THE EFFECTS OF CHROMIUM ON SKELETAL MUSCLE MEMBRANE/CYTOSKELETAL PARAMETERS AND INSULIN SENSITIVITY

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Dedication

This dissertation is dedicated to my family. I would not be where I am today without the continued love, support and guidance I have received from my family. I would like to thank my mother and father for always being there for me and encouraging me to work up to my potential. They have been wonderful role models who have taught me to have a strong work ethic, integrity, compassion for others and a passion for my career. I would also like to thank my brother, sister and extended family for always being there for me and being great sources of advice, encouragement and support throughout the years.

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Abstract

Nolan John Hoffman

THE EFFECTS OF CHROMIUM ON SKELETAL MUSCLE MEMBRANE/CYTOSKELETAL PARAMETERS AND INSULIN SENSITIVITY

A recent review of randomized controlled trials found that trivalent chromium (Cr^{3+}) supplementation significantly improved glycemia among patients with diabetes, consistent with a long-standing appreciation that this micronutrient optimizes carbohydrate metabolism. Nevertheless, a clear limitation in the current evidence is a lack of understanding of Cr^{3+} action. We tested if increased AMP-activated protein kinase (AMPK) activity, previously observed in Cr^{3+}-treated cells or tissues from Cr^{3+}-supplemented animals, mediates improved glucose transport regulation under insulin-resistant hyperinsulinemic conditions. In L6 myotubes stably expressing the glucose transporter GLUT4 carrying an exofacial *myc*-epitope tag, acute insulin stimulation increased GLUT4*myc* translocation by 69% and glucose uptake by 97%. In contrast, the hyperinsulinemic state impaired insulin stimulation of these processes. Consistent with Cr^{3+}'s beneficial effect on glycemic status, chromium picolinate (CrPic) restored insulin's ability to fully regulate GLUT4*myc* translocation and glucose transport. Insulin-resistant myotubes did not display impaired insulin signaling, nor did CrPic amplify insulin signaling. However, CrPic normalized elevated membrane cholesterol that impaired cortical filamentous actin (F-actin)

structure. Mechanistically, data support that CrPic lowered membrane cholesterol via AMPK. Consistent with this data, siRNA-mediated AMPK silencing blocked CrPic's beneficial effects on GLUT4 and glucose transport regulation. Furthermore, the AMPK agonist 5-aminoimidazole-4-carboxamide-1-ß-D-ribonucleoside (AICAR) protected against hyperinsulinemia-induced membrane/cytoskeletal defects and GLUT4 dysregulation. To next test Cr^{3+} action *in vivo*, we utilized obesity-prone C57Bl/6J mice fed a low fat (LF) or high fat (HF) diet for eight weeks without or with CrPic supplementation administered in the drinking water (8 µg/kg/day). HF feeding increased body weight beginning four weeks after diet intervention regardless of CrPic supplementation and was independent of changes in food consumption. Early CrPic supplementation during a five week acclimation period protected against glucose intolerance induced by the subsequent eight weeks of HF feeding. As observed in other insulin-resistant animal models, skeletal muscle from HF-fed mice displayed membrane cholesterol accrual and loss of F-actin. Skeletal muscle from CrPic-supplemented HF-fed mice showed increased AMPK activity and protection against membrane cholesterol accrual and F-actin loss. Together these data suggest a mechanism by which Cr^{3+} may positively impact glycemic status, thereby stressing a plausible beneficial action of Cr^{3+} in glucose homeostasis.

Jeffrey S. Elmendorf, Ph.D., Chair

Table of Contents

List of Figures

Abbreviations

2-DG	2-deoxy-D-glucose
ABC	ATP-binding cassette transporter
ACAT	Acyl-coenzyme A:cholesterol acyltransferase
ACC	Acetyl-CoA carboxylase
ACTN4	Alpha-actinin-4
AICAR	5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleoside
AMP	5’ adenosine monophosphate
AMPK	5’ AMP-activated protein kinase
APS	Adaptor protein containing PH and SH domains
Arp2/3	Actin-related proteins 2/3
AS160	Akt substrate of 160-kDa
ATP	5’ adenosine triphosphate
ATV	Atorvastatin
AUC	Area under the curve
CaMKKβ	Calmodulin-dependent protein kinase kinase β
CAP	Cbl-associated protein
Cav-actin	Caveolin-associated filamentous actin
CBS	Cystathionine-β-synthase
CDK5	Cyclin-dependent kinase-5
Chol	Cholesterol
CoA	Coenzyme A
CPT-1	Carnitine palmitoyltransferase 1

Cr^{3+}	Trivalent chromium
Cr^{6+}	Hexavalent chromium
CrCIT	Chromium citrate
$CrCl_3$	Chromium chloride
$Cr(D\text{-}Phe)_3$	Chromium (D-phenylalanine)$_3$
CrN	Chromium nicotinate/niacin
CRP	C-reactive protein
CrPic	Chromium picolinate
CrY	Chromium yeast
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DNP	2,4-dinitrophenol
ER	Endoplasmic reticulum
FA	Fatty acid
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
F-actin	Filamentous actin
GAP	GTPase activating domain
GEF	GLUT4 enhancer factor
GFAT	Glutamine:fructose-6-phosphate amidotransferase
GLUT	Glucose transporter
GSV	GLUT4 storage vesicle
HBP	Hexosamine biosynthesis pathway

HDL	High density lipoprotein
HF	High fat
HMG-CoA	3-hydroxymethyl-3-glutaryl coenzyme A
HMGR	HMG-CoA reductase
IL-6	Interleukin-6
INSIG	Insulin-induced protein
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
IR	Insulin receptor
IRAP	Insulin-responsive amino peptidase
IRS	Insulin receptor substrate
IVGTT	Intravenous glucose tolerance test
kDa	Kilodalton
LDL	Low density lipoprotein
LF	Low fat
LXR	Liver X receptor
L6-GLUT4*myc*	L6 muscle cells stably expressing GLUT4 that carries an exofacial *myc*-epitope tag
MEF-2	Myocyte enhancer factor-2
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NRF1	Nuclear respiratory factor 1
OGA	O-GlcNAcase

OGT	O-linked N-acetylglucosamine transferase
PA	Phosphatidic acid
PAS	Phosho-Akt substrate
PBS	Phosphate buffered saline
PGC-1α	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5 bisphosphate
PIP_3	Phosphatidylinositol 3,4,5 triphosphate
PLD1	Phospholipase D1
PM	Plasma membrane
PP2A	Protein phosphatase 2A
RXR	Retinoic X receptor
SCAP	SREBP cleavage-activating protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SNAP23	Synaptosomal-associated protein 23
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
SRE	Sterol response element
SREBP	Sterol response element binding protein
T2D	Type 2 diabetes
TBC1D	Tre-2 BUB2 CDC16, 1 domain family member

TIRFM	Total internal reflection microscopy
TNFα	Tumor necrosis factor alpha
t-SNARE	Target SNARE
TUG	Tether containing UBX domain for GLUT4
Ubc9	Ubiquitin-conjugating enzyme 9
UDP-GlcNAc	Uridine diphosphate-N-acetylglucosamine
v-SNARE	Vesicle SNARE
VAMP2	Vesicle-associated membrane protein 2
ZA	Zaragozic acid A
α-MEM	Alpha minimum essential medium
βCD	Methyl-beta-cyclodextrin
βCD:Chol	Methyl-beta-cyclodextrin preloaded with cholesterol

Chapter I. Introduction

Humans have evolved to fight starvation. It is ironic that food has become modern man's foe contributing to the increasing worldwide prevalence of obesity and type 2 diabetes (T2D). Despite enormous medical progress in infectious diseases leading to a dramatic increase in human life expectancy, for the first time we are witnessing a reversal of this trend. This is mainly being driven by non-infectious diseases including diabetes, cardiovascular disease and cancer. Overnutrition and lack of physical activity in the developed world have contributed to the growing incidence of obesity and T2D, which have now reached epidemic proportions. According to the United States Centers for Disease Control and Prevention 2011 National Diabetes Fact Sheet (1), 25.8 million Americans and 79 million American adults now have diabetes and pre-diabetes, respectively. T2D accounts for over 90% of those afflicted with diabetes. Diabetes has become a major healthcare and economic burden in the United States and worldwide with an estimated total annual cost of $174 billion in the United States in 2007. As one of the world's fastest growing chronic diseases, diabetes is a leading cause of heart failure, kidney failure, stroke, lower limb amputations and blindness in adults (1). Importantly, there is also a hidden burden whereby diabetes can lead to other chronic diseases such as cancer, heart disease and Alzheimer's disease.

Insulin resistance is a well-recognized pathophysiological feature of pre-diabetes and T2D. Insulin resistance is known to drive the progression of T2D and has been found to be highly correlative with cardiovascular risk factors that

often contribute to morbidity in T2D patients (2). A comprehensive understanding of the cellular and molecular mechanisms contributing to insulin resistance has not been deciphered. However, it is well-appreciated that nutrient excess and obesity due to overeating and lack of physical activity predispose individuals for insulin resistance and T2D. While new drugs continue to be introduced and have shown some promise for patients, these strategies as a whole are not effectively curbing the worldwide epidemic. This is mainly due to the complexity of insulin resistance and adaptable nature of obesity. For example, when a person goes on a diet and reduces energy intake, this can be accompanied by a compensatory reduction in whole body energy expenditure (3). The shift towards a positive energy balance during the progression of obesity not only involves an increased energy intake, but also a concomitant lack of physical activity to utilize this excess energy (3). Achieving patient compliance with recommended programs involving increased physical activity and reduced energy intake remains a major obstacle in curbing the obesity and T2D epidemics. Therefore, it is crucial to continue dissecting the cellular and molecular mechanisms involved in insulin resistance to identify new drug targets of therapeutic interest and develop novel strategies for the treatment and/or prevention of obesity and T2D.

While the complex links between obesity and insulin resistance are still incompletely understood, increased levels of glucose, insulin and fatty acids (FAs) have been shown to negatively impact insulin sensitivity in both *in vitro* and *in vivo* experimental models (4-15). For example, high levels of glucose and lipids have been shown to prevent the ability of insulin to activate key signaling

intermediates resulting in insulin resistance (5, 6, 9). Interestingly, studies have demonstrated that pathophysiologically-relevant nutrient toxicity can result in insulin resistance without altering key insulin signaling intermediates (7, 8, 13, 16). Collectively, several studies have established membrane and cytoskeletal derangements (i.e. alterations in the plasma membrane (PM) lipid environment and/or cellular cytoskeletal structure) as key distal aspects of insulin resistance that can impair insulin sensitivity independent of insulin signaling abnormalities (7, 8, 10, 17-21). Interestingly, trivalent chromium (Cr^{3+}) has been shown to positively impact GLUT4 regulation by lowering PM cholesterol (22-24). Cr^{3+} is a micronutrient that has been appreciated to be beneficial for optimal glucose and lipid metabolism since the 1950s. However, whether Cr^{3+} can protect against membrane cholesterol accrual and whether this prevents cortical filamentous actin (F-actin) loss and GLUT4 dysregulation remains unknown.

Building upon fundamental findings in the field presented next, my thesis research focused on determining the effects of Cr^{3+} on skeletal muscle membrane/cytoskeletal parameters and insulin sensitivity. The following introductory sections will highlight insulin-regulated glucose homeostasis, GLUT4 regulation by insulin, insulin resistance, Cr^{3+}, and AMP-activated protein kinase (AMPK).

I.A. Insulin-Regulated Glucose Homeostasis

Insulin is a pancreatic hormone produced by β-cells in the pancreatic islets of Langerhans. Insulin regulates a plethora of cellular functions in many tissues

throughout the body. A primary function of insulin entails the regulation of post-prandial glucose homeostasis. In the post-prandial state, an elevation of blood glucose triggers release of insulin from β-cells. Once released into the bloodstream, insulin acts on the adipose tissue, skeletal muscle and liver to clear excess circulating glucose and restore glucose homeostasis. Insulin acts on the liver to inhibit hepatic glucose output by turning off glycogenolysis and gluconeogenesis. In adipose tissue and striated muscle (i.e. skeletal and cardiac muscle) insulin signals to stimulate glucose transport out of the bloodstream and into these target tissues. The combined effects of insulin on suppressing hepatic glucose output by the liver and stimulation of glucose uptake into adipose tissue and skeletal muscle are essential in maintaining whole body glucose homeostasis.

In adipose and striated muscle tissues, insulin-mediated glucose transport is achieved by the ability of insulin to stimulate the redistribution of the insulin-responsive glucose transporter GLUT4 from intracellular pools to the PM (17, 25-27). In the absence of insulin, GLUT4 primarily resides in intracellular membrane pools. Upon insulin binding to the insulin receptor on the surface of muscle and fat cells, insulin triggers a signaling cascade that stimulates an increase in the exocytosis rate of GLUT4-containing vesicles. These complex trafficking events orchestrated by insulin populates the PM with GLUT4 to allow glucose transport. The inability of insulin to properly stimulate glucose transport into muscle/fat and inhibit hepatic glucose output, termed insulin resistance, is a central feature of obesity, pre-diabetes and T2D. Insulin resistance initially results in glucose

intolerance, and the hyperinsulinemic response from the pancreatic β-cells immediately compensates to account for the reduced peripheral sensitivity to insulin action. While increased insulin secretion from the β-cells can be effective in maintaining blood glucose levels over time, this hyperinsulinemia and insulin resistance can lead to β-cell expansion and eventual exhaustion/death. Once the remaining β-cells can no longer secrete sufficient insulin to maintain glucose homeostasis, blood glucose levels increase indicating progression of insulin resistance to frank T2D.

At the cellular and molecular levels, insulin resistance is very complicated and involves many different mechanisms varying between different tissues. In adipose tissue and skeletal muscle, one certain definition of insulin resistance is the failure of insulin to properly recruit, mobilize and insert GLUT4 into the PM to stimulate glucose transport in the setting of normal GLUT4 protein expression. Together these two tissues account for over 90% of post-prandial glucose disposal (28). The complex derangements observed in insulin resistance stress the importance for efforts to dissect these mechanisms of GLUT4 dysregulation to develop new drug targets and therapeutic strategies for the treatment and/or prevention of insulin resistance and T2D. While a complete understanding of how insulin regulates GLUT4 translocation to the PM and glucose transport does not yet exist, significant advances have been made to help us understand the actions of insulin how these processes become deranged in insulin resistance.

The focus of this thesis research was to dissect membrane/cytoskeletal parameters of skeletal muscle insulin sensitivity altered in insulin resistance and

determine the mechanisms amendable to Cr^{3+} action. This research is primarily focused on skeletal muscle, as this tissue is responsible for a large majority of post-prandial glucose disposal (29) and is regarded as a major site of insulin resistance (28). Therefore, the following sections and subsections will provide a pertinent outline and analysis of our current state of knowledge regarding insulin regulation of glucose transport and insulin resistance, primarily in skeletal muscle. Expanded information on hepatic and/or adipocyte insulin action can be found in several detailed reviews on these topics (30-32). While skeletal muscle will be the major focus of the background and research outlined in this thesis, it is important to note that skeletal muscle is by no means the only tissue involved with maintenance of glucose homeostasis and development of insulin resistance.

I.B. Signaling, Cytoskeletal and Membrane-Based GLUT4 Regulation

Solving how insulin regulates glucose transport into skeletal muscle and adipose tissue remains a fundamental challenge in biology and a significant issue in medicine. A central feature of this process is the coordinated accumulation of the glucose transporter GLUT4 into the plasma membrane. New signaling and cytoskeletal mechanisms of insulin-stimulated GLUT4 exocytosis are of emerging interest, particularly those at or just beneath the plasma membrane. The following subsections examine signals that functionally engage GLUT4 exocytosis, consider cytoskeletal regulation of the stimulated GLUT4 itinerary, and appraise involvement of plasma membrane parameters in GLUT4 control. Explored further are how these newly defined signaling, cytoskeletal, and

membrane mechanisms may be of therapeutic interest in the treatment and/or prevention of GLUT4 dysregulation in disease.

I.B.1. Recruiting GLUT4

Under normal insulin responsiveness, insulin promotes the removal of excess glucose from the circulation by stimulating the exocytic recruitment of intracellular GLUT4 storage vesicles (GSVs) to the plasma membrane (PM) of skeletal muscle and fat cells (25, 27). This stimulated redistribution of intracellular GSVs results in PM GLUT4 accrual that facilitates cellular glucose uptake (**Fig. 1**). Activation of GSVs by insulin requires a phosphatidylinositol 3-kinase (PI3K) signal involving the upstream insulin receptor (IR) and insulin receptor substrate (IRS) activators and the downstream Akt2 target enzyme (25, 27, 33).

