented to be increased in the presence of hyperinsulinemia [267-269] with a prediction being that the machinery of cholesterol synthesis would be elevated. Further exacerbating the insulin-driven accumulation of cholesterol, the activity of HMGR is itself enhanced in the presence of insulin [271]. Observations from our group suggest that hyperinsulinemia is associated with elevated PM cholesterol and insulin resistance in 3T3-L1 adipocytes that can be rescued by cholesterol removal. Excitingly, removal of cholesterol from the PM is associated with GLUT4 accumulation and increased insulin-stimulated glucose uptake [11, 124, 125], further confirming a role for PM cholesterol in insulin and GLUT4 action. An interesting postulate is whether other forms of insulin resistance (i.e. excess FAs) are marked by increased PM cholesterol and as such is a primary focus of my work presented in Chapter IIA.

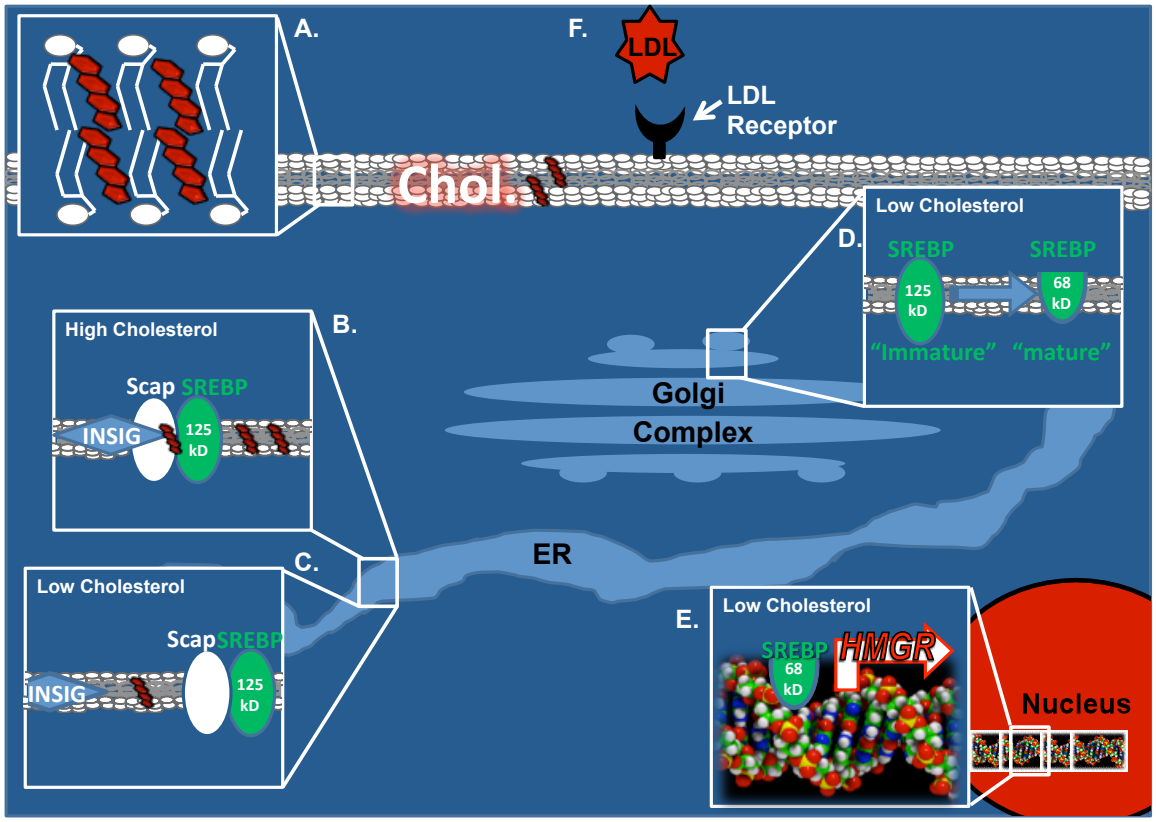

Fig. 2 Regulation of cellular cholesterol accrual. Shown above is a schematic of several cellular components necessary for the regulation of cholesterol homeostasis. The largest pool of cellular cholesterol is found in the PM at ~ 1:1 ratio with phospholipids (A.). Under normal to elevated concentrations of cholesterol Scap and SREBP are bound to INSIG and retained in the ER (B.). In conditions of decreased cellular cholesterol Scap and SREBP are released from INSIG and translocate to the Golgi complex (C.). In the Golgi complex SREBP is cleaved from the "immature" 125 kD form to the mature transcription factor of 68 kD (D.). This 68 kD fragment translocates to the nucleus where it binds to promoter regions containing SRE (i.e., *HMGR* [E.]). Cells may also accumulate cholesterol via LDL-mediated cholesterol transport (F.). Detailed discussion of each component shown above can be found in the previous sections.

The accumulation of PM cholesterol during states of insulin resistance is of great interest to our group. However, an often asked addendum to this postulate involves the mechanism responsible for the production of the cholesterol (i.e. where does the cholesterol come from?). A possibility that we favor involves nutrient-mediated excess glucose flux through the hexosamine biosynthesis pathway (HBP), resulting in transcriptional reprogramming of the cell for a more cholesterogenic phenotype. With this hypothesis in mind the following section and subsections will detail the HBP, its regulation, and dysregulation in states of insulin resistance.

E. The Hexosamine Biosynthetic Pathway

In the context of modern lifestyle, with abundant nutrients and reduced physical activity, it is of interest whether excess nutrients are acting directly to decrease skeletal muscle, as well as whole body, insulin responsiveness. Given that the etiologies of obesity-associated insulin resistance are complex and likely involve an imperfectly understood interplay of many factors, the concept of "chronic over-nutrition" is an attractive and integrative explanation for the global abnormalities in organs and pathways that are associated with T2D and insulin resistance. The mechanistic basis of nutrient sensing is not well known and is likely to be both complex and redundant. However, concerted effort has been made to identify fuel sensing pathways that control the distribution of nutrients for long term storage. The flux of HBP is known to parallel substrate (glucose) availability and thus can be considered a nutrient sensor [283, 284]. Further

solidifying its importance in human pathophysiology, the HBP has been implicated in the development and progression of insulin resistance. With this in mind, the HBP becomes a candidate of great interest.

E.1. Regulation of the HBP

Under normal nutrient load, 2-5% of glucose taken into the cell is shunted from the glycolytic pathway and into to HBP [285-287] through glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate limiting enzyme of this pathway. These molecules are ultimately converted to uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), before transfer of single GlcNAc moieties to hydroxyl groups on Ser/Thr residues of target proteins by O-linked N-acetylglucosamine transferase (OGT)[288]. The reverse reaction, removal of the O-GlcNAc moiety, is catalyzed by the O-GlcNAcase (OGA) enzyme[289, 290]. Thus the cycling of this post translational modification is rapid, transient, and suitable for signaling. O-GlcNAc is common in cytoplasmic and nuclear proteins and has been implicated in multiple cellular processes including transcriptional regulation, signal transduction, and metabolism [291, 292]. Interestingly, OGT is primarily found in the nucleus, while OGA is localized in the cytoplasm. However a considerable fraction of OGA (~20%) is found in the nucleus and a reciprocal portion of OGT is located in the cytoplasm [290]. Furthermore, OGT and OGA are often found in the same complexes, suggesting coordinated regulation of these opposing enzymes [293].

Although our current knowledge of pathway regulation is somewhat superficial; it is known that UDP-GlcNAc is a potent inhibitor of GFAT, establishing a potential feedback mechanism of regulation. Unfortunately this inhibition is somewhat difficult to understand considering UDP-GlcNAc concentrations can rise to millimolar levels in the cell. It is also of interest that OGT activity is sensitive to UDP-GlcNAc concentrations and increases as the concentration of substrate increases [294]. Recent *in vivo* and *in vitro* study has shown that OGT is phosphorylated and activated by Calmodulin-dependent protein kinase IV (CaMKIV) [295]. This activation is associated with regulation of transcription, signaling, protein localization, and cytoskeletal reorganization [288, 292]. Unfortunately, at this time, little is known about the regulation of OGA activity in response to common cellular stimuli.

E.2. Dysregulation of HBP flux and Insulin Resistance

The HBP is an example of a normal evolutionary adaptation, however, chronic and excessive flux through this pathway has been documented to induce a diabetic-like phenotype. The detrimental effect of excess flux through this pathway was first described by Marshall *et al.* when they documented the induction of insulin resistance to glucose transport in cultured adipocytes exposed to hyperglycemia [286]. Additional studies suggest this resistance is due to defects in GLUT4 translocation and subsequent glucose transport [296, 297]. These effects were also induced by glucosamine, an intermediate of the pathway. Further implicating this mechanism, glucosamine-induced defects were not

additive to those induced by hyperglycemia suggesting a shared pathway [298]. In confirmation of these findings, our group has documented HBP-mediated insulin resistance induced by hyperinsulinemia [299], likely to increase glucose flux even in euglycemic conditions. This observation may be especially important in light of the compensatory hyperinsulinemia observed in pre-diabetes.

In addition to acting as a sensor of carbohydrate supply, it is likely that HBP flux is also influenced by excess lipid load [285]. In addition to these *in vitro* studies, several *in vivo* studies have shown that increased HBP flux through overexpression of GFAT resulted in the development of insulin resistance [283, 300, 301]. Furthermore, the resistance inducing capacity of this pathway was established when it was found that even a subtle (20%) overexpression was sufficient to induce resistance [302]. Similarly, inhibition of OGA induces an increase in O-GlcNAc levels that is associated with and induction of insulin resistance [303]. Conversely, overexpression of OGA in diabetic hearts improved calcium cycling, an index of resistance that is induced by OGT overexpression [304].

While elevated levels of O-GlcNAc are associated with insulin resistance in multiple cell types [302, 303], the exact link between excess HBP flux and insulin resistance is not well understood. As such, several mechanisms have been put forth to describe this cellular phenomenon. Recent study by the Evans group suggests OGT is targeted to the PM by phosphoinositides, where it increases O-GlcNAcylation and inhibition of key insulin-signaling proteins [305]. Alternatively, the observation that increases in global phosphorylation are associated with global

decreases in O-GlcNAcylation, and vice versa, has lead to the “ying yang” hypothesis. This postulate states that O-GlcNAcylation may be competitive to phosphorylation on many Ser/Thr residues important for cellular signaling events [306]. Additionally, work from our group has put forth the hypothesis that increased O-GlcNAcylation of key transcription factors induces PM and cytoskeletal defects leading to an insulin-resistant state [299]. The details of this work are the primary focus of my work presented in Chapter IIB.

F. Thesis Hypothesis and Specific Aims

On the basis of these fundamental findings in the field, and the gaps in knowledge that I observed, I formulated the following hypothesis for my thesis work. Saturated Fatty Acids induce insulin resistance through defects in membrane and cytoskeletal dynamics; specifically, elevated PM cholesterol and decreased cortical actin polymerization. Conversely, activation of AMPK increases insulin sensitivity and GLUT4 action via a lowering of PM cholesterol. To test this hypothesis, I constructed the following specific aims: 1) To determine the effects of excess FFAs on membrane and cytoskeletal aspects of insulin-resistance; and 2) to test if changes in PM cholesterol contribute to AMPK-mediated improvements in insulin and GLUT4 action.

Chapter II

Results

II. A.

Fatty Acid-Induced Plasma Membrane Cholesterol Accrual and Glucose Transport Dysfunction

Summary

Study has shown that PM cholesterol and cortical F-actin influence the regulation of the insulin-responsive glucose transporter GLUT4. As exposure to excess FAs induce glucose intolerance by mechanisms imperfectly understood, we tested here if PM cholesterol accrual could contribute to the pathogenesis of FA-induced defects in skeletal muscle glucose transport. Skeletal muscle from high-fat fed animals and insulin-sensitive and insulin-resistant human subjects were evaluated. Study also utilized L6 myotubes stably expressing GLUT4 that carries an exofacial myc-epitope tag (L6-GLUT4myc) to directly determine the impact of excess FAs on the status of membrane cholesterol, F-actin, and GLUT4 regulation. High-fat fed, insulin-resistant animals displayed an elevated level of skeletal muscle membrane cholesterol and a loss in cortical F-actin, compared to normal-chow fed animals. Consistent with a cholesterol component of glucose intolerance, human skeletal muscle biopsies revealed an inverse correlation between membrane cholesterol and whole-body glucose disposal. Mechanistically, exposure of L6-GLUT4myc myotubes to the saturated FA

palmitate induced a 27% increase in PM cholesterol that destabilized F-actin and decreased insulin-stimulated PM GLUT4 and glucose transport. Cholesterol normalization protected against the palmitate-induced defects, whereas characteristically measured defects in insulin signaling were not corrected. Conversely, experimental loading of L6-GLUT4myc-myotube membranes with exogenous cholesterol provoked a palmitate-like cytoskeletal/GLUT4 derangement, without inducing signaling defects. Our results show that palmitate promotes PM cholesterol accrual with cytoskeletal defects that compromise GLUT4 function, which can be reversed with cholesterol lowering strategies. Moreover, this potentially targetable membrane/cytoskeletal defect is present in skeletal muscle from high-fat fed animal models and glucose-intolerant humans.

Results

Muscle membrane cholesterol is elevated in glucose-intolerant animals and humans

To examine the effect of high-fat diet on muscle membrane cholesterol content we obtained muscle biopsies from normal chow and high-fat fed C57BL/6J mice and Ossabaw swine. High-fat feeding of C57BL/6J mice for 4 weeks, which is known to induce glucose intolerance [307-309], induced an increase in membrane cholesterol compared to controls (Fig. 3a). This membrane cholesterol accrual was also witnessed in high-fat fed Ossabaw swine (Fig. 3b).

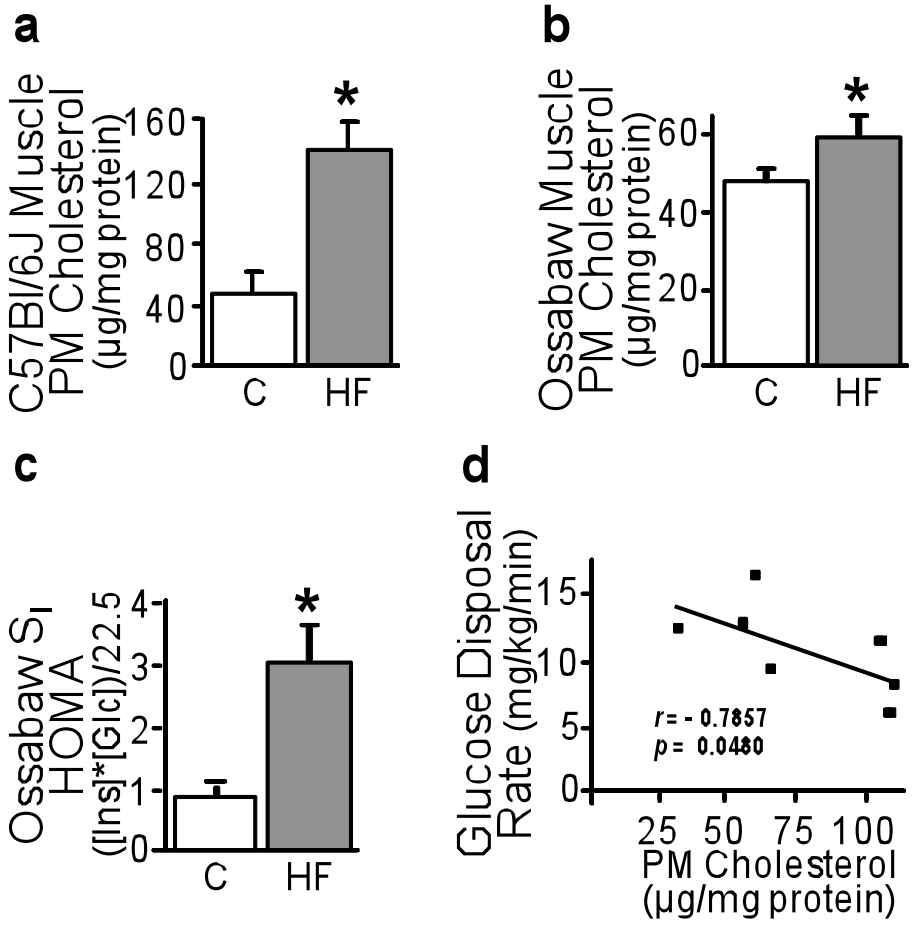

Fig. 3 Insulin-resistant human subjects and animal models display skeletal muscle membrane cholesterol accrual. Skeletal muscle membrane cholesterol from control and high-fat (HF) fed C57/B6 mice (a) and Ossabaw swine (b). HOMA values in the Ossabaw swine (c). Plot of glucose disposal rate (GDR) and skeletal membrane cholesterol from human subjects with a range of insulin sensitivities assessed by hyperinsulinemic, euglycemic clamp (d). Values are means ±SE from 3 mice and 6-8 swine per experimental group. *P<0.05 vs. control group

.

HOMA values shown in Fig. 3c confirm the insulin-resistant phenotype of this Ossabaw swine model [310, 311]. We also obtained human muscle biopsies from individuals across a range of insulin sensitivities. As shown in Fig. 3d, there was an inverse correlation between membrane cholesterol content and glucose disposal rate.

Palmitate induces glucose transport system dysregulation in muscle cells

Exposure of L6-GLUT4myc myotubes to increasing concentrations of palmitate (C16:0, a saturated FA documented to be a prevalent lipid in rat muscle [77] and to desensitize muscle and cultured myotubes to the metabolic effects of insulin [13, 312]) resulted in a dose-dependent impairment in insulin-stimulated GLUT4myc translocation (Fig. 4a). Recapitulating findings of others the palmitate-induced defect was significant at 300 μM, a concentration in the upper range of human physiology [313, 314]. Whereas higher concentrations of this lipid were associated with a trend for a dose-dependent increase in basal GLUT4 translocation and glucose transport (Fig. 4b), this was not the case with the physiological concentration of palmitate (Fig. 4c). All subsequent evaluations of insulin action and membrane/cytoskeletal features used 300 μM palmitate. Consistent with the negative effect of palmitate on insulin-regulated GLUT4 translocation, insulin-stimulated 2-deoxyglucose (2-DG) transport was impaired 36% (Fig. 4d).

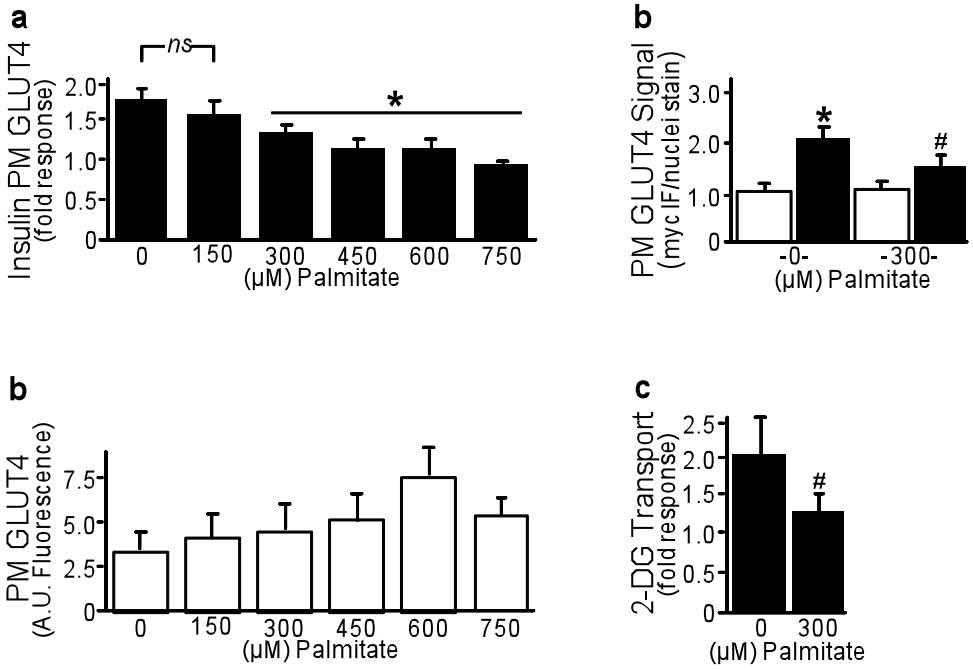

Fig. 4 Palmitate induces insulin resistance in L6-GLUT4myc myotubes. Incubation of L6-GLUT4myc myotubes in the presence of palmitate (C16:0) for 16 hr impairs insulin-stimulated GLUT4 translocation (a & c), induces a basal, dose-dependent increase in GLUT4 (b), and 2-deoxyglucose (2DG) uptake (d). Open bars, basal; closed bars, 20 min insulin stimulated. Values are means ±SE of GLUT4myc/nucleus quantification (a & b) and 2-DG uptake (c). *P<0.05 vs. control group, #P<0.05 vs. control insulin group. Values are means ±SE of GLUT4myc/nucleus quantification. *P<0.05 vs. control group.

Palmitate-induced insulin-resistant myotubes display increased membrane cholesterol

The preceding data suggested that excess FAs may contribute to PM cholesterol accrual. To test this hypothesis, we prepared PM from control- and palmitate-treated myotubes. Analogous to the animal and human findings, the membranes prepared from the palmitate-induced insulin-resistant myotubes displayed a 27% increase in cholesterol compared to control (Fig. 5a, bar 1). We next tested if methyl-β-cyclodextrin (βCD)-mediated cholesterol removal of this excess cholesterol mitigated the palmitate-induced insulin resistance. A low-dose (1 mM) βCD-treatment decreased membrane cholesterol in palmitate-treated myotubes to levels witnessed in control (Fig. 5a, bar 2). Strikingly, the 26% decrease in insulin-stimulated PM GLUT4 induced by palmitate was completely mitigated by the normalization of PM cholesterol content by βCD (Fig. 5c, compare bars 1 and 2). βCD treatment of control cells tended to increase basal and insulin-stimulated PM GLUT4, yet this did not reach statistical significance (Figs. 5b and 5c, bar 3), consistent with observations showing a small, if any, effect of 1 mM βCD on basal and insulin-stimulated PM GLUT4 [124, 125]. It is important to note, however, that in the presence of palmitate, the βCD treatment increased basal PM GLUT4 (Fig. 5b, bar 2). The basis for this gain is unclear, yet likely explains the correction in insulin-stimulated PM GLUT4 content shown in Fig. 5c (bar 2). Regardless of the precise mechanism(s), these data show that normalizing PM cholesterol during palmitate exposure favorably affects PM GLUT4 content and, in turn, permits an insulin-stimulated glucose transport

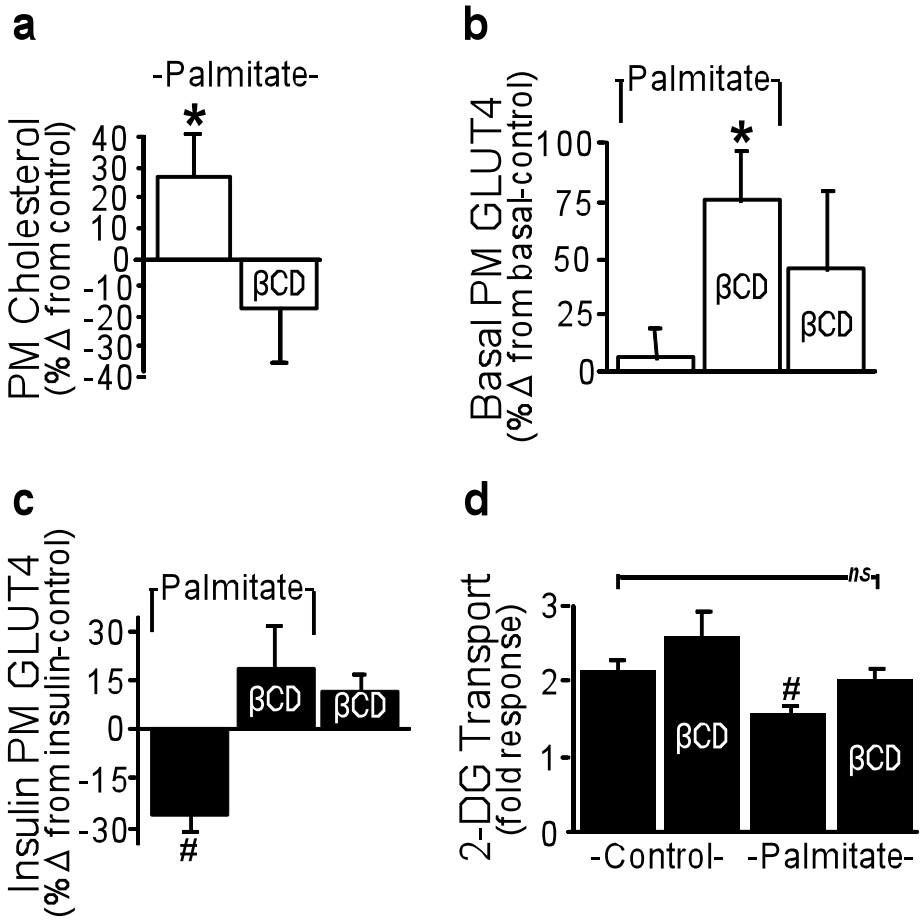

Fig. 5 Cholesterol lowering protects against palmitate-induced insulin resistance. Membrane cholesterol (a) GLUT4myc (b & c) and 2-DG uptake (d) from control and palmitate-treated L6-GLUT4myc myotubes co-treated without or with βCD. Values are means ±SE of membrane cholesterol (a) GLUT4myc/nucleus quantification (b & c) and 2-DG uptake (d). Open bars, basal; closed bars, 20 min insulin stimulated. *P<0.05 vs. control basal group, #P<0.05 vs. control insulin group.

response similar to that witnessed in control cells (Fig. 5d, compare bars 1 and 4).