Until the discovery of AS160 (Akt substrate of 160-kilodaltons (kDa)) in 2002 (34), how the IR/IRS1/PI3K/Akt2 signal coupled to GSVs remained unclear. This protein, also known as TBC1D4 (Tre-2 BUB2 CDC16, 1 domain family member 4), contains a GTPase activating domain (GAP) for Rabs, small G proteins implicated in vesicle trafficking (35, 36). In the basal state, the Rab-GAP function of TBC1D4 is thought to contribute to the intracellular retention of GSVs by promoting the inactive GDP-bound state of Rabs; whereas insulin-stimulated Akt2 suppresses the Rab-GAP activity of the TBC1D4 and thus increases the active GTP-bound form of Rabs on GSVs to promote exocytosis (**Fig. 1A**). Consistent with this localized functionality, TBC1D4 associates with GSVs via binding to the insulin-responsive amino peptidase (IRAP), a GSV cargo

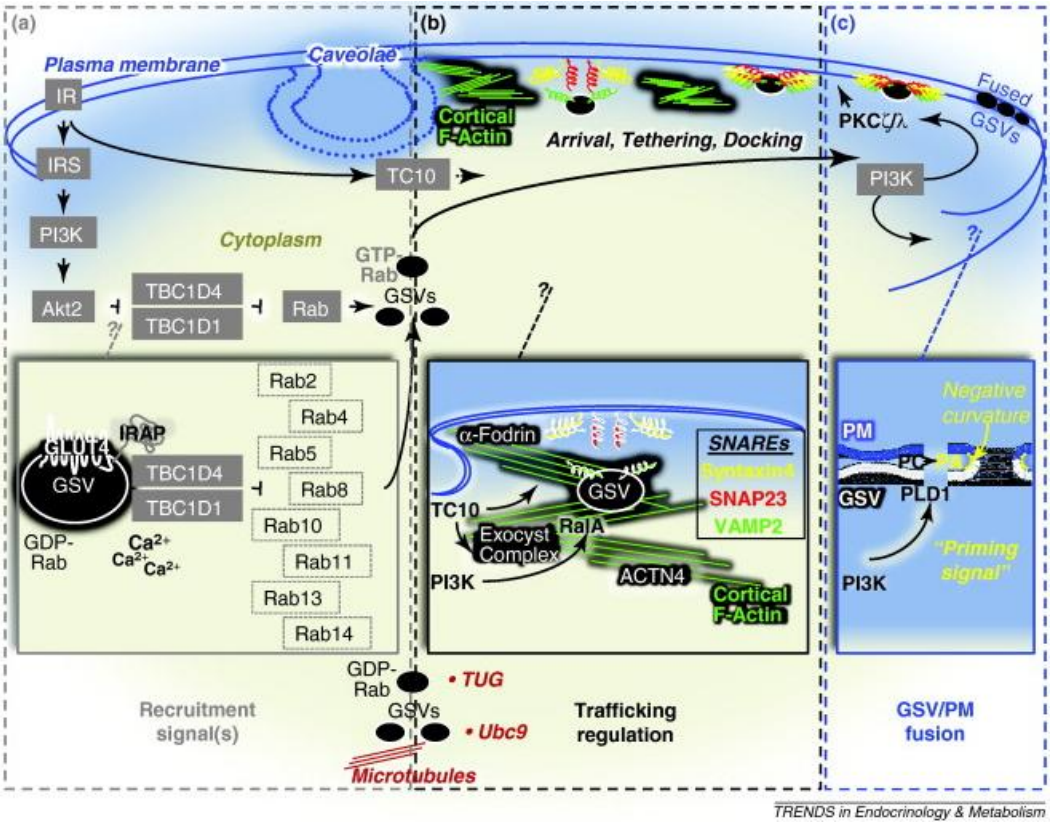

Figure 1. Schematic illustration of putative signals, cytoskeletal mechanisms, and plasma membrane parameters involved in insulin-stimulated GLUT4 storage vesicle exocytosis (17)**.**

(A) Activation of GSVs by insulin requires a PI3K signal involving the upstream IR and IRS activators and the downstream Akt2 target enzyme. TBC1D4 and TBC1D1, substrates of Akt2, have been suggested to couple the PI3K/Akt2 signal to GSVs via its action on one or more critical Rab proteins. The basal intracellular pool of GDP-Rab GSVs shown associated with several putative anchoring systems (e.g., microtubules, Ubc9 (ubiquitin-conjugating enzyme 9), TUG (tether containing UBX domain for GLUT4)) are activated by the

suppression of the Rab-GAP activity of TBC1D4/TBC1D1 by Akt2. Several putative Rab proteins, the existence of possible calcium regulation, and mechanisms associating TBC1D4 (and presumably TBC1D1) to the GSV via IRAP have been suggested (see inset). (B) Cortical F-actin, likely originating at the neck region of caveolae PM microdomains, plays a critical role in GSV trafficking. Reorganization of the cortical F-actin meshwork by insulin signaling to TC10 allows GSV/PM arrival, tethering, and docking. A large number of proposed insulin-regulated processes occur in this PM vicinity such as TC10-regulated formation of the exocyst complex and cortical F-actin remodeling, PI3K/RalA-stimulated transition of trafficking GSVs to tethered GSVs, a role of ACTN4 and/or the exocyst complex in tethering, and an α-fodrin-mediated rearrangement of cortical actin filaments in the area of syntaxin 4 to facilitate GSV/PM SNARE protein interaction and docking (see inset). (C) Insulin signaling, through two putative PI3K signals that activate PKCζ/λ and PLD1, prepares GSVs for fusion with the PM. The first PKCζ/λ signal has been implicated in promoting the dissociation of Munc18c from syntaxin4, contributing to the fusion-competent SNARE complex. The second PLD1 signal primes the GSV and PM for fusion by generating PA, which has been suggested to act as a fusogenic lipid in biophysical modeling studies by lowering the activation energy for membrane bending (i.e., negative membrane curvature) during generation and expansion of fusion pores (see inset).

protein (37, 38). Another Rab-GAP known as TBC1D1 with identical Rab specificity as TBC1D4 (39) also displays similar regulation of GLUT4 in 3T3-L1 adipocytes (39), skeletal muscle myotubes (40), and mouse skeletal muscle (41). Interestingly, expression of a TBC1D1 genetic variant (R125W, linked with human obesity (42)), was found to impair insulin-stimulated glucose transport in mouse skeletal muscle (41). However, studies of TBC1D1 function in 3T3-L1 adipocytes showing that expression of wild-type TBC1D1 displays a similar inhibitory effect as R125W, and that GLUT4 regulation is intact following TBC1D1 knockdown, raises questions on the importance of TBC1D1 and R125W in health and disease (40), (43). Nevertheless, given the high expression levels of TBC1D1 in skeletal muscle compared to adipocytes (43), future attention on TBC1D1 functionality in GLUT4 regulation has merit. Another important area of current investigation is aimed at identifying which Rab protein(s) are targeted by TBC1D4 and TBC1D1 Rab-GAP activity (**Fig. 1A, inset**).

Using immunoblotting and mass spectrometry techniques to analyze GLUT4-containing intracellular vesicles, the Rab proteins Rabs 4, 5, and 11 have been shown to associate with GSVs (35, 36). Additional evidence from proteomic analysis (44) and mass spectrometry (45) demonstrates that Rabs 2, 8, 10, and 14 are associated with GSVs, raising questions as to which Rabs are important. Historically, Rab4 has been a major Rab of focus in GLUT4 regulation (36); however, new evidence suggests that other Rabs may play roles in regulating GLUT4. The highly homologous nature of Rab proteins, lack of antibodies specific for Rabs, and potential false positives represent ongoing challenges in

dissecting specific Rabs associated with GSVs in subcellular fractionation and immunlocalization studies (36). Moreover, establishing a functional significance for one or more Rabs in GSV trafficking has been challenging. Functionally, Rabs 2, 8A, 10, and 14 have been identified as putative targets of TCB1D4 *in vitro* (45). Recent studies have utilized siRNA (small interfering RNA) knockdown of Rabs to more precisely dissect roles in GLUT4 regulation, as interpretation of data from overexpression-based analyses are complicated by potential off target effects on other Rabs and Rab effectors. Knockdown of Rab10 in adipocytes has suggested a role for Rab10 in insulin-stimulated GLUT4 translocation (46). These results are supported by findings showing that only knockdown of Rab10, not that of Rabs 8A, 8B, and 14, prevented insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (47). In line with earlier findings regarding Rab4 (36), Rab 4B knockdown in adipocytes supports its role in regulating GLUT4 translocation (48). In muscle cells the Rabs 8A and 14, but not Rabs 8B and 10, rescue the inhibition of GLUT4 translocation by a constitutively-active TBC1D4 (49). Consistent with these findings, knockdown of Rabs 8A and 14 inhibits insulin-stimulated GLUT4 translocation (50) implicating these Rabs in muscle GLUT4 regulation. Although Rab specificity is not completely dissected, together these studies begin to establish tissue-specific roles for Rab proteins in GLUT4 translocation and highlight the crucial need for future efforts to fill this gap in our understanding.

Although it was hoped that the discovery of TBC1D4 would lead to the precise identification of the GSV-regulatory Rab protein, the finding of TBC1D1

and several GSV-associated Rabs that are targeted by TBC1D4 and TBC1D1 have provided additional advances in our understanding that will require further study. In this regard it is intriguing that calmodulin has been reported to bind to a small domain just amino terminal to the GAP domain of TBC1D4 (51). The association was calcium dependent. Nevertheless, study of a point mutant of TBC1D4 lacking calmodulin binding did not seem to indicate a requirement for calmodulin/TBC1D4 binding in GLUT4 regulation. Perhaps this calmodulin-binding domain regulates contraction-, but not insulin-, stimulated GLUT4 regulation (52). Despite these data fitting a calcium-independent model of insulin-regulated GLUT4 translocation, intermittent study through the years seems to support a role of calcium (53). Interestingly, new studies have identified requirements for inositol 1,4,5-triphosphate-receptor and calcium/calmodulin-dependent protein kinase II pathways in GLUT4 regulation by insulin (54, 55). These new insights support the need for future investigation into calcium-based aspects of GLUT4 control.

In summary, new additions to our understanding of GSV recruitment have been made. Namely, data implicate that insulin-mediated suppression of the Rab-GAP activity of GSV-localized TBC1D4 (and presumably TBC1D1) activates a critical GSV-regulatory Rab protein. Although the precise identification of the Rab protein or proteins involved in GSV recruitment needs continued delineation, data from several studies have framed key cytoskeletal events distal to Rab functionality in the itinerary of the activated GSV.

I.B.2. Motoring GSVs

Conceptually, a long-standing view has been that microtubules coordinate long-range, whereas actin orchestrates short-range, GSV movement (21, 56, 57). Findings implicate microtubules in mediating basal subcellular distribution of GSVs, but not the accelerated rate of GLUT4 translocation stimulated by insulin (58). For example, basally GSVs display long-range movements beneath the PM, with their trajectories extensively spread on the entire PM (59). This is consistent with findings that insulin stimulation halts this long-range GSV basal itinerary and stimulates GSV/PM tethering, docking, and fusion in rat primary adipocytes (60). Recent mounting evidence discussed below supports that this insulin-stimulated switch from a basal GSV trajectory to an insulin-regulated PM-bound track occurs at a microtubule/actin junction beneath the PM.

It is well-documented that insulin elicits a rapid, dynamic remodeling of actin filaments into a cortical mesh, and this mesh is necessary for GLUT4 translocation in both cultured and primary skeletal muscle and fat cells (21). At a functional level, new studies by several groups continue to point to important roles of several recognized and new insulin-regulated proteins (e.g., TC10 (61), actin-related proteins 2/3 (Arp2/3) and Cofilin (62), Myo1c (55), Rac1 (63), focal adhesion kinase (FAK) (64)) that control actin dynamics to influence GLUT4 translocation, some of which we provide more detail on below. More relevant to the present discussion are data that place GSVs in the meshwork and suggest a functionality of the actin mesh in GLUT4 translocation (**Fig. 1B**). For example, alpha-actinin-4 (ACTN4), a protein responsible for linking actin filaments to

intracellular structures, is required for insulin-stimulated GLUT4 translocation in L6 myotubes (65). Mechanistically, ACTN4 co-precipitated and co-localized with GLUT4 along actin filaments induced by insulin stimulation, suggesting that ACTN4 may play a role in tethering GSVs to the actin cytoskeleton (**Fig. 1B, inset**). The tethering function of the cortical actin mesh likely plays a critical role in the final steps of GSV/PM docking and fusion regulated by syntaxin 4 and synaptosomal-associated protein 23 (SNAP23) target (t-) soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) and the GSV vesicle (v-) SNARE vesicle-associated membrane protein 2 (VAMP2) (66). Together these findings provide evidence that ACTN4 may direct GSVs to the insulin-organized cortical actin meshwork to facilitate GSV delivery to the appropriate PM-localized SNARE-mediated docking machinery.

In cultured and primary adipocytes an evolutionarily conserved tethering complex termed the exocyst complex has been described for GSV/PM targeting (67, 68). This complex, comprised of eight subunits, mediates the initial recognition of the exocytic vesicle and target membranes for fusion. The assembly of the adipocyte GSV exocyst complex occurs at PM caveolae/raft regions and requires insulin activation of the Rho family member GTPase TC10, which mediates recruitment of exocyst components Exo70, Sec6, and Sec8 (67). Interestingly, activation of TC10 also appears necessary for the regulation of cortical filamentous actin (F-actin) reorganization (**Fig. 1B, inset**), which we discuss further below. Along with insulin activation of a Rab GTPase for GSV recruitment and a Rho GTPase for GSV/PM arrival, insulin also activates a GSV-

associated Ral GTPase termed RalA that induces GSV/exocyst association juxtaposed to the PM (**Fig. 1B, inset**) (69). The observation that RalA interacts with Myo1c, a molecular motor implicated in GSV trafficking, implicates a convergence of vesicle trafficking to tethering (55, 69).

Of interest are the insulin signals to each of the GTPases. Similar to Rab activation, insulin signaling to RalA is PI3K-mediated (69). Whereas the PI3K/Rab signal involves Akt2 signaling to TBC1D4 and/or TBC1D1, it remains unclear if PI3K signaling to RalA involves Akt2 stimulation and distal signaling to TBC1D4, TBC1D1, or a yet to be determined substrate. Unlike Rab and RalA activation, insulin is appreciated to use a PI3K-independent signal to activate the Rho GTPase TC10. Although initial study delineated several component proteins (e.g. APS (adaptor protein containing PH and SH domains), c-Cbl, CAP (Cbl-associated protein), CrkII/C3G) involved in this TC10 activation, subsequent siRNA knockdown studies challenged their proximal importance in TC10-regulated GLUT4 translocation (70). Subsequent study revealed that TC10 has two isoforms (TC10α and TC10β), and that insulin-stimulated TC10α, not TC10β, regulates GLUT4 translocation (61, 71). With the use of pharmacological inhibitors and siRNA-mediated knockdown, study has delineated that proximal insulin signaling to TC10α-, but not TC10β-, involves the activation of cyclin-dependent kinase-5 (CDK5) in caveolae/raft domains via proximal non-receptor tyrosine kinase Fyn activation by insulin (71). Furthermore, this work shows that active CDK5 maintains TC10α in caveolae/rafts where it functions to disrupt cortical F-actin.

With regards to actin, identification of α- and β-fodrin isoforms as abundant components of rat primary adipocyte PM caveolae/rafts is of interest (72). Fodrin is a nonerythroid spectrin that forms filamentous α-β heterodimers and binds to actin at both ends, forming a repeating “corral”-like network beneath the PM. Interestingly, α-fodrin and syntaxin 4 co-localize and interact in rat adipocytes, and insulin enhances this interaction. In contrast, disruption of cortical actin by latrunculin A reduces the α-fodrin-syntaxin 4 interaction, blocks α-fodrin remodeling, and inhibits GLUT4 translocation. In this case, the regulated remodeling of the fodrin-actin network apparently plays a key role in permitting GSV/PM fusion by allowing GSV-VAMP2 access to syntaxin 4 (**Fig. 1B, inset**). Once insulin-stimulated α-fodrin remodeling had occurred GLUT4 translocation became insensitive to latrunculin A, suggesting that only the exocytic step of GLUT4 translocation at or near the PM requires this aspect of cortical F-actin remodeling (72).

In summary, these data are consistent with the concept that the insulin-stimulated motoring of the GSV, subsequent to its Rab recruitment signal, requires insulin signaling to RalA by an undefined PI3K mechanism and to TC10α via a Fyn/CDK5 pathway. Whereas RalA activation stimulates the trafficking GSV to tether with the caveolae/raft-localized exocyst complex, active TC10α regulates the formation of the exocyst complex for GSV tethering and also reorganizes the cortical F-actin for GSV docking. Together these studies may suggest that the exocyst-tethered GSV transitions to a syntaxin 4/SNAP23-

docked GSV by the possibility that fodrin couples syntaxin 4 to caveolae-localized F-actin, in which data presented below seem to support.

I.B.3. Bilayering GSVs

It is clear that the regulated meshwork of actin filaments beneath the PM plays a critical role in several steps of the GSV itinerary, particularly GSV arrival, tethering, and docking. In addition to a likely role of bilayer parameters in GSV recruitment and mobilization steps, new data indicate that insulin-regulated changes in PM lipids promote GSV/PM fusion. As lipids are key pathophysiological players in disorders of glucose metabolism, studies demonstrating an impact of PM lipids on insulin action and GLUT4 translocation warrant consideration. The next section describes data from several microscopy-based explorations of PM functionality that provide new insight into PM/F-actin coupling and GSV/PM fusion regulation by insulin.

Caveolae represent specialized, morphologically distinct sphingolipid-cholesterol microdomains of the PM, which are stabilized by caveolin proteins (73). Through the years many functions for caveolae have been postulated in insulin and GLUT4 action. Although caveolae functionality needs to be cautiously interpreted because problems are associated with each of the numerous strategic approaches used to study these structures, a caveolae-based TC10α/exocyst complex tethering and cortical F-actin dispersion mechanism in this vicinity is consistent with data implicating a caveolae-actin association (74, 75). Particularly, fluorescence confocal labeling of caveolae and cortical F-actin

revealed actin filaments emanating from caveolae microdomains (74). Whereas disruption of this caveolin-associated F-actin, termed Cav-actin, structure with latrunculin B, Clostridium difficile toxin B or a dominant-interfering TC10 mutant (TC10/T31N) did not affect the organization of clustered caveolae; disruption of the clustered caveolae with methyl-beta-cyclodextrin (β-CD) dispersed the Cav-actin structure (74). Quantitative electron microscopy and freeze-fracture analyses later revealed that cytoskeletal components, including actin, are highly enriched in the membrane area underlying the neck part of caveolae (75). Together, these findings assign caveolae with a critical functionality in cortical F-actin organization. Given the unequivocal importance of cortical F-actin in insulin-regulated GLUT4 translocation, these findings also emphasize the importance of caveolae in GLUT4 regulation. Of interest to our understanding of Cav-actin structure regulation are new electron microscopic data that show phosphatidylinositol 4,5 bisphosphate (PIP_2) is highly concentrated at the rim of caveolae (76). This localization of PIP_2 is consistent with its regulation of the cytoskeleton where this lipid's availability is recognized to modulate membrane/cytoskeleton interaction, the stability of cortical F-actin, and the turnover of cytoplasmic stress fibers (77). Interestingly, reduced PM PIP_2 and cortical F-actin structure are observed in hyperinsulinemia-induced insulin-resistant 3T3-L1 adipocytes and L6 myotubes where insulin-stimulated GLUT4 translocation is impaired, but corrected with exogenous PIP_2 addition to the PM by provoking a restoration of cortical F-actin structure (7, 78).

Given the emerging evidence of PM functionality in GLUT4 regulation, several recent studies have employed total internal reflection microscopy (TIRFM) to critically examine GSV/GLUT4 regulation. This microscopy uses an evanescent wave to selectively illuminate and excite fluorophores in a restricted region of the cell immediately adjacent to the PM. This further experimental scrutiny has expanded upon findings from cell-free reconstitution assays showing insulin activation of the PM fraction of the in vitro reaction is the essential step in GSV/PM fusion (79). For example, several TIRFM analyses have provided strong evidence for a critical PM signal that appears to prime the PM and/or the GSVs for fusion (80-82). With a combination of live cell and steady-state TIRFM analyses with PI3K and Akt inhibition, Akt was suggested to be a crucial regulator of insulin-stimulated GSV/PM pre-fusion (i.e., recruitment, tethering, docking) events (80). In contrast, insulin-stimulated GSV/PM fusion seemed to occur independently of Akt activity. That is, although GSV/PM prefusion was inefficient with Akt blockade, fusion of this lower level of docked GSVs with the PM was properly stimulated by insulin. However, based on inhibition of this GSV/PM fusion with wortmannin, it appears this postdocking step is PI3K dependent. Dynamic tracking of single GSVs with computational analysis of thousands of events lends strong support to this model whereby insulin uses a PI3K/Akt signal to accelerate GSV/PM prefusion and another signal to prepare GSVs and/or the PM for GSV/PM fusion (81). Although the steady-state analysis suggested this second signal required PI3K, this conclusion could not be made with live cell GSV tracking (80, 81).

Certainly, the putative role of PI3K in priming GSV/PM fusion requires confirmation, yet is of great interest as PI3K signaling to PKCζ/λ/Munc18c (83, 84) and/or PLD1 (phospholipase D1) (85) could promote GSV/PM fusion (**Fig. 1C, inset**). Whereas insulin signaling through PKCζ/λ has been proposed to dissociate Munc18c from syntaxin 4, a necessary postdocking/prefusion event (83, 84); PLD1 activation by insulin has been implicated in generating fusion-competent membranes (86). Mechanistically, PLD1 generates the lipid phosphatidic acid (PA), which has been suggested to act as a fusogenic lipid in biophysical modeling studies by lowering the activation energy for membrane bending (i.e., negative membrane curvature) during generation and expansion of fusion pores (**Fig. 1C, inset**) (87). Together these data suggest PI3K may use two distinct signals to regulate critical mechanisms of GSV/PM fusion post GSV/PM docking.

I.C. Obesity, Insulin Resistance and GLUT4 Dysregulation

New additions to the molecular details of GLUT4 regulation by insulin attest to the great progress being made in our mechanistic understanding of insulin-stimulated glucose transport in health and disease. As highlighted earlier, the prevalence of diabetes in the United States continues to rise, but more troubling is the escalating global impact of this disease. Despite the increase in knowledge, global prevalence of diabetes in 2010 was 284 million people worldwide, constituting around 6.4% of the world population. Projections for 2030 estimate the prevalence reaching 439 million individuals, comprising ~7.7% of

the world population (88). This is attributed in large part to the rising incidence of obesity worldwide, which makes it essential to focus attention on molecular mechanisms underlying insulin resistance that are fueled by obesity. Insulin resistance is a complex, progressive syndrome with many associated pathologies. Extensive research efforts are underway to continue unraveling these complex mechanisms linking nutrient oversupply to insulin resistance. The subsections below will outline fundamental findings that have uncovered various mechanisms underlying the complex nature of insulin resistance in skeletal muscle.

I.C.1. Lipid-induced insulin resistance

In the context of abundant nutrient supply and lack of physical activity in the developed world, it has become apparent that excess FAs have a negative effect on skeletal muscle insulin sensitivity. Insulin resistance is highly correlated with obesity (89, 90), increased circulating FAs and the accumulation of lipids in skeletal muscle and adipose tissue (91, 92). Outlined below are several proposed mechanisms that begin to explain the role FA's play in the development of insulin resistance (93).

Numerous hypotheses have been proposed throughout the years in an effort to explain how FAs and their metabolites directly induce insulin resistance. The first major hypothesis linking FAs to glucose metabolism was proposed by Randle and colleagues. The Randle hypothesis suggested that FAs directly compete with glucose for the same oxidative pathway, which impairs glucose

metabolism in the face of increased FAs (94). More recent studies have disproven this early hypothesis. Using ^{13}C and ^{31}P nuclear magnetic resonance (NMR) spectroscopy to analyze insulin-resistant humans, it has been shown that the major defect underlying insulin resistance is glucose uptake (95) and glycogen synthesis (96) in skeletal muscle, rather than impairment in glucose catabolism (97). In further support of defective glucose uptake accounting for insulin resistance, lipid infusion has been shown to impair tyrosine phosphorylation of IRS. Lipid infusion also increases activation of PKCθ, which is a Ser/Thr kinase which is also expressed higher in HF-fed rats compared to low fat (LF)-fed rats (98). Based on these observations, Shulman and colleagues hypothesized that serine phosphorylation of IRS is the primary mechanism involved in FA-induced insulin resistance (99). This model implicates FAs and their metabolic intermediates (i.e. acyl-Co enzyme A (CoA)s, ceramides and diacylglycerides (DAGs)) as signaling molecules. These signaling lipids can phosphorylate serine residues of IRS, which causes defects in tyrosine phosphorylation of IRS and disrupts downstream insulin signaling (95, 99).

A related hypothesis has been proposed by Summers and colleagues based on the observation that saturated FAs have a more deleterious effect on insulin sensitivity compared to unsaturated FAs (100). This model was supported by the observation that palmitate, the most prevalent saturated FA in the circulation and skeletal muscle (101), stimulates *de novo* synthesis of ceramide. The sphingolipid ceramide has a potent negative effect on insulin signaling at the level of Akt phosphorylation (6, 9, 102). In further support of this mechanism,

ceramide levels are negatively correlated with insulin sensitivity in humans (103). When cultured skeletal muscle cells and adipocytes are treated with ceramide analogues *in vitro*, these cells become insulin-resistant, as evidenced by findings from several independent studies showing defects in insulin-stimulated glucose uptake and glycogen synthesis (6, 9, 104, 105). Palmitate-induced defects in Akt phosphorylation in cultured C2C12 myotubes and human myotubes are prevented by treatment with inhibitors of ceramide synthesis (6, 106-108). Moreover, depletion of ceramide pools by overexpressing acid ceramidase also confers protection against palmitate-induced defects in Akt activation (109). The mechanisms by which ceramide inhibits insulin signaling at the level of Akt phosphorylation appear to involve the protein phosphatase 2A (PP2A), inhibition of Akt translocation to the PM and activation of Ser/Thr kinases including JNK and IKK. PP2A was identified as a target for ceramide-induced insulin resistance (110), as PP2A has been shown to dephosphorylate Akt and reduce insulin signaling resulting in insulin resistance (111). In addition, ceramides have been shown to prevent the translocation event of Akt to the PM to allow Akt phosphorylation and activation. The prevention of Akt translocation has been shown to be mediated by PKCζ phosphorylating Akt on its PH domain at Ser34 (112). This phosphorylation event prevents the binding of Akt with phosphatidylinositol 3,4,5 triphosphate (PIP_3) at the PM (112). These findings are supported by the observations that PKCζ inhibitors and expression of a dominant negative form of PKC prevents the negative effects of ceramide on insulin sensitivity (106). Finally, ceramides have also been shown to induce insulin

resistance by activating Ser/Thr kinases such as JNK and IKK mediated by the inflammatory cytokine tumor necrosis factor alpha (TNFα) (113-115).

It is well appreciated that chronic, systemic inflammation is associated with insulin resistance. There are a multitude of effects of the inflammatory state associated with obesity, not just including ceramide-associated defects, which are implicated in the development of insulin resistance (116-118). Systemic inflammation is known to result in macrophage accumulation in adipose tissue (119, 120) and more recently skeletal muscle (121), for example. The expanding fat mass in obesity leads to an activation of these macrophages that stimulate the production of inflammatory cytokines such as TNFα, C-reactive protein (CRP) and interleukin-6 (IL-6). These inflammatory cytokines wreak havoc on insulin signaling by activating JNK in skeletal muscle, leading to increased serine phosphorylation of IRS and insulin resistance (95, 99). Cytokines are also associated with activation of SOCS proteins (122, 123), which decrease IRS tyrosine phosphorylation and target IRS for proteasomal degradation (124, 125). Although the mechanisms will not be discussed in detail, mitochondrial dysfunction is another important aspect of insulin resistance of emerging interest. Defects in mitochondria are known to increase oxidative stress and production of reactive oxygen species that negatively impact insulin sensitivity (126, 127). The mechanisms described in this section likely contribute to insulin resistance interdependently, as insulin resistance is well accepted to be a complex interplay of many different factors (93). While lipid-induced mechanisms have been shown to involve signaling defects leading to insulin resistance, several groups have

also identified states of insulin resistance induced by excess insulin and glucose exposure, for example, that do not involve defects in key insulin signaling proteins. The emerging appreciation of these complementary aspects of GLUT4 regulation and insulin sensitivity will be described in the following subsections.

I.C.2. Insulin- and glucose-induced insulin resistance

Similarly to FAs, increased levels of insulin and glucose have been shown to negatively impact insulin sensitivity in both *in vitro* and *in vivo* experimental models (4-15). The pathophysiology of insulin resistance involves a compensatory increase in insulin secretion by the β-cells to account for reduced insulin sensitivity in skeletal muscle. Although hyperinsulinemia is typically viewed as a compensatory response by the pancreas to offset insulin resistance, studies in man (128, 129) and in cultured cells (130, 131) have clearly shown that insulin resistance can be induced by hyperinsulinemia. These data, coupled with the fact that it is difficult to find a study in which insulin resistance and hyperinsulinemia are clearly separated in time (132), suggest a scenario where hyperinsulinemia may actually cause insulin resistance. Regardless of the exact temporal sequence in which these derangements appear, hyperinsulinemia is widely viewed to accelerate diabetes progression and worsen prognosis (132, 133). In fact, plasma insulin has been measured in multiple population-based studies which demonstrate an important clinical impact of hyperinsulinemia (134). For example, obesity, impaired glucose tolerance, T2D, hypertension and hypertriglyceridemia were found to be two to three times more prevalent in

individuals with fasting insulin levels of greater than 31 µU/ml (186 pm) than in subjects with fasting insulin below that level. Cell studies testing the effects of chronic insulin exposure have documented attenuated expression levels and/or activity states of insulin signaling intermediates. However, these studies used concentrations of insulin >16,666 µU/ml (100 nM), concentrations ~500-fold greater than the 31 µU/ml-defined hyperinsulinemic cutoff. Several groups (14, 15), including ours (7, 8), show that 12-18 hour exposure of cells to more physiologically relevant insulin concentrations [100-833 µU/ml; 600-5000 pM] (134) induces insulin resistance while proximal insulin signaling remains intact.

Similarly to hyperinsulinemia, exposure of muscle and fat cells to excess glucose has also been shown to negatively impact insulin sensitivity. The fuel sensing pathways involved in chronic overnutrition are not well understood and likely very complex. An emerging appreciation in the context of excess nutrients and obesity contributing to insulin resistance is the involvement of the hexosamine biosynthesis pathway (HBP) in regulating glucose flux. The HBP is considered as a nutrient sensor, as HBP flux parallels glucose availability (135, 136). Under normal nutrient load, 2-5% of glucose that enters the cells is shunted from the glycolytic pathway to the HBP (137, 138). HBP flux is regulated by the pathway's rate limiting enzyme, glutamine:fructose-6-phosphate amidotransferase (GFAT) (139). Flux through the HBP ultimately results in conversion of substrate to uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc). Single GlcNAc moieties are transferred to hydroxyl groups on Ser/Thr residues of target proteins by O-linked N-acetylglucosamine transferase (OGT)

(140). Removal of the O-GlcNAc moiety is achieved by the enzyme O-GlcNAcase (OGA) (141, 142). Therefore, the rapid and transient cycling of this post translational modification is a rapid and transient nutrient-sensing signaling mechanism. O-GlcNAc has been implicated in cellular processes such as transcriptional regulation, signal transduction and metabolism (143, 144). These O-GlcNAc modifications are common for both cytoplasmic and nuclear proteins (142, 145). While the HBP pathway and its regulation is not the major focus of my thesis research, the relevant implications of increased HBP flux and impact on membrane/cytoskeletal parameters of insulin sensitivity will be described in further detail below.

The nutrient-sensing HBP is a classical example of a normal evolutionary adaptation; however, chronic HBP flux has been shown to induce defects associated with the pathophysiology of insulin resistance. Marshall and colleagues first described the detrimental effects of excess HBP flux (138). This study showed that exposure of cultured adipocytes with hyperglycemia induced an insulin-resistant phenotype (138). This hyperglycemia-induced insulin resistance has been shown to involve defects in GLUT4 translocation and glucose transport (146, 147). Studies have shown that the effects of hyperglycemia and glucosamine treatments used to drive the HBP pathway are not additive, suggesting a shared pathway (148). *In vivo* studies have also implicated the HBP in the development of insulin resistance. For example, overexpression of GFAT has been shown to result in insulin resistance (135, 149-151). In addition, inhibition of OGA increases O-GlcNAc levels and induces

insulin resistance (152). Although elevated levels of O-GlcNAc are also associated with insulin resistance in a variety of cell types (151, 152), the mechanisms by which increased HBP flux induces insulin resistance are not completely understood. Recent findings have suggested that OGT is targeted to the PM by phosphoinositides where OGT increases O-GlcNAcylation and inhibits key insulin signaling proteins (153). Alternative studies have suggested that O-GlcNAcylation competes with phosphorylation on Ser/Thr residues to impact cellular signaling events (154).

Published data, as well as new unpublished data, from our group have demonstrated that HBP flux through palmitate, hyperglycemia and glucosamine treatments promotes key transcription factors that induce membrane cholesterol accrual and F-actin defects associated with cellular insulin resistance (10, 11, 23, 24). To further support a mechanism of HBP flux inducing insulin resistance, palmitate-, hyperglycemia- and glucosamine-induced membrane/cytoskeletal defects and insulin resistance were prevented by HBP inhibition (10, 11, 24). Our results suggest that compensatory hyperinsulinemia is likely to increase glucose flux even in euglycemic conditions, highlighting an important aspect of pre-diabetes that may be prevented by modulating the HBP. In addition to its roles as a sensor of glucose load, HBP flux is also likely influenced by excess lipids (10, 137). The HBP is emerging as a key fuel sensing pathway in the regulation of cellular cholesterol accrual. A possibility we favor involves increased HBP flux resulting in a transcriptional reprogramming of the cell to a more cholesterogenic phenotype (10, 11, 23). To further describe the involvement of cholesterol in

insulin resistance, the following subsection will briefly describe how cellular cholesterol levels are regulated and how cellular cholesterol becomes dysregulated in the development of insulin resistance.

I.C.3. Cholesterol dysregulation in insulin resistance

A role for cholesterol in the pathogenesis of cardiovascular disease is well recognized (155). Furthermore, an appreciation is emerging for the role of cholesterol in glucose homeostasis and development of metabolic and neurodegenerative diseases (156-158). Cholesterol regulation is a very tightly-regulated process. However, increased cellular, and specifically membrane, cholesterol levels are implicated in provoking insulin resistance. Cellular membranes are composed of cholesterol and phospholipids, and these lipids are essential for membrane structure and function (159-161). Cholesterol organization in the membrane is complexly regulated and depends on both cholesterol concentration and membrane composition (162, 163). Cholesterol has been shown to have preferential interactions with particular membrane lipids and proteins to form microdomains, known as lipid rafts, essential for cell function, signaling, adhesion and motility (73, 164-166). The majority of cellular cholesterol (65-90%) is found in the PM (159) as opposed to intracellular membranes, and cholesterol comprises 35-45% of all PM lipid species (167). Low density lipoprotein (LDL) delivers extracellular cholesterol to cells; however, all nucleated cells can synthesize cholesterol *de novo*. The cholesterol biosynthesis pathway utilizes acetyl CoA to convert mevalonate to lanosterol,

which is eventually modified to form cholesterol by several downstream enzymatic reactions (168).

Cholesterol is essential for cellular structure and function; however, it can also have detrimental effects. Therefore, cholesterol regulation is complexly controlled to maintain a balance between synthesized cholesterol and external cholesterol delivered by LDL. Cholesterol regulation is primarily regulated by a feedback mechanism to control 3-hydroxy-3-methyl-glutaryl coenzyme A reductase (HMGR) protein levels. HMGR is the rate limiting enzyme of cholesterol synthesis (169). Protein levels of HMGR are regulated by the sterol response element binding protein (SREBP) 2, SREBP cleavage-activating protein (SCAP) and insulin-induced protein (INSIG) 1 and 2 (170). An extremely small portion of cellular cholesterol (0.5%) is contained in the endoplasmic reticulum (ER) membrane and functions as a major regulatory mechanism (171, 172).

The continued rapid exchange of cholesterol between the ER and PM allows the cell to monitor cellular and membrane cholesterol levels (160, 173, 174). Specifically, in a state of sufficient sterol levels (>5% of total ER lipids), HMGR and SCAP bind to INSIG (175). HMGR is ubiquitinated and degraded following binding to INSIG, while SCAP and the bound SREBP are retained in the ER (176, 177). Because SREBP-2 is a transcription factor responsible for activation of transcription of all cholesterogenic genes (178, 179), this retention of SREBP-2 prevents further sterol synthesis to maintain cellular sterol homeostasis. However, when sterol levels drop below 5% of total ER lipids, HMGR and SCAP no longer bind INSIGs (175). The release of HMGR and SCAP prevents HMGR

degradation and promotes the trafficking of SCAP/SREBP from the ER to the Golgi complex to form the mature forms of the SREBP transcription factors that translocate to the nucleus (170, 180, 181). Mature SREBPs bind to the sterol response element (SRE) in the nucleus and activate genes necessary for sterol synthesis (178, 179), including up to a 200-fold increase in HMGR transcription (182, 183). It is of interest that the expression of specific SREBP isoforms, including SREBP-1c, is enhanced by insulin in liver, fat and skeletal muscle (184-187). In addition, hyperinsulinemia has been shown to increase SREBP-1 expression (188-190), which is responsible for regulating genes involved in fatty acid synthesis and to a lesser extent genes involved in cholesterol synthesis (178). Expression of SREBP-2, on the other hand, is mainly regulated by cellular cholesterol levels (191).

Another aspect of cholesterol regulation involves phosphorylation events targeting enzymes in the cholesterol synthesis pathway (192). For example, HMGR activity is regulated by phosphorylation, and insulin exposure has been specifically shown to increase HMGR activity (192). As described in detail below, the cellular energy sensor AMPK phosphorylates HMGR to regulate cholesterol levels depending on cellular energy status (193, 194). HMGR is also regulated by the cholesterol synthesis pathway itself by a feedback inhibition loop by which a downstream intermediate of cholesterol synthesis, lanosterol, can target HMGR for proteasomal degradation to decrease cholesterol levels (195). Another mechanism by which cells decrease cholesterol levels is through Acyl-coenzyme

A:cholesterol acyltransferase (ACAT) mediated esterification and storage of cholesterol (196).