Defective signal transduction appears independent of PM cholesterol accrual

Myotubes treated with 100 nM insulin for 5 min displayed an approximately 3-fold increase in Akt-2 phosphorylation at serine 474 (Fig. 6, lanes/bars 1 and 2). As is well-documented [13], palmitate treatment caused a decrease in phosphorylation of Akt-2 (Fig. 6, lanes/bars 3 and 4), the isoform primarily responsible for insulin-stimulated glucose transport [40]. Strikingly, the positive effect of βCD on GLUT4 translocation was not associated with a rescue of the palmitate-induced defect in Akt-2 phosphorylation (Fig. 6, lanes/bars 5 and 6).

Exogenously added cholesterol promotes F-actin loss and GLUT4 dysregulation

To confirm the negative impact of excess membrane cholesterol on GLUT4 we used βCD to load exogenous cholesterol into the membrane as we have previously documented [124]. Treatment of control myotubes with βCD conjugated to cholesterol markedly increased membrane cholesterol (Fig. 7a). This cholesterol loading decreased cortical F-actin (27±.072%, p = 0.0005) and insulin-stimulated PM GLUT4 (Fig. 7b). The loss in insulin responsiveness was comparable to that witnessed in the palmitate-treated myotubes. Accordingly, we found that palmitate-treated cells displayed a 32% decrease in F-actin (Fig. 8a)

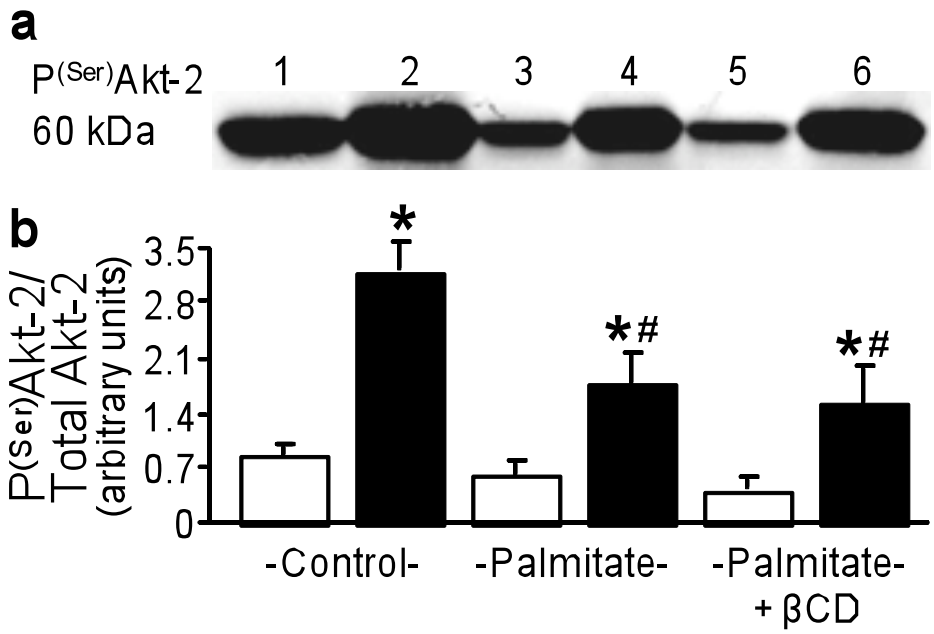

Fig. 6 Impaired insulin signaling is not rescued by cholesterol lowering. Basal and insulin-stimulated Akt-2 phosphorylation in control and palmitate-treated L6-GLUT4myc myotubes co-treated without or with βCD. Values are means ±SE of phospho-Akt-2. Open bars, basal; closed bars, 5 min insulin stimulated. *P<0.05 vs. control basal, #P<0.05 vs. control 5' insulin

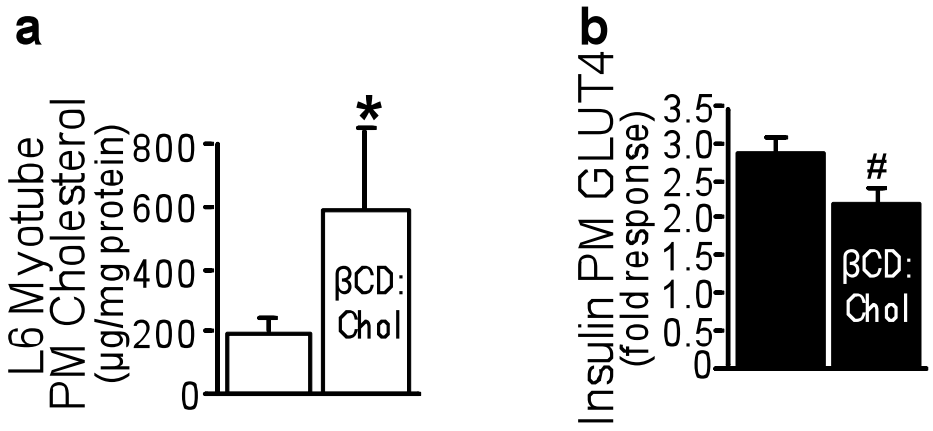

Fig. 7 Exogenous cholesterol membrane loading induces cellular insulin resistance. Membrane cholesterol (a) and GLUT4myc (b) of L6-GLUT4myc myotubes treated with or without 5 mM βCD:cholesterol Values are means ± SE of membrane cholesterol(a) and GLUT4myc/nucleus quantification (b). Open bars, basal; closed bars, 20 min insulin stimulated. *P<0.05 vs. control basal, #P<0.05 vs. control 5'insulin.

and that soleus muscle removed from the same mice we used to measure membrane cholesterol (*see Fig. 3a*), revealed the predicted reciprocal change in F-actin (Fig. 8b). Consistent with this cytoskeletal disruption resulting from excess membrane cholesterol, the βCD-induced rescue of GLUT4 in the palmitate-treated myotubes was associated with a gain in F-actin (Fig. 8a).

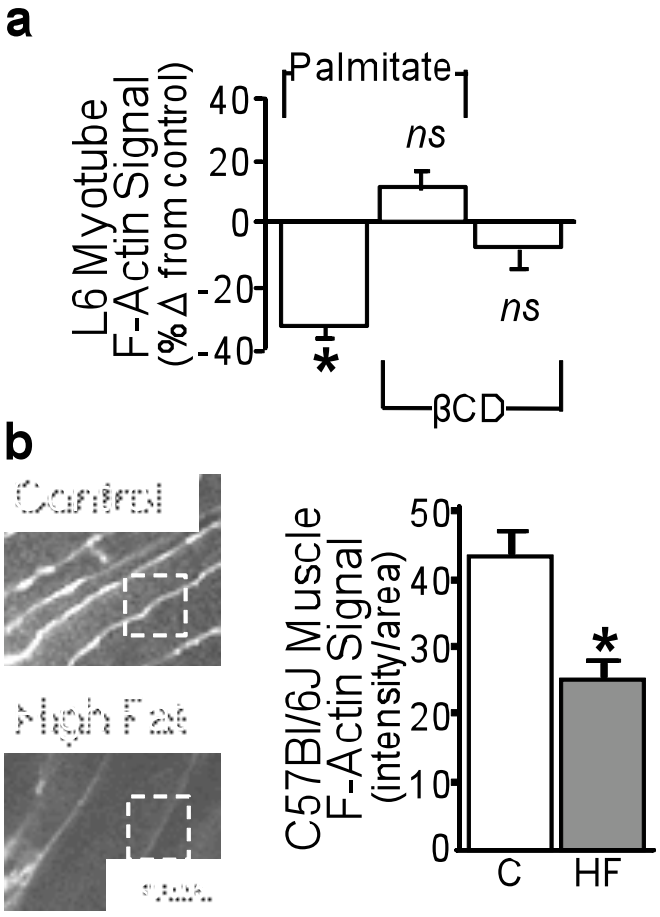

Fig. 8 Cytoskeletal derangement is induced by palmitate and high-fat diet regimens. Cortical F-actin from control and palmitate-treated L6-GLUT4myc myotubes co-treated without or with βCD (a) and skeletal muscle from control (C) and high-fat fed (HF) C57/B6 mice (b & c). Values are means ± SE of F-actin/area.*P<0.05 vs. control.

II. B.

The Role of the Hexosamine Biosynthetic Pathway in Fat- and Hyperinsulinemia-Induced Insulin Resistance

Summary

Hyperinsulinemia, hyperlipidemia, and hyperglycemia are conditions known to induce insulin resistance and accelerate diabetes progression. Recent study suggests mechanisms linking increased HBP activity to PM cholesterol- and cortical F-actin-associated impairment of glucose transporter GLUT4 regulation. These changes in PM and cytoskeletal architecture and insulin sensitivity were reminiscent of those observed in the FA-induced model of insulin resistance, and as such, a collaborative project was initiated between myself and another student, Guru Pattar, to elucidate a possible common mechanism of insulin resistance. Here we tested the hypothesis that PM cholesterol accrual results from elevated HBP flux induced by physiological hyperinsulinemia, hyperlipidemia, hyperglycemia, or experimental manipulations of the HBP. By increasing the flux of glucose through the HBP, PM cholesterol content was increased in 3T3-L1 adipocytes and similar trends were observed in L6-GLUT4myc myotubes. This increase in PM cholesterol occurred concomitantly with a loss of F-actin and an induction of GLUT4 dysregulation. Nuclear extracts revealed an increase in the cholesterogenic transcription factors specificity protein 1 (Sp1), SREBP-1, and NFY, with Sp1 showing increased O-linked glycosylation. Consistent with this Sp1 modification eliciting a maximal

transcriptional activation of SREBP-1, the mRNA and protein expression levels of 3-hydroxy-3-methylglutaryl CoA reductase and SREBP-1 itself were elevated. The cholesterogenic response associated with increased HBP activity was completely prevented in cells where O-linked N-acetylglucosamine transferase (OGT) expression was suppressed by siRNA-mediated knockdown. Concomitantly, these cells were protected against membrane/cytoskeletal abnormalities and insulin resistance. These data causally link increased HBP activity to PM cholesterol accrual and cortical F-actin loss that compromises the efficient regulation of GLUT4 by insulin. Analogous cholesterol-laden PM and actin filament loss in skeletal muscle from streptozotocin-induced hyperglycemic mice provide in vivo evidence that membrane and cytoskeletal abnormalities may be a contributing distal derangement induced by the HBP that impairs insulin and GLUT4 action.

Results

Hyperinsulinemia, hyperlipidemia, hyperglycemia, and glucosamine increase PM cholesterol

Previous study from our group as well as others has shown that physiological hyperinsulinemia [11, 315, 316] [14, 15, 317] and hyperglycemia [14, 315] treatments result in an insulin resistant phenotype and loss of cortical F-actin. Furthermore, pharmacological manipulation of the HBP with glucosamine recapitulates this resistant state [15, 315, 318, 319]. Confirming a role for the HBP, this

resistance was associated with an increase in O-linked glycosylation [315]. Concomitant with this resistance we found that 3T3-L1 adipocytes cultured in the presence of 25 mM glucose or treated with 5 nM insulin or 2 mM glucosamine display a 20-30% increase in PM cholesterol (Fig. 9a-c). Furthermore, pilot data from L6-GLUT4myc myotubes cultured in the presence of 25 mM glucose or treated with 5 nM insulin, likewise display trends for 12 and 20% elevations in membrane cholesterol, respectively (Fig. 9d & e).

These initial findings were of interest concerning the previous study on fat-induced glucose transport dysfunction, as the FA-induced defects were also associated with elevated PM cholesterol (Fig. 5a). Further confirming these findings, previous study has shown that lipid infusion in rats [320], and direct palmitate, high glucose, or glucosamine treatments in myotubes [16, 321], elevates glucose flux through the HBP. Interestingly, we found that conditions which activate this pathway are associated with a decrease in F-actin in 3T3-L1 adipocytes [322] and L6-GLUT4myc myotubes [316]. In the context of the present work, an intriguing question is whether palmitate, like the other diabetogenic insults, increases HBP activity and does this promote a cholesterogenic response. To probe this idea we measured *O*-linked glycosylation, an end product of the HBP, and found that palmitate-treated myotubes displayed a statistically significant increase in *O*-linked glycosylation, as determined via anti-RL2 immunofluorescence (Fig. 10).

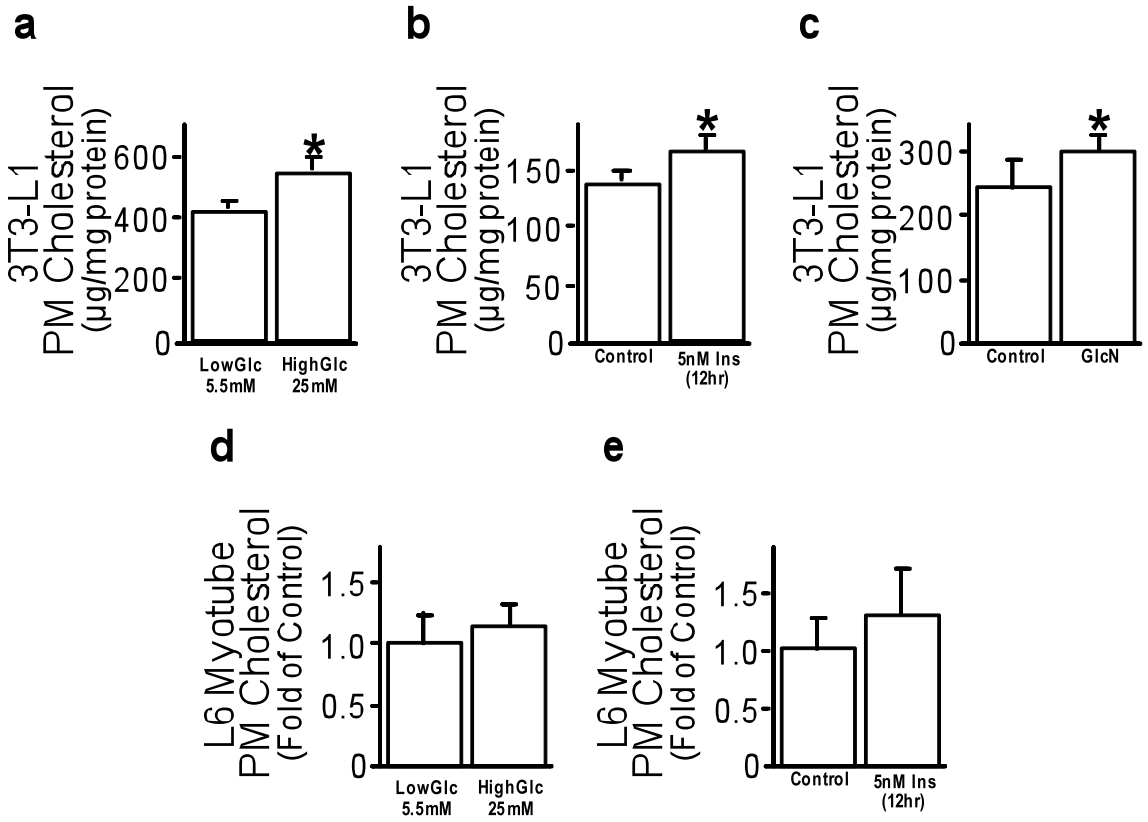

Fig 9. Excess glucose stimulates PM Cholesterol accrual. Plasma membrane fractions from hyperglycemia- (a), hyperinsulinemia- (b), and glucosamine-treated adipocytes (c) as well as hyperglycemia- (d), hyperinsulinemia- (e) treated L6-GLUT4myc myotubes, exhibit increased cholesterol content. Values are means ± SE of membrane cholesterol. *P<0.05 vs. control

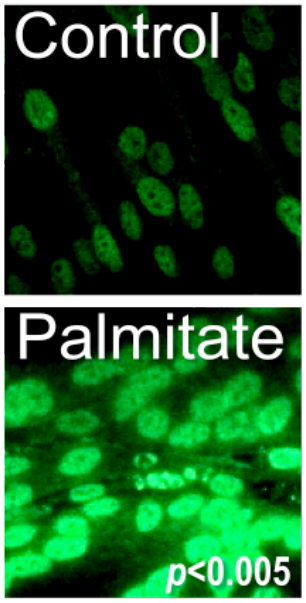

Fig. 10 Palmitate induces HBP flux in L6-GLUT4myc myotubes. Anti-RL2-detected O-linked glycosylation immunofluorescence from control and palmitate-treated L6-GLUT4myc myotubes.

Diabetogenic insults are associated with activation of cholesterogenic factors.

Nuclear extracts from 3T3-L1 adipocytes challenged with hyperinsulinemia, revealed an increase in the cholesterogenic transcription factors Sp1, SREBP-1, and NFY (Fig 11a). Of particular interest in adipocytes cultured under hyperglycemic conditions, co-IP of Sp1 with RL-2 revealed a trend for an increase in O-linked glycosylation (Fig 11b), a modification associated with elevated transcriptional activity of this transcription factor [323-325]. Consistent with this Sp1 modification eliciting a maximal transcriptional activation of SREBP-1 [326, 327], the mRNA expression levels of *Hmgr* were elevated and there was a trend for the elevation of HMGR protein (Fig 11b and c). While we were unable to detect the protein levels due to poor antibody banding in this cell type, L6-GLUT4myc myotubes treated with palmitate display an increase in the *Hmgr* transcript (Fig. 11d). Collectively, these pilot data hint at an interesting cholesterogenic mechanism for physiological hyperinsulinemia-, hyperlipidemia-, and hyperglycemia-induced defects in GLUT4 regulation.

HBP regulation of the Cholesterogenic Response

The previous observations hinted that the accumulation of PM cholesterol, and the resulting insulin resistance, was linked to increased flux through the HBP and subsequent glycosylation of targets that are deleterious to insulin and GLUT4 action. To more clearly elucidate the role of HBP flux in the development of PM cholesterol-mediated insulin resistance, we utilized siRNA to knockdown

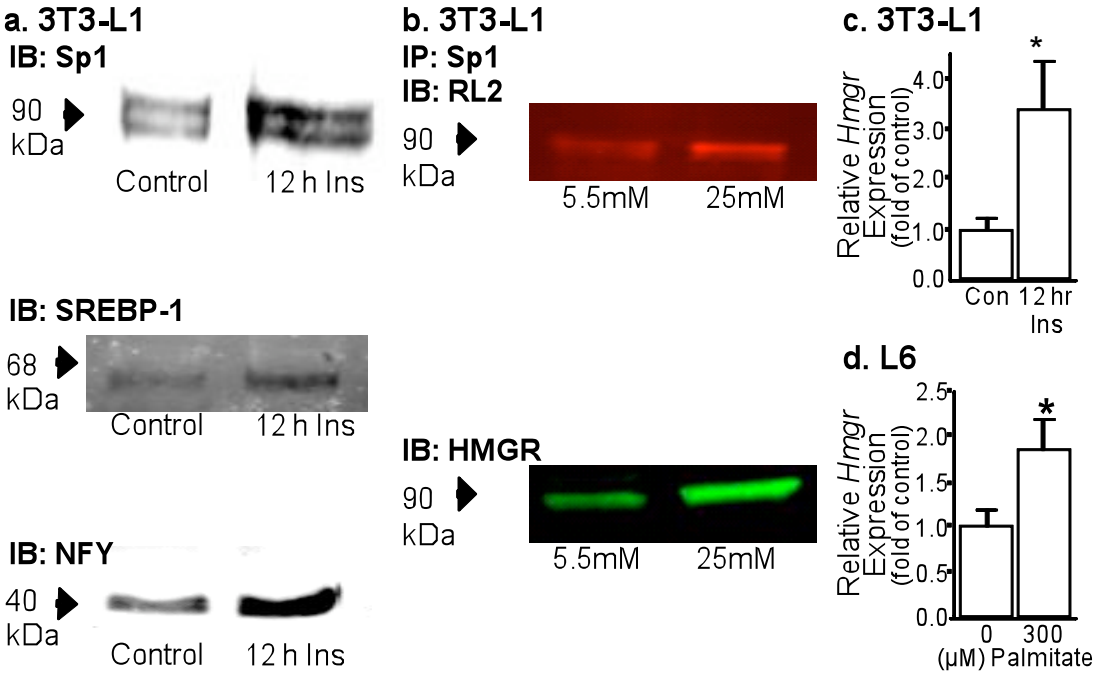

Fig. 11. Hyperinsulinemia, hyperglycemia, and hyperlipidemia drive cholesterogenic transcription activity. Nuclear fractions reveal an increase in Sp1, SREBP-1, and NFY in 3T3-L1 adipocytes cultured in hyperinsulinemic conditions (a). O-linked glycosylation of Sp1 is elevated in 3T3-L1 adipocytes cultured in hyperglycemic conditions and this corresponds to an increase in HMGR protein (b). Hyperinsulinemic 3T3-L1 adipocytes (c) and hyperlipidemic L6-GLUT4myc myotubes (d) display elevated *Hmgr* expression. Values are means ± SE of fold *Hmgr* expression from control. *P<0.05 vs. control

OGT. L6-GLUT4myc myotubes were transfected with a calcium-phosphate based protocol that displayed high transfection efficiency as determined by the detection of the fluorescently labeled siGLO oligos (Fig. 12a). Specific oligonucleotides targeted to OGT (#75, 76, & 77) reduced detectable OGT protein by 81, 63, and 54%, respectively, as compared to the control cells transfected with scramble oligos (Fig. 12b). All subsequent assays utilized oligo # s130675 (#75), as it produced the greatest knockdown. This reduction in OGT was associated with a trend for the ablation of palmitate induced increase in O-linked glycosylation (Fig. 12c), confirming the blockade of this pathway by knockdown of the transferase. It should be noted that the global repression of O-linked glycosylation is expected as we are knocking down all enzymatic transfer, thus only non-enzymatic glycosylation events should be occurring in these cells. Concomitant with the reduction of OGT, we observed a trend for prevention of both hyperinsulinemia- and palmitate-stimulated PM cholesterol accrual as compared to the control cells transfected with scramble oligos (Fig. 10d). An interesting observation is that both of the siOGT control samples displayed a slight increase in PM cholesterol over the siScrm controls (Fig. 12d & e). However additional replicates will be needed to confirm this trend. Furthermore, in cells where OGT was depleted, there was a trend for the rescue of the cortical F-actin defect in both hyperinsulinemia and palmitate treated myotubes (Fig. 12f). Consistent with an HBP-mediated increase in cholesterol through *Hmgr* induction, there was a trend for the prevention of hyperinsulinemia-induced increase in *Hmgr* in OGT (Fig. 12g, compare bars 2 and 5)

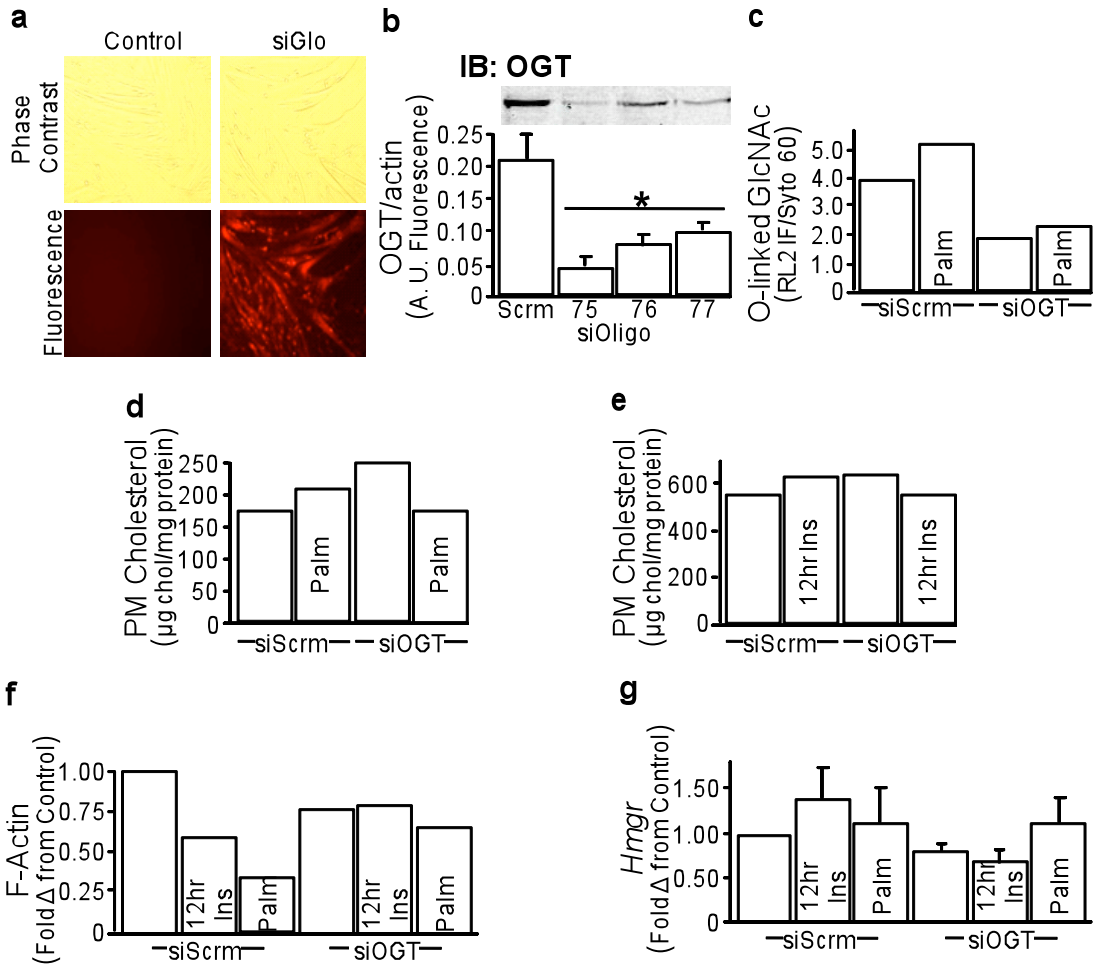

Fig. 12. Knockdown of O-Glycosyltransferase (OGT) with siRNA in L6-GLUT4myc Myotubes. Calcium-phosphate based transfectionof L6-GLUT4myc myotubes with siGlo oligos revealed substantial transfection efficiency (a). siRNA-mediated knockdown of OGT (b). O-linked GlcNAc of palmitate-treated siOGT myotubes (c). PM cholesterol in palmitate- and hyperinsulinemia-treated siOGT myotubes (d & e). F-actin (f) and *Hmgr* expression (g) in palmitate- and hyperinsulinemia-treated siOGT myotubes Values are means ± SE. *P<0.05 vs. Scramble (Scrm) control.

knockdown myotubes. Surprisingly, there was no trend for increased transcription in palmitate treated cells, and no change in the absence of OGT (Fig. 12g, compare bars 3 and 6).