Trafficking events involving the ATP-binding cassette transport proteins (ABCA1 and ABCG1) mediate efflux of cholesterol from cells (197, 198). The nuclear factor liver X receptor (LXR) facilitates cellular cholesterol efflux by binding in heterodimers to the retinoic X receptor (RXR) (199). LXR becomes activated by ligand binding, which has been suggested to involve oxysterols or intermediates from the steroid hormone and cholesterol synthesis pathways (200, 201). When ligands bind LXR, this nuclear factor binds to target promoters such as ABCA1 to induce gene transcription and promote cellular cholesterol efflux (202). As described above, the balance of cholesterol synthesis and efflux tightly regulates cellular cholesterol levels. However, studies suggest that cholesterol can become dysregulated and contribute to cellular insulin resistance.

Insulin resistance and the metabolic syndrome are closely associated with dyslipidemia, and specifically alterations in circulating and cellular cholesterol levels. Specifically, insulin resistance is associated with elevated LDL and decreased high density lipoprotein (HDL) levels. These alterations suggest that insulin resistance is closely related to dysregulation of cholesterol homeostasis (203). As previously described, insulin enhances expression of SREBP-1c (184-187). Furthermore, hyperinsulinemia has been shown to increase SREBP activity (188-190), and presumably cholesterol synthesis machinery. Along with SREBP-1c, insulin has also been shown to enhance HMGR activity (192). Of interest is recent evidence from our group demonstrating that hyperinsulinemia induces

membrane cholesterol accrual that impairs the trafficking of the cholesterol transporter ABCA1 and cholesterol efflux in 3T3-L1 adipocytes (24), potentially involving the HBP activation. Furthermore, our recent data highlights the role of membrane cholesterol accrual in the pathophysiology of insulin resistance (10, 11, 23). New data demonstrate increased membrane cholesterol levels in skeletal muscles membranes obtained from insulin-resistant mice, rats, swine and humans (10). Importantly, our analyses suggest an inverse relationship between membrane cholesterol content in human skeletal muscle biopsies and glucose disposal rate (10). It is now apparent that excess nutrient exposure in the forms of hyperinsulinemia, hyperglycemia and dyslipidemia can increase membrane cholesterol content and perturb GLUT4 regulatory components leading to insulin resistance. However, it remains unclear if skeletal muscle membrane cholesterol accrual contributes to cytoskeletal-based defects associated with hyperinsulinemia-induced insulin resistance and whether membrane/cytoskeletal defects can be therapeutically reversed by Cr^{3+} and AMPK action. Based on the fundamental concepts implicating cholesterol dysregulation and insulin resistance, the following subsection will specifically consider membrane/cytoskeletal derangements that impair GLUT4 regulation by insulin.

I.C.4. Membrane/cytoskeletal derangements in insulin resistance

Of particular interest to our group are the PM lipid environment and cortical actin cytoskeleton, which are required for the tethering, docking and fusion of

GSVs with the PM upon insulin stimulation. Based on evidence from multiple groups, it is well known that cortical F-actin dynamics are essential for insulin-regulated glucose transport in skeletal muscle and adipose tissue (72, 204-211). The cortical F-actin cytoskeleton, located immediately beneath the PM, is a highly-dynamic meshwork. The proximity of the cortical F-actin meshwork with the PM suggests an interplay between the PM and regulation of actin remodeling required for GLUT4 translocation (18, 21, 205-207, 212, 213). Pharmacological disruption of the actin cytoskeleton with latrunculin (208, 214, 215), cytochalasin D (207) and botulinum toxin C2 (216) inhibits insulin-stimulated GLUT4 translocation to the PM, supporting a crucial role for actin integrity in insulin action. It is well documented that insulin induces a very rapid reorganization of the cortical F-actin cytoskeleton, as early as 20 seconds following insulin stimulation, into a meshwork that involves membrane ruffling (204, 217, 218). In addition, actin comet-tails have been observed in adipocytes and skeletal muscle cells following insulin stimulation (215, 219, 220). Multiple groups have clearly shown that cytoskeletal derangements occurring in insulin resistance can disturb GLUT4 trafficking (7, 8, 16, 18-20), as recently reviewed (17, 21, 221). Studies from our group demonstrated that skeletal muscle insulin sensitivity critically relies on F-actin integrity (8, 18). In addition, our group has demonstrated that exposure of 3T3-L1 adipocytes and L6 myotubes to a physiologically relevant dose of insulin induces a loss of cortical F-actin and insulin resistance without detectable insulin signaling abnormalities (7, 8).

Several studies showing GLUT4 dysregulation without apparent defects in proximal insulin signaling point to the existence of other disabling factors of insulin-regulated GLUT4 exocytosis. With new signaling, cytoskeletal and bilayer additions available to our understanding, a major goal now is to precisely and accurately assess the functional status of the GLUT4 regulatory system. Whether critical flaws in cytoskeletal F-actin organization, lipid bilayer composition and/or GSV/PM fusion priming contribute to insulin resistance is not known, yet a clear association has been demonstrated between the diabetic milieu, cytoskeletal disorganization and bilayer abnormalities. For example, isolated neutrophils from patients with type 2 diabetes display decreased actin polymerization compared to neutrophils from non-diabetic control subjects (222). This impairment is associated with persistent expression of the endothelial adhering beta 2-integrin CD11b/CD18, potentially exacerbating vascular dysfunction in diabetic patients. Also, diabetic rat retinal endothelial cells have a prominent reduction in F-actin integrity, a finding closely linked to vascular leakage (223). Loss of rat mesangial cell F-actin has also been reported after exposure to early-diabetic state-like conditions (224), possibly causing diabetic hyperfiltration. Further support has been derived from examination of erythrocytes from overweight, insulin-resistant individuals which show marked changes in the phospholipid composition of the PM (225).

With regards to insulin action and glucose transport, cholesterol complexing drug experiments have demonstrated that removal of cholesterol from the PM augments basal glucose uptake and metabolism (226, 227). Consistent with

cholesterol causing highly-ordered gel-like states, moderate increases in PM fluidity increase glucose transport in adipocytes (228). Consistent with membrane fluidity influencing GLUT4 regulation, insulin-stimulated glucose transport declines when fluidity diminishes (228). Mechanistically, it is now appreciated that non-physiological PM cholesterol depletion greater than 50% reduces the rate of internalization of PM GLUT4 by more than 85% (229)**.** Although this certainly explains the gain in PM GLUT4, it is an artificial, experimentally-induced gain. In contrast, reductions of PM cholesterol less than 50% do not affect the rate of endocytosis, but do increase PM GLUT4 content (230). These later findings suggest moderate PM cholesterol lowering augments GSV exocytosis. In further support of a role for membrane cholesterol in regulating GSV exocytosis, several groups have demonstrated that hydrolysis of sphingomyelin by sphingomyelinase stimulates GLUT4 translocation to the PM and glucose transport (5, 230, 231). Our group further demonstrated that this insulin independent effect on GLUT4 and glucose transport was associated with a loss of PM cholesterol (230). Studies using methyl-β-cyclodextrin (βCD) (230), nystatin, filipin (230) and more recently Cr^{3+} treatment to lower membrane cholesterol have confirmed a key role of PM cholesterol in GLUT4 regulation (22, 23). How these PM cholesterol-based aspects of GLUT4 regulation intermingle with insulin signaling and/or GSV regulation is unknown, yet coordinated signaling events and/or F-actin reorganization are areas of interest (21). Perhaps PM cholesterol toxicity contributes to impaired PIP_2-regulated cortical F-actin

organization observed in insulin-induced insulin resistant 3T3-L1 adipocytes and L6 myotubes.

Although speculative, we view this as a possible indication that membrane/cytoskeletal defects in skeletal muscle and/or adipose tissue could negatively impinge on GLUT4 translocation, perhaps before defects are induced in proximal insulin signaling. Supporting this view are studies using various cell model systems of insulin resistance that demonstrate insulin signaling to Akt2/TBC1D4 is not impaired (7, 13, 16, 19, 78, 232). Collectively these data point to the notion that some insulin-resistant states may result from a membrane/cytoskeletal-based mechanical defect in GSV/PM arrival, tethering, docking and/or fusion. In summary, several new essential features of insulin-stimulated GLUT4 regulation have been revealed. From IR activation to GSV/PM fusion, multiple signals and a large number of processes apparently occur. Beyond GSV/PM fusion recent data also suggest that insulin continues to control the postfusion dispersal of GLUT4 in the PM (233). Although complete discussion of this new putative level of insulin regulation is out of the scope of this background, it exemplifies the fact that other unknown signals and processes likely await discovery. In the context of insulin resistance it becomes excruciatingly apparent why the failure of insulin-regulated glucose transport into skeletal muscle and adipose tissue is such an extremely difficult problem to solve. In conjunction with pinning down the complete mechanisms of the Akt2/GSV recruitment signal, the TC10/GSV tethering and actin reorganization system, and several putative PI3K transition signals (i.e., the RalA GSV

trafficking-to-tethering signal; the PKCζ/λ docking disassembly signal; and the PLD priming GSV/PM fusibility signal), important additional advances should include delineating the spatial and temporal aspects of all these signals and associated processes. Such additions will create a parallel opportunity to define the molecular events responsible for GLUT4 dysregulation in insulin resistance.

The studies described above have established membrane and cytoskeletal derangements as key distal aspects of insulin resistance that can impair insulin sensitivity independent of insulin signaling defects (7, 8, 10, 17-21). Interestingly, trivalent chromium (Cr^{3+}) is a micronutrient that has been shown to have beneficial effects on glucose and lipid metabolism and has been suggested to impact membrane/cytoskeletal parameters of skeletal muscle insulin sensitivity; however, the cellular and molecular mechanism(s) of Cr^{3+} action are still not known. The following subsections consider the history of Cr^{3+} in health and disease, clinical benefits of Cr^{3+} supplementation and outline several proposed mechanisms for Cr^{3+} action in adipose tissue and skeletal muscle.

I.D. Chromium: History and Effects on Glucose/Lipid Metabolism

The case for Cr^{3+} in the management of T2D began in 1957 with the discovery by Schwarz and Mertz that an unidentified substance in brewer's yeast prevented the age-related decline of glucose tolerance in rats (234, 235). Two years later, Schwarz and Mertz identified this unknown "glucose tolerance factor" as chromium (234, 236, 237). Based on this identification, chromium was originally established as an essential nutrient in animals required for optimal

glucose and lipid metabolism (234), although this has recently been challenged (238). Regardless, humans are unable to produce Cr^{3+}. Therefore, this nutrient must be obtained through dietary sources.

In 1977, Cr^{3+} was declared an essential nutrient for humans when significant elevations in blood glucose, peripheral neuropathy and unexpected weight loss were first observed in a patient receiving total parenteral nutrition (239). This patient was found to be glucose intolerant as assessed by intravenous glucose tolerance test (IVGTT). Regular insulin and glucose infusions were effective at restoring normal body weight in this patient; however, the patient's glucose intolerance was not improved. Levels of Cr^{3+} in the blood and hair were analyzed, and it was revealed that Cr^{3+} levels were extremely low compared to normally observed ranges. Based on the emerging roles of Cr^{3+} in glucose and lipid metabolism and the fact that the total parenteral nutrition was found to be devoid of Cr^{3+}, it was supplemented with Cr^{3+}. Addition of Cr^{3+} to the nutrition regimen for two weeks normalized the patient's glucose tolerance, and this supplementation reduced insulin requirements and subsided peripheral neuropathy in the following five months of hospitalization (239).

Several additional studies have supported the concept of Cr^{3+} essentiality in humans by showing that Cr^{3+} supplementation resulted in similar beneficial effects in other hyperglycemic patients (240-242). In further support of Cr^{3+} essentiality, evidence suggests that Cr^{3+} deficiency can lead to defects characteristic of cardiovascular disease such as elevated serum cholesterol, elevated triglycerides and decreased HDL levels (243-245). It is important to note

that Cr^{3+} has been well documented to have beneficial effects in *diabetic* animals and humans. As mentioned above, the essentiality of Cr^{3+} and effects on glucose metabolism in *healthy* animals has come into question based on findings in lean healthy rats (238, 246). These findings further support the concept that Cr^{3+} action is dependent upon the diabetic milieu. Nevertheless, increasing Cr^{3+} content in the diet of these healthy rats significantly improved insulin sensitivity (238), which suggests a beneficial effect of Cr^{3+} on glucose metabolism.

Nutritional Cr^{3+} in the trivalent form is found in a variety of foods such as brewer's yeast, whole grains, meats and vegetables. The popularity of Cr^{3+} supplements is significant, as chromium picolinate (CrPic) supplements are currently the second highest-selling health supplement behind sales of calcium supplements (247). Unlike hexavalent chromium (Cr^{6+}), which is a byproduct of stainless steel and pigment processing and is classified as carcinogenic for humans (248), Cr^{3+} is known to be stable and relatively non-toxic with a large safety range (249). A variety of forms of Cr^{3+} are ingested including chromium yeast (CrY), chromium chloride ($CrCl_3$) and CrPic, the most stable and bioavailable form of Cr^{3+} (250). Recently pharmacological compounds and Cr^{3+} complexes have been introduced such as chromium nicotinate/niacin (CrN) (251), chromium (D-phenylalanine)$_3$ (Cr(D-Phe)$_3$) (252), and chromium citrate (CrCIT) (253). These newly-designed forms of Cr^{3+} have been demonstrated to have beneficial effects on glucose and lipid metabolism (252, 254-258) and appear to be specific for Cr^{3+} as opposed to other minerals such as magnesium, manganese and iron. Together these fundamental findings demonstrate the

potential value of Cr^{3+} as a micronutrient and prompted future research efforts to determine the cellular and molecular mechanism(s) of Cr^{3+} action that impact glucose and lipid metabolism.

I.D.2. Effects of Cr^{3+} on glucose and lipid metabolism

Several *in vivo* studies have started to characterize the impact of Cr^{3+} on regulatory mechanisms of glucose and lipid metabolism and the basis for Cr^{3+}'s nutritive value. In spite of these arduous efforts since the original concept of Cr^{3+} essentiality, the mechanism(s) of Cr^{3+} action are still not completely understood nearly fifty years later (259). Animal and human studies have provided encouraging findings regarding the effects of Cr^{3+} in T2D (260-263). Several studies have demonstrated beneficial effects of Cr^{3+} on glucose metabolism, lipid metabolism and specifically insulin sensitivity in a variety of genetic and streptozotocin/alloxan-induced diabetic mouse (264) and rat models (253, 263, 265-268). Furthermore, studies have also shown that Cr^{3+} supplementation improves insulin sensitivity in hyperinsulinemic, insulin-resistant (262, 269) and diet-induced insulin-resistant rats (270). In addition, animal studies have shown that maternal chromium restriction induces glucose intolerance (271) and adiposity (272) in offspring, and deficiency of dietary Cr^{3+} can disrupt insulin sensitivity (273, 274).

An association between Cr^{3+} status and insulin resistance in humans has also been demonstrated (275). Several studies of Cr^{3+} supplementation in humans have yielded encouraging results regarding the clinical impact of this

micronutrient. For example, multiple human studies demonstrate that Cr^{3+} supplementation can improve aspects of glucose and lipid metabolism in patients with T2D. Interestingly, taken together the available human data seem to suggest that Cr^{3+} supplementation is most effective in T2D patients (276-280) compared to obese, insulin-resistant patients at risk for T2D (281-283). Adding another level of complexity to the observations of Cr^{3+} action in humans, it has been recently proposed that patient phenotype can be important in predicting clinical response (284), and that humans can potentially be classified into "responders" and "non-responders" based on prior T2D progression and glucose metabolism variables (285, 286). Regardless of the contradictory findings in humans studies with various strengths and weaknesses in study design (250, 287, 288), a recent meta-analysis conducted through August 2006 concluded that Cr^{3+} significantly improves glycosylated hemoglobin by -0.6% (-0.9 to -0.2) and fasting glucose by -1.0 mM (-1.4 to -0.5) in T2D subjects (289). No significant effect was found in people without diabetes, in accord with our *in vitro* findings described below that Cr^{3+}-effectiveness is contingent on the presence of a diabetic milieu (23).

I.D.2. Cellular and molecular mechanisms of Cr^{3+} action

Several mechanisms have been proposed for the interactions between Cr^{3+} and insulin action. For example, studies have shown a direct interaction of Cr^{3+} with insulin (290, 291). In addition, it has been shown that Cr^{3+} enhances insulin action by increasing IR number (292) and increasing the tyrosine kinase activity of IRs that have been previously activated by insulin (293, 294). Studies have

implicated transferrin in the transport of Cr^{3+} from the blood to insulin-sensitive tissues, as the loading of transferrin with Cr^{3+} has been demonstrated *in vitro* (295) and is increased following addition of Cr^{3+} to the bloodstream (296). Interestingly, insulin has been shown to stimulate the movement of transferrin receptors to the PM, which supports the idea that transferrin may deliver Cr^{3+} to insulin-sensitive tissues from the blood upon insulin stimulation (297, 298). However, the mechanisms involved in Cr^{3+} transport and interactions with transferrin are not completely understood. A naturally occurring oligopeptide known as low-molecular weight chromium-binding substance, or chromodulin, has been demonstrated to bind chromic ions in response to the insulin-stimulated activation of IR (293, 299). Chromodulin and its binding to chromium have recently been characterized after many years of arduous efforts to further understand this mediator in chromium action (300). The binding of chromodulin to chromic ions was shown to robustly stimulate IR's tyrosine kinase activity in the presence of insulin. Stimulation of IR activity induced by this binding event would be expected to increase insulin-stimulated GLUT4 translocation to the PM, as has been previously observed (22, 262). However, the insulin signaling mediators involved in this process have not been identified.

In further support of Cr^{3+} positively impacting GLUT4 regulation, published studies from our group demonstrate that Cr^{3+} at a clinically efficacious dose mobilized GLUT4 to the PM and enhanced insulin-stimulated glucose transport in 3T3-L1 adipocytes (22). Cr^{3+} did not increase the activation states of any known signaling mediators of insulin action. Reports have shown that Cr^{3+} increases

membrane fluidity and the rate of insulin internalization (301). While insulin-stimulated GLUT4 translocation does not appear to require insulin first being transported into the cell (302), studies have suggested that IR internalization may function to compartmentalize and promote efficient interaction with downstream insulin signaling substrate(s) associated with internal cellular membranes (303-305). In line with an effect of Cr^{3+} on membrane fluidity (301), our group has shown that Cr^{3+} treatment decreases PM cholesterol in 3T3-L1 adipocytes (22). Mechanistically, Cr^{3+} action was rendered ineffective by replenishment of cells with exogenous cholesterol (22). Since Cr^{3+} has the greatest benefit on glucose metabolism in hyperglycemic insulin-resistant individuals (289), we tested if this PM cholesterol-lowering Cr^{3+} effect was dependent on the diabetic milieu. Studies revealed that the GLUT4 redistribution to the PM in 3T3-L1 adipocytes treated with Cr^{3+} only occurred in cells cultured under high glucose (25 mM) conditions and not in cells cultured under non-diabetic (5.5 mM glucose) conditions (23). Cr^{3+} selectively lowered excess PM cholesterol in the high glucose cells, which contained more PM cholesterol than low glucose cells (23). New data from our group also suggest that Cr^{3+} protects against hyperinsulinemia-induced cholesterol accrual in 3T3-L1 adipocytes (24).

The actions of Cr^{3+} on PM cholesterol levels described in this subsection are consistent with its effects on membrane fluidity. As described above, moderate changes in membrane fluidity have been shown to increase glucose transport (228). In addition, augmentation of membrane fluidity has been documented to further increase basal glucose transport in fat cells, which is not fully active under

basal conditions (306). Interestingly, the antidiabetic drug metformin has also been shown to increase membrane fluidity and subsequently enhance insulin action (307, 308). Similarly to Cr^{3+}, this effect of metformin on membrane fluidity may underlie the mechanism by which metformin increases GLUT4 translocation (309, 310).

While studies reviewed in this section suggest that Cr^{3+} positively impacts GLUT4 via PM cholesterol lowering and potentially other cellular/molecular mechanisms, the antidiabetic actions of Cr^{3+} against hyperinsulinemia- and obesity-induced insulin resistance in skeletal muscle have not been studied extensively either *in vitro* or *in vivo*. Furthermore, whether Cr^{3+} improves cholesterol-based aspects of GLUT4 regulation (e.g. F-actin) is not known. Although Cr^{3+} is thought to be an essential nutrient in animals and humans by optimizing glucose and lipid metabolism, much work remains to dissect the mechanism(s) by which Cr^{3+} may have nutritional value. The rationale for dissecting mechanism(s) of Cr^{3+} action in this thesis research is that once the biochemical basis for Cr^{3+} action is completely dissected, its use as a supplement could be optimized and/or development of pharmacologically innovative approaches to the prevention and treatment of glucose/lipid metabolism disorders could be initiated.

With regards to Cr^{3+}, it is of further interest that AMPK activity has been observed to be increased in Cr^{3+}-treated cells or tissues from Cr^{3+}-supplemented animals (22, 24, 255, 311-315). Interestingly, it was recently demonstrated that Cr^{3+}-mediated activation of AMPK is involved in Cr^{3+} inhibiting the release of

resistin, which has been implicated in insulin resistance, in insulin-resistant 3T3-L1 adipocytes (311). Given the importance of membrane cholesterol regulation in insulin action and the development of insulin resistance, of interest is the fact that AMPK phosphorylates/inhibits HMGR, the rate-limiting enzyme in cholesterol synthesis. Arduous research efforts have focused on identifying cellular targets of therapeutic interest and new drug therapies to prevent or slow the progression of insulin resistance in obesity and T2D. AMPK represents a promising cellular target that has received much attention in the contexts of obesity, insulin resistance, dyslipidemia and T2D. The following section will focus on studies implicating AMPK in the regulation of glucose transport and cholesterol synthesis. This section will outline AMPK structure/function, AMPK regulation of insulin action and downstream cellular targets of AMPK that are of therapeutic interest in the treatment and/or prevention of insulin resistance.

I.E. AMPK Regulation of Glucose Transport and Cholesterol Synthesis

As described above, a major challenge in curbing the epidemics of obesity and T2D involves a lack of physical activity in the developed world in the face of overeating. It is well documented that physical exercise has positive effects on glucose homeostasis in healthy individuals as well as those with T2D (316, 317). The benefits of exercise are based on the concept that muscle contraction is known to be a potent stimulus of glucose transport (318, 319) and has been shown to increase skeletal muscle insulin sensitivity (320-323). The insulin-sensitizing effects of exercise in skeletal muscle are significant as skeletal

muscle is responsible for a large majority of post-prandial glucose disposal (29) and is regarded as a major site of insulin resistance (28). The mechanism(s) by which exercise elicits these effects on glucose transport and insulin sensitivity is not completely understood; however, several effectors have been implicated in contraction/exercise-induced glucose transport. These putative mechanisms include, but are not limited to, calcium (53, 324, 325), nitric oxide (NO) (326, 327), bradykinin (328, 329) and AMPK (324, 330, 331). Studies have now established AMPK as a central player of the energy-sensing mechanisms involved in contraction/exercise-stimulated glucose transport. In addition to contraction/exercise, AMPK has also been proposed as a mediator of insulin-sensitizing and antidiabetic therapies such as metformin (332, 333), resveratrol (334-337), epigallocatechin gallate (338, 339), berberine (340-342), bitter melon (343), the plant sterol β-sitosterol (344), nigella sativa seed ethanol extract (345) and Cr^{3+} (22, 24, 255, 311-315). AMPK has also been shown to be involved as a mediator of the actions of insulin-sensitizing adipokines and cytokines such as adiponectin (346-348), leptin (349, 350) and IL-6 (348, 351-353). Taken together, our current understanding of AMPK-based regulation of insulin sensitivity and glucose transport in skeletal muscle establishes AMPK as a therapeutic target of interest for insulin resistance and T2D.

I.E.1. AMPK Structure and Regulation

AMPK has been referred to as a “fuel gauge” for maintaining cellular energy regulation (354). AMPK is a heterotrimeric Ser/Thr protein kinase and is

conserved from yeast to humans (355, 356). This kinase is composed of three subunits; a catalytic α subunit and regulatory β and γ subunits (357). AMPK is found in many tissues throughout the body (358) and functions as an energy sensor to maintain cellular energy homeostasis (330, 359). One isoform of the α subunit is the α1 isoform. Complexes containing α1 are ubiquitously expressed, while α2-containing complexes are more highly expressed in skeletal muscle, heart and liver (360-362). These isoforms are thought to play different roles in maintaining cellular energy homeostasis due to variations in their target specificity and cellular locations (363, 364). Kinase activity is primarily possessed by the α subunit, but the β and γ subunits contribute to regulation of kinase activity along with their roles in stabilizing the heterotrimeric kinase complex (362). The β subunit contains a glycogen binding domain that is associated with inhibition of AMPK when glycogen is bound (365-367). In addition, the γ subunit contains cystathionine-β-synthase (CBS) binding domains that are important for binding molecules containing adenosine (368). CBS binding domains specifically bind 5’ adenosine monophosphate (AMP), which results in allosteric activation of AMPK (369).

AMPK is activated in skeletal muscle by cellular energy stresses that lead to depletion of high-energy molecules such as 5’ adenosine triphosphate (ATP), phosphocreatine and glycogen. An increase in the AMP/ATP ratio results in accumulated AMP binding to the CBS domain of the γ subunit (369) and subtly increases kinase activity of AMPK. Along with this allosteric activation, AMP binding also causes a conformational change that favors the α subunit’s

activation loop being targeted by upstream kinases on Thr172 (370-372). Two kinases, including LKB1 (373-375) and calmodulin-dependent protein kinase kinase β (CaMKKβ) (376, 377) have been implicated as the kinases upstream of AMPK that target Thr172. Phosphorylation of AMPK at Thr172 has been shown to be essential for kinase activity (371, 378, 379), and phosphorylation levels are highly associated with kinase activity level (379).

I.E.2. Downstream Targets of AMPK

AMPK induces both acute and chronic metabolic adaptations once it becomes activated to restore cellular energy homeostasis. Acutely, AMPK directly regulates downstream targets by phosphorylation. Chronic effects of AMPK activation involve regulating expression of genes involved in glucose and lipid metabolism (380-383), for example. Activation of AMPK is now recognized as being partially responsible for health benefits from exercise (384). Similarly to endurance training, AMPK elicits adaptive metabolic responses such as increased uptake and metabolism of glucose and FAs. AMPK has also been shown to regulate enzymes central to the catabolism of glucose and FAs, including mitochondrial enzymes (380-383, 385-388). AMPK activation acutely functions to restore cellular energy homeostasis by inhibiting anabolic pathways that consume energy and activating catabolic pathways that increase energy in the cell (specifically ATP) (330, 354). It is well documented that AMPK phosphorylates the downstream target acetyl-CoA carboxylase (ACC) on Ser79. Phosphorylation of ACC by AMPK inhibits this enzyme resulting in a switch from

synthesizing to oxidizing FAs. Inhibition of ACC by AMPK-mediated phosphorylation decreases the product of this enzyme, malonyl-CoA, a key intermediate in FA synthesis (389-391). Furthermore, it has been demonstrated that AMPK activation results in activation of malonyl-CoA decarboxylase, which further depletes malonyl-CoA levels (392). The decrease in malonyl-CoA levels relieves the inhibition of carnitine palmitoyltransferase 1 (CPT-1), which allows for increased transport of FAs into the mitochondria for oxidation (393-396). The increased FA oxidation is also accompanied by a reciprocal decrease in the amount of substrate for FA esterification.

Another important downstream target that is phosphorylated by AMPK at Ser872 is HMGR (397-399). Of interest is the fact that AMPK can directly inactivate the rate limiting enzyme in cholesterol synthesis and decrease lipid biosynthesis given our understanding of how cellular cholesterol accrual perturbs GLUT4 regulation and insulin sensitivity. AMPK-based regulation of cholesterol synthesis will be described below in more detail. Explored in the next section are fundamental findings that begin to dissect how AMPK regulates glucose transport and set the stage for our studies to determine the role of membrane cholesterol in AMPK and Cr^{3+} action.

I.E.3. AMPK Regulation of Glucose Transport

As described in detail above, muscle contraction during exercise is known to stimulate glucose transport (318, 319) and increase skeletal muscle insulin sensitivity (320-323). Furthermore, AMPK activation has been demonstrated to

acutely increase glucose transport in skeletal muscle both *in vivo* and *in vitro* (400-409). Specifically, AMPK activation increases glucose transport in skeletal muscles obtained from animal models (402) (400, 401) and cultured skeletal muscle myotube lines such as L6 (403-406), C2C12 (407) and H-2K (408, 409). This insulin-mimetic stimulation of glucose transport by AMPK has been shown to be mediated by increased GLUT4 delivery and accumulation in the PM (410). However, the exact mechanisms by which AMPK activation acutely affects GLUT4 regulation and insulin sensitivity are not fully understood. In addition to its insulin mimetic actions, AMPK has also been shown to have insulin-sensitizing effects (411-413). Putative mechanisms tying AMPK's insulin-mimetic and insulin-sensitizing actions to GLUT4 trafficking will be described in detail below.

In addition to acutely stimulating glucose transport, AMPK activation expands the long-term cellular capacity for glucose transport and oxidation by positively regulating transcription of genes involved in these processes (414). For example, AMPK activation has been shown to positively regulate transcription of GLUT4 (381, 400, 415, 416), citrate synthase, succinate dehydrogenase (385), cytochrome C and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) (417-419). Mechanisms involved in the AMPK-mediated increase in GLUT4 expression include the specific activation of transcription factors such as myocyte enhancer factor-2 (MEF-2), GLUT4 enhancer factor (GEF) (420) and nuclear respiratory factor 1 (NRF1) (382, 421).

A well-accepted hypothesis relating AMPK activation to GLUT4 accumulation in the PM involves AMPK-mediated phosphorylation of AS160/TBC1D4 and its

homolog TBC1D1. These Rab GAPs, which are distal players in the insulin signaling pathway of GSV recruitment, are emerging as a putative nexus between the AMPK and insulin signaling pathways to explain the insulin-mimetic and insulin-sensitizing actions of AMPK. AMPK activation and insulin have both been shown to increase phosphorylation levels of TBC1D4 and TBC1D1 (422-424) to regulate GSV trafficking (37, 331). Study has shown that mutation of these putative phosphorylation residues on TBC1D4 prevents AMPK's insulin-mimetic action (37). In further support of AMPK's dual actions on mimicking and sensitizing insulin action, recent kinetic evidence demonstrates that AMPK can regulate a separate pool of GLUT4 as insulin to further potentiate PM GLUT4 accumulation and glucose transport (404).

Mechanistically, AMPK has well accepted roles in cellular, tissue-specific and whole body energy balance (359). AMPK knockout studies have been helpful in establishing roles for AMPK isoforms in skeletal muscle. Whole body knockout of the AMPK α2 subunit prevented pharmacological AMPK activation-induced increases in skeletal muscle glucose uptake, while knockout of the α1 subunit had no effect (425). In addition, mice lacking the α1 isoform present no defect in glucose homoeostasis, as assessed by glucose tolerance testing (426). In support of a role for the AMPK α2 subunit regulating glucose homeostasis, mice lacking the α2 isoform clearly exhibit reduced insulin sensitivity (426) and the α2 isoform is the major catalytic isoform expressed in skeletal muscle (360, 362). The role of AMPK in insulin resistance has been studied using muscle-specific transgenic mice expressing an inactive form of the AMPK α2 catalytic subunit

(427). These studies demonstrated that ablation of muscle AMPK α2 activity worsens the glucose intolerance induced by high fat (HF) feeding. This exacerbated glucose intolerance was associated with a decrease in insulin-stimulated muscle glucose transport, measured in isolated muscles *in vitro* (427). Together these results demonstrate that AMPK α2 activity is an important factor contributing to insulin action on glucose transport in skeletal muscle. While the mechanisms linking AMPK to glucose transport outlined in this subsection are still not completely understood, an appreciation is emerging for the role of AMPK-mediated regulation of cellular cholesterol in modulating insulin sensitivity. Recent work from our group has provided evidence that targeting AMPK may be beneficial for cholesterol-based aspects of GLUT4 dysregulation in insulin resistance.

I.E.4. AMPK Regulation of Cholesterol Synthesis

As described above, an important downstream target phosphorylated by AMPK at Ser872 is HMGR (397-399). HMGR is the rate-limiting enzyme in cholesterol synthesis, and phosphorylation of HMGR at Ser872 inhibits this enzyme to shut off cholesterol biosynthesis. AMPK is well appreciated to play roles in improving plasma lipids and cellular lipid stores (359, 428). While regulation of cellular cholesterol is a tightly controlled process in mammalian cells, cellular cholesterol accrual can become dysregulated in states of obesity and insulin resistance as detailed above. For example, tissues obtained from insulin-resistant mice, rats, swine and humans all display an increase in

membrane cholesterol content (10). Exposure of adipocytes and skeletal muscle cells to excess glucose, insulin and FAs has been shown to induce membrane cholesterol accrual (10, 11, 23, 24). Recent evidence suggests that AMPK activation and Cr^{3+}, potentially via activation of AMPK, can restore elevated cellular membrane cholesterol in insulin-resistant 3T3-L1 adipocytes leading to beneficial effects on cellular cholesterol efflux machinery (24). Furthermore, our group recently determined that treatment of L6 skeletal muscle myotubes with excess FA's results in an increase in membrane cholesterol content that perturbs GLUT4 regulation and involves increased expression of *Hmgcr*, the gene that encodes for HMGR (10).

Based on the hypothesis that AMPK and Cr^{3+} action inhibit cholesterol synthesis via phosphorylation of HMGR and may improve key membrane/cytoskeletal parameters of insulin sensitivity, my thesis research explored the potential role(s) of the AMPK-HMGR cholesterol regulatory axis in Cr^{3+} action and control of PM GLUT4 accumulation. An improved mechanistic understanding of how AMPK and Cr^{3+}-induced AMPK activity affect membrane/cytoskeletal aspects of GLUT4 regulation and dysregulation in insulin resistance is of paramount importance because it opens novel cellular avenues for nutritional AMPK-targeted therapies for insulin resistance and T2D.

I.F. Thesis Hypothesis and Specific Aims

Based on the fundamental research findings described above and current gap in understanding regarding the mechanism(s) of chromium action, especially in

skeletal muscle, I formulated the following hypothesis for my thesis research. My central hypothesis is that chromium, via AMPK activation, protects against GLUT4 and glucose transport dysfunction in skeletal muscle cells by protecting against membrane cholesterol accrual that induces F-actin loss and insulin resistance. Furthermore, I hypothesized that the actions of chromium and AMPK would improve skeletal muscle insulin sensitivity and glucose metabolism *in vivo*. Using cellular and animal models of insulin resistance, I tested my central hypothesis by pursuing the following two specific aims: 1) To define chromium's action(s) against membrane/cytoskeletal derangements and insulin resistance; and 2) to dissect the mechanism(s) by which AMPK activity positively impacts GLUT4 regulation.

Chapter II. Results

II.A. AMPK is Involved in a Membrane/Cytoskeletal Pathway of Chromium Action that Improves Glucose Transport Regulation in Insulin-Resistant Skeletal Muscle Cells

II.A.1. Summary

A recent review of randomized controlled trials found that Cr^{3+} supplementation significantly improved glycemia among patients with diabetes, consistent with a long-standing appreciation that this micronutrient optimizes carbohydrate metabolism. Nevertheless, a clear limitation in the current evidence is a lack of understanding of Cr^{3+} action. Here we tested if increased AMPK activity, previously observed in cells treated with Cr^{3+} or tissues from animals supplemented with Cr^{3+}, mediates improved glucose transport regulation under insulin-resistant hyperinsulinemic conditions. In L6 myotubes stably expressing the glucose transporter GLUT4 that carries an exofacial *myc*-epitope tag, acute insulin-stimulation (20 min, 100 nM) increased GLUT4*myc* translocation by 69% and glucose uptake by 97%. In contrast, a hyperinsulinemic state, induced by inclusion of 5 nM insulin in the medium for 12 h, impaired stimulation of these processes by insulin. Consistent with Cr^{3+}'s beneficial effects on glycemic status, supplementing the medium with 100 nM chromium picolinate (CrPic) for 16 h restored insulin's ability to fully regulate GLUT4*myc* translocation and glucose transport. The insulin-resistant myotubes did not display impaired insulin

signaling, nor did CrPic amplify insulin signaling. However, CrPic normalized elevated membrane cholesterol that impaired cortical F-actin structure. Mechanistically, data support that CrPic lowered membrane cholesterol via AMPK. Consistent with this data, siRNA-mediated AMPK silencing blocked CrPic's beneficial effects on GLUT4 and glucose transport regulation. These data identify a mechanism by which Cr^{3+} may positively impact glycemic status, thereby stressing a plausible, essential action of this micronutrient in glucose homeostasis.