In vivo hyperglycemia is associated with increased PM Cholesterol and cortical F-actin loss

As an *in vivo* confirmation of our cell culture findings, hyperglycemia was induced in mice via low dose streptozotocin (STZ)-mediated ablation of β cells. Following five-six weeks of hyperglycemia, analogous trends for cholesterol-laden PM and actin filament loss were observed in skeletal muscle from these animals (Fig. 13a & b). These data suggest that membrane and cytoskeletal abnormalities may be a contributing distal derangement induced by the HBP. Furthermore, these defects may encompass a mechanistic node of impaired insulin and GLUT4 action.

While still preliminary, these exciting pilot studies may provide a mechanistic link between nutrient excess and PM cholesterol-mediated insulin resistance, establishing a long sought after common defect for multiple diabetogenic insults. Although defects in glucose tolerance and insulin resistance were the focus of the two previous sections of this chapter, an equally interesting question is whether states of increased insulin sensitivity/glucose transport are associated with reduced levels of PM cholesterol. As such, the following section will describe study on the antidiabetic AMPK, and its role in the regulation of PM cholesterol, as well as insulin and GLUT4 action.

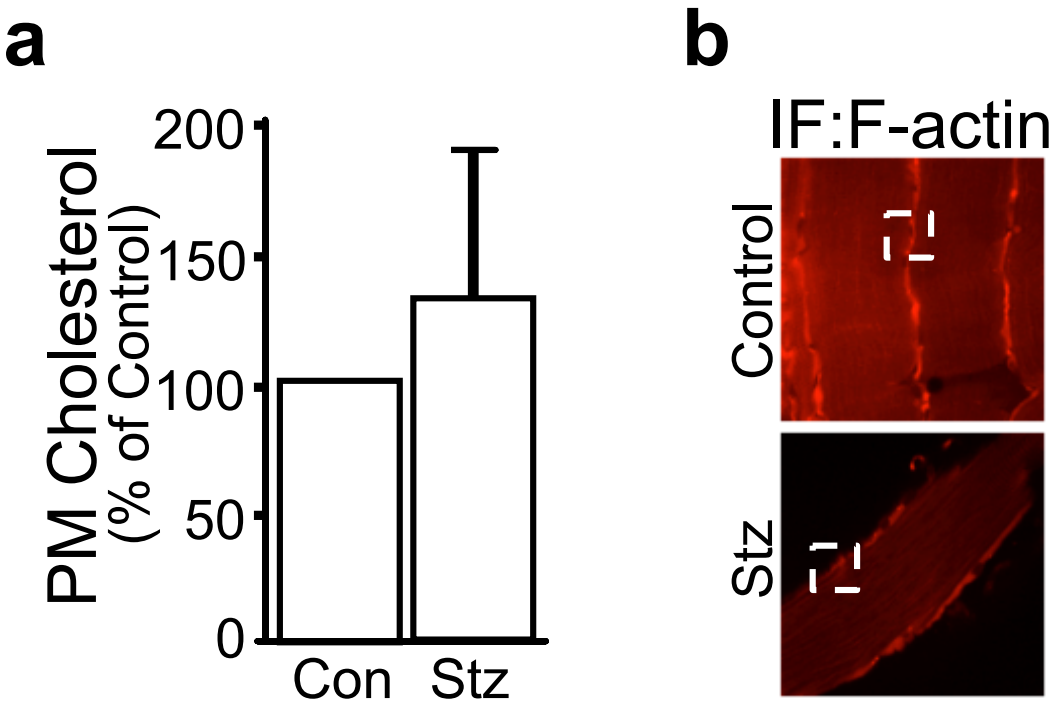

Fig. 13. Hyperglycemic mice display PM and cytoskeletal defects. Skeletal muscle from STZ-treated mice display a reciprocal increase in PM cholesterol (a) and decrease in F-actin structure (b).

II. C.

Activation of AMPK Enhances Insulin but Not Basal Regulation of GLUT4 Translocation via Lowering Membrane Cholesterol: Evidence for Divergent AMPK GLUT4 Regulatory Mechanisms

Summary

The antidiabetic mechanism of AMPK remains imperfectly understood. However, unequivocal evidence documents that enhanced basal and insulin-stimulated translocation of GLUT4 to the PM is a critical step in AMPK action. Furthermore, the ability of AMPK to inhibit energy-consuming biosynthetic pathways, such as fatty acid and sterol synthesis appears to be an essential aspect of decelerating diabetes progression. Here we tested the prediction that AMPK stimulation regulates PM cholesterol content, a membrane parameter documented to influence GLUT4 trafficking. Activation of AMPK by 5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleoside (AICAR) or 2,4-dinitrophenol (DNP) increased basal and insulin-stimulated GLUT4 translocation to the PM in L6-GLUT4myc. Key insulin signaling effectors (*e.g., insulin receptor, insulin receptor substrate 1, Akt-2, AS160*) were not engaged, however, both agents diminished PM cholesterol by 25% ($P<0.05$). Interestingly, PM cholesterol replenishment abrogated the AMPK effect on insulin, but not basal, regulation of GLUT4 translocation. Conversely, AMPK knockdown prevented the enhancement of both basal and insulin-stimulated GLUT4 translocation. These data suggest that regulation of basal GLUT4 translocation by AMPK is

independent of PM cholesterol lowering, yet this AMPK-mediated loss of PM cholesterol positively impacts the regulation of GLUT4 translocation by insulin. We propose that divergent signals from AMPK regulate basal and insulin-stimulated GLUT4 translocation. Perhaps PM cholesterol accrual could be an important contributor to impaired insulin responsiveness and glucose intolerance and ultimately the diabetic condition that pharmaceutical targeting of AMPK may effectively prevent.

Results

AMPK activation in L6-GLUT4myc myotubes

L6-GLUT4myc myotubes treated with either AICAR or DNP for 45 or 30 min, respectively, resulted in a characteristic increase in AMPK Thr^{172} phosphorylation (Fig. 14a). The phosphorylation of this residue has been shown to be associated with activation of the kinase [190]. Consistent with AICAR and DNP stimulating AMPK Thr^{172} phosphorylation via an ATP-independent [*i.e., AICAR conversion to the 5'-AMP analogue 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranotide (ZMP)*] and an ATP-dependent mechanism (*i.e., mitochondrial uncoupling that prevents cellular production of ATP*), Fig. 14b shows that intracellular ATP was unchanged by AICAR, yet decreased ~40% following DNP

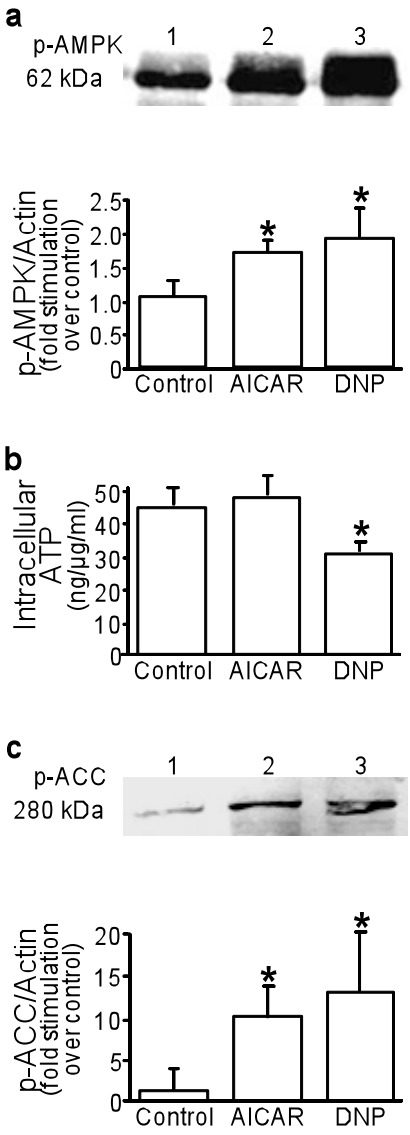

Fig. 14 AMPK activation in L6-GLUT4myc myotubes. Myotubes were left untreated or treated with AICAR or DNP, and cell lysates were subjected to SDS/PAGE and immunoblot analysis using anti-phospho AMPK (a) and anti-phospho ACC (c) antibodies. Treatments and procedures, as well as ATP determinations (b) were performed as described in Methods. Values are means ±SE, *P<0.05 vs. control group.

treatment. Consistent with the increase in AMPK activity; the phosphorylation of ACC Ser^{79}, the most well-characterized phosphorylation target of AMPK, increased after AICAR and DNP treatment (Fig. 14c). Use of an antibody that recognizes HMGR when phosphorylated on Ser^{872} also suggested that AICAR- and DNP-induced phosphorylation of this target, yet the intensity of the bands detected were too faint for accurate analysis.

AMPK signaling enhances basal and insulin-stimulated GLUT4 translocation

AICAR and DNP treatment of the L6-GLUT4myc-GLUT4myc myotubes elicited an increase in basal PM GLUT4 (Fig. 15a, compare bars 1, 3, and 5), as well as an increase in insulin response at the level of GLUT4 translocation to the PM (Fig. 15a, compare bars 2, 4, and 6). As documented by others [328], this increase in basal and insulin-stimulated GLUT4 translocation was not associated with phosphorylation/activation of key insulin signaling molecules such as the insulin receptor substrate 1 and Akt-2 (Fig. 15b). We also did not witness an AICAR- or DNP-induced increase in basal or insulin-stimulated phosphorylation of AS160 (Fig. 15c).

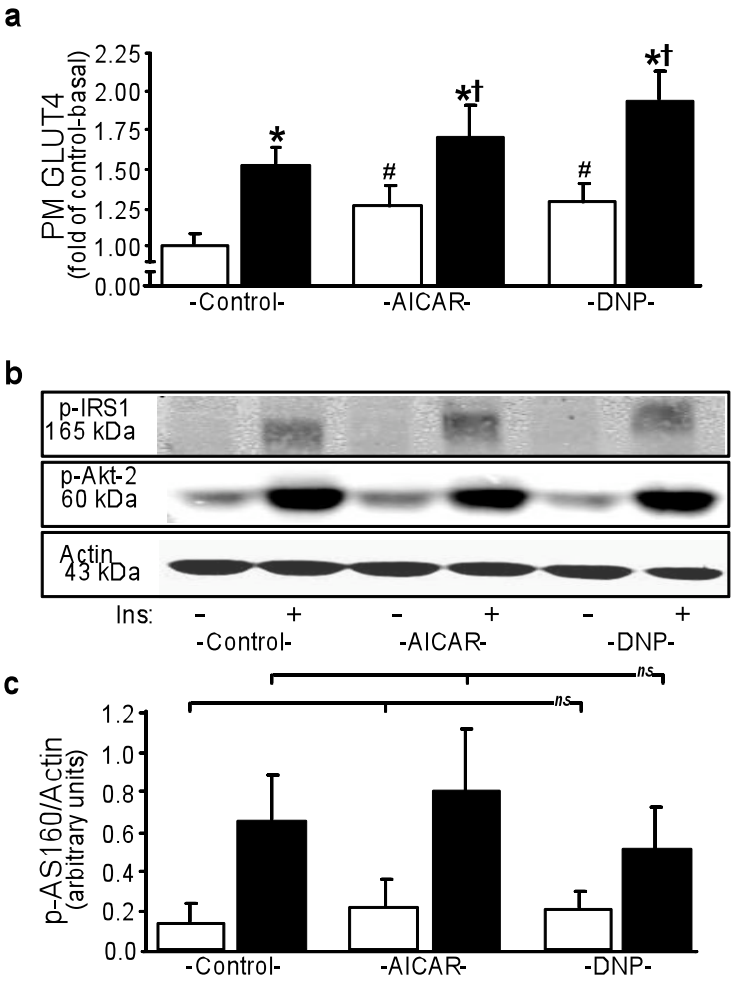

Fig. 15 AMPK activation increases PM GLUT4 independent of insulin signaling enhancement. PM GLUT4 from myotubes were treated as described in Fig. 12 in the absence (open bars) or presence of insulin (closed bars). Values are means ±SE of GLUT4myc/nucleus quantification (a). phospho-IRS-1 and phospho-Akt2 (b), and phospho-AS160 (c) levels were determined as described in Methods. Values are means ±SE of phospho-AS160 quantification (c)*P<0.05 vs. basal- treatment group, #P<0.05 vs. basal-control group, †P<0.05 vs. insulin-control group.

AICAR- and DNP-treated L6-GLUT4myc myotubes display a loss in PM cholesterol

Concomitant to their effects on GLUT4 trafficking, both AICAR and DNP elicited a 25% decrease in PM cholesterol (Fig. 16). As we have previouslyreported [124], extraction of ~25% of PM cholesterol with a low dose of βCD (Fig. 16b) also resulted in a reciprocal increase in basal and insulin-stimulated PM GLUT4 (Fig. 16c). To further dissect the role of PM cholesterol in the AMPK mediated translocation events, L6-GLUT4myc myotubes were treated with AICAR and DNP in the presence of exogenous cholesterol conjugated to βCD. Fig. 15a shows that this cholesterol addition strategy mitigated the enhancement of insulin-stimulated GLUT4 translocation induced by DNP (Fig. 17a, compare bars 2, 4, 6 and 8). Interestingly, the increase in basal PM GLUT4 induced by DNP was not prevented (Fig.17a, compare bars 1, 3, 5 and 7). Basal and insulin-stimulated GLUT4 translocation in the absence of DNP was not affected by the exogenous cholesterol (Fig. 17a, compare bars 1, 2, 5 and 6). Similar trends were observed with AICAR as a stimulant (Fig. 17b).

AMPK mediates cholesterol-dependent and -independent regulation of GLUT4

In contrast to our expectation, the increase in basal PM GLUT4 induced by DNP and AICAR was not eliminated by replenishing the PM cholesterol, hinting at a potential divergent AMPK-mediated pathway that controls basal PM GLUT4 independently of cholesterol. If true, we reasoned that siRNA-mediated

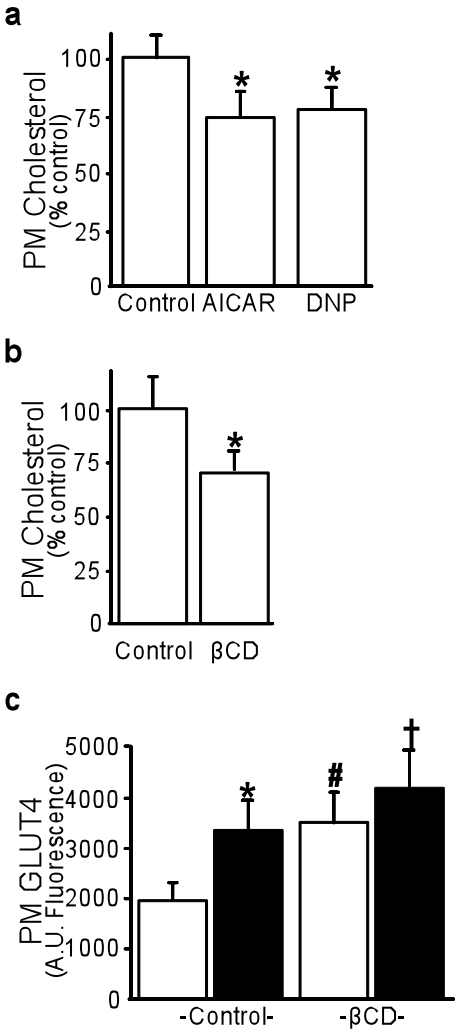

Fig. 16 AMPK activation decreases PM cholesterol PM cholesterol from myotubes treated as described in Fig. 12 (a) or with 5 mM βCD (b). PM cholesterol content was determined as described in Methods. Values are means ±SE of PM cholesterol. PM GLUT4 from myotubes were treated with or without 5 mM βCD in the absence (open bars) or presence of insulin (closed bars). *P<0.05 vs. basal- treatment group, #P<0.05 vs. basal-control group, †P<0.05 vs. insulin-control group.

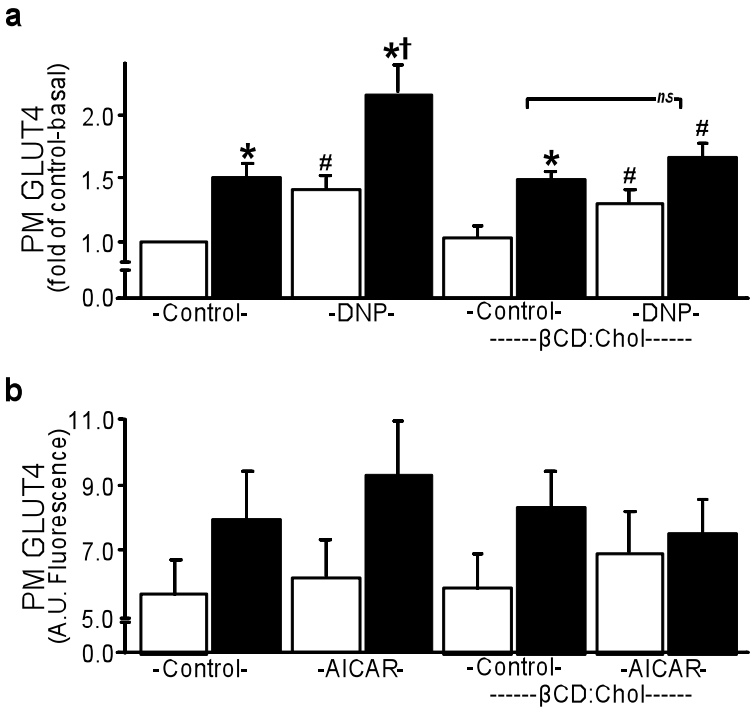

Fig. 17 AMPK-enhanced insulin action is cholesterol dependent. Myotubes were treated as described in Fig. 12 in the absence or presence of exogenous cholesterol (βCD:Chol; 1 mM 8:1 molar ratio). GLUT4 content was determined as described in Methods. Values are means ±SE of GLUT4myc/nucleus quantification. *P<0.05 vs. respective-basal, #P<0.05 vs. control-basal, and †P<0.05 vs. control-insulin.

knockdown of the catalytic α subunits of AMPK should prevent the enhancement of both basal and insulin-stimulated GLUT4 translocation by DNP. Since these cells express α1 and α2 isoforms of AMPK, oligonucleotides against both were used simultaneously for knockdown. The combination of α1 and α2 isoform specific oligonucleotides reduced the detectable pan-AMPKα protein by 90% (Fig. 18a). This reduction in AMPKα was associated with an ablation of DNP-stimulated PM cholesterol lowering as compared to the control cells transfected with scramble oligos (Fig. 18b). Both basal and insulin-stimulated enhancements in PM GLUT4 by DNP were absent in AMPK deficient cells (Fig. 18c).

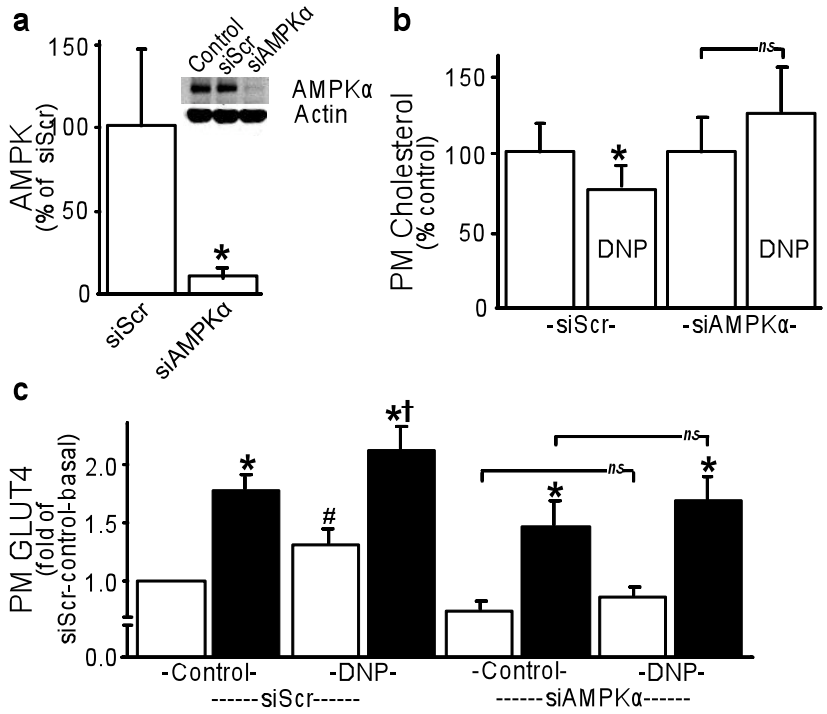

Fig. 18 AMPK knockdown abrogated DNP-induced PM cholesterol lowering and increased basal- and insulin-stimulated PM GLUT4. Transfected myotubes were prepared and AMPK protein (a), PM cholesterol (b), and PM GLUT4 (c) were determined as described in Methods. Values are means ±SE of GLUT4myc/nucleus quantification (b). *P<0.05 vs. basal- treatment group, #P<0.05 vs. basal-control group, †P<0.05 vs. insulin-control group.

Chapter III

Perspectives

The studies described in the previous chapter suggest that PM cholesterol and cortical F-actin influence skeletal muscle glucose transport. The importance of these studies is of fundamental and clinical interest as they describe diabetogenic insults that promote membrane/cytoskeletal dysfunction which may be amendable for therapy. Study on the effects of exposure of skeletal muscle to excess FAs tested if PM cholesterol/F-actin changes could contribute to FA-induced glucose transporter GLUT4 dysregulation in skeletal muscle. Furthermore this work was extended to investigate excess-nutrient stimulation of HBP flux and cholesterogenic reprogramming, providing preliminary evidence for the mechanism that may drive these PM/cytoskeletal defects in FA-induced resistance as well as other diabetogenic insults. Conversely, study investigating lipid-lowering action of AMPK suggested a beneficial influence on PM cholesterol balance as well as stimulating accumulation of GLUT4 at the PM and increasing insulin response. Although only one study was focused solely on a mechanism of insulin resistance, both describe the role of PM cholesterol in the regulation of GLUT4 trafficking.

While the derangements that contribute to insulin resistance are certainly numerous and complex, work throughout the field has highlighted the detrimental effects of saturated FAs, and particularly palmitate, on insulin sensitivity in skeletal muscle. Data presented in Chapter II.A. suggest a new consideration

that membrane cholesterol accrual may play a contributing role in obesity-associated insulin resistance. Mechanistically, the cholesterol-laden membrane compromises cortical F-actin structure, documented by several laboratories to be an essential feature of insulin and GLUT4 action [105, 316, 329, 330]. A prediction, based on the myotube and animal analyses, is that in insulin-resistant humans F-actin disruption is occurring concomitantly with PM cholesterol accrual, and this may play a causal role in skeletal muscle insulin resistance.

In the context of signaling defects, perhaps the decrease in insulin signaling to Akt-2 that we see induced by palmitate was not as damaging as would be predicted and/or not yet advanced to a level to compromise GLUT4 regulation. As these studies progressed, an alternative hypothesis involving the stimulation of PKCθ by FA of was suggested. As detailed in Chapter I, this stimulation, results in serine phosphorylation of IRS1 and inhibition of the insulin signaling cascade [71, 75, 331]. In the presence of FAs this kinase has been shown to be phosphorylated on serine residue 538. This activation loop phosphorylation has been shown to induce a robust activation leading to serine phosphorylation of its targets [332], Phosphorylation of one of these targets, IRS1 on serine residue 1101, has been shown to directly inhibit tyrosine phosphorylation and thus induce insulin resistance [331]. We tested this postulate in our system of palmitate-induced GLUT4 dysfunction, and found that, palmitate treatment resulted in a trend for an 11% increase in phosphorylation of PKCθ on Ser^{538} (Fig. 19a). Furthermore, while there was a trend for a 73% increase in phosphorylation of IRS1 on $Serine^{1101}$ (Fig. 19b), this was not associated with any defects in tyrosine

phosphorylation of IRS1 as determined by the PY20 antibody (Fig. 19c). Taken together these data suggest that PKCθ is unlikely to be the mediator of signaling defects observed in Akt-2 phosphorylation or the FA-induced insulin resistance in this system. This view of a non-signaling based defect in GLUT4 regulation is supported by recent analysis showing that insulin's maximal effect on GLUT4 translocation in L6-GLUT4myc myotubes occurs at insulin concentrations where only 5% of the total Akt pool is phosphorylated [333]. Moreover, that work also revealed that palmitate concentrations below 300 μM did not induce substantial defects in insulin signaling. Therefore, this model of insulin resistance points to a major node of insulin resistance beyond proximal insulin signaling steps.

We speculate that palmitate drives PM cholesterol accrual that directly inhibits GLUT4 trafficking. Furthermore, it is our hypothesis that the correction of PM cholesterol to levels witnessed in control cells restores an insulin-stimulated exocytosis process, such as an F-actin-dependent event, but does not inhibit endocytosis, which requires a larger decrease in PM cholesterol content [334, 335]. Alternatively, it is possible that PM cholesterol accrual increases endocytosis and perhaps normalization of the endocytic rate of GLUT4 retrieval accounts for its basal and insulin-stimulated PM accumulation with βCD. Regardless of the precise GLUT4 trafficking step influenced by palmitate- or βCD-induced changes in PM cholesterol, these data seem to unequivocally support that excess FAs induce PM cholesterol accrual that compromises F-actin, documented to be

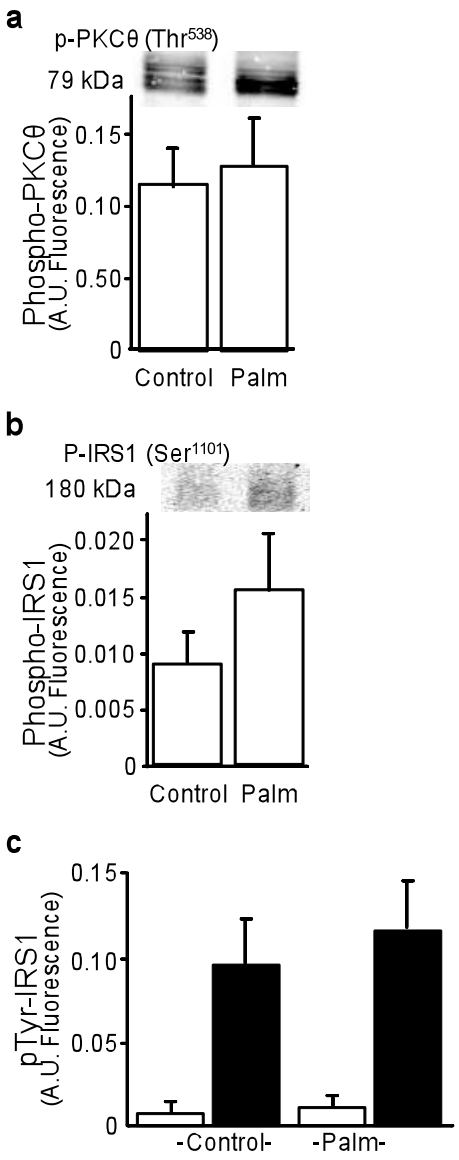

Fig. 19 PKCθ does not mediate Palmitate-induced Insulin resistance in L6-GLUT4myc myotubes. Myotubes were treated as in Fig and phosphorylation of PKCθ (Thr^{538}), IRS (Ser^{1101}), and tyrosine phosphorylation of IRS1were assessed using phosphor-specific antibodies(a b&cb), Values are means ±SE of phospho-PKCθ, -IRS1 (Ser^{1101} or tyrosine).

essential for normal GLUT4 responsiveness. In further support of a cholesterol-associated event, Shigematsu *et al*. found that PM caveolae, cholesterol-enriched membrane domains, influence GLUT4 endocytosis [335]. Based on these and the current findings, further caveolae study and detailed kinetic analyses of GLUT4 exocytosis and endocytosis trafficking patterns during states of insulin sensitivity and insulin resistance are warranted.

It should be noted that the high-fat diets we employed for the swine and mice contained 2% or 0.2% cholesterol, respectively. Interestingly, this difference in dietary cholesterol amount did not equate to a similar difference in skeletal muscle membrane cholesterol accrual. For example, the high-fat fed swine that consumed more cholesterol displayed a lower increase in skeletal muscle membrane cholesterol than that witnessed in the mice fed a high-fat diet containing far less cholesterol. As mammalian cells contain an intricate feedback system that senses the level of membrane cholesterol and modulates the transcription of genes that mediate cholesterol synthesis and uptake [250], it is likely that circulating cholesterol does not contribute to skeletal muscle membrane cholesterol content. Rather a scenario that we favor is that hyperlipidemia drives PM cholesterol accrual via cellular processes, resulting in GLUT4 dysregulation. Further confirming the hypothesis that excess cholesterol is synthesized, not acquired; inhibition of HMGR with the potent pharmacological inhibitor atorvistatin (ATV 0.5 μM), prevents palmitate-induced accumulation of PM cholesterol (Fig. 20a) and restores insulin-stimulated PM GLUT4 to control levels (Fig. 20b, compare bars 2, 4, and 6). It is important to note, however, that

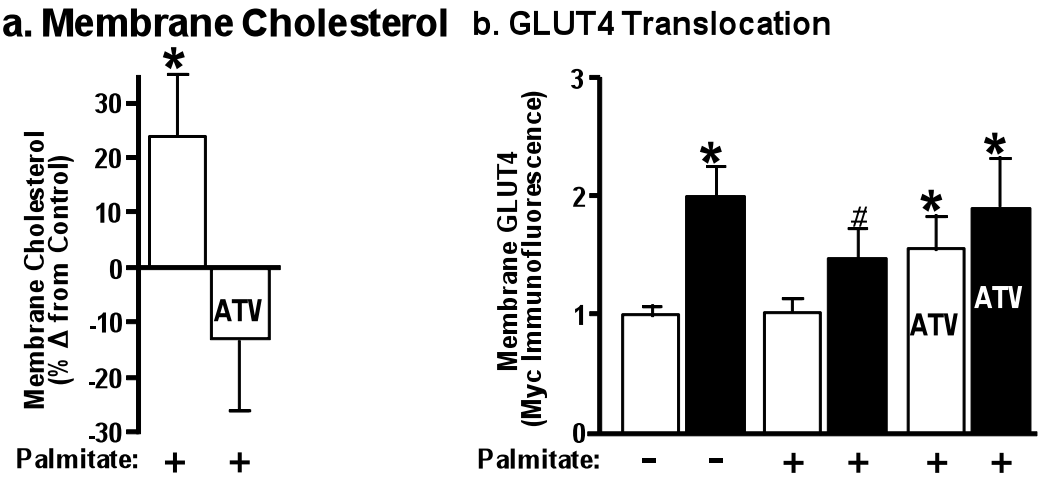

Fig 20. Cholesterol lowering protects against palmitate-induced insulin resistance. Membrane cholesterol (a) and GLUT4myc (b) from control and palmitate-treated L6-GLUT4myc myotubes co-treated without or with 0.5 μM ATV. Values are means ±SE of membrane cholesterol (a) or GLUT4myc/nucleus quantification (b). Open bars, basal; closed bars, 20 min insulin stimulated. *P<0.05 vs. control basal group, #P<0.05 vs. control insulin group.

in the presence of palmitate, the ATV treatment increased basal PM GLUT4 (Fig. 20b, bar 5). This effect was similar to that witnessed in the βCD-mediated cholesterol lowering. As stated previously, the basis for these gains are unclear, yet likely explains the correction in insulin-stimulated PM GLUT4 content. Regardless of the precise mechanism(s), these data show that normalizing PM cholesterol during palmitate exposure favorably affects PM GLUT4 content.

In striking similarity to our myotube findings, an approximate 40% reduction in insulin-stimulated skeletal muscle glucose transport has been seen as early as 5 weeks in C57Bl/6J mice fed a high-fat diet [307], and at the 4-week interval skeletal muscle insulin resistance in these animals is also suggested by a marked decrease in glucose disposal rate with no change in hepatic glucose production [308]. Interestingly, insulin-stimulated Akt phosphorylation tends to be decreased by 4 weeks of high-fat feeding, although this effect did not reach statistical significance until after 8 weeks, and a similar pattern was observed in liver tissue [336]. Ongoing studies are now specifically evaluating the temporal sequence of membrane/cytoskeletal and signal transduction derangements in skeletal muscle from high-fat fed animals. A prediction we favor is that membrane/cytoskeletal derangement occurs before signal dysfunction and this early event contributes to the initial loss of insulin sensitivity. In summary, these data suggest that a contributing factor in the pathogenesis of insulin resistance in skeletal muscle might involve an accrual of PM cholesterol and a resultant defect in membrane/cytoskeletal function. Interestingly, this cell-surface localized defect seems to concomitantly occur with insulin signal propagation impairment in the

L6-GLUT4myc myotube system. Nevertheless, the later derangement was not limiting, as correction of membrane cholesterol excess mitigates cytoskeletal dysfunction and GLUT4 responsiveness, while signaling remained impaired.

For several years our group has reported both associated and causal links between excess PM cholesterol, decreased cortical F-actin, and multiple pathophysiological insults resulting in insulin resistance[11, 105, 316, 337, 338]. However, a mechanistic link between the various diabetogenic insults and the accumulation of cholesterol remained elusive. Recently we found that increased glucose flux through the HBP, stimulated by hyperinsulinemic conditions, induces the same loss of F-actin and insulin sensitivity as is seen with other diabetogenic insults that increased PM cholesterol [299].The possibility that the diabetic milieu promotes PM cholesterol accrual and F-actin loss via increased HBP activity is a question that has become of great interest to our group, and was the focus of work presented in Chapter II. B. Study in 3T3-L1 adipocytes and myotubes presented here suggest evidence for a common mechanism of insulin resistance that is dependent on HBP-mediated accumulation of PM cholesterol. Hyperglycemia, hyperlipidemia, and hyperinsulinemia treatments resulted in trends for elevated PM cholesterol and O-linked glycosylation, while inducing defects in cortical F-actin as well as insulin and GLUT4 action in both cell types. These findings are further confirmed by preliminary study in L6-GLUT4myc myotubes that have been transiently transfected with a plasmid that induces the overexpression of GFAT. In these cells we observed a trend for an increase (31±17% p=0.21) in O-linked glycosylation and a reciprocal decrease (48±25% p=0.20) in cortical F-

actin. Together these observations suggest a possible link between various diabetogenic insults and cholesterol accumulation.

A role for excess glucose flux through the HBP in insulin resistance was first proposed by Marshall *et al.* [286]. In the time since this finding, multiple *in vitro* and *in vivo* studies have suggested that glucose flux through this pathway is stimulated by states of excess nutrient supply including hyperglycemia, hyperinsulinemia [315], and hyperlipidemia [16, 285]. Further bolstering these initial observations, recent clinical evidence has documented that GFAT expression and activity is elevated in T2D patients [339], and specifically in insulin resistant skeletal muscle [340]. Several mechanisms for the HBP mediated induction of insulin resistance have been proposed including activation of JNK [321], induction of ER stress [341], O-linked glycosylation of components within the insulin-signaling pathway [305], and modulation of leptin expression [342]. A possibility we tested was that accrual of excess PM cholesterol, and the resulting induction of insulin resistance, is due to HBP-mediated activation of the SREBP transcription factor. It has been shown that SREBP is regulated by glucose, through the HBP [343]. In support of this hypothesis, increased HBP flux has recently shown to induce the transactivating capacity of Sp1; [323], a transcription factor shown to promote the maximal transcriptional activation of SREBP-1c [326, 327]. SREBP-1c is encoded from the SREBP-1 gene that also encodes an almost identical protein designated SREBP-1a. Interestingly, Weigert and colleagues reported a study linking palmitate with the activation of the HBP and increased Sp1 DNA binding activity in human myotubes [16], while Hawkins *et al.* described an increase in

intermediates of the glucosamine pathway in fat-induced insulin resistance [285]. As SREBP-1 plays an active role in the transcriptional regulation of FA synthesis and, albeit to a lesser extent, cholesterol synthesis genes [253], we predict that an unappreciated downfall of increased SREBP-1 regulation via HBP-modified Sp1 activity is increased cholesterol synthesis resulting in PM cholesterol accrual. In line with this reasoning, Yang *et al*. recently observed that increased hepatic cholesterol content resulted from increasing the activity of key enzymes of the HBP. These findings are confirmed by trends for elevated levels of *Hmgr* mRNA observed in L6-GLUT4myc myotubes treated under hyperinsulinemic and hyperlipidemic conditions, as well as the elevated PM cholesterol and depleted cortical F-actin levels observed in streptozotocin treated mice. Although preliminary at this time, collectively these findings implicate a common and reversible abnormality (*i.e., HBP-mediated PM cholesterol accrual*) that can be a novel target to slow the progression/worsening of insulin resistance. Future studies will focus on the effects of diabetogenic insults on parameters of PM, cytoskeletal, and GLUT4 regulation in the presence or absence of OGT.

Along the lines that this PM/cytoskeletal defect may be reversible, the study described in Chaper II.C. provides evidence that PM cholesterol levels are regulated by AMPK and that this membrane-based event augments regulation of GLUT4 by insulin. In particular, AMPK activation was found to decrease PM cholesterol. Strikingly, the expected AMPK-enhanced insulin-stimulated PM GLUT4 translocation was prevented by replenishing PM cholesterol. This experimental strategy did not mitigate the AMPK-regulated gain in basal PM

GLUT4, suggesting that AMPK regulates basal and insulin-stimulated PM GLUT4 content by cholesterol-independent and -dependent mechanisms, respectively.

Of particular interest is that AMPK has been implicated in at least a portion (~30%) of the antidiabetic effect of exercise [344]. Although numerous mechanisms have been suggested in the insulin-mimetic and insulin-sensitizing action of exercise [345, 346], these mechanisms are incompletely understood. In the context of our studies, the AMPK-mediated decrease in PM cholesterol accounted for a slight (<50%) increase in insulin response seen in cells treated with AICAR and DNP. Whether skeletal muscle contraction and/or exercise are associated with a reduction in PM cholesterol is of interest and future studies are planned to investigate this possibility.

The inability of cholesterol add-back to prevent the AMPK-associated increase in basal GLUT4 was unexpected. A prediction was that the insulin mimetic activity of AMPK stimulation entailed the reported stimulation of AS160 [231, 347, 348], yet this signaling event was not observed in the present study. Perhaps AS160 phosphorylation detected with the anti-phopso-Akt substrate (PAS) antibody failed to detect the Thr^{642} PAS phosphorylation motif demonstrated to be specifically phosphorylated in L6-GLUT4myc myotubes treated with AICAR; whereas it recognizes the Ser^{588} PAS phosphorylation motif that is phosphorylated in tandem with the Thr^{642} site in response to insulin stimulation [231]. However, study in mouse skeletal muscle suggests the reverse scenario; i.e., AICAR-stimulated increases in AS160 phosphorylation occur at the Ser^{588} PAS phosphorylation motif [349]. Use of $pThr^{642}$ and $pSer^{588}$ specific

antibodies and an immunoprecipitation approach to enhance examination of endogenous AS160 phosphorylation might demonstrate AMPK-mediated phosphorylation.

We are currently performing these studies to carefully dissect if the AMPK/AS160 signal mediates the basal increase in PM GLUT4. Nevertheless, the present findings show PM cholesterol regulation as a novel target of AMPK that contributes to its insulin-sensitizing action. Mechanistically, we suggest that this may result from the AMPK-mediated phosphorylation and subsequent inactivation of HMGR. Although it is also possible that AMPK may lower PM cholesterol via suppression of SREBP-1c expression [350-352], the rapidity of the DNP- and AICAR-induced decrease in PM cholesterol likely suggests an instant inhibition of HMGR. However, chronic AMPK stimulation may also influence PM cholesterol content via suppressing SREBP-1c. In any case, given the rapid kinetics of newly synthesized cholesterol reaching the PM ($t_{1/2}$=10-20 min) [353-355] and recycling of cholesterol between the PM and endoplasmic reticulum ($t_{1/2}$=40 min) [244], it is entirely possible that HMGR and/or SREBP-1c suppression by AMPK accounts, at least in part, for the reduction in PM cholesterol. Therefore, we are now exploring both of these possibilities as avenues to PM cholesterol regulation by AMPK.

A second interest is that we [124] and others [334, 356] have previously documented that extensive ≥50% PM cholesterol depletion clearly inhibits endocytosis, yet smaller 20-30% reductions in PM cholesterol [124] do not. This suggests that small reductions in PM cholesterol increase PM GLUT4 via

augmenting an aspect of GLUT4 exocytosis. Detailed kinetic analyses of GLUT4 exocytosis and endocytosis trafficking patterns are currently underway. Regardless of whether the elevated PM GLUT4 content results from increased exocytosis, decreased endocytosis, or a combination of both, increased insulin-stimulated PM GLUT4 content is a beneficial result associated with PM cholesterol lowering. In the context of vesicular trafficking, perhaps the AMPK-mediated loss of PM cholesterol does not entail a regulated decrease in cholesterol synthesis and a subsequent lack of cholesterol delivery to the PM as we suggested above, but rather reflects an accelerated internalization of cholesterol-enriched domains. We do not necessarily favor this possibility, yet such an event could account for the loss of PM cholesterol and have a similar beneficial effect on the GLUT4 response. For example, hydrolysis of PM sphingomyelin by sphingomyelinase results in the movement of cholesterol from the PM to intracellular compartments [357-359] and we have witnessed that this sphingomyelinase-induced loss in PM cholesterol enhances basal and insulin-stimulated PM GLUT4 exocytosis [124].

In terms of human health, we know that insulin resistance is a progressive syndrome, with many associated pathologies appearing as insulin sensitivity worsens. We speculate that at least a portion of the beneficial effects of exercise/contraction, and the effects of many AMPK agonists, are mediated through an AMPK mechanism that counterbalances PM cholesterol accrual. In direct support of this notion and the findings herein; beta-sitosterol, a potent AMPK activator recently identified from a medicinal plant extract library, was

shown to lower the intracellular level of cholesterol in L6-GLUT4myc myotubes [152]. That work also demonstrated an associated increase in basal PM GLUT4, yet did not dissect whether the increase resulted from a PM cholesterol-dependent and/or -independent effect. Also, the effect of this plant sterol on insulin-stimulated PM GLUT4 was not evaluated. Although it was demonstrated that pharmacological inhibition of AMPK rendered the effect of beta-sitosterol on increasing basal PM GLUT4 ineffective, our data would predict that this was not a PM cholesterol mediated event; whereas any AMPK-mediated enhancement of insulin-stimulated PM GLUT4 would be regulated by PM cholesterol lowering. Future studies should focus on elucidating the relationship of AMPK to GLUT4 regulation and glucose transport within the context of a cholesterol-regulatory signaling pathway.

An interesting caveat to these studies is that L6-GLUT4myc myotubes show a trend for expressing predominately the α1 isoform of AMPK and very little of the α2 isoform (Fig. 21a). This is surprising in light of reports showing α2 is the isoform most highly expressed in skeletal muscle tissue, while the α1 isoform is ubiquitously expressed [173, 174]. This observation prompted us to investigate the expression of the AMPKα subunits throughout differentiation. To our surprise, we found that AMPKα1 protein levels display a tendency to increase from plating to full differentiation (day 8-10) before a gradual decline (Fig. 21b). Conversely, AMPKα2 protein levels show a tendency to be initially high in the myoblasts, but drop precipitously through differentiation, becoming nearly undetectable (Fig.

21c). A trend was also found for total AMPKα protein levels to follow those of the α1 subunit (Fig. 19d).

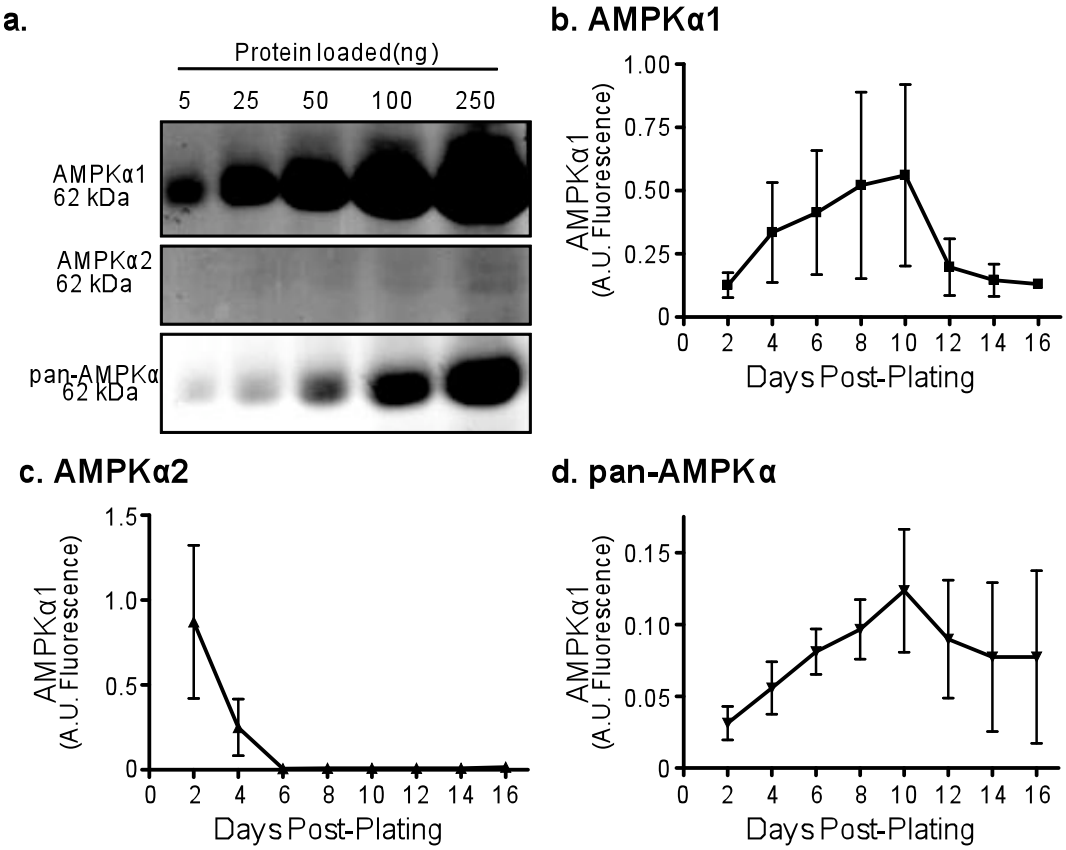

Fig. 21 AMPKα subunits are differentially expressed in L6-GLUT4myc myotubes. IB analysis of α1 and α2 isoforms in myotubes from peak differentiation (day 8-10) (Fig 18a). α subunit protein levels from L6-GLUT4myc myotube throughout differentiation (Fig 18b-d). Values are means ±SE α subunit protein levels.

Conclusions

As a whole these studies show PM cholesterol accrual and cortical F-actin loss uniformly in skeletal muscle from glucose-intolerant mice, swine, and humans. *In vivo* and *in vitro* dissection demonstrated this membrane/cytoskeletal derangement induces GLUT4 dysregulation and glucose intolerance and is promoted by excess FAs. Preliminary pilot studies presented here suggest exciting new evidence for a common mechanism of insulin resistance that is dependent on HBP-mediated accumulation of PM cholesterol. Parallel studies unveiled that the action of AMPK entailed lowering PM cholesterol that enhanced the regulation of GLUT4/glucose transport by insulin. In conclusion, these data are consistent with PM cholesterol regulation being an unappreciated aspect of AMPK signaling that benefits insulin-stimulated GLUT4 translocation during states of nutrient excess promoting PM cholesterol accrual. A schematic representation of these findings is shown below in Fig. 22.