II.A.2. Results

Cr^{3+} protects against hyperinsulinemia-induced GLUT4/glucose transport dysfunction: We first performed studies to determine if Cr^{3+} protects against hyperinsulinemia-induced insulin resistance in skeletal muscle cells using the L6-*GLUT4myc* myotube cell system. The L6 myotube cell line stably expresses GLUT4 carrying a *myc* epitope on the first exofacial domain of GLUT4 and allows for detection of GLUT4 transporters at the PM with glucose transport functionality (429). This *myc*-tagged transporter segregates, cycles, and responds to insulin similarly to endogenous GLUT4 (430, 431). Myotubes cultured with or without hyperinsulinemia (12 h, 5 nM) and/or CrPic (16 h, 100 nM) were left untreated or acutely stimulated with insulin (20 min, 100 nM) to determine the ability of insulin to stimulate GLUT4 translocation to the PM. As shown in **Figure 2A**, *myc* epitope labeling at the PM was increased 69% ($P<0.05$, compare bars 1 and 2) in response to acute insulin stimulation.

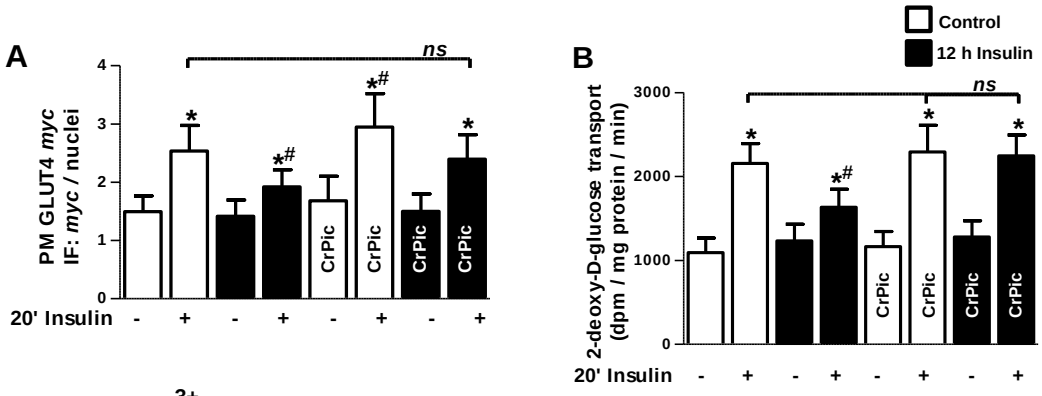

Figure 2. Cr^{3+} protects against hyperinsulinemia-induced GLUT4/glucose transport dysfunction. L6-GLUT4*myc* myotubes were pretreated without (white bars) or with (black bars) 5 nM insulin for 12 h in the absence or presence of 100 nM CrPic, which was added to the culturing medium 4 h before the 12-h insulin pretreatment for a total time of 16 h. After the 16-h and 12-h pretreatments, cells were either left untreated (A, panels 1, 3, 5, and 7) or treated (A, panels 2, 4, 6, and 8) with 100 nM insulin for 20 min. GLUT4*myc* immunofluorescence (A) was assessed and normalized using Syto 60 nuclear stain. Mean values ± SEM of GLUT4*myc* are shown from 6-7 independent experiments. All insulin-stimulated GLUT4*myc* values were significantly (*, P<0.05) elevated over their respective controls. *#, P<0.05 vs. insulin-stimulated control. For 2-DG uptake experiments (B), cells were pretreated as described above. After the 16-h and 12-h pretreatments, cells were either left untreated (B, panels 1, 3, 5, and 7) or treated (B, panels 2, 4, 6, and 8) with 100 nM insulin for 20 min. 2-DG transport values were normalized to protein content in each sample. Mean values ± SEM of 2-DG transport are shown from 6-7 independent experiments. All insulin-stimulated 2-DG transports were significantly (*, P<0.05) elevated over their respective controls. *#, P<0.05 vs. insulin-stimulated control.

The ability of insulin to stimulate GLUT4 translocation was inhibited by ~50% ($P<0.05$) in myotubes exposed to hyperinsulinemia compared to insulin-stimulated control myotubes (compare bars 2 and 4). Neither hyperinsulinemia nor CrPic had no significant effect on basal PM GLUT4 detection (compare bars 1, 3, and 5); however, CrPic-treated myotubes displayed a 16% ($P<0.05$) increase in insulin-stimulated PM GLUT4 levels versus control insulin-stimulated myotubes (compare bars 2 and 6). Importantly, CrPic protected against the hyperinsulinemia-induced loss in GLUT4 regulation by insulin (compare bars 2, 4, and 8). To directly test the ability of these PM-localized GLUT4 transporters to transport glucose following the acute insulin stimulation, we performed 2-deoxy-D-glucose (2-DG) uptake assays (**Fig. 2B**). Glucose transport was increased 97% ($P<0.05$; compare bars 1 and 2) in response to acute insulin stimulation. The ability of insulin to stimulate glucose transport was inhibited by 67% ($P<0.05$) in myotubes exposed to hyperinsulinemia compared to insulin-stimulated control myotubes (compare bars 2 and 4) with no change in basal glucose transport levels (compare bars 1 and 3). CrPic had no significant effect on basal or insulin-stimulated glucose transport rate in control myotubes (compare bars 1 and 5 and 2 and 6). However, and in line with the measured increase in PM GLUT4, CrPic restored the hyperinsulinemia-induced loss in glucose transport regulation by insulin (compare bars 2, 4, and 8).

Cr^{3+} suppresses PM cholesterol accrual accompanying cortical F-actin loss: Previously we documented F-actin loss in these myotubes contributed to

insulin-induced insulin resistance (8). We now find in this hyperinsulinemic cell model of insulin resistance that a 20% ($P<0.05$) increase in PM cholesterol accompanies the 11% ($P<0.05$) decrease in F-actin (**Figs. 3A and 3B**, bars 2). Moreover, these hyperinsulinemia-induced changes were prevented by CrPic treatment (**Fig. 3A and 3B**, bars 4) with no effect in control myotubes (bars 3). To probe this observation further, we next tested the effect of zaragozic acid A (ZA), which inhibits the enzyme squalene synthase downstream of HMGR and effectively blocks cholesterol synthesis (432). An advantage to using this strategy versus direct blockade of HMGR activity with a statin is that statins have recently been reported to also increase AMPK activity (433). Similarly to CrPic, ZA (16 h, 10 μM) pretreatment protected against hyperinsulinemia-induced PM cholesterol accrual and F-actin loss (**Figs. 4A and 4B**, bars 4) with no effect observed in control myotubes (bars 3). Consistent with these hyperinsulinemia induced membrane/cytoskeletal changes and insulin resistance being corrected by the cholesterol-lowering action of Cr^{3+}, ZA treatment protected against the hyperinsulinemia-induced loss in insulin-stimulated GLUT4 translocation (**Fig. 4C**, compare bars 2, 3, and 5). ZA tended to increase PM GLUT4 in control myotubes (compare bars 2 and 4) similarly to CrPic; however this did not reach statistical significance.

Insulin signaling-independent action of Cr^{3+}: Our previous studies suggested that neither hyperinsulinemia exposure nor effects of CrPic could be attributed to

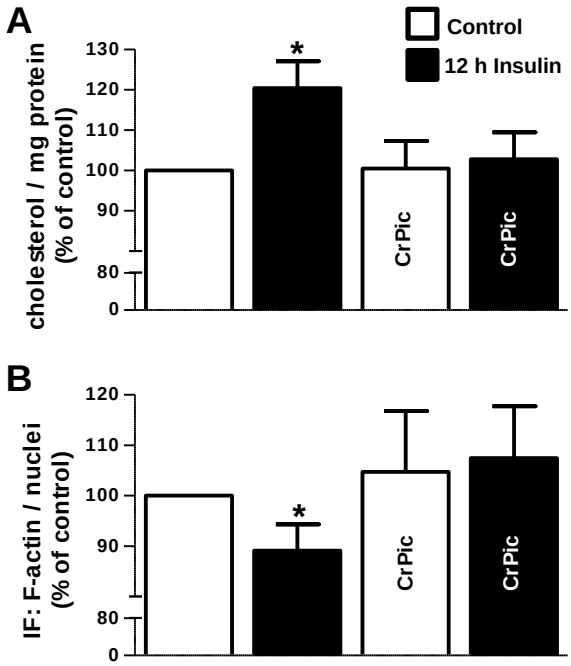

Figure 3. Cr^{3+} protects against hyperinsulinemia-induced membrane/cytoskeletal derangements. L6-GLUT4*myc* myotubes were pretreated without (white bars) or with (black bars) 5 nM insulin for 12 h in the absence or presence of 100 nM CrPic, which was added to the culturing medium 4 h before the 12-h insulin pretreatment for a total time of 16 h. Cholesterol measurement following membrane fractionation (A) and quantification of immunofluorescent labeling of F-actin (B) were determined, respectively. Mean values ± SEM of membrane cholesterol and F-actin are shown from 4-11 independent experiments. *, $P<0.05$ vs. control.

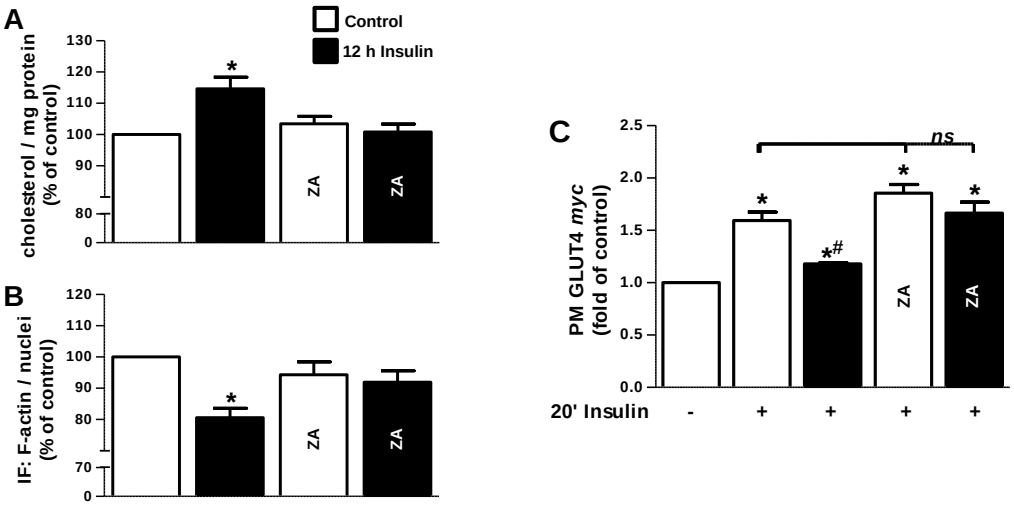

Figure 4. Cholesterol synthesis inhibition protects against hyperinsulinemia-induced membrane/cytoskeletal derangements and insulin resistance. L6-GLUT4*myc* myotubes were pretreated without (white bars) or with (black bars) 5 nM insulin for 12 h in the absence or presence of 10 µM zaragozic acid A (ZA), which was added to the culturing medium 4 h before the 12-h insulin pretreatment for a total time of 16 h. Cholesterol measurement following membrane fractionation (A) and quantification of immunofluorescent labeling of F-actin (B) were determined, respectively. Mean values ± SEM of membrane cholesterol and F-actin are shown from 3-4 independent experiments. *, P<0.05 vs. control. To assess the effects of ZA on hyperinsulinemia-induced GLUT4 dysregulation, cells were pretreated as described in Figure 2. After the 16-h and 12-h pretreatments, cells were either left untreated or treated with 100 nM insulin for 20 min and GLUT4*myc* immunofluorescence was determined (C). Mean values ± SEM of GLUT4*myc* are shown from 3 independent experiments. Insulin-stimulated GLUT4*myc* were significantly (*, P<0.05) elevated over their respective controls. *#, P<0.05 vs. insulin-stimulated control.

decreases or increases, respectively, in insulin signaling (7, 8, 22, 23), although studies have suggested that CrPic amplifies insulin signaling (294, 434, 435). Therefore, we further evaluated key insulin signaling parameters of insulin action. As previously reported in this model system (8), hyperinsulinemia did not impair basal or insulin-stimulated phosphorylation of Akt2 (**Fig. 5A**, compare bars 1-4) or phosphorylation of its downstream substrate AS160 (**Fig. 5B**, compare bars 1-4). In addition, similar to published observations in 3T3-L1 adipocytes **(22)**, CrPic treated control or hyperinsulinemic cells did not display any change in basal or insulin-stimulated phosphorylation of Akt2 or AS160 (**Figs. 5A and 5B**, bars 4-8).

Increased AMPK activity mediates Cr^{3+} action: Consistent with previous observations in Cr^{3+}-treated cells (22, 24, 311-313) or tissue from Cr^{3+}-supplemented animals (255, 314, 315), AMPK activity was increased as evidenced by increased phosphorylation of the catalytic alpha subunit at Thr172 (**Fig. 6A**, compare bars 1 and 2). Although this increase was very slight (14%) it was statistically significant. Treatment with hyperinsulinemia had no significant effect on Thr172 phosphorylation (**Fig. 6A**, compare bars 1 and 3). Consistent with the concept that Cr^{3+} action is dependent on the diabetic milieu, we observed that CrPic-induced phosphorylation of AMPK was 30% ($P<0.05$) more robust in hyperinsulinemia-induced insulin-resistant myotubes compared to control myotubes (**Fig. 6A**, *$P<0.05$; compare bars 2 and 4). The maximal increase in AMPK activity observed in CrPic-treated myotubes exposed to hyperinsulinemia is more similar to the level of AMPK phosphorylation we

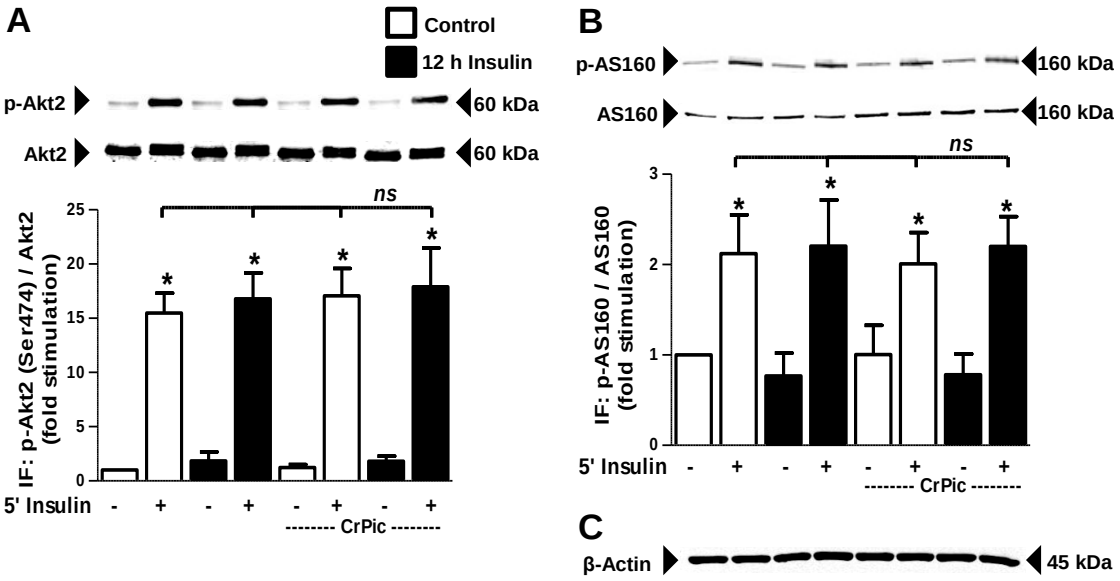

Figure 5. Insulin signaling-independent action of Cr^{3+}. Cells were treated as described in Figure 2. After the 16-h and 12-h pretreatments, cells were either left untreated (panels 1, 3, 5, and 7) or treated (panels 2, 4, 6, and 8) with 100 nM insulin for 5 min. After treatment, whole-cell lysates were prepared and subjected to Western blot analyses to assess Akt2(Ser474) phosphorylation (A), phospho-Akt substrate phosphorylation (B), and β-actin (C). White bars and black bars represent control and hyperinsulinemic groups, respectively. Immunoblots shown in A and B are representative of 3-8 experiments, and all quantitated values presented as means ± SEM from # 3-8 experiments were determined by densitometry and normalized to total protein (Akt2, AS160). All insulin-stimulated values were significantly (*, $P<0.05$) elevated over their respective controls.

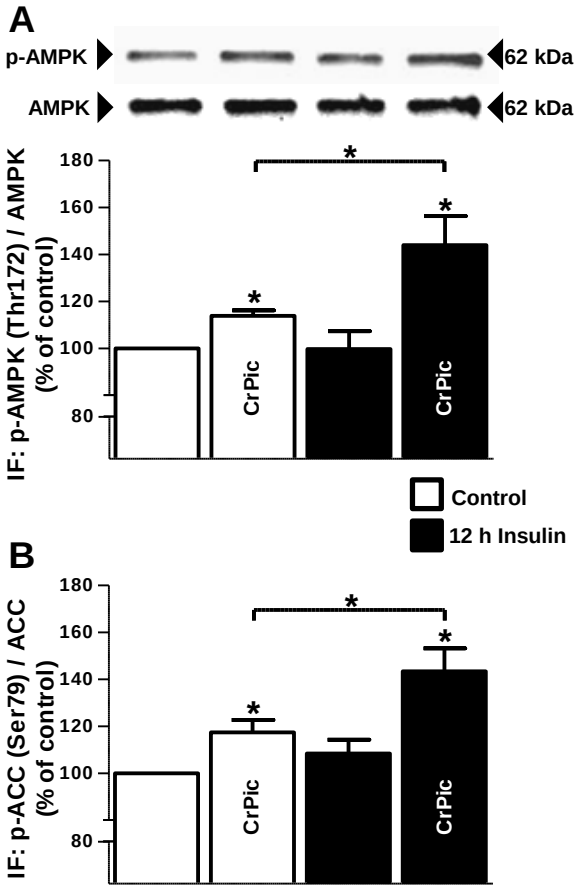

Figure 6. Cr^{3+} increases activation of AMPK. Cells were treated as described in Figure 3. After treatment, whole-cell lysates were prepared and subjected to Western blot analyses to assess AMPK(Thr172) phosphorylation (A) and ACC(Ser79) phosphorylation (B). White bars and black bars represent control and hyperinsulinemic groups, respectively. Immunoblots shown in A and B are representative of 5 independent experiments, and all quantitated values presented as means ± SEM from 5 independent experiments were determined by densitometry and normalized to total protein (AMPK and ACC, respectively). *, $P<0.05$ vs. respective control.

observe with the widely-used experimental activator of AMPK, 5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleoside (*AICAR*). To confirm that CrPic treatment increased AMPK activity, we next determined the effect of CrPic on phosphorylation of acetyl-CoA carboxylase (ACC) at serine residue 79 (Ser79), a well characterized downstream substrate of AMPK. **Fig. 6B** shows that CrPic induced a similar 17% increase ($P<0.05$) in phosphorylation of ACC at Ser79 in CrPic-treated myotubes compared to control (compare bars 1 and 2), and hyperinsulinemia had no significant effect on ACC Ser79 (compare bars 1 and 3). In line with our AMPK Thr172 findings, CrPic treatment robustly increased phosphorylation of ACC at Ser79 in myotubes exposed to hyperinsulinemia by 43% ($P<0.05$, compare bars 2 and 4). Note that data were normalized to respective total protein amounts using α-AMPK alpha subunit and α-ACC antibodies.