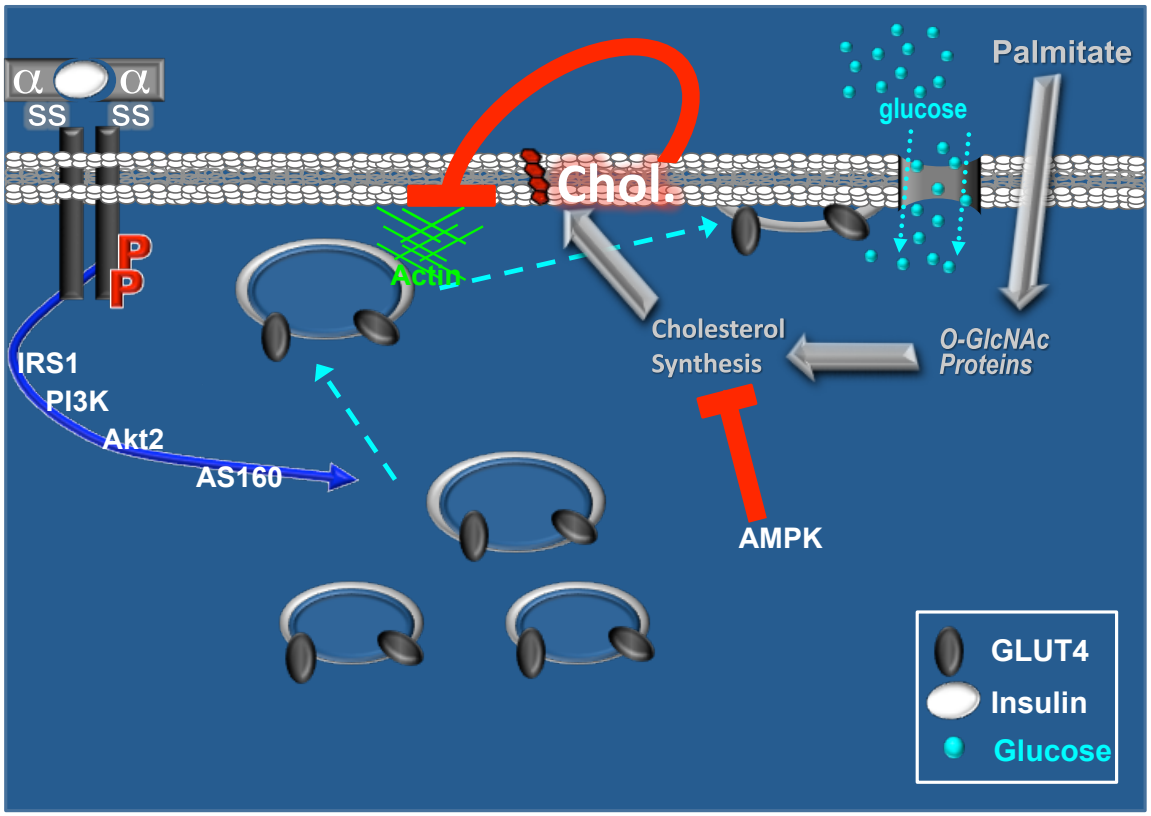

Fig. 22 Insulin mediated regulation of glucose transport in skeletal muscle.

Shown above is a schematic of the findings described in this thesis. GLUT4 trafficking is increased in response to insulin or AMPK stimulation. The increase in PM GLUT4 is regulated via a PM cholesterol-mediated event. Conversely, high fat diets/palmitate treatments lead to the accumulation of PM cholesterol and a distruption of cortical F-Actin, which induces GLUT4 dysregulation and inhibits insulin-stimulated glucose transport. Detailed discussion of each component shown above can be found in the previous sections.

Chapter IV

Experimental Procedures

Animals

Four week old, male C57/BL6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). For the hyperlipidemic study mice were assigned to two groups for 4 weeks of treatment. Control mice (N=3) were fed a normal chow diet containing 18% kcal from protein, 78% kcal from carbohydrates, and 4% kcal from fat (#7017; Harlan, Indianapolis, IN). High-fat fed mice (n=3) were fed a diet containing 17% kcal from protein, 43% kcal from carbohydrates, and 41% kcal from fat (D12079B; Research Diets Inc., New Brunswick, NJ). For the hyperglycemic study, mice were assigned to two groups for 5-6 weeks of treatment. STZ mice were injected with 0.3 ml per day of 4.6 mg/ml STZ in peritoneal cavity for five days. Control mice were injected with saline at the same interval. STZ mice developed hyperglycemia at approximately one week post-final STZ dose. Mice were maintained in hyperglycemic state for 5-6 weeks before sacrifice and analysis. Hindlimb muscles were dissected out and blotted on gauze, quickly rinsed in saline, and either immersed in 4% paraformaldehyde/phosphate buffered saline (PBS) (soleus) or frozen in liquid nitrogen (gastrocnemius). Three month old Ossabaw miniature swine were assigned to two groups for 55 weeks of treatment. Control swine (n=9) were fed standard, lean chow diet containing 22% kcal from protein, 70% kcal from carbohydrates, and 8% kcal from fat (TestDiet, Richmond, IN). Pigs in the lean

group ate 1500 kcal/day until 6 months of age, then 2500 kcal/day until sacrifice. Obese swine (n=9) were fed excess calorie high-fat diet. Similar to that in previous reports [310, 311], the high-fat feed was composed of lean chow supplemented with (% by weight): cholesterol 2.0, hydrogenated soybean oil 47.6 (contains 56% trans FAs), corn oil 2.5, and sodium cholate 0.7. This mixture yielded a composition of 10% kcal from protein, 17% kcal from carbohydrates and 73% kcal from fat. Obese group pigs ate ~3000-3200 kcal/day until 6 months of age, then 7000-7800 kcal/day until sacrifice. All animals were housed in individual cages/pens and provided a 12-hr light/12-hr dark cycle. Water was provided ad libitum. Fasting plasma glucose and insulin were determined from blood samples drawn from conscious pigs to calculate the homeostatic model assessment (HOMA) value for insulin resistance [311]. All animal protocols were approved by the IUSM Institutional Animal Care and Use Committee.

Human biopsies

Percutaneous needle biopsies of the vastus lateralus were obtained from seven subjects (5 men/2 women; Body Mass Index [BMI] 27.9±1.7 kg/m^2, [range 22.5-34]; age 37.29±4.49 years, [range 19-53]) in whom insulin sensitivity was determined by hyperinsulinemic-euglycemic clamp as described [360]. All subjects gave informed consent for these studies which were approved by the Indiana University-Purdue University Institutional Review Board.

Cell culture

L6-GLUT4myc culture and induction of insulin resistance. Rat L6-GLUT4myc (generously obtained from Dr. Amira Klip, The Hospital for Sick Children, Toronto, Canada) were cultured as previously described [316]. Myoblasts were maintained in α-Minimum essential medium (α-MEM) containing 5 mM glucose and 10% fetal bovine serum (FBS; HyClone Laboratories, Grand Island, NY), and differentiated into multinucleated myotubes with 2% FBS. All studies used myotubes between 4 and 6 days post-initiation of differentiation. Hyperinsulinemia induction of insulin resistance was performed by treating cells with 5 nM insulin for 12 hr in DMEM supplemented with 1%FBS. Hyperglycemia induction of insulin resistance was performed by treating cells with 25 mM insulin for 12 hr in DMEM supplemented with 1%FBS. Palmitate induction of insulin resistance was performed by treating cells with palmitate conjugated to FA-free bovine serum albumin (BSA; MP Biomedicals, Solon, OH) as detailed by Chavez *et al*. [83]. Briefly, palmitate was dissolved in ethanol at a concentration of 75 mM. For each experiment this stock solution was diluted (1:25) to 3 mM in 1% FBS-Dulbecco's modified Eagle's medium (DMEM) containing 2% BSA (w/v), briefly sonicated, and incubated at 55°C for 10 min. Samples were then diluted to the appropriate concentration in 1% FBS-DMEM-2% BSA, cooled to room temperature, and filter sterilized. Myotubes were incubated for 15-16 hr in either the presence or absence of palmitate. Prior to all experiments, cells were serum starved for 60 minutes. All acute treatments occurred during the final 20-45 minutes of serum starvation. To activate AMPK cells were either treated or left untreated for 45 or 30 min with 1 mM AICAR or 200 μM DNP, respectively.

During the final 20 min of treatment, cells were stimulated with 100 nM insulin or left in the basal state.

Adipocyte culture and induction of insulin resistance. Murine 3T3-L1 preadipocytes were cultured in DMEM containing 25 mM glucose and 10% calf serum at 37°C in an 8% CO2 atmosphere. Confluent cultures were induced to differentiate into adipocytes. Adipocytes cultured/differentiated under the standard 25 mM glucose protocol were switched into DMEM/5.5 mM glucose medium containing 10% FBS for two days prior to inducing insulin resistance. Three experimental conditions were utilized routinely: i) control, ii) 5 nM insulin, and iii) 2 mM GlcN. In each of these conditions the culture medium for overnight induction of insulin resistance contained 1 mM pyruvate as an additional energy source. Overnight incubations were limited to 12 h to minimize complications due to effects of glucose deprivation on glucose transport [361]. The three experimental conditions are as follows:

(i) Control. Control adipocytes were incubated for 12 h in DMEM/5.5 mM glucose medium containing 10% FBS in the absence of insulin. The concentration of glutamine in the incubation medium was 2 mM (referred to as control).

(ii) Insulin. Impairment of the glucose transport system was induced by 12 h treatment of cells in DMEM/5.5 mM glucose containing 10% FBS, 5 nM insulin, and 2 mM glutamine (referred to as 12 h Ins).

(iii) GlcN. To induce insulin resistance, adipocytes were treated with DMEM containing 10% FBS and 2 mM GlcN for 12 h, in the absence of glucose, glutamine, and insulin (referred to as 12 h GlcN).

Glucose transport

Uptake assays were performed as previously described [316]. Briefly, treated cells were incubated in glucose-free buffer (125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2.6 mM$MgSO_4$, 25 mM HEPES, 2 mM pyruvate, and 2% BSA) for 30 min, then either left in the basal state or stimulated with 100 nM insulin for 20 min. Uptake was initiated with the addition of 2-deoxy-[1,2,-^{3}H]-glucose (0.055 µCi/µl; PerkinElmer, Boston, MA). Nonspecific uptake was quantitated via cell-associated radioactivity in the presence of 20 µM cytochalasin B. After 5 min, uptake was terminated via four quick washes with ice-cold PBS. Cells were solubilized in 1 N NaOH and [^{3}H] was measured by liquid scintillation. Counts were normalized to total cellular protein, as determined by the Bradford method.

Membrane analyses

L6-GLUT4myc myotubes. Crude plasma membrane fractions were obtained using a differential centrifugation method as detailed by Khayat *et al.* [328], with slight modifications. Briefly, skeletal muscle or L6-GLUT4myc myotubes were washed, resuspended in HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, and 255 mM sucrose containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml pepstatin, 10 µg/ml aprotinin, and 5 µg/ml leupeptin), and processed by

polytron homogenization (15 s) or shearing the sample through a 22-gauge needle 10 times, respectively. Lysates were then centrifuged at 5,000 ×g for 20 min at 4°C. The supernatant was subjected to centrifugation at 100,000 ×g for 30 min. The pellet was resuspended in HES buffer and assayed for protein (Bradford) and cholesterol (Amplex Red) content as previously described [126].

3T3-L1 adipocytes. Adipocyte subcellular membrane fractions were obtained using the differential centrifugation method previously described [362] with slight modification. Briefly, control and insulin-stimulated 3T3-L1 adipocytes were washed and resuspended in HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA and 255 mM sucrose containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin and 5 μg/ml leupeptin). Cell lysates were prepared by shearing the cells through a 22-gauge needle 10 times. Lysates were then centrifuged at 19,000 × g for 20 min at 4°C. The intracellular membrane pellet was obtained by centrifugation of the resulting supernatant at 180,000 × g for 75 min at 4°C. The plasma membrane pellet was obtained by resuspending the pellet from the initial 19,000 × g centrifugation in HES buffer followed by layering onto a 1.12 M sucrose cushion for centrifugation at 100,000 × g for 60 min. The plasma membrane layer was removed from the sucrose cushion and centrifuged at 40,000 × g for 20 min. All pelleted fractions were resuspended in a detergent-containing lysis buffer and assayed for soluble protein content.

Preparation of nuclear extracts

Nuclear extracts were collected as previously described [363]. Briefly, 3T3-L1 adipocytes were washed with PBS and 2 ml of hypotonic buffer (10 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM DTT containing 20 mM leupeptin, 2 mM pepstatin, 2 mM aprotinin and 0.5 M PMSF). Cell lysates were prepared by shearing the cells through a 22-gauge needle 5 times. Prepared lysates were centrifuged at 800 g for 10 min at 4°C. The pellet was then carefully dried and resuspended in 0.5 ml of buffer C (10 mM HEPES, pH 7.4, 0.42 M NaCl, 25% glycerol (v/v), 1.5 mM MgCl2, 0.5 mM EDTA containing 20 mM leupeptin, 2 mM pepstatin, 2 mM aprotinin and 0.5 M PMSF). Nuclei were then visualized by staining with 0.2% trypan blue. Proteins were allowed to swell out of the nuclei by vortexing for 15 s every 10 min for 40 min. The mixture was then centrifuged at full speed in a microcentrifuge at 4°C for 15 min. Supernatant was collected and assayed for soluble protein content.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE analyses)

Total cell extracts were prepared from 100-mm dishes. Myotubes were washed two times with ice-cold PBS and scraped into 1 ml lysis buffer (25 mM Tris, pH 7.4, 50 mM NaF, 10 mM $Na_3P_2O_7$, 137 mM NaCl, 10% glycerol, and 1% Nonidet P-40) containing 2 mM PMSF, 2 mM Na_3VO_4, 5 µg/ml aprotinin, 10 µM leupeptin, and 1 µM pepstatin A then rotated for 15 min at 4°C. Insoluble material was separated from the soluble extract by centrifugation for 15 min at 4°C.

Protein concentrations were determined via the Bradford method and equivalent protein amounts were loaded onto a 7.5% acrylamide gel. The resolved fractions were transferred to nitrocellulose (Bio-Rad, Hercules, CA). Phospho-Akt-2 was detected with anti-phospho-Akt-2 (Ser474) (Genscript, Piscataway, NJ). Phospho-IR and IRS1 were detected with a monoclonal phosphotyrosine antibody (PY20; Transduction Laboratories, San Diego, CA). We also used phosphospecific antibodies to AS160 (PAS; Cell Signaling, Danvers, MA), HMGR and ACC (Millipore, Temecula, CA). AMPK was detected with anti-AMPK (Cell Signaling Technology, Danvers, MA) OGT was detected with anti-OGT (Santa Cruz Biotechnology, Santa Cruz, CA) Equal protein loading was confirmed by Ponceau staining and by immunoblot analysis with anti-actin antibody (Cytoskeleton, Denver, CO). All immunoblots were labeled with IR-conjugated secondary antibody and analyzed via the Odyssey imaging system (LI-COR, Lincoln, NE).

Immunocytochemistry

Labeling of isolated soleus muscle was performed as previously described [316]. Briefly, tissues were fixed for 2 h and then permeabilized. Paired tissue sections were rinsed three times in PBS and then blocked in 5% milk/PBS/0.05% Tween. Tissues were then incubated in anti-F-actin antibody and extensively washed in PBS prior to incubation for 60 min at room temperature in rhodamine red-X-conjugated donkey anti-mouse IgM. Tissues were again subjected to an extensive PBS wash and then a quick ddH_2O rinse. Tissues were mounted in

Vectashield and analyzed via confocal microscopy (model LSM 510 NLO; Zeiss, Thornwood, NY). All microscope/camera settings were identical between groups. Labeling of myotubes was performed as previously described [316]. Briefly, myotubes were fixed and left unpermeabilized (GLUT4myc) or permeabilized (F-actin) in 0.2% Triton-X 100/PBS and blocked in Odyssey Blocking Buffer. The blocked samples were then incubated in primary antibody. GLUT4myc was detected with anti-cMyc (Santa Cruz Biotechnology, Santa Cruz, CA). Following the overnight labeling, cells were washed and incubation with infrared-conjugated secondary antibody. Images were collected and quantitated with the LI-COR infrared imaging system as previously described [316]. Immunofluorescent intensity was normalized to intensity from Syto60, a fluorescent nucleic acid stain (Molecular Probes).

RNA isolation and measurement using RT-PCR

L6-GLUT4myc myotubes were treated with or without palmitate for 16 hours, washed twice in ice cold PBS. Cells were lysed using a Qiagen QIAshredder and RNA was isolated using a Qiagen RNeasy mini kit. RNA was reversed transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for production of a RT-PCR template. Reactions were performed in a 96-well plate format using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each reaction contained the following: 12.5 µL of SYBR GREEN (Applied Biosystems), 500 nM of each primer, cDNA, and RNase free water up to a total volume of 25 µL. The ΔΔCt method of relative

quantification using real-time quantitative PCR was used. Data are expressed as the ratio of *Hmgr* to *Gapdh* using primers specific for the rat *Hmgr* gene (forward 5'-TGT GGG AAC GGT GAC ACT TA-3'; reverse 5'-CTT CAA ATT TTG GGC ACT CA-3') and rat *Gapdh* gene (forward 5'-ATG GCC AGC CTC GTC CCG TAG ACC AAA ATG-3'; reverse 5'-AAG TGG GCC CCG GCC TTC TCC AT-3') with an annealing temperature of 60^{o}C.

ATP measurement.

Intracellular ATP content was measured using a luminescence ATP detection assay system (ATPlite^{TM}, PerkinElmer Inc.) as we have previously described [315]. Briefly, following experimental treatments, L6-GLUT4myc myotubes were lysed using a mammalian cell lysis buffer which inactivates endogenous ATPase. Subsequently whole cell lysate was incubated with substrate buffer and luminescence was detected using the SpectraMax M2 (Molecular Devices). Luminescence was converted to actual ATP content (nM) by use of an ATP standard curve. ATP concentrations were normalized for protein concentration determined by the Bradford method.

siRNA design and transfection.

Three independent oligonuceotide sequences, designed and purchased from Ambion (Austin, TX), were tested for each of the two alpha subunit isoforms of AMPK or OGT. The oligonucleotides with the highest knockdown efficiency for α1, α2, and OGT were respectively: CGA GUU GAC UGG ACA UAA Att (siRNA

ID#: 194424) GCA ACU AUC AAA GAC AUA Ctt (siRNA ID#: 194794), and CAU UUA UGA CAA UCG AAU Att (siRNA ID#: s130675). As these cells express both AMPK alpha isoforms, the combination of the two nucleotides led to the greatest knockdown efficiency of total AMPK, while only one oligo was necessary for OGT knockdown studies. Ambion's Negative Control #1 siRNA (Cat #:4635) was used as a control in all experiments. For all knockdown experiments cells were seeded as described before. Cells were first transfected at approximately 48 hr post seeding (or ~60% confluency). Calcium phosphate transfection protocol was utilized as follows, 60 pmol of siRNA was added to siRNA mix: 15 µl ddH_2O, 15 µl Buffer A (0.5 M $CaCl_2$, 0.1 M HEPES (pH 7.0)), and 30 µl Buffer B (0.28 M NaCl, 0.75 mM NaH_2PO_4, 0.75 mM Na_2HPO_4, 0.05 M HEPES (pH 7.0)) and after 10 min at room temperature, the siRNA mix was added to each well of a 12 well plate containing 600 µl DMEM + 5% FBS and incubated 12-16 hr. Following 12-16 hr incubation, media was aspirated and replaced with DMEM+2% FBS. Additional transfection was repeated 72 hr after initial transfection. Cells were treated and assayed 72 hr after final transfection.

Statistics

Values are presented as means ±SE. The significance of differences between means was evaluated by analysis of variance. Where differences among groups were indicated, the Neumann-Keuls test was used for post hoc comparison between groups. GraphPad Prism 4 software was used for all analyses. $P < 0.05$ was considered significant.

Chapter V

References

1. ADA. http://www.diabetes.org/diabetes-statistics.jsp. Accessed Mar 31, 2009.
2. Cefalu WT. Insulin resistance: cellular and clinical concepts. Exp Biol Med (Maywood) 2001;226(1):13-26.
3. Nash DT. Relationship of C-reactive protein, metabolic syndrome and diabetes mellitus: potential role of statins. J Natl Med Assoc 2005;97(12):1600-7.
4. Nigro J, Osman N, Dart AM, Little PJ. Insulin resistance and atherosclerosis. Endocr Rev 2006;27(3):242-59.
5. Reaven GM. Insulin resistance, the insulin resistance syndrome, and cardiovascular disease. Panminerva Med 2005;47(4):201-10.
6. Olsen GS, Hansen BF. AMP kinase activation ameliorates insulin resistance induced by free fatty acids in rat skeletal muscle. Am J Physiol Endocrinol Metab 2002;283(5):E965-70.
7. Kraegen EW, Saha AK, Preston E, Wilks D, Hoy AJ, Cooney GJ, Ruderman NB. Increased malonyl-CoA and diacylglycerol content and reduced AMPK activity accompany insulin resistance induced by glucose infusion in muscle and liver of rats. Am J Physiol Endocrinol Metab 2006;290(3):E471-9.
8. Tzatsos A, Kandror KV. Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. Mol Cell Biol 2006;26(1):63-76.
9. Wang ZQ, Zhang XH, Russell JC, Hulver M, Cefalu WT. Chromium picolinate enhances skeletal muscle cellular insulin signaling in vivo in obese, insulin-resistant JCR:LA-cp rats. J Nutr 2006;136(2):415-20.
10. Chavez JA, Knotts TA, Wang LP, Li G, Dobrowsky RT, Florant GL, Summers SA. A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. J Biol Chem 2003;278(12):10297-303.
11. Chen G, Raman P, Bhonagiri P, Strawbridge AB, Pattar GR, Elmendorf JS. Protective effect of phosphatidylinositol 4,5-bisphosphate against cortical filamentous actin loss and insulin resistance induced by sustained exposure of 3T3-L1 adipocytes to insulin. J Biol Chem 2004;279(38):39705-9.
12. Rossetti L, Giaccari A, DeFronzo RA. Glucose toxicity. Diabetes Care 1990;13(6):610-30.
13. Chavez JA, Summers SA. Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. Arch Biochem Biophys 2003;419(2):101-9.

14. Nelson BA, Robinson KA, Buse MG. High glucose and glucosamine induce insulin resistance via different mechanisms in 3T3-L1 adipocytes. Diabetes 2000;49(6):981-91.
15. Ross SA, Chen X, Hope HR, Sun S, McMahon EG, Broschat K, Gulve EA. Development and comparison of two 3T3-L1 adipocyte models of insulin resistance: increased glucose flux vs glucosamine treatment. Biochem Biophys Res Commun 2000;273(3):1033-41.
16. Weigert C, Klopfer K, Kausch C, Brodbeck K, Stumvoll M, Haring HU, Schleicher ED. Palmitate-induced activation of the hexosamine pathway in human myotubes: increased expression of glutamine:fructose-6-phosphate aminotransferase. Diabetes 2003;52(3):650-6.
17. Czech MP, Buxton JM. Insulin action on the internalization of the GLUT4 glucose transporter in isolated rat adipocytes. J Biol Chem 1993;268(13):9187-90.
18. Kandror KV, Pilch PF. Compartmentalization of protein traffic in insulin-sensitive cells. Am J Physiol 1996;271(1 Pt 1):E1-14.
19. Rea S, James DE. Moving GLUT4: the biogenesis and trafficking of GLUT4 storage vesicles. Diabetes 1997;46(11):1667-77.
20. Weickert MO, Pfeiffer AF. Signalling mechanisms linking hepatic glucose and lipid metabolism. Diabetologia 2006;49(8):1732-41.
21. Watson RT, Kanzaki M, Pessin JE. Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. Endocr Rev 2004;25(2):177-204.
22. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. Diabetes 1981;30(12):1000-7.
23. Bjornholm M, Zierath JR. Insulin signal transduction in human skeletal muscle: identifying the defects in Type II diabetes. Biochem Soc Trans 2005;33(Pt 2):354-7.
24. Luo RZ, Beniac DR, Fernandes A, Yip CC, Ottensmeyer FP. Quaternary structure of the insulin-insulin receptor complex. Science 1999;285(5430):1077-80.
25. Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 2006;7(2):85-96.
26. Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S, et al. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. Nature 1994;372(6502):182-6.
27. Araki E, Lipes MA, Patti ME, Bruning JC, Haag B, 3rd, Johnson RS, Kahn CR. Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. Nature 1994;372(6502):186-90.
28. Huang C, Thirone AC, Huang X, Klip A. Differential contribution of insulin receptor substrates 1 versus 2 to insulin signaling and glucose uptake in l6 myotubes. J Biol Chem 2005;280(19):19426-35.