We next tested the effect of siRNA-mediated knockdown of the catalytic α subunits of AMPK on the ability of AICAR and DNP CrPic to modulate GLUT4 and glucose transport regulation. 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 75% (**Fig. 7**, $P<0.05$). Similar to non-transfected myotubes, exposure of both siScramble control and siAMPK myotubes to hyperinsulinemia significantly impaired insulin-stimulated fold increase in PM GLUT4 (**Fig. 8A**, $P<0.05$; compare bars 1 and 2, 5 and 6) and 2-DG uptake (**Fig. 8B**, $P<0.05$; compare bars 1 and 2, 5 and 6). Cr^{3+} had no

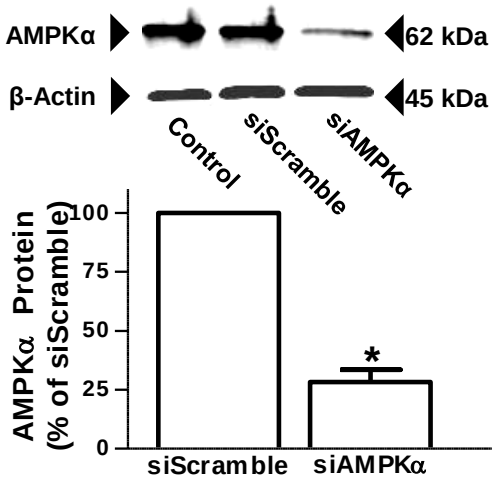

Figure 7. AMPKα knockdown efficiency. Following transient siRNA transfection of scrambled control oligos (siScramble) or oligos targeting the α catalytic subunits of AMPK (siAMPK) as described in *Materials and Methods,* whole-cell lysates were prepared and subjected to Western blot analyses to assess pan-AMPKα knockdown efficiency of pan-AMPKα. Immunoblots are representative of 4 independent experiments, and all quantitated values presented as means ± SEM from 4 independent experiments were determined by densitometry and normalized to β-actin. *, $P<0.05$ vs. respective control.

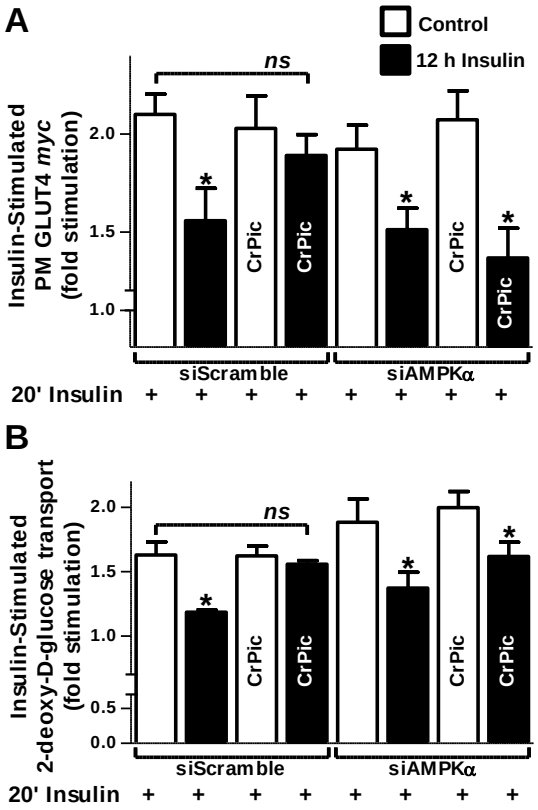

Figure 8. AMPKα is required for Cr^{3+}'s protection against hyperinsulinemia-induced GLUT4 and glucose transport dysfunction. Following transient siRNA transfection of scrambled control oligos (siScramble) or oligos targeting the α catalytic subunits of AMPK (siAMPK) as described in *Materials and Methods*, GLUT4*myc* and 2-DG transport was analyzed. Cells were pretreated as described in Figure 2. After the 16-h and 12-h pretreatments, cells were either left untreated or treated with 100 nM insulin for 20 min. The fold increase in insulin-stimulated GLUT4*myc* immunofluorescence was determined (A) or 2-DG transport was assessed (B). Mean values ± SEM of GLUT4*myc* and 2-DG transport are shown from 6-8 independent experiments. *, $P<0.05$ vs. insulin-stimulated control.

significant effect on insulin-stimulated PM GLUT4 (**Fig. 8A**, compare bars 1 and 3, 5 and 7) and 2-DG uptake (**Fig. 8B**, compare bars 1 and 3, 5 and 7) in insulin-sensitive myotubes. Importantly, CrPic pretreatment of insulin-resistant siScramble myotubes protected against GLUT4 (**Fig. 8A**, compare bars 1 and 4) and 2-DG transport dysregulation (**Fig. 8B**, compare bars 1 and 4); however, the beneficial, protective effects of CrPic against GLUT4 (**Fig. 8A**, $P<0.05$; compare bars 5 and 8) and 2-DG transport dysregulation (**Fig. 8B**, $P<0.05$; compare bars 5 and 8) were not present in siAMPK myotubes. Note we have not measured membrane cholesterol or F-actin in these transfected cells based on technical difficulties and the high cost associated with these studies, which require a large amount of cells for starting material. However, future studies are planned to delineate the effects of CrPic on membrane cholesterol and F-actin in both wild-type and AMPK knockout mice, as described in detail below in the *Perspectives* section.

In summary, data from the *in vitro* studies presented in Chapter IIA offer several novel observations. First, accompanying the hyperinsulinemia-induced decrease in F-actin structure we have previously observed in insulin-resistant L6 skeletal muscle myotubes (8) is an increase in membrane cholesterol content (**Fig. 9A**). Second, Cr^{3+} effectively protects against GLUT4 and glucose transport dysfunction induced by hyperinsulinemia (**Fig. 9B**). Third, we provide important insight into the mechanism of Cr^{3+} action by demonstrating that CrPic increases phosphorylation/activation of AMPK in both control and insulin-resistant myotubes and that AMPK is required for the protective effect of CrPic against

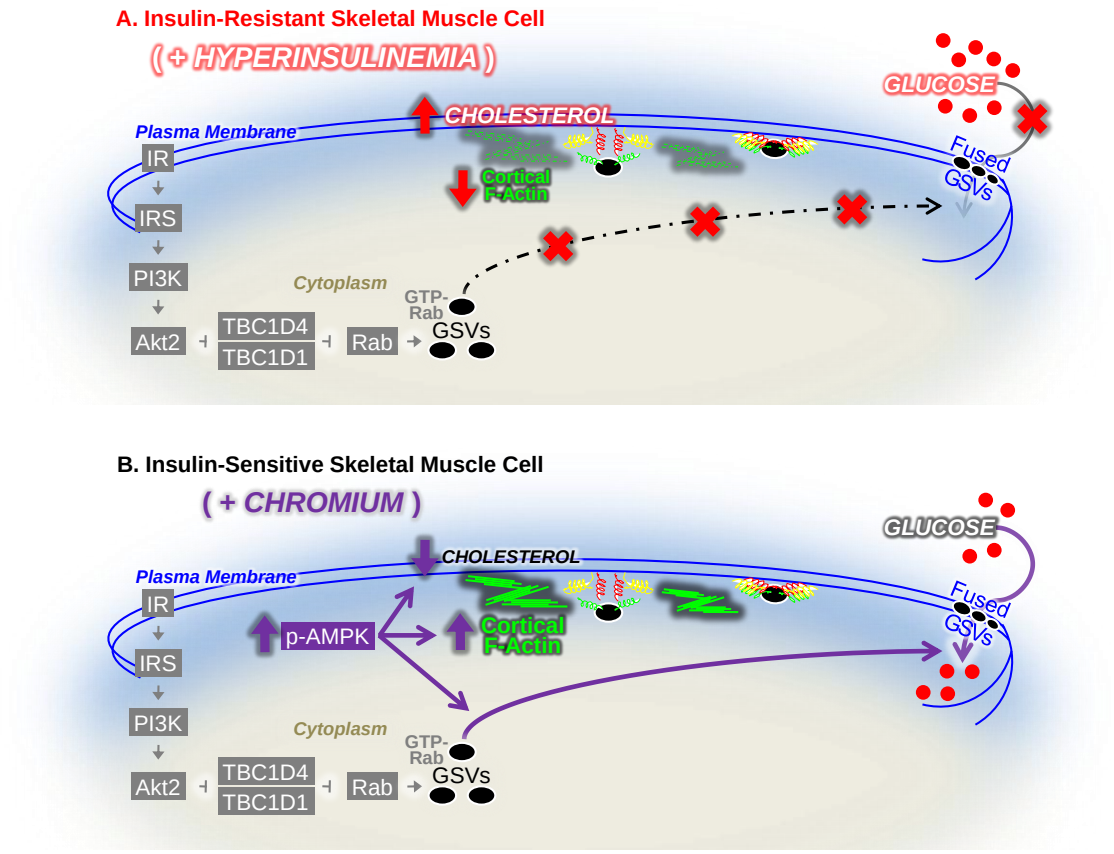

Figure 9. Model of hyperinsulinemia-induced insulin resistance and chromium action in skeletal muscle cells. Exposure of L6-GLUT4*myc* myotubes to hyperinsulinemia induces insulin resistance. Specifically, exposure to hyperinsulinemia results in membrane cholesterol accrual and a reciprocal loss of F-actin, which induces GLUT4 and glucose transport dysregulation without detectable abnormalities in insulin signaling (A). Pretreatment of L6-GLUT4*myc* myotubes with chromium protects against hyperinsulinemia-induced insulin resistance. Chromium, via activation of AMPK, prevents hyperinsulinemia-induced membrane cholesterol accrual, F-actin loss and GLUT4/glucose transport dysregulation without enhancing insulin signaling (B).

GLUT4/glucose transport dysfunction induced by hyperinsulinemia (**Fig. 9B**). Fourth, the cholesterol-lowering action of CrPic we first reported in control 3T3-L1 adipocytes (22) effectively lowered the insulin-induced elevation in membrane cholesterol to cholesterol levels observed in control, insulin-sensitive myotubes. In addition, we provide evidence that membrane cholesterol normalization restores F-actin levels and insulin-stimulated GLUT4 translocation to that observed in control, insulin-sensitive myotubes independent of insulin signaling enhancement (**Fig. 9B**). In continuation of these studies, we aimed to further dissect the mechanism(s) by which the cholesterol-lowering action of AMPK positively impacts GLUT4 regulation below in Chapter IIB.

II.B. AMPK Enhances Insulin-Stimulated GLUT4 Regulation via Lowering Membrane Cholesterol: Evidence for AMPK Activity Countering Membrane Cholesterol-Induced Insulin Resistance

II.B.1. Summary

Studies presented in the previous chapter suggested a novel mechanism of AMPK activity that enhances insulin action. Interestingly, it is also known that AMPK activity can have insulin-mimetic actions. The studies presented in this chapter focused on using well-characterized AMPK activators to further probe the cholesterol-lowering mechanism(s) of AMPK activity that impact GLUT4 regulation. In L6 myotubes stably expressing an exofacial *myc*-epitope tagged GLUT4, AMPK activation by 5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleoside (AICAR; 45 min, 1 mM) or 2,4-dinitrophenol (DNP; 30 min, 200 μM) increased surface *myc*-epitope labeling by ~25%. Insulin stimulation (20 min, 100 nM) of control cells increased surface GLUT4myc labeling by ~50% and this was enhanced by ~25% in the presence of AICAR or DNP pretreatments. Consistent with AMPK-mediated suppression of cholesterol synthesis, AICAR and DNP decreased membrane cholesterol by ~20%. Surprisingly, whereas AMPK knockdown prevented the effect of AICAR and DNP on the increased basal and insulin responses, cholesterol add-back only suppressed the AMPK-associated enhancement in insulin action. Cells cultured in a hyperinsulinemic milieu, resembling conditions *in vivo* that promote the progression/worsening of insulin resistance, displayed an increase in membrane cholesterol. This increase

in membrane cholesterol occurred concomitant with a loss of cortical F-actin and defects in GLUT4 regulation by insulin. These membrane/cytoskeletal derangements as well as GLUT4 dysregulation were prevented by AICAR. Taken together, these data suggest that AMPK activity protects against excess membrane cholesterol, an underappreciated component of insulin resistance that we have found to compromise the normal insulin regulation of GLUT4.

II.B.2. Results

AMPK activation in L6 myotubes: Treatment of L6-GLUT4*myc* myotubes with AICAR (1 mM, 45 min) or DNP (200 μM, 30 min) resulted in an increase in AMPK Thr172 phosphorylation (**Fig. 10A**), an event characteristic of increased kinase activity. Consistent with this surrogate of increased activity (379); the phosphorylation of acetyl CoA carboxylase (ACC) Ser79, a well-characterized phosphorylation target of AMPK, was increased by the AICAR and DNP treatments (**Fig. 10B**). Note that the quantification of these analyses represent the ratio of immunoblot band intensities detected with anti-phosphorylation antibodies [*i.e., α-pAMPK, α-pACC*] to that measured with specific protein antibodies [*i.e., α-AMPK, α-ACC*]. Finally, in line with AICAR and DNP stimulating AMPK Thr172 phosphorylation via an ATP-independent [*i.e., AICAR conversion to the 5'-AMP analogue ZMP*] and an ATP- dependent mechanism [*i.e., mitochondrial uncoupling that prevents cellular production of ATP*], respectively; intracellular ATP levels were unchanged by AICAR, yet decreased 40% following DNP treatment (**Fig. 11**).

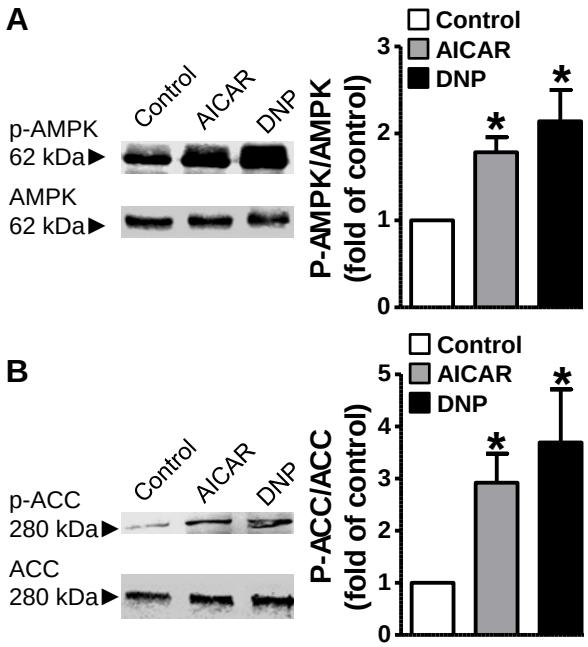

Figure 10. AMPK activation in L6 myotubes. Myotubes were left untreated or treated with AICAR (1 mM, 45 min) or DNP (200 µM, 30 min). Total cell extracts were prepared and protein concentrations were determined by the Bradford method. Equivalent protein amounts were separated by 7.5% SDS/PAGE. The resolved fractions were transferred to nitrocellulose, and immunoblot analyses were performed using anti-phospho AMPK and anti-pan AMPK (A) and anti-phospho ACC and anti-pan ACC antibodies (B). Quantification of these analyses represent the ratio of immunoblot band intensities detected with anti-phosphorylation antibodies [*i.e., α-pAMPK, α-pACC*] to that measured with specific protein antibodies [*i.e., α-AMPK, α-ACC*]. Values are means ±SEM of 6-12 independent experiments, *P<0.05 vs. control group.

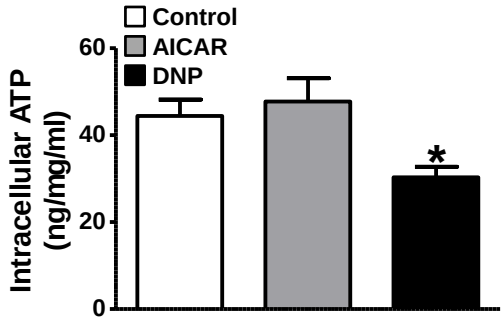

Figure 11. AMPK activation by ATP-independent and ATP-dependent mechanism. Myotubes were left untreated or treated with AICAR (1 mM, 45 min) or DNP (200 µM, 30 min). Intracellular ATP content was measured using a luminescence ATP detection assay system as described in *Experimental Procedures*. ATP concentrations were normalized for protein concentration determined by the Bradford method. Values are means ±SEM of 4 independent experiments. *P<0.05 vs. control group.

AMPK signaling enhances basal and insulin-stimulated GLUT4 translocation: We next measured cell surface GLUT4 levels under the various treatment conditions using the L6-GLUT4*myc* cell system stably expressing GLUT4 tagged with an exofacial *myc*-epitope (GLUT4*myc*). In this modified cell line the tagged transporter segregates, cycles, and responds to insulin in a manner similar to endogenous GLUT4 (431, 436). As shown in **Figure 12A** (panels 1 and 2), acute insulin stimulation of control cells resulted in increased immunologic labeling of the *myc*-epitope at the surface of intact cells. Red fluorescent co-staining of nucleic acids with Syto60 was employed as a means to normalize the *myc* signal in quantitative, cell-population based analyses using the LI-COR imaging system (**Fig. 12A**). Acute AICAR and DNP treatments of control cells increased surface GLUT4*myc* labeling (**Figs. 12A and 12B**, compare panels and bars 1, 3, and 5). Furthermore, these treatments resulted in an increase in insulin-stimulated PM GLUT4 accumulation (**Figs. 12A and 12B**, compare panels and bars 2, 4, and 6). As documented by others (437), the AMPK-stimulated gain of PM GLUT4 in the absence or presence of insulin was not associated with phosphorylation/activation of key insulin signaling molecules such as the insulin receptor substrate-1 (IRS-1) and Akt-2 (**Fig. 13A**). In addition, an AICAR- or DNP-induced increase in the phosphorylation of AS160 was not observed (**Fig. 13B**, bars 1, 3, and 5), nor did these stimuli affect the insulin-stimulated phosphorylation of AS160 (**Fig. 13B**, bars 2, 4, and 6).