29. White MF, Kahn CR. The insulin signaling system. J Biol Chem 1994;269(1):1-4.
30. Backer JM, Myers MG, Jr., Shoelson SE, Chin DJ, Sun XJ, Miralpeix M, Hu P, Margolis B, Skolnik EY, Schlessinger J, et al. Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. EMBO J 1992;11(9):3469-79.
31. Yu J, Zhang Y, McIlroy J, Rordorf-Nikolic T, Orr GA, Backer JM. Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. Mol Cell Biol 1998;18(3):1379-87.
32. Funaki M, Katagiri H, Inukai K, Kikuchi M, Asano T. Structure and function of phosphatidylinositol-3,4 kinase. Cell Signal 2000;12(3):135-42.
33. Clarke JF, Young PW, Yonezawa K, Kasuga M, Holman GD. Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. Biochem J 1994;300 (Pt 3):631-5.
34. Scheid MP, Woodgett JR. Unravelling the activation mechanisms of protein kinase B/Akt. FEBS Lett 2003;546(1):108-12.
35. Dong LQ, Liu F. PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle. Am J Physiol Endocrinol Metab 2005;289(2):E187-96.
36. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 2005;307(5712):1098-101.
37. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell 2006;124(3):471-84.
38. Kohn AD, Summers SA, Birnbaum MJ, Roth RA. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 1996;271(49):31372-8.
39. Jiang ZY, Zhou QL, Coleman KA, Chouinard M, Boese Q, Czech MP. Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. Proc Natl Acad Sci U S A 2003;100(13):7569-74.
40. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem 2001;276(42):38349-52.
41. Bae SS, Cho H, Mu J, Birnbaum MJ. Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B. J Biol Chem 2003;278(49):49530-6.
42. Roach WG, Chavez JA, Miinea CP, Lienhard GE. Substrate specificity and effect on GLUT4 translocation of the Rab GTPase-activating protein Tbc1d1. Biochem J 2007;403(2):353-8.

43. Kane S, Sano H, Liu SC, Asara JM, Lane WS, Garner CC, Lienhard GE. A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. J Biol Chem 2002;277(25):22115-8.
44. Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. J Biol Chem 2003;278(17):14599-602.
45. Eguez L, Lee A, Chavez JA, Miinea CP, Kane S, Lienhard GE, McGraw TE. Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein. Cell Metab 2005;2(4):263-72.
46. Elmendorf JS, Pessin JE. Insulin signaling regulating the trafficking and plasma membrane fusion of GLUT4-containing intracellular vesicles. Exp Cell Res 1999;253(1):55-62.
47. Larance M, Ramm G, Stockli J, van Dam EM, Winata S, Wasinger V, Simpson F, Graham M, Junutula JR, Guilhaus M, James DE. Characterization of the role of the Rab GTPase-activating protein AS160 in insulin-regulated GLUT4 trafficking. J Biol Chem 2005;280(45):37803-13.
48. Miinea CP, Sano H, Kane S, Sano E, Fukuda M, Peranen J, Lane WS, Lienhard GE. AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. Biochem J 2005;391(Pt 1):87-93.
49. Sano H, Roach WG, Peck GR, Fukuda M, Lienhard GE. Rab10 in insulin-stimulated GLUT4 translocation. Biochem J 2008;411(1):89-95.
50. Sano H, Eguez L, Teruel MN, Fukuda M, Chuang TD, Chavez JA, Lienhard GE, McGraw TE. Rab10, a target of the AS160 Rab GAP, is required for insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane. Cell Metab 2007;5(4):293-303.
51. Ishikura S, Bilan PJ, Klip A. Rabs 8A and 14 are targets of the insulin-regulated Rab-GAP AS160 regulating GLUT4 traffic in muscle cells. Biochem Biophys Res Commun 2007;353(4):1074-9.
52. Welsh GI, Leney SE, Lloyd-Lewis B, Wherlock M, Lindsay AJ, McCaffrey MW, Tavare JM. Rip11 is a Rab11- and AS160-RabGAP-binding protein required for insulin-stimulated glucose uptake in adipocytes. J Cell Sci 2007;120(Pt 23):4197-208.
53. Standaert ML, Bandyopadhyay G, Perez L, Price D, Galloway L, Poklepovic A, Sajan MP, Cenni V, Sirri A, Moscat J, Toker A, Farese RV. Insulin activates protein kinases C-zeta and C-lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. J Biol Chem 1999;274(36):25308-16.
54. Imamura T, Huang J, Usui I, Satoh H, Bever J, Olefsky JM. Insulin-induced GLUT4 translocation involves protein kinase C-lambda-mediated functional coupling between Rab4 and the motor protein kinesin. Mol Cell Biol 2003;23(14):4892-900.

55. Braiman L, Alt A, Kuroki T, Ohba M, Bak A, Tennenbaum T, Sampson SR. Activation of protein kinase C zeta induces serine phosphorylation of VAMP2 in the GLUT4 compartment and increases glucose transport in skeletal muscle. Mol Cell Biol 2001;21(22):7852-61.
56. Hodgkinson CP, Mander A, Sale GJ. Protein kinase-zeta interacts with munc18c: role in GLUT4 trafficking. Diabetologia 2005;48(8):1627-36.
57. Ribon V, Saltiel AR. Insulin stimulates tyrosine phosphorylation of the proto-oncogene product of c-Cbl in 3T3-L1 adipocytes. Biochem J 1997;324 (Pt 3):839-45.
58. Liu J, DeYoung SM, Hwang JB, O'Leary EE, Saltiel AR. The roles of Cbl-b and c-Cbl in insulin-stimulated glucose transport. J Biol Chem 2003;278(38):36754-62.
59. Liu J, Kimura A, Baumann CA, Saltiel AR. APS facilitates c-Cbl tyrosine phosphorylation and GLUT4 translocation in response to insulin in 3T3-L1 adipocytes. Mol Cell Biol 2002;22(11):3599-609.
60. Chiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, Macara IG, Pessin JE, Saltiel AR. Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. Nature 2001;410(6831):944-8.
61. Watson RT, Shigematsu S, Chiang SH, Mora S, Kanzaki M, Macara IG, Saltiel AR, Pessin JE. Lipid raft microdomain compartmentalization of TC10 is required for insulin signaling and GLUT4 translocation. J Cell Biol 2001;154(4):829-40.
62. Jiang ZY, Chawla A, Bose A, Way M, Czech MP. A phosphatidylinositol 3-kinase-independent insulin signaling pathway to N-WASP/Arp2/3/F-actin required for GLUT4 glucose transporter recycling. J Biol Chem 2002;277(1):509-15.
63. Inoue M, Chang L, Hwang J, Chiang SH, Saltiel AR. The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. Nature 2003;422(6932):629-33.
64. JeBailey L, Rudich A, Huang X, Di Ciano-Oliveira C, Kapus A, Klip A. Skeletal muscle cells and adipocytes differ in their reliance on TC10 and Rac for insulin-induced actin remodeling. Mol Endocrinol 2004;18(2):359-72.
65. DeFronzo RA, Simonson D, Ferrannini E. Hepatic and peripheral insulin resistance: a common feature of type 2 (non-insulin-dependent) and type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1982;23(4):313-9.
66. Kolterman OG, Gray RS, Griffin J, Burstein P, Insel J, Scarlett JA, Olefsky JM. Receptor and postreceptor defects contribute to the insulin resistance in noninsulin-dependent diabetes mellitus. J Clin Invest 1981;68(4):957-69.

67. Perseghin G, Scifo P, De Cobelli F, Pagliato E, Battezzati A, Arcelloni C, Vanzulli A, Testolin G, Pozza G, Del Maschio A, Luzi L. Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. Diabetes 1999;48(8):1600-6.
68. Manco M, Mingrone G, Greco AV, Capristo E, Gniuli D, De Gaetano A, Gasbarrini G. Insulin resistance directly correlates with increased saturated fatty acids in skeletal muscle triglycerides. Metabolism 2000;49(2):220-4.
69. Qatanani M, Lazar MA. Mechanisms of obesity-associated insulin resistance: many choices on the menu. Genes Dev 2007;21(12):1443-55.
70. Randle PJ, Garland PB, Hales CN, Newsholme EA. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet 1963;1(7285):785-9.
71. Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI. Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. J Clin Invest 1999;103(2):253-9.
72. Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, Shulman GI. Mechanism of free fatty acid-induced insulin resistance in humans. J Clin Invest 1996;97(12):2859-65.
73. Rothman DL, Shulman RG, Shulman GI. 31P nuclear magnetic resonance measurements of muscle glucose-6-phosphate. Evidence for reduced insulin-dependent muscle glucose transport or phosphorylation activity in non-insulin-dependent diabetes mellitus. J Clin Invest 1992;89(4):1069-75.
74. Schmitz-Peiffer C, Browne CL, Oakes ND, Watkinson A, Chisholm DJ, Kraegen EW, Biden TJ. Alterations in the expression and cellular localization of protein kinase C isozymes epsilon and theta are associated with insulin resistance in skeletal muscle of the high-fat-fed rat. Diabetes 1997;46(2):169-78.
75. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. J Biol Chem 2002;277(52):50230-6.
76. Rivellese AA, De Natale C, Lilli S. Type of dietary fat and insulin resistance. Ann N Y Acad Sci 2002;967:329-35.
77. Gorski J, Nawrocki A, Murthy M. Characterization of free and glyceride-esterified long chain fatty acids in different skeletal muscle types of the rat. Mol Cell Biochem 1998;178(1-2):113-8.
78. Summers SA, Garza LA, Zhou H, Birnbaum MJ. Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide. Mol Cell Biol 1998;18(9):5457-64.

79. Straczkowski M, Kowalska I, Nikolajuk A, Dzienis-Straczkowska S, Kinalska I, Baranowski M, Zendzian-Piotrowska M, Brzezinska Z, Gorski J. Relationship between insulin sensitivity and sphingomyelin signaling pathway in human skeletal muscle. Diabetes 2004;53(5):1215-21.
80. Powell DJ, Turban S, Gray A, Hajduch E, Hundal HS. Intracellular ceramide synthesis and protein kinase Czeta activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. Biochem J 2004;382(Pt 2):619-29.
81. Pickersgill L, Litherland GJ, Greenberg AS, Walker M, Yeaman SJ. Key role for ceramides in mediating insulin resistance in human muscle cells. J Biol Chem 2007;282(17):12583-9.
82. Sabin MA, Stewart CE, Crowne EC, Turner SJ, Hunt LP, Welsh GI, Grohmann MJ, Holly JM, Shield JP. Fatty acid-induced defects in insulin signalling, in myotubes derived from children, are related to ceramide production from palmitate rather than the accumulation of intramyocellular lipid. J Cell Physiol 2007;211(1):244-52.
83. Chavez JA, Holland WL, Bar J, Sandhoff K, Summers SA. Acid ceramidase overexpression prevents the inhibitory effects of saturated fatty acids on insulin signaling. J Biol Chem 2005;280(20):20148-53.
84. Dobrowsky RT, Kamibayashi C, Mumby MC, Hannun YA. Ceramide activates heterotrimeric protein phosphatase 2A. J Biol Chem 1993;268(21):15523-30.
85. Ugi S, Imamura T, Maegawa H, Egawa K, Yoshizaki T, Shi K, Obata T, Ebina Y, Kashiwagi A, Olefsky JM. Protein phosphatase 2A negatively regulates insulin's metabolic signaling pathway by inhibiting Akt (protein kinase B) activity in 3T3-L1 adipocytes. Mol Cell Biol 2004;24(19):8778-89.
86. Powell DJ, Hajduch E, Kular G, Hundal HS. Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKCzeta-dependent mechanism. Mol Cell Biol 2003;23(21):7794-808.
87. Bruce CR, Dyck DJ. Cytokine regulation of skeletal muscle fatty acid metabolism: effect of interleukin-6 and tumor necrosis factor-alpha. Am J Physiol Endocrinol Metab 2004;287(4):E616-21.
88. Steinberg GR, Michell BJ, van Denderen BJ, Watt MJ, Carey AL, Fam BC, Andrikopoulos S, Proietto J, Gorgun CZ, Carling D, Hotamisligil GS, Febbraio MA, Kay TW, Kemp BE. Tumor necrosis factor alpha-induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. Cell Metab 2006;4(6):465-74.
89. Dyck DJ, Heigenhauser GJ, Bruce CR. The role of adipokines as regulators of skeletal muscle fatty acid metabolism and insulin sensitivity. Acta Physiol (Oxf) 2006;186(1):5-16.
90. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest 1995;95(5):2409-15.

91. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW, Jr. CCR2 modulates inflammatory and metabolic effects of high-fat feeding. J Clin Invest 2006;116(1):115-24.
92. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol 2004;25(1):4-7.
93. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003;112(12):1796-808.
94. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 2003;112(12):1821-30.
95. Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van Obberghen E. SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. J Biol Chem 2001;276(51):47944-9.
96. Mooney RA, Senn J, Cameron S, Inamdar N, Boivin LM, Shang Y, Furlanetto RW. Suppressors of cytokine signaling-1 and -6 associate with and inhibit the insulin receptor. A potential mechanism for cytokine-mediated insulin resistance. J Biol Chem 2001;276(28):25889-93.
97. Ueki K, Kondo T, Kahn CR. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol Cell Biol 2004;24(12):5434-46.
98. Rui L, Yuan M, Frantz D, Shoelson S, White MF. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem 2002;277(44):42394-8.
99. Zorzano A, Liesa M, Palacin M. Mitochondrial dynamics as a bridge between mitochondrial dysfunction and insulin resistance. Arch Physiol Biochem 2009;115(1):1-12.
100. Roberts CK, Sindhu KK. Oxidative stress and metabolic syndrome. Life Sci 2009;84(21-22):705-12.
101. Kanzaki M, Pessin JE. Insulin-stimulated GLUT4 translocation in adipocytes is dependent upon cortical actin remodeling. J Biol Chem 2001;276(45):42436-44.
102. Patel N, Rudich A, Khayat ZA, Garg R, Klip A. Intracellular segregation of phosphatidylinositol-3,4,5-trisphosphate by insulin-dependent actin remodeling in L6 skeletal muscle cells. Mol Cell Biol 2003;23(13):4611-26.
103. Tsakiridis T, Vranic M, Klip A. Disassembly of the actin network inhibits insulin-dependent stimulation of glucose transport and prevents recruitment of glucose transporters to the plasma membrane. J Biol Chem 1994;269(47):29934-42.
104. Peyrollier K, Hajduch E, Gray A, Litherland GJ, Prescott AR, Leslie NR, Hundal HS. A role for the actin cytoskeleton in the hormonal and growth-factor-mediated activation of protein kinase B. Biochem J 2000;352 Pt 3:617-22.

105. Brozinick JT, Jr., Hawkins ED, Strawbridge AB, Elmendorf JS. Disruption of cortical actin in skeletal muscle demonstrates an essential role of the cytoskeleton in glucose transporter 4 translocation in insulin-sensitive tissues. J Biol Chem 2004;279(39):40699-706.
106. Bose A, Cherniack AD, Langille SE, Nicoloro SM, Buxton JM, Park JG, Chawla A, Czech MP. G(alpha)11 signaling through ARF6 regulates F-actin mobilization and GLUT4 glucose transporter translocation to the plasma membrane. Mol Cell Biol 2001;21(15):5262-75.
107. Kanzaki M, Watson RT, Hou JC, Stamnes M, Saltiel AR, Pessin JE. Small GTP-binding protein TC10 differentially regulates two distinct populations of filamentous actin in 3T3L1 adipocytes. Mol Biol Cell 2002;13(7):2334-46.
108. Omata W, Shibata H, Li L, Takata K, Kojima I. Actin filaments play a critical role in insulin-induced exocytotic recruitment but not in endocytosis of GLUT4 in isolated rat adipocytes. Biochem J 2000;346 Pt 2:321-8.
109. Aktories K, Barmann M, Ohishi I, Tsuyama S, Jakobs KH, Habermann E. Botulinum C2 toxin ADP-ribosylates actin. Nature 1986;322(6077):390-2.
110. Janmey PA, Stossel TP. Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. Nature 1987;325(6102):362-4.
111. Lu PJ, Shieh WR, Rhee SG, Yin HL, Chen CS. Lipid products of phosphoinositide 3-kinase bind human profilin with high affinity. Biochemistry 1996;35(44):14027-34.
112. Tong P, Khayat ZA, Huang C, Patel N, Ueyama A, Klip A. Insulin-induced cortical actin remodeling promotes GLUT4 insertion at muscle cell membrane ruffles. J Clin Invest 2001;108(3):371-81.
113. Tsakiridis T, Tong P, Matthews B, Tsiani E, Bilan PJ, Klip A, Downey GP. Role of the actin cytoskeleton in insulin action. Microsc Res Tech 1999;47(2):79-92.
114. Kanzaki M, Watson RT, Khan AH, Pessin JE. Insulin stimulates actin comet tails on intracellular GLUT4-containing compartments in differentiated 3T3L1 adipocytes. J Biol Chem 2001;276(52):49331-6.
115. Kanzaki M, Furukawa M, Raab W, Pessin JE. Phosphatidylinositol 4,5-bisphosphate regulates adipocyte actin dynamics and GLUT4 vesicle recycling. J Biol Chem 2004;279(29):30622-33.
116. Kandror KV, Pilch PF. gp160, a tissue-specific marker for insulin-activated glucose transport. Proc Natl Acad Sci U S A 1994;91(17):8017-21.
117. Kandror KV, Yu L, Pilch PF. The major protein of GLUT4-containing vesicles, gp160, has aminopeptidase activity. J Biol Chem 1994;269(49):30777-80.
118. Mastick CC, Aebersold R, Lienhard GE. Characterization of a major protein in GLUT4 vesicles. Concentration in the vesicles and insulin-stimulated translocation to the plasma membrane. J Biol Chem 1994;269(8):6089-92.
119. Waters SB, D'Auria M, Martin SS, Nguyen C, Kozma LM, Luskey KL. The amino terminus of insulin-responsive aminopeptidase causes Glut4 translocation in 3T3-L1 adipocytes. J Biol Chem 1997;272(37):23323-7.

120. Chen F, Ma L, Parrini MC, Mao X, Lopez M, Wu C, Marks PW, Davidson L, Kwiatkowski DJ, Kirchhausen T, Orkin SH, Rosen FS, Mayer BJ, Kirschner MW, Alt FW. Cdc42 is required for PIP(2)-induced actin polymerization and early development but not for cell viability. Curr Biol 2000;10(13):758-65.
121. Miki H, Miura K, Takenawa T. N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. EMBO J 1996;15(19):5326-35.
122. Horvath EM, Tackett L, Elmendorf JS. A novel membrane-based anti-diabetic action of atorvastatin. Biochem Biophys Res Commun 2008;372(4):639-43.
123. Horvath EM, Tackett L, McCarthy AM, Raman P, Brozinick JT, Elmendorf JS. Antidiabetogenic Effects of Chromium Mitigate Hyperinsulinemia-Induced Cellular Insulin Resistance via Correction of Plasma Membrane Cholesterol Imbalance. Mol Endocrinol 2008;22(4):937-50.
124. Liu P, Leffler BJ, Weeks LK, Chen G, Bouchard CM, Strawbridge AB, Elmendorf JS. Sphingomyelinase activates GLUT4 translocation via a cholesterol-dependent mechanism. Am J Physiol Cell Physiol 2004;286(2):C317-29.
125. Chen G, Liu P, Pattar GR, Tackett L, Bhonagiri P, Strawbridge AB, Elmendorf JS. Chromium activates glucose transporter 4 trafficking and enhances insulin-stimulated glucose transport in 3T3-L1 adipocytes via a cholesterol-dependent mechanism. Mol Endocrinol 2006;20(4):857-70.
126. Pattar GR, Tackett L, Liu P, Elmendorf JS. Chromium picolinate positively influences the glucose transporter system via affecting cholesterol homeostasis in adipocytes cultured under hyperglycemic diabetic conditions. Mutat Res 2006;610(1-2):93-100.
127. Michikawa M. Neurodegenerative disorders and cholesterol. Curr Alzheimer Res 2004;1(4):271-5.
128. Lukiw WJ, Pappolla M, Pelaez RP, Bazan NG. Alzheimer's disease--a dysfunction in cholesterol and lipid metabolism. Cell Mol Neurobiol 2005;25(3-4):475-83.
129. Sjogren M, Blennow K. The link between cholesterol and Alzheimer's disease. World J Biol Psychiatry 2005;6(2):85-97.
130. Pilch PF, Thompson PA, Czech MP. Coordinate modulation of D-glucose transport activity and bilayer fluidity in plasma membranes derived from control and insulin-treated adipocytes. Proc Natl Acad Sci U S A 1980;77(2):915-8.
131. Vainio S, Bykov I, Hermansson M, Jokitalo E, Somerharju P, Ikonen E. Defective insulin receptor activation and altered lipid rafts in Niemann-Pick type C disease hepatocytes. Biochem J 2005;391(Pt 3):465-72.
132. Younsi M, Quilliot D, Al-Makdissy N, Delbachian I, Drouin P, Donner M, Ziegler O. Erythrocyte membrane phospholipid composition is related to hyperinsulinemia in obese nondiabetic women: effects of weight loss. Metabolism 2002;51(10):1261-8.

133. David TS, Ortiz PA, Smith TR, Turinsky J. Sphingomyelinase has an insulin-like effect on glucose transporter translocation in adipocytes. Am J Physiol 1998;274(5 Pt 2):R1446-53.
134. Pattar G, Tackett L, Liu P, Elmendorf JS. Chromium picolinate positively influences the glucose transporter system via affecting cholesterol homeostasis in adipocytes cultured under hyperglycemic diabetic conditions. Mutation Research (in press) 2006.
135. Kennedy JW, Hirshman MF, Gervino EV, Ocel JV, Forse RA, Hoenig SJ, Aronson D, Goodyear LJ, Horton ES. Acute exercise induces GLUT4 translocation in skeletal muscle of normal human subjects and subjects with type 2 diabetes. Diabetes 1999;48(5):1192-7.
136. Holloszy JO, Narahara HT. Studies of tissue permeability. X. Changes in permeability to 3-methylglucose associated with contraction of isolated frog muscle. J Biol Chem 1965;240(9):3493-500.
137. Ivy JL, Holloszy JO. Persistent increase in glucose uptake by rat skeletal muscle following exercise. Am J Physiol 1981;241(5):C200-3.
138. Richter EA, Garetto LP, Goodman MN, Ruderman NB. Muscle glucose metabolism following exercise in the rat: increased sensitivity to insulin. J Clin Invest 1982;69(4):785-93.
139. Garetto LP, Richter EA, Goodman MN, Ruderman NB. Enhanced muscle glucose metabolism after exercise in the rat: the two phases. Am J Physiol 1984;246(6 Pt 1):E471-5.
140. Wallberg-Henriksson H, Constable SH, Young DA, Holloszy JO. Glucose transport into rat skeletal muscle: interaction between exercise and insulin. J Appl Physiol 1988;65(2):909-13.
141. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med 2002;346(6):393-403.
142. Tuomilehto J, Lindstrom J, Eriksson JG, Valle TT, Hamalainen H, Ilanne-Parikka P, Keinanen-Kiukaanniemi S, Laakso M, Louheranta A, Rastas M, Salminen V, Uusitupa M. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. N Engl J Med 2001;344(18):1343-50.
143. Holloszy JO, Constable SH, Young DA. Activation of glucose transport in muscle by exercise. Diabetes Metab Rev 1986;1(4):409-23.
144. Holloszy JO, Hansen PA. Regulation of glucose transport into skeletal muscle. Rev Physiol Biochem Pharmacol 1996;128:99-193.
145. Balon TW, Nadler JL. Evidence that nitric oxide increases glucose transport in skeletal muscle. J Appl Physiol 1997;82(1):359-63.
146. Roberts CK, Barnard RJ, Scheck SH, Balon TW. Exercise-stimulated glucose transport in skeletal muscle is nitric oxide dependent. Am J Physiol 1997;273(1 Pt 1):E220-5.
147. Dietze G, Wicklmayr M. Evidence for a participation of the kallikrein-kinin system in the regulation of muscle metabolism during muscular work. FEBS Lett 1977;74(2):205-8.

148. Sakamoto K, Goodyear LJ. Invited review: intracellular signaling in contracting skeletal muscle. J Appl Physiol 2002;93(1):369-83.
149. Winder WW, Hardie DG. AMP-activated protein kinase, a metabolic master switch: possible roles in type 2 diabetes. Am J Physiol 1999;277(1 Pt 1):E1-10.
150. Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, Zhou G, Williamson JM, Ljunqvist O, Efendic S, Moller DE, Thorell A, Goodyear LJ. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. Diabetes 2002;51(7):2074-81.
151. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001;108(8):1167-74.
152. Hwang SL, Kim HN, Jung HH, Kim JE, Choi DK, Hur JM, Lee JY, Song H, Song KS, Huh TL. Beneficial effects of beta-sitosterol on glucose and lipid metabolism in L6 myotube cells are mediated by AMP-activated protein kinase. Biochem Biophys Res Commun 2008;377(4):1253-8.
153. Breen DM, Sanli T, Giacca A, Tsiani E. Stimulation of muscle cell glucose uptake by resveratrol through sirtuins and AMPK. Biochem Biophys Res Commun 2008;374(1):117-22.
154. Penumathsa SV, Thirunavukkarasu M, Zhan L, Maulik G, Menon VP, Bagchi D, Maulik N. Resveratrol enhances GLUT-4 translocation to the caveolar lipid raft fractions through AMPK/Akt/eNOS signalling pathway in diabetic myocardium. J Cell Mol Med 2008;12(6A):2350-61.
155. Zang M, Xu S, Maitland-Toolan KA, Zuccollo A, Hou X, Jiang B, Wierzbicki M, Verbeuren TJ, Cohen RA. Polyphenols stimulate AMP-activated protein kinase, lower lipids, and inhibit accelerated atherosclerosis in diabetic LDL receptor-deficient mice. Diabetes 2006;55(8):2180-91.
156. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R, Sinclair DA. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 2006;444(7117):337-42.
157. Collins QF, Liu HY, Pi J, Liu Z, Quon MJ, Cao W. Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis through 5'-AMP-activated protein kinase. J Biol Chem 2007;282(41):30143-9.
158. Murase T, Misawa K, Haramizu S, Hase T. Catechin-induced activation of the LKB1/AMP-activated protein kinase pathway. Biochem Pharmacol 2009;78(1):78-84.
159. Cheng Z, Pang T, Gu M, Gao AH, Xie CM, Li JY, Nan FJ, Li J. Berberine-stimulated glucose uptake in L6 myotubes involves both AMPK and p38 MAPK. Biochim Biophys Acta 2006;1760(11):1682-9.

160. Tan MJ, Ye JM, Turner N, Hohnen-Behrens C, Ke CQ, Tang CP, Chen T, Weiss HC, Gesing ER, Rowland A, James DE, Ye Y. Antidiabetic activities of triterpenoids isolated from bitter melon associated with activation of the AMPK pathway. Chem Biol 2008;15(3):263-73.
161. Fang X, Palanivel R, Zhou X, Liu Y, Xu A, Wang Y, Sweeney G. Hyperglycemia- and hyperinsulinemia-induced alteration of adiponectin receptor expression and adiponectin effects in L6 myoblasts. J Mol Endocrinol 2005;35(3):465-76.
162. Hardie DG. The AMP-activated protein kinase pathway--new players upstream and downstream. J Cell Sci 2004;117(Pt 23):5479-87.
163. Janovska A, Hatzinikolas G, Staikopoulos V, McInerney J, Mano M, Wittert GA. AMPK and ACC phosphorylation: effect of leptin, muscle fibre type and obesity. Mol Cell Endocrinol 2008;284(1-2):1-10.
164. Steinberg GR, Rush JW, Dyck DJ. AMPK expression and phosphorylation are increased in rodent muscle after chronic leptin treatment. Am J Physiol Endocrinol Metab 2003;284(3):E648-54.
165. Glund S, Deshmukh A, Long YC, Moller T, Koistinen HA, Caidahl K, Zierath JR, Krook A. Interleukin-6 directly increases glucose metabolism in resting human skeletal muscle. Diabetes 2007;56(6):1630-7.
166. Carey AL, Steinberg GR, Macaulay SL, Thomas WG, Holmes AG, Ramm G, Prelovsek O, Hohnen-Behrens C, Watt MJ, James DE, Kemp BE, Pedersen BK, Febbraio MA. Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. Diabetes 2006;55(10):2688-97.
167. Febbraio MA, Hiscock N, Sacchetti M, Fischer CP, Pedersen BK. Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction. Diabetes 2004;53(7):1643-8.
168. Hardie DG, Carling D. The AMP-activated protein kinase--fuel gauge of the mammalian cell? Eur J Biochem 1997;246(2):259-73.
169. Kemp BE, Mitchelhill KI, Stapleton D, Michell BJ, Chen ZP, Witters LA. Dealing with energy demand: the AMP-activated protein kinase. Trends Biochem Sci 1999;24(1):22-5.
170. Davies SP, Carling D, Hardie DG. Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. Eur J Biochem 1989;186(1-2):123-8.
171. Gao G, Widmer J, Stapleton D, Teh T, Cox T, Kemp BE, Witters LA. Catalytic subunits of the porcine and rat 5'-AMP-activated protein kinase are members of the SNF1 protein kinase family. Biochim Biophys Acta 1995;1266(1):73-82.
172. Stapleton D, Woollatt E, Mitchelhill KI, Nicholl JK, Fernandez CS, Michell BJ, Witters LA, Power DA, Sutherland GR, Kemp BE. AMP-activated protein kinase isoenzyme family: subunit structure and chromosomal location. FEBS Lett 1997;409(3):452-6.

173. Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T, House CM, Fernandez CS, Cox T, Witters LA, Kemp BE. Mammalian AMP-activated protein kinase subfamily. J Biol Chem 1996;271(2):611-4.
174. Woods A, Azzout-Marniche D, Foretz M, Stein SC, Lemarchand P, Ferre P, Foufelle F, Carling D. Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. Mol Cell Biol 2000;20(18):6704-11.
175. Salt I, Celler JW, Hawley SA, Prescott A, Woods A, Carling D, Hardie DG. AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform. Biochem J 1998;334 (Pt 1):177-87.
176. Woods A, Salt I, Scott J, Hardie DG, Carling D. The alpha1 and alpha2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity in vitro. FEBS Lett 1996;397(2-3):347-51.
177. Polekhina G, Gupta A, Michell BJ, van Denderen B, Murthy S, Feil SC, Jennings IG, Campbell DJ, Witters LA, Parker MW, Kemp BE, Stapleton D. AMPK beta subunit targets metabolic stress sensing to glycogen. Curr Biol 2003;13(10):867-71.
178. Hudson ER, Pan DA, James J, Lucocq JM, Hawley SA, Green KA, Baba O, Terashima T, Hardie DG. A novel domain in AMP-activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. Curr Biol 2003;13(10):861-6.
179. Scott JW, Hawley SA, Green KA, Anis M, Stewart G, Scullion GA, Norman DG, Hardie DG. CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. J Clin Invest 2004;113(2):274-84.
180. Cheung PC, Salt IP, Davies SP, Hardie DG, Carling D. Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. Biochem J 2000;346 Pt 3:659-69.
181. Sanders MJ, Grondin PO, Hegarty BD, Snowden MA, Carling D. Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. Biochem J 2007;403(1):139-48.
182. Stein SC, Woods A, Jones NA, Davison MD, Carling D. The regulation of AMP-activated protein kinase by phosphorylation. Biochem J 2000;345 Pt 3:437-43.
183. Riek U, Scholz R, Konarev P, Rufer A, Suter M, Nazabal A, Ringler P, Chami M, Muller SA, Neumann D, Forstner M, Hennig M, Zenobi R, Engel A, Svergun D, Schlattner U, Wallimann T. Structural properties of AMP-activated protein kinase: dimerization, molecular shape, and changes upon ligand binding. J Biol Chem 2008;283(26):18331-43.
184. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, Alessi DR, Hardie DG. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J Biol 2003;2(4):28.

185. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, Cantley LC. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc Natl Acad Sci U S A 2004;101(10):3329-35.
186. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, Schlattner U, Wallimann T, Carlson M, Carling D. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr Biol 2003;13(22):2004-8.
187. Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG, Hardie DG. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab 2005;2(1):9-19.
188. Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D. Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab 2005;2(1):21-33.
189. Scott JW, Norman DG, Hawley SA, Kontogiannis L, Hardie DG. Protein kinase substrate recognition studied using the recombinant catalytic domain of AMP-activated protein kinase and a model substrate. J Mol Biol 2002;317(2):309-23.
190. Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D, Hardie DG. Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. J Biol Chem 1996;271(44):27879-87.
191. Holmes BF, Kurth-Kraczek EJ, Winder WW. Chronic activation of 5'-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. J Appl Physiol 1999;87(5):1990-5.
192. Ojuka EO, Jones TE, Nolte LA, Chen M, Wamhoff BR, Sturek M, Holloszy JO. Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca(2+). Am J Physiol Endocrinol Metab 2002;282(5):E1008-13.
193. Bergeron R, Ren JM, Cadman KS, Moore IK, Perret P, Pypaert M, Young LH, Semenkovich CF, Shulman GI. Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. Am J Physiol Endocrinol Metab 2001;281(6):E1340-6.
194. Winder WW, Holmes BF, Rubink DS, Jensen EB, Chen M, Holloszy JO. Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. J Appl Physiol 2000;88(6):2219-26.
195. Holloszy JO. Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. J Biol Chem 1967;242(9):2278-82.
196. Jones TE, Baar K, Ojuka E, Chen M, Holloszy JO. Exercise induces an increase in muscle UCP3 as a component of the increase in mitochondrial biogenesis. Am J Physiol Endocrinol Metab 2003;284(1):E96-101.

197. Ojuka EO, Jones TE, Han DH, Chen M, Wamhoff BR, Sturek M, Holloszy JO. Intermittent increases in cytosolic Ca2+ stimulate mitochondrial biogenesis in muscle cells. Am J Physiol Endocrinol Metab 2002;283(5):E1040-5.
198. Winder WW, Hardie DG. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. Am J Physiol 1996;270(2 Pt 1):E299-304.
199. Vavvas D, Apazidis A, Saha AK, Gamble J, Patel A, Kemp BE, Witters LA, Ruderman NB. Contraction-induced changes in acetyl-CoA carboxylase and 5'-AMP-activated kinase in skeletal muscle. J Biol Chem 1997;272(20):13255-61.
200. Chen ZP, McConell GK, Michell BJ, Snow RJ, Canny BJ, Kemp BE. AMPK signaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. Am J Physiol Endocrinol Metab 2000;279(5):E1202-6.
201. Assifi MM, Suchankova G, Constant S, Prentki M, Saha AK, Ruderman NB. AMP-activated protein kinase and coordination of hepatic fatty acid metabolism of starved/carbohydrate-refed rats. Am J Physiol Endocrinol Metab 2005;289(5):E794-800.
202. Velasco G, Geelen MJ, Guzman M. Control of hepatic fatty acid oxidation by 5'-AMP-activated protein kinase involves a malonyl-CoA-dependent and a malonyl-CoA-independent mechanism. Arch Biochem Biophys 1997;337(2):169-75.
203. McGarry JD, Brown NF. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem 1997;244(1):1-14.
204. Ruderman NB, Saha AK, Vavvas D, Witters LA. Malonyl-CoA, fuel sensing, and insulin resistance. Am J Physiol 1999;276(1 Pt 1):E1-E18.
205. Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur J Biochem 1995;229(2):558-65.
206. Gibson DM, Parker RA, Stewart CS, Evenson KJ. Short-term regulation of hydroxymethylglutaryl coenzyme A reductase by reversible phosphorylation: modulation of reductase phosphatase in rat hepatocytes. Adv Enzyme Regul 1982;20:263-83.
207. Beg ZH, Allmann DW, Gibson DM. Modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity with cAMP and wth protein fractions of rat liver cytosol. Biochem Biophys Res Commun 1973;54(4):1362-9.
208. Ojuka EO, Nolte LA, Holloszy JO. Increased expression of GLUT-4 and hexokinase in rat epitrochlearis muscles exposed to AICAR in vitro. J Appl Physiol 2000;88(3):1072-5.
209. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ. Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. Diabetes 1998;47(8):1369-73.

210. Zheng D, MacLean PS, Pohnert SC, Knight JB, Olson AL, Winder WW, Dohm GL. Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. J Appl Physiol 2001;91(3):1073-83.
211. Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T, Tabata I. Effects of low-intensity prolonged exercise on PGC-1 mRNA expression in rat epitrochlearis muscle. Biochem Biophys Res Commun 2002;296(2):350-4.
212. Ojuka EO. Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in muscle. Proc Nutr Soc 2004;63(2):275-8.
213. Suwa M, Nakano H, Kumagai S. Effects of chronic AICAR treatment on fiber composition, enzyme activity, UCP3, and PGC-1 in rat muscles. J Appl Physiol 2003;95(3):960-8.
214. Holmes BF, Sparling DP, Olson AL, Winder WW, Dohm GL. Regulation of muscle GLUT4 enhancer factor and myocyte enhancer factor 2 by AMP-activated protein kinase. Am J Physiol Endocrinol Metab 2005;289(6):E1071-6.
215. Ramachandran B, Yu G, Gulick T. Nuclear respiratory factor 1 controls myocyte enhancer factor 2A transcription to provide a mechanism for coordinate expression of respiratory chain subunits. J Biol Chem 2008;283(18):11935-46.
216. Merrill GF, Kurth EJ, Hardie DG, Winder WW. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol 1997;273(6 Pt 1):E1107-12.
217. Bergeron R, Russell RR, 3rd, Young LH, Ren JM, Marcucci M, Lee A, Shulman GI. Effect of AMPK activation on muscle glucose metabolism in conscious rats. Am J Physiol 1999;276(5 Pt 1):E938-44.
218. Patel N, Khayat ZA, Ruderman NB, Klip A. Dissociation of 5' AMP-activated protein kinase activation and glucose uptake stimulation by mitochondrial uncoupling and hyperosmolar stress: differential sensitivities to intracellular Ca2+ and protein kinase C inhibition. Biochem Biophys Res Commun 2001;285(4):1066-70.
219. Chen HC, Bandyopadhyay G, Sajan MP, Kanoh Y, Standaert M, Farese RV, Jr., Farese RV. Activation of the ERK pathway and atypical protein kinase C isoforms in exercise- and aminoimidazole-4-carboxamide-1-beta-D-riboside (AICAR)-stimulated glucose transport. J Biol Chem 2002;277(26):23554-62.
220. Sakoda H, Ogihara T, Anai M, Fujishiro M, Ono H, Onishi Y, Katagiri H, Abe M, Fukushima Y, Shojima N, Inukai K, Kikuchi M, Oka Y, Asano T. Activation of AMPK is essential for AICAR-induced glucose uptake by skeletal muscle but not adipocytes. Am J Physiol Endocrinol Metab 2002;282(6):E1239-44.
221. Abbud W, Habinowski S, Zhang JZ, Kendrew J, Elkairi FS, Kemp BE, Witters LA, Ismail-Beigi F. Stimulation of AMP-activated protein kinase (AMPK) is associated with enhancement of Glut1-mediated glucose transport. Arch Biochem Biophys 2000;380(2):347-52.

222. Fryer LG, Foufelle F, Barnes K, Baldwin SA, Woods A, Carling D. Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells. Biochem J 2002;363(Pt 1):167-74.
223. Fryer LG, Hajduch E, Rencurel F, Salt IP, Hundal HS, Hardie DG, Carling D. Activation of glucose transport by AMP-activated protein kinase via stimulation of nitric oxide synthase. Diabetes 2000;49(12):1978-85.
224. Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW. 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes 1999;48(8):1667-71.
225. Jessen N, Pold R, Buhl ES, Jensen LS, Schmitz O, Lund S. Effects of AICAR and exercise on insulin-stimulated glucose uptake, signaling, and GLUT-4 content in rat muscles. J Appl Physiol 2003;94(4):1373-9.
226. Fisher JS, Gao J, Han DH, Holloszy JO, Nolte LA. Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. Am J Physiol Endocrinol Metab 2002;282(1):E18-23.
227. Iglesias MA, Ye JM, Frangioudakis G, Saha AK, Tomas E, Ruderman NB, Cooney GJ, Kraegen EW. AICAR administration causes an apparent enhancement of muscle and liver insulin action in insulin-resistant high-fat-fed rats. Diabetes 2002;51(10):2886-94.
228. Arias EB, Kim J, Funai K, Cartee GD. Prior exercise increases phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle. Am J Physiol Endocrinol Metab 2007;292(4):E1191-200.
229. Chen S, Murphy J, Toth R, Campbell DG, Morrice NA, Mackintosh C. Complementary regulation of TBC1D1 and AS160 by growth factors, insulin and AMPK activators. Biochem J 2008;409(2):449-59.
230. Taylor EB, An D, Kramer HF, Yu H, Fujii NL, Roeckl KS, Bowles N, Hirshman MF, Xie J, Feener EP, Goodyear LJ. Discovery of TBC1D1 as an insulin-, AICAR-, and contraction-stimulated signaling nexus in mouse skeletal muscle. J Biol Chem 2008;283(15):9787-96.
231. Stockli J, Davey JR, Hohnen-Behrens C, Xu A, James DE, Ramm G. Regulation of glucose transporter 4 translocation by the Rab guanosine triphosphatase-activating protein AS160/TBC1D4: role of phosphorylation and membrane association. Mol Endocrinol 2008;22(12):2703-15.
232. Lange Y, Swaisgood MH, Ramos BV, Steck TL. Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. J Biol Chem 1989;264(7):3786-93.
233. Lange Y, Steck TL. Cholesterol homeostasis. Modulation by amphiphiles. J Biol Chem 1994;269(47):29371-4.
234. Thewke D, Kramer M, Sinensky MS. Transcriptional homeostatic control of membrane lipid composition. Biochem Biophys Res Commun 2000;273(1):1-4.
235. Almeida PF, Vaz WL, Thompson TE. Percolation and diffusion in three-component lipid bilayers: effect of cholesterol on an equimolar mixture of two phosphatidylcholines. Biophys J 1993;64(2):399-412.

236. Ahmed SN, Brown DA, London E. On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. Biochemistry 1997;36(36):10944-53.
237. Yeagle PL. Cholesterol and the cell membrane. Biochim Biophys Acta 1985;822(3-4):267-87.
238. Schroeder F, Woodford JK, Kavecansky J, Wood WG, Joiner C. Cholesterol domains in biological membranes. Mol Membr Biol 1995;12(1):113-9.
239. Simons K, Ikonen E. Functional rafts in cell membranes. Nature 1997;387(6633):569-72.
240. Maxfield FR, Wustner D. Intracellular cholesterol transport. J Clin Invest 2002;110(7):891-8.
241. Lange Y, Ye J, Steck TL. Circulation of cholesterol between lysosomes and the plasma membrane. J Biol Chem 1998;273(30):18915-22.
242. Lange Y. Disposition of intracellular cholesterol in human fibroblasts. J Lipid Res 1991;32(2):329-39.
243. Lange Y, Steck TL. Quantitation of the pool of cholesterol associated with acyl-CoA:cholesterol acyltransferase in human fibroblasts. J Biol Chem 1997;272(20):13103-8.
244. Lange Y, Ye J, Rigney M, Steck TL. Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol. J Lipid Res 1999;40(12):2264-70.
245. Ikonen E. Cellular cholesterol trafficking and compartmentalization. Nat Rev Mol Cell Biol 2008;9(2):125-38.
246. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990;343(6257):425-30.
247. Brown MS, Goldstein JL. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc Natl Acad Sci U S A 1999;96(20):11041-8.
248. Lange Y, Echevarria F, Steck TL. Movement of zymosterol, a precursor of cholesterol, among three membranes in human fibroblasts. J Biol Chem 1991;266(32):21439-43.
249. Lange Y, Steck TL. The role of intracellular cholesterol transport in cholesterol homeostasis. Trends Cell Biol 1996;6(6):205-8.
250. Radhakrishnan A, Goldstein JL, McDonald JG, Brown MS. Switch-like control of SREBP-2 transport triggered by small changes in ER cholesterol: a delicate balance. Cell Metab 2008;8(6):512-21.
251. Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, Goldstein JL, Brown MS. Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell 2002;110(4):489-500.
252. Sever N, Yang T, Brown MS, Goldstein JL, DeBose-Boyd RA. Accelerated degradation of HMG CoA reductase mediated by binding of insig-1 to its sterol-sensing domain. Mol Cell 2003;11(1):25-33.

253. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 1997;89(3):331-40.
254. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 2002;109(9):1125-31.
255. Nohturfft A, Yabe D, Goldstein JL, Brown MS, Espenshade PJ. Regulated step in cholesterol feedback localized to budding of SCAP from ER membranes. Cell 2000;102(3):315-23.
256. Espenshade PJ, Li WP, Yabe D. Sterols block binding of COPII proteins to SCAP, thereby controlling SCAP sorting in ER. Proc Natl Acad Sci U S A 2002;99(18):11694-9.
257. Cheng D, Espenshade PJ, Slaughter CA, Jaen JC, Brown MS, Goldstein JL. Secreted site-1 protease cleaves peptides corresponding to luminal loop of sterol regulatory element-binding proteins. J Biol Chem 1999;274(32):22805-12.
258. Espenshade PJ, Cheng D, Goldstein JL, Brown MS. Autocatalytic processing of site-1 protease removes propeptide and permits cleavage of sterol regulatory element-binding proteins. J Biol Chem 1999;274(32):22795-804.
259. Zelenski NG, Rawson RB, Brown MS, Goldstein JL. Membrane topology of S2P, a protein required for intramembranous cleavage of sterol regulatory element-binding proteins. J Biol Chem 1999;274(31):21973-80.
260. Brown MS, Faust JR, Goldstein JL, Kaneko I, Endo A. Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase. J Biol Chem 1978;253(4):1121-8.
261. Nakanishi M, Goldstein JL, Brown MS. Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. J Biol Chem 1988;263(18):8929-37.
262. Goldstein JL, DeBose-Boyd RA, Brown MS. Protein sensors for membrane sterols. Cell 2006;124(1):35-46.
263. Foretz M, Guichard C, Ferre P, Foufelle F. Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. Proc Natl Acad Sci U S A 1999;96(22):12737-42.
264. Guillet-Deniau I, Mieulet V, Le Lay S, Achouri Y, Carre D, Girard J, Foufelle F, Ferre P. Sterol regulatory element binding protein-1c expression and action in rat muscles: insulin-like effects on the control of glycolytic and lipogenic enzymes and UCP3 gene expression. Diabetes 2002;51(6):1722-8.
265. Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, Solanes G, Lowell BB, Spiegelman BM. Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. J Clin Invest 1998;101(1):1-9.

266. Sewter C, Berger D, Considine RV, Medina G, Rochford J, Ciaraldi T, Henry R, Dohm L, Flier JS, O'Rahilly S, Vidal-Puig AJ. Human obesity and type 2 diabetes are associated with alterations in SREBP1 isoform expression that are reproduced ex vivo by tumor necrosis factor-alpha. Diabetes 2002;51(4):1035-41.
267. Boizard M, Le Liepvre X, Lemarchand P, Foufelle F, Ferre P, Dugail I. Obesity-related overexpression of fatty-acid synthase gene in adipose tissue involves sterol regulatory element-binding protein transcription factors. J Biol Chem 1998;273(44):29164-71.
268. Kakuma T, Lee Y, Higa M, Wang Z, Pan W, Shimomura I, Unger RH. Leptin, troglitazone, and the expression of sterol regulatory element binding proteins in liver and pancreatic islets. Proc Natl Acad Sci U S A 2000;97(15):8536-41.
269. Shimomura I, Bashmakov Y, Horton JD. Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. J Biol Chem 1999;274(42):30028-32.
270. Eberle D, Hegarty B, Bossard P, Ferre P, Foufelle F. SREBP transcription factors: master regulators of lipid homeostasis. Biochimie 2004;86(11):839-48.
271. Ingebritsen TS, Geelen MJ, Parker RA, Evenson KJ, Gibson DM. Modulation of hydroxymethylglutaryl-CoA reductase activity, reductase kinase activity, and cholesterol synthesis in rat hepatocytes in response to insulin and glucagon. J Biol Chem 1979;254(20):9986-9.
272. Brown MS, Brunschede GY, Goldstein JL. Inactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in vitro. An adenine nucleotide-dependent reaction catalyzed by a factor in human fibroblasts. J Biol Chem 1975;250(7):2502-9.
273. Hardie DG. Metabolic control: a new solution to an old problem. Curr Biol 2000;10(20):R757-9.
274. Song BL, Javitt NB, DeBose-Boyd RA. Insig-mediated degradation of HMG CoA reductase stimulated by lanosterol, an intermediate in the synthesis of cholesterol. Cell Metab 2005;1(3):179-89.
275. Chang TY, Chang CC, Cheng D. Acyl-coenzyme A:cholesterol acyltransferase. Annu Rev Biochem 1997;66:613-38.
276. Oram JF, Lawn RM, Garvin MR, Wade DP. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. J Biol Chem 2000;275(44):34508-11.
277. Lorkowski S, Kratz M, Wenner C, Schmidt R, Weitkamp B, Fobker M, Reinhardt J, Rauterberg J, Galinski EA, Cullen P. Expression of the ATP-binding cassette transporter gene ABCG1 (ABC8) in Tangier disease. Biochem Biophys Res Commun 2001;283(4):821-30.
278. Song C, Kokontis JM, Hiipakka RA, Liao S. Ubiquitous receptor: a receptor that modulates gene activation by retinoic acid and thyroid hormone receptors. Proc Natl Acad Sci U S A 1994;91(23):10809-13.

279. Fu X, Menke JG, Chen Y, Zhou G, MacNaul KL, Wright SD, Sparrow CP, Lund EG. 27-hydroxycholesterol is an endogenous ligand for liver X receptor in cholesterol-loaded cells. J Biol Chem 2001;276(42):38378-87.
280. Spencer TA, Li D, Russel JS, Collins JL, Bledsoe RK, Consler TG, Moore LB, Galardi CM, McKee DD, Moore JT, Watson MA, Parks DJ, Lambert MH, Willson TM. Pharmacophore analysis of the nuclear oxysterol receptor LXRalpha. J Med Chem 2001;44(6):886-97.
281. Edwards PA, Kast HR, Anisfeld AM. BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis. J Lipid Res 2002;43(1):2-12.
282. Reaven G. Insulin resistance, type 2 diabetes mellitus, and cardiovascular disease: the end of the beginning. Circulation 2005;112(20):3030-2.
283. Hebert LF, Jr., Daniels MC, Zhou J, Crook ED, Turner RL, Simmons ST, Neidigh JL, Zhu JS, Baron AD, McClain DA. Overexpression of glutamine:fructose-6-phosphate amidotransferase in transgenic mice leads to insulin resistance. J Clin Invest 1996;98(4):930-6.
284. Nelson BA, Robinson KA, Koning JS, Buse MG. Effects of exercise and feeding on the hexosamine biosynthetic pathway in rat skeletal muscle. Am J Physiol 1997;272(5 Pt 1):E848-55.
285. Hawkins M, Barzilai N, Liu R, Hu M, Chen W, Rossetti L. Role of the glucosamine pathway in fat-induced insulin resistance. J Clin Invest 1997;99(9):2173-82.
286. Marshall S, Bacote V, Traxinger RR. Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. J Biol Chem 1991;266(8):4706-12.
287. Traxinger RR, Marshall S. Coordinated regulation of glutamine:fructose-6-phosphate amidotransferase activity by insulin, glucose, and glutamine. Role of hexosamine biosynthesis in enzyme regulation. J Biol Chem 1991;266(16):10148-54.
288. Wells L, Vosseller K, Hart GW. Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc. Science 2001;291(5512):2376-8.
289. Dong DL, Hart GW. Purification and characterization of an O-GlcNAc selective N-acetyl-beta-D-glucosaminidase from rat spleen cytosol. J Biol Chem 1994;269(30):19321-30.
290. Gao Y, Wells L, Comer FI, Parker GJ, Hart GW. Dynamic O-glycosylation of nuclear and cytosolic proteins: cloning and characterization of a neutral, cytosolic beta-N-acetylglucosaminidase from human brain. J Biol Chem 2001;276(13):9838-45.
291. Slawson C, Housley MP, Hart GW. O-GlcNAc cycling: how a single sugar post-translational modification is changing the way we think about signaling networks. J Cell Biochem 2006;97(1):71-83.
292. Hart GW, Housley MP, Slawson C. Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. Nature 2007;446(7139):1017-22.

293. Whisenhunt TR, Yang X, Bowe DB, Paterson AJ, Van Tine BA, Kudlow JE. Disrupting the enzyme complex regulating O-GlcNAcylation blocks signaling and development. Glycobiology 2006;16(6):551-63.
294. Kreppel LK, Hart GW. Regulation of a cytosolic and nuclear O-GlcNAc transferase. Role of the tetratricopeptide repeats. J Biol Chem 1999;274(45):32015-22.
295. Song M, Kim HS, Park JM, Kim SH, Kim IH, Ryu SH, Suh PG. o-GlcNAc transferase is activated by CaMKIV-dependent phosphorylation under potassium chloride-induced depolarization in NG-108-15 cells. Cell Signal 2008;20(1):94-104.
296. Buse MG. Hexosamines, insulin resistance, and the complications of diabetes: current status. Am J Physiol Endocrinol Metab 2006;290(1):E1-E8.
297. Heart E, Choi WS, Sung CK. Glucosamine-induced insulin resistance in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 2000;278(1):E103-12.
298. Rossetti L, Hawkins M, Chen W, Gindi J, Barzilai N. In vivo glucosamine infusion induces insulin resistance in normoglycemic but not in hyperglycemic conscious rats. J Clin Invest 1995;96(1):132-40.
299. Bhonagiri P, Pattar GR, Horvath EM, Habegger KM, McCarthy AM, Elmendorf JS. Hexosamine Biosynthesis Pathway Flux Contributes to Insulin Resistance Via Altering Membrane Pip2 and Cortical F-Actin. Endocrinology 2008.
300. Tang J, Neidigh JL, Cooksey RC, McClain DA. Transgenic mice with increased hexosamine flux specifically targeted to beta-cells exhibit hyperinsulinemia and peripheral insulin resistance. Diabetes 2000;49(9):1492-9.
301. McClain DA, Alexander T, Cooksey RC, Considine RV. Hexosamines stimulate leptin production in transgenic mice. Endocrinology 2000;141(6):1999-2002.
302. McClain DA, Lubas WA, Cooksey RC, Hazel M, Parker GJ, Love DC, Hanover JA. Altered glycan-dependent signaling induces insulin resistance and hyperleptinemia. Proc Natl Acad Sci U S A 2002;99(16):10695-9.
303. Vosseller K, Wells L, Lane MD, Hart GW. Elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation in 3T3-L1 adipocytes. Proc Natl Acad Sci U S A 2002;99(8):5313-8.
304. Hu Y, Belke D, Suarez J, Swanson E, Clark R, Hoshijima M, Dillmann WH. Adenovirus-mediated overexpression of O-GlcNAcase improves contractile function in the diabetic heart. Circ Res 2005;96(9):1006-13.
305. Yang X, Ongusaha PP, Miles PD, Havstad JC, Zhang F, So WV, Kudlow JE, Michell RH, Olefsky JM, Field SJ, Evans RM. Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance. Nature 2008;451(7181):964-9.

306. Wells L, Kreppel LK, Comer FI, Wadzinski BE, Hart GW. O-GlcNAc transferase is in a functional complex with protein phosphatase 1 catalytic subunits. J Biol Chem 2004;279(37):38466-70.
307. Turner N, Bruce CR, Beale SM, Hoehn KL, So T, Rolph MS, Cooney GJ. Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid oxidation in lipid-induced insulin resistance in rodents. Diabetes 2007;56(8):2085-92.
308. de Leeuw van Weenen JE, Hu L, Jansen-Van Zelm K, de Vries MG, Tamsma JT, Romijn JA, Pijl H. Four weeks high fat feeding induces insulin resistance without affecting dopamine release or gene expression patterns in the hypothalamus of C57Bl6 mice. Brain Res 2009;1250:141-8.
309. Kim F, Pham M, Maloney E, Rizzo NO, Morton GJ, Wisse BE, Kirk EA, Chait A, Schwartz MW. Vascular inflammation, insulin resistance, and reduced nitric oxide production precede the onset of peripheral insulin resistance. Arterioscler Thromb Vasc Biol 2008;28(11):1982-8.
310. Sturek M, Wenzel J, Byrd JP, Edwards JM, Lloyd PG, Tune JD, March KL, Miller MA, Mokelke EA, Brisbin IL. Ossabaw island miniture swine: cardiometabolic syndrome assessment. In: Swindle M, editor. Swine in the laboratory: Surgery, anesthesia, imaging and experimental techniques. Boca Raton: CRC Press; 2007. p. 397-402.
311. Dyson MC, Alloosh M, Vuchetich JP, Mokelke EA, Sturek M. Components of metabolic syndrome and coronary artery disease in female Ossabaw swine fed excess atherogenic diet. Comp Med 2006;56(1):35-45.
312. Aas V, Rokling-Andersen M, Wensaas AJ, Thoresen GH, Kase ET, Rustan AC. Lipid metabolism in human skeletal muscle cells: effects of palmitate and chronic hyperglycaemia. Acta Physiol Scand 2005;183(1):31-41.
313. Vaccaro O, Mancini FP, Ruffa G, Sabatino L, Iovine C, Masulli M, Colantuoni V, Riccardi G. Fasting plasma free fatty acid concentrations and Pro12Ala polymorphism of the peroxisome proliferator-activated receptor (PPAR) gamma2 gene in healthy individuals. Clin Endocrinol (Oxf) 2002;57(4):481-6.
314. Kim F, Tysseling KA, Rice J, Pham M, Haji L, Gallis BM, Baas AS, Paramsothy P, Giachelli CM, Corson MA, Raines EW. Free fatty acid impairment of nitric oxide production in endothelial cells is mediated by IKKbeta. Arterioscler Thromb Vasc Biol 2005;25(5):989-94.
315. Bhonagiri P, Pattar GR, Horvath EM, Habegger KM, McCarthy AM, Elmendorf JS. Hexosamine biosynthesis pathway flux contributes to insulin resistance via altering membrane phosphatidylinositol 4,5-bisphosphate and cortical filamentous actin. Endocrinology 2009;150(4):1636-45.
316. McCarthy AM, Spisak KO, Brozinick JT, Elmendorf JS. Loss of cortical actin filaments in insulin-resistant skeletal muscle cells impairs GLUT4 vesicle trafficking and glucose transport. Am J Physiol Cell Physiol 2006;291(5):C860-8.

317. Krook A, Bjornholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG, Jr., Wallberg-Henriksson H, Zierath JR. Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. Diabetes 2000;49(2):284-92.
318. Chen G, Liu P, Thurmond DC, Elmendorf JS. Glucosamine-induced insulin resistance is coupled to O-linked glycosylation of Munc18c. FEBS Lett 2003;534(1-3):54-60.
319. Kralik SF, Liu P, Leffler BJ, Elmendorf JS. Ceramide and glucosamine antagonism of alternate signaling pathways regulating insulin- and osmotic shock-induced glucose transporter 4 translocation. Endocrinology 2002;143(1):37-46.
320. Hawkins M, Angelov I, Liu R, Barzilai N, Rossetti L. The tissue concentration of UDP-N-acetylglucosamine modulates the stimulatory effect of insulin on skeletal muscle glucose uptake. J Biol Chem 1997;272(8):4889-95.
321. Srinivasan V, Tatu U, Mohan V, Balasubramanyam M. Molecular convergence of hexosamine biosynthetic pathway and ER stress leading to insulin resistance in L6 skeletal muscle cells. Mol Cell Biochem 2009;328(1-2):217-24.
322. Bhonagiri P, Pattar GR, Horvath EM, Habegger KM, McCarthy AM, Elmendorf JS. Hexosamine Biosynthesis Pathway Flux Contributes to Insulin Resistance Via Altering Membrane Pip2 and Cortical F-Actin. Endocrinology 2008;150:1636-1645.
323. Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M. Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. Proc Natl Acad Sci U S A 2000;97(22):12222-6.
324. Goldberg HJ, Scholey J, Fantus IG. Glucosamine activates the plasminogen activator inhibitor 1 gene promoter through Sp1 DNA binding sites in glomerular mesangial cells. Diabetes 2000;49(5):863-71.
325. Han I, Kudlow JE. Reduced O glycosylation of Sp1 is associated with increased proteasome susceptibility. Mol Cell Biol 1997;17(5):2550-8.
326. Cagen LM, Deng X, Wilcox HG, Park EA, Raghow R, Elam MB. Insulin activates the rat sterol-regulatory-element-binding protein 1c (SREBP-1c) promoter through the combinatorial actions of SREBP, LXR, Sp-1 and NF-Y cis-acting elements. Biochem J 2005;385(Pt 1):207-16.
327. Deng X, Yellaturu C, Cagen L, Wilcox HG, Park EA, Raghow R, Elam MB. Expression of the rat sterol regulatory element-binding protein-1c gene in response to insulin is mediated by increased transactivating capacity of specificity protein 1 (Sp1). J Biol Chem 2007;282(24):17517-29.
328. Khayat ZA, Tsakiridis T, Ueyama A, Somwar R, Ebina Y, Klip A. Rapid stimulation of glucose transport by mitochondrial uncoupling depends in part on cytosolic Ca2+ and cPKC. Am J Physiol 1998;275(6 Pt 1):C1487-97.

329. Omata W, Shibata H, Li L, Takata K, Kojima I. Actin filaments play a critical role in insulin-induced exocytotic recruitment but not in endocytosis of GLUT4 in isolated rat adipocytes. Biochem J 2000;346(Pt 2):321-8.
330. Wang Q, Bilan PJ, Tsakiridis T, Hinek A, Klip A. Actin filaments participate in the relocalization of phosphatidylinositol3-kinase to glucose transporter-containing compartments and in the stimulation of glucose uptake in 3T3-L1 adipocytes. Biochem J 1998;331 (Pt 3):917-28.
331. Li Y, Soos TJ, Li X, Wu J, Degennaro M, Sun X, Littman DR, Birnbaum MJ, Polakiewicz RD. Protein kinase C Theta inhibits insulin signaling by phosphorylating IRS1 at Ser(1101). J Biol Chem 2004;279(44):45304-7.
332. Keranen LM, Dutil EM, Newton AC. Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. Curr Biol 1995;5(12):1394-1403.
333. Hoehn KL, Hohnen-Behrens C, Cederberg A, Wu LE, Turner N, Yuasa T, Ebina Y, James DE. IRS1-independent defects define major nodes of insulin resistance. Cell Metab 2008;7(5):421-33.
334. Blot V, McGraw TE. GLUT4 is internalized by a cholesterol-dependent nystatin-sensitive mechanism inhibited by insulin. EMBO J 2006;25(24):5648-58.
335. Shigematsu S, Watson RT, Khan AH, Pessin JE. The adipocyte plasma membrane caveolin functional/structural organization is necessary for the efficient endocytosis of GLUT4. J Biol Chem 2003;278(12):10683-90.
336. Kim F, Pham M, Maloney E, Rizzo NO, Morton GJ, Wisse BE, Kirk EA, Chait A, Schwartz MW. Vascular Inflammation, Insulin Resistance, and Reduced Nitric Oxide Production Precede the Onset of Peripheral Insulin Resistance. Arterioscler Thromb Vasc Biol 2008.
337. Strawbridge AB, Elmendorf JS. Phosphatidylinositol 4,5-bisphosphate reverses endothelin-1-induced insulin resistance via an actin-dependent mechanism. Diabetes 2005;54(6):1698-705.
338. Strawbridge AB, Elmendorf JS. Endothelin-1 impairs glucose transporter trafficking via a membrane-based mechanism. J Cell Biochem 2006;97(4):849-56.
339. Srinivasan V, Sandhya N, Sampathkumar R, Farooq S, Mohan V, Balasubramanyam M. Glutamine fructose-6-phosphate amidotransferase (GFAT) gene expression and activity in patients with type 2 diabetes: inter-relationships with hyperglycaemia and oxidative stress. Clin Biochem 2007;40(13-14):952-7.
340. Yki-Jarvinen H, Daniels MC, Virkamaki A, Makimattila S, DeFronzo RA, McClain D. Increased glutamine:fructose-6-phosphate amidotransferase activity in skeletal muscle of patients with NIDDM. Diabetes 1996;45(3):302-7.
341. Werstuck GH, Khan MI, Femia G, Kim AJ, Tedesco V, Trigatti B, Shi Y. Glucosamine-induced endoplasmic reticulum dysfunction is associated with accelerated atherosclerosis in a hyperglycemic mouse model. Diabetes 2006;55(1):93-101.

342. Wang J, Liu R, Hawkins M, Barzilai N, Rossetti L. A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. Nature 1998;393(6686):684-8.
343. Hasty AH, Shimano H, Yahagi N, Amemiya-Kudo M, Perrey S, Yoshikawa T, Osuga J, Okazaki H, Tamura Y, Iizuka Y, Shionoiri F, Ohashi K, Harada K, Gotoda T, Nagai R, Ishibashi S, Yamada N. Sterol regulatory element-binding protein-1 is regulated by glucose at the transcriptional level. J Biol Chem 2000;275(40):31069-77.
344. Mu J, Brozinick JT, Jr., Valladares O, Bucan M, Birnbaum MJ. A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. Mol Cell 2001;7(5):1085-94.
345. Fisher JS. Potential Role of the AMP-activated Protein Kinase in Regulation of Insulin Action. Cellscience 2006;2(3):68-81.
346. Fujii N, Jessen N, Goodyear LJ. AMP-activated protein kinase and the regulation of glucose transport. Am J Physiol Endocrinol Metab 2006;291(5):E867-77.
347. Treebak JT, Glund S, Deshmukh A, Klein DK, Long YC, Jensen TE, Jorgensen SB, Viollet B, Andersson L, Neumann D, Wallimann T, Richter EA, Chibalin AV, Zierath JR, Wojtaszewski JF. AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. Diabetes 2006;55(7):2051-8.
348. Bruss MD, Arias EB, Lienhard GE, Cartee GD. Increased phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. Diabetes. 2005;54(1):41-50.
349. Kramer HF, Witczak CA, Fujii N, Jessen N, Taylor EB, Arnolds DE, Sakamoto K, Hirshman MF, Goodyear LJ. Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. Diabetes 2006;55(7):2067-76.
350. Awazawa M, Ueki K, Inabe K, Yamauchi T, Kaneko K, Okazaki Y, Bardeesy N, Ohnishi S, Nagai R, Kadowaki T. Adiponectin suppresses hepatic SREBP1c expression in an AdipoR1/LKB1/AMPK dependent pathway. Biochem Biophys Res Commun 2009;382(1):51-6.
351. Yang J, Maika S, Craddock L, King JA, Liu ZM. Chronic activation of AMP-activated protein kinase-alpha1 in liver leads to decreased adiposity in mice. Biochem Biophys Res Commun 2008;370(2):248-53.
352. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001;108(8):1167-74.
353. Kaplan MR, Simoni RD. Transport of cholesterol from the endoplasmic reticulum to the plasma membrane. J Cell Biol 1985;101(2):446-53.
354. Liscum L, Munn NJ. Intracellular cholesterol transport. Biochim Biophys Acta 1999;1438(1):19-37.
355. DeGrella RF, Simoni RD. Intracellular transport of cholesterol to the plasma membrane. J Biol Chem 1982;257(23):14256-62.

356. Antonescu CN, Diaz M, Femia G, Planas JV, Klip A. Clathrin-dependent and independent endocytosis of glucose transporter 4 (GLUT4) in myoblasts: regulation by mitochondrial uncoupling. Traffic 2008;9(7):1173-90.
357. Zha X, Pierini LM, Leopold PL, Skiba PJ, Tabas I, Maxfield FR. Sphingomyelinase treatment induces ATP-independent endocytosis. J Cell Biol 1998;140(1):39-47.
358. Skiba PJ, Zha X, Maxfield FR, Schissel SL, Tabas I. The distal pathway of lipoprotein-induced cholesterol esterification, but not sphingomyelinase-induced cholesterol esterification, is energy-dependent. J Biol Chem 1996;271(23):13392-400.
359. Wojtanik KM, Liscum L. The transport of low density lipoprotein-derived cholesterol to the plasma membrane is defective in NPC1 cells. J Biol Chem 2003;278(17):14850-6.
360. Garvey WT, Maianu L, Zhu JH, Brechtel-Hook G, Wallace P, Baron AD. Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. J Clin Invest 1998;101(11):2377-86.
361. van Putten JP, Krans HM. Glucose as a regulator of insulin-sensitive hexose uptake in 3T3 adipocytes. J Biol Chem 1985;260(13):7996-8001.
362. Elmendorf JS. Fractionation analysis of the subcellular distribution of GLUT-4 in 3T3-L1 adipocytes. Methods Mol Med 2003;83:105-11.
363. Dugail I. Transfection of adipocytes and preparation of nuclear extracts. Methods Mol Biol 2001;155:141-6.

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Indiana University, Indianapolis, Indiana
Ph.D. Biochemistry and Molecular Biology
Concentration: Diabetes and Obesity
Thesis: Membrane Cholesterol Balance in Exercise and Insulin Resistance

1998-2002
Indiana University, Bloomington, Indiana
B.S. Biochemistry, minor in Biology

Work Experience

2002-2005
Research Technician, Alcoholism Research Department, Indiana University School of Medicine

Honors and Awards

2001
Dean's List, Indiana University, Bloomington, Indiana

2007
Jack Davis Award (best student seminar), Department of Biochemistry and Molecular Biology, Indiana University School of Medicine

Grants and Fellowships

Indiana University Diabetes & Obesity Research Training Program DeVault Fellowship

Publications

Carr LG, **Habegger K**, Spence JP, Liu L, Lumeng L, Foroud T. Development of congenic rat strains for alcohol consumption derived from the alcohol-preferring and nonpreferring rats. Behav Genet. 2006 Mar; 36(2):285-90. Epub 2006 Feb 10.

Spence JP, Liang T, **Habegger K**, Carr LG. Effect of polymorphism on expression of the neuropeptide Y gene in inbred alcohol-preferring and -nonpreferring rats. Neuroscience. 2005;131(4):871-6.

Liang T, **Habegger K**, Spence JP, Foroud T, Ellison JA, Lumeng L, Li TK, Carr LG. Glutathione S-transferase 8-8 expression is lower in alcohol-preferring than in alcohol-nonpreferring rats. Alcohol Clin Exp Res. 2004 Nov;28(11):1622-8.

Carr LG, **Habegger K**, Spence J, Ritchotte A, Liu L, Lumeng L, Li TK, and Foroud T. Analyses of Quantitative Trait Loci Contributing to Alcohol Preference in HAD1/LAD1 and HAD2/LAD2 Rats. Alcohol Clin Exp Res (11):1710-7, 2003.

Bhonagiri P, Horvath EM, Pattar GR, **Habegger KM**, McCarthy AM, and Elmendorf JS. Hexosamine Biosynthesis Pathway Flux Contributes to Insulin Resistance via Altering Membrane PIP_2 and Cortical F-Actin. Endocrinology (150): 1636-45, 2008

Habegger KM, Tackett L, Sealls W, Bell LN, Awad MY, Blue E, Gallagher PJ, Sturek M, Alloosh MA, Steinberg H, Considine R, and Elmendorf JS. Fatty Acid-Induced Plasma Membrane Cholesterol Accrual and Insulin Resistance. (Under editorial review)

Habegger KM and Elmendorf JS. 5'-AMP Dependent Protein Kinase Positively Influences the Insulin-Regulated Glucose Transport System in L6 Myotubes via Lowering Membrane Cholesterol. (Under editorial review)

Habegger KM*, Pattar GR*, Bhonagiri P, and Elmendorf JS. Plasma Membrane Cholesterol Accrual Elicited by Increased Hexosamine Biosynthesis Promotes Insulin Resistance in Fat and Skeletal Muscle Cells (In Preparation) * co-first authorship.

Abstracts

Carr LG, **Habegger K**, Liu L, Lumeng L, Li TK, and Foroud T. Development of Congenic Rat Strains for Alcohol Preference. (RSA, 2004)

Liang TB, **Habegger K**, Spence J, Ellison JA, Lumeng L, Li TK, and Carr LG. Glutathione-S-Tansferase 8 Expression is Lower in Alcohol -Preferring than in -Nonpreferring Rats. (RSA, 2004)

Liu L, Spence J, **Habegger K**, Lumeng L, Li TK, Foroud T, Carr LG. Quantitative trait loci influencing alcohol consumption in the high alcohol drinking and low alcohol drinking rats were confirmed in the replicate rat lines. (RSA, 2003).

Habegger KM, Horvath E, and Elmendorf JS. DNP-Stimulated GLUT4 Translocation via 5'-AMP Activated Protein Kinase is Dependent on Plasma Membrane Cholesterol and Potentially Coupled Phosphoinositide-Regulated Actin Polymerization Events. Medicine & Science in Sports & Exercise. 38(11) Suppl 1:S8, November 2006.

Habegger KM, McCarthy AM, Brozinick JT, and Elmendorf JS. Membrane and Cytoskeletal Dysfunction as a Novel Basis of Lipid-Induced Insulin Resistance. (ADA, 2006)

Habegger KM, Horvath EM, and Elmendorf JS. DNP-Stimulated GLUT4 Translocation via 5'-AMP Activated Protein Kinase is Dependent on Plasma Membrane Cholesterol and Potentially Coupled Phosphoinositide-Regulated Actin Polymerization Events. (FASEB-AMPK, 2006)

Habegger KM and Elmendorf JS. Activators of 5'-AMP Activated Protein Kinase Stimulate GLUT4 Translocation via a Plasma Membrane Cholesterol Dependent Event. (FASEB-Glucose Transporters, 2007)

Habegger KM, Tackett L, Bell LN, Brozinick JT, Gallagher PJ, Blue E, Sturek M, and Elmendorf JS. Evidence That Insulin-Resistant/Cholesterol-Laden Plasma Membrane Results From Hyperlipidemia in L6-Myotubes. (ADA, 2008)

Habegger KM and Elmendorf JS. AMPK Enhances Insulin and GLUT4 action in L6 Myotubes via Lowering Plasma Membrane Cholesterol. (ADA, 2009)

Pattar GR, **Habegger KM**, Bhonagiri P, and Elmendorf JS. Plasma Membrane Cholesterol Accrual Elicited by Increased Hexosamine Biosynthesis Promotes Insulin Resistance in Fat and Skeletal Muscle Cells. (ADA, 2009)

Research Experience

Preparation and analysis of Protein:
SDS-PAGE, Western blot analysis, whole-cell immunofluorescence, immunoprecipitation, fluorescent and confocal microscopy in cultured cells and intact tissue, subcellular fractionation
Preparation and analysis of DNA and RNA:
PCR, RT-PCR, qRT-PCR, sequencing, microsatellite mapping, agarose and acrylamide gel electrophoresis, plasmid isolation, restriction digest, ligation, vector construction, isolation of genomic DNA from various tissues, nucleic acid precipitation, *In situ* hybridization
Cell culture:
Growth of neural (SK-N-SH) and skeletal muscle (L6 & C2C12) cell lines, transient plasmid transfection, luciferase and renilla assays, transient siRNA knockdown, 2DG uptake
Animal Studies:
Development and maintenance of congenic breeding colonies, dissection of rodent brain, liver, heart, kidney, skeletal muscle, and fat pads.