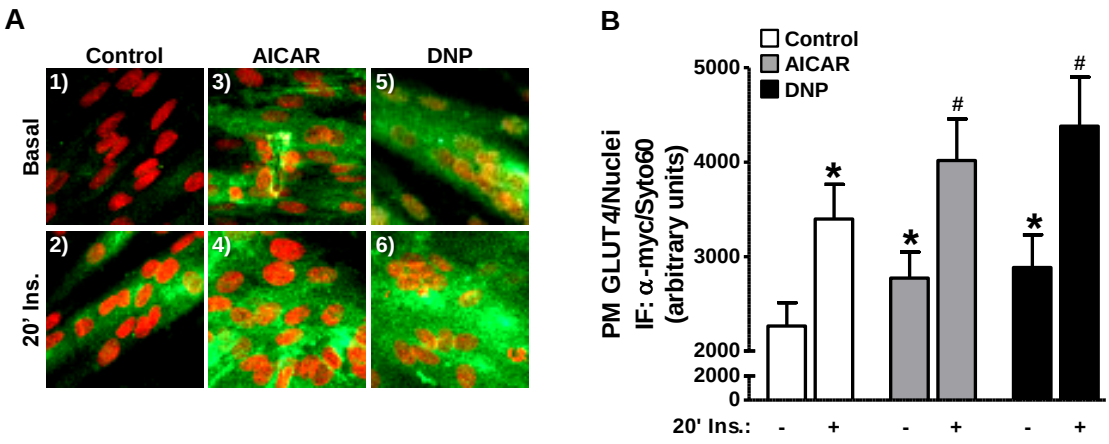

Figure 12. AMPK activation increases PM GLUT4. Myotubes were left untreated or treated with AICAR (1 mM, 45 min) or DNP (200 µM, 30 min) in the absence (Basal, panels and bars 1, 3, and 5) or presence (Insulin, panels and bars 2, 4, and 6) of acute insulin stimulation (100 nM, 20 min). After treatment, cells were fixed and left unpermeabilized. The samples were then labeled with anti-*myc* antibody, washed, and incubated with an infrared-conjugated secondary antibody (LiCor) or with FITC-conjugated anti-mouse (confocal). Immunofluorescent intensity was normalized to intensity from Syto60, a fluorescent nucleic acid stain. Images were collected and quantified with the Odyssey system. Representative α-*myc* immunofluorescent and Syto60 staining images of treated myotubes are shown (A). Values are means ±SEM of α-*myc*/Syto 60 signal quantification from 20-40 independent experiments (B). *P<0.05 vs. control-basal group; #P<0.05 vs. control-insulin group.

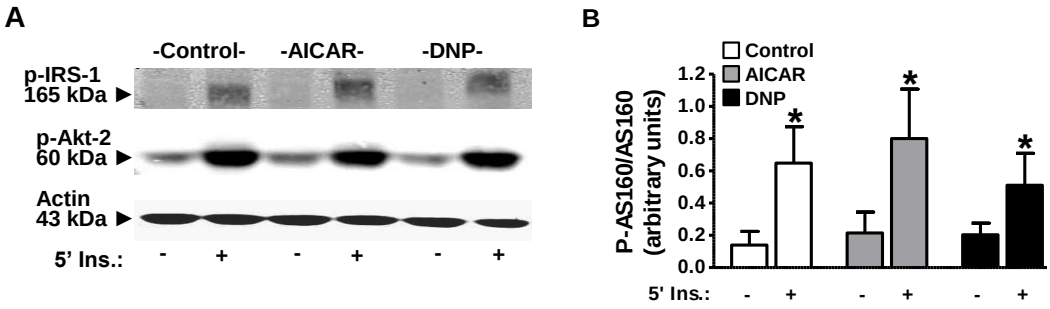

Figure 13. AMPK activation does not engage or enhance insulin signaling. Myotubes were left untreated or treated with AICAR (1 mM, 45 min) or DNP (200 µM, 30 min) in the absence (Basal, panels and bars 1, 3, and 5) or presence (Insulin, panels and bars 2, 4, and 6) of acute insulin stimulation (100 nM, 5 min). Total cell extracts were prepared and protein concentrations were determined via the Bradford method. Equivalent protein amounts were separated by 7.5% SDS/PAGE. The resolved fractions were transferred to nitrocellulose, and immunoblot analyses were performed using phospho-IRS-1 and phospho-Akt2 (A) and phospho-AS160 (B) antibodies. Equal protein loading was confirmed by Ponceau staining and by immunoblot analysis with anti-actin antibody. Representative immunoblots are shown from 3-6 independent experiments (A). Quantification of these analyses represent the ratio of immunoblot band intensities detected with anti-phosphorylation antibody [*i.e., α-pAS160*] to that measured with specific protein antibody [*i.e., α-AS160*]. Values presented in (B) are means ±SEM from 6 independent experiments. *P<0.05 vs. control-basal group.

AICAR- and DNP-treated L6 myotubes display a loss in membrane cholesterol: Concomitant to their effects on GLUT4 trafficking, both AICAR and DNP elicited a 20% decrease in membrane cholesterol (**Fig. 14A**, compare bars 1-3). As seen in **Figure 14B**, the AICAR- and DNP-induced loss of membrane cholesterol could be prevented with methyl-β-cyclodextrin (βCD) preloaded with cholesterol (βCD:Chol). This experimental strategy was found to mitigate AICAR- and DNP-enhanced insulin-stimulated GLUT4 translocation (**Figs. 14C and 14D**, compare bars 2, 4, 6, and 8). Interestingly, the insulin-mimetic increase in PM GLUT4 induced by AICAR and DNP was not prevented (**Figs. 14C and 14D**, compare bars 1, 3, 5, and 7). Neither basal nor insulin-stimulated PM GLUT4 levels in control cells were affected by the exogenously added cholesterol (Figs. **14C and 14D**, compare bars 1, 2, 5 and 6). These data suggest that the insulin-like action of AICAR and DNP on mobilizing GLUT4 to the cell surface results from a cholesterol-independent mechanism; whereas membrane cholesterol lowering by AICAR and DNP contributes to the enhancement of insulin regulation of the transporter.

AMPK mediates cholesterol-dependent and -independent regulation of GLUT4: To further test if increased AMPK activity played a causal role in enhancing both basal and insulin-stimulated GLUT4 translocation via PM cholesterol regulation, we tested the effect of siRNA-mediated knockdown of the catalytic α subunits of AMPK. Since these cells express α1 and α2 isoforms of AMPK, oligonucleotides against both were used simultaneously for knockdown.

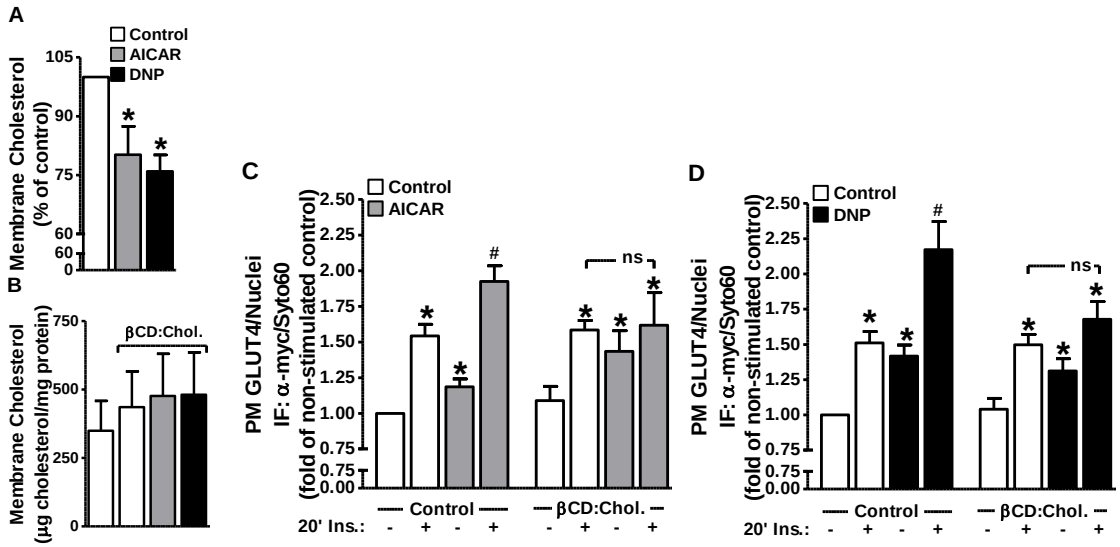

Figure 14. AMPK activation decreases membrane cholesterol and AMPK-enhanced insulin action is cholesterol-dependent. Myotubes were left untreated or treated with AICAR (1 mM, 45 min) or DNP (200 µM, 30 min) in the absence (A) or presence (B) of exogenous cholesterol (βCD:Chol; 1 mM 8:1 molar ratio). A plasma membrane-enriched fraction was prepared as described in *Experimental Procedures*. Membrane cholesterol contents (A and B) were determined by the Amplex Red cholesterol assay and were normalized to protein content as determined by the Bradford method. Basal and insulin-stimulated (100 nM, 20 min) PM GLUT4 contents (C and D) were determined in myotubes treated as described in Figure 12 in the absence or presence of exogenous cholesterol (βCD:Chol; 1 mM 8:1 molar ratio, 45 min). Values are means ±SEM of 9-13 independent experiments. *P<0.05 vs. control (or control-basal) groups, #P<0.05 vs. control-insulin group.

The combination of α1 and α2 isoform specific oligonucleotides reduced the detectable pan-AMPKα protein by 90% (**Fig. 15A**). This reduction in AMPKα was associated with an ablation of DNP-stimulated membrane cholesterol lowering as compared to control cells transfected with scramble oligos (**Fig. 15B**). In addition, consistent with increased AMPK activity regulating basal and insulin-stimulated GLUT4 translocation, DNP-stimulated GLUT4 translocation and enhanced insulin regulation were both absent in AMPK deficient cells (**Fig. 15C**). Although we did not measure the effect of AICAR on membrane cholesterol in siScramble and siAMPK cells, we did observe the same loss of AICAR-stimulated GLUT4 translocation and enhanced insulin regulation in AMPK deficient cells (**Fig. 15D**).

AICAR mitigates membrane cholesterol accrual, cortical F-actin loss, and GLUT4 dysregulation in hyperinsulinemia-induced insulin-resistant L6 myotubes: We next performed membrane cholesterol, cortical F-actin and GLUT4 analyses using hyperinsulinemia-induced insulin-resistant L6 myotubes, a model system previously observed to have a loss in actin filaments and defective GLUT4 regulation by insulin (8). **Figure 16A** (compare bars 1 and 2) shows a small (14%), yet significant increase in membrane cholesterol induced by hyperinsulinemia accompanying a 14% loss of cortical F-actin (**Fig. 16B**, compare bars 1 and 2). Consistent with a beneficial effect of AMPK activation on insulin responsiveness, AICAR treatment prevented the hyperinsulinemia-induced membrane cholesterol accrual (**Fig. 16A**, compare bars 2 and 3) and cortical F-actin loss (**Fig. 16B**, compare bars 2 and 3).

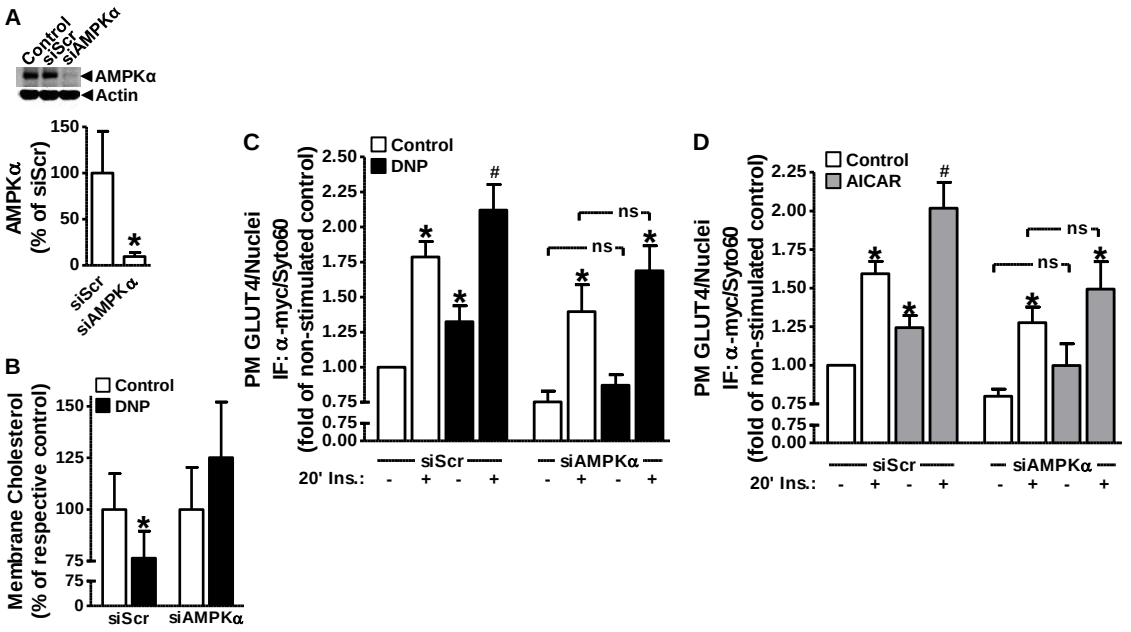

Figure 15. AMPK knockdown abrogated DNP-induced membrane cholesterol lowering and increased basal- and insulin-stimulated PM GLUT4. Myotubes received transient transfections targeting the AMPK α1 and α2 catalytic subunits as described in *Experimental Procedures*. Myotubes were then left untreated or treated with AICAR (1 mM, 45 min) or DNP (200 µM, 30 min). Total cell extracts were prepared from non-transfected control, siScramble-transfected (siScr), and siAMPKα-transfected myotubes, and SDS-PAGE and immunoblot analyses were performed as described in Figure 13 using anti-AMPK and anti-actin antibodies (A). Membrane cholesterol content was determined as described in Figure 14 (B). Basal and insulin-stimulated (100 nM, 20 min) PM GLUT4 contents (C and D) were determined in myotubes treated as described in Figure 12. Values are means ±SEM of 6-11 independent experiments. *P<0.05 vs. siScr (or control-basal) groups, #P<0.05 vs. control-insulin groups.

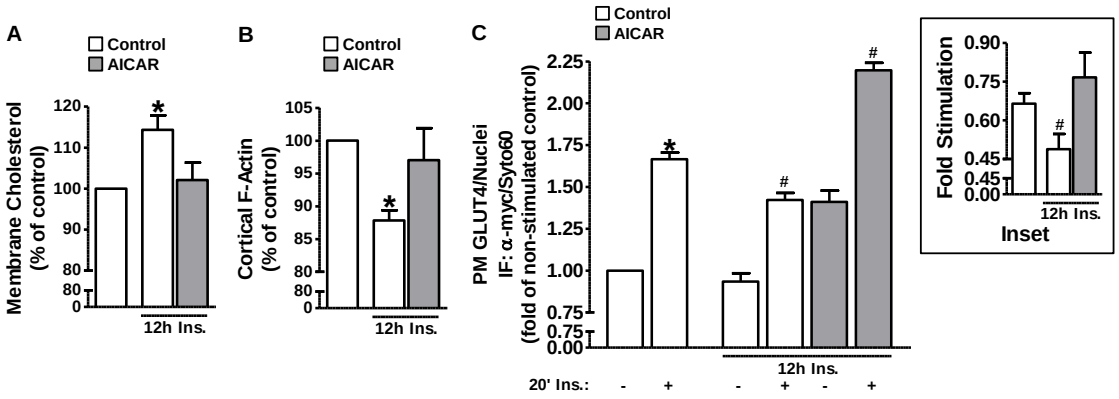

Figure 16. AMPK activation protects against hyperinsulinemia-induced membrane cholesterol accrual, F-actin loss, and GLUT4 dysregulation. Myotubes were left untreated or treated with AICAR (1 mM, 45 min) in the absence or presence of hyperinsulinemia (5 nM, 12 h). Membrane cholesterol content was determined as described in Figure 14 (A). Cortical F-actin immunofluorescence was performed as described in *Experimental Procedures*. Samples were then labeled with anti-F-actin antibody, washed, and incubated with an infrared-conjugated secondary antibody. Immunofluorescent intensity was normalized to intensity from Syto60 and quantified with the Odyssey system (B). Basal and insulin-stimulated (100 nM, 20 min) PM GLUT4 contents (C) were determined in myotubes treated as described in Figure 12. Values are means ±SEM of 4-8 independent experiments. *P<0.05 vs. control (or control-basal) groups; #P<0.05 vs. control-insulin groups. Fold insulin stimulation for each treatment condition are presented in panel C inset.

These AMPK-induced membrane/cytoskeletal improvements restored regulation of GLUT4 by insulin (**Fig. 16C**, compare bars 4 and 6). Note that the AICAR-stimulated gain in PM GLUT4 was still observed in cells exposed to hyperinsulinemia. Importantly, the AICAR treatment protected against the hyperinsulinemia-induced loss of fold insulin stimulation (see **Fig. 16C, inset**).

Skeletal muscle from insulin-resistant animals display membrane cholesterol accrual: A model whereby membrane cholesterol lowering enhances regulation of GLUT4 by insulin suggests that membrane cholesterol accrual may contribute to impaired insulin action. Consistent with this model, exogenous cholesterol loading has been shown to induce an insulin-resistant state in L6 myotubes (10). As a start to translating our cell-based membrane cholesterol findings to the whole animal, we extended these analyses to critically test whether cholesterol overload contributed to the skeletal muscle cytoskeletal abnormalities and insulin resistance. We first evaluated cortical F-actin structure in epitrochlearis skeletal muscle, a small flat muscle in the rat forelimb optimal for visual inspection, to ensure replication of earlier findings. Results were similar to previous observations (8) with the muscles of obese rats showing markedly lower F-actin structure (**Fig. 17A**). Aligned with cholesterol compromising the F-actin structure, soleus muscle membrane cholesterol from these same obese rats was 23% higher (**Fig. 17B**) than lean controls. Although the epitrochlearis skeletal muscle provides a better sample for imaging, we were able to thinly slice an imaging sample from the same soleus muscle we used to measure membrane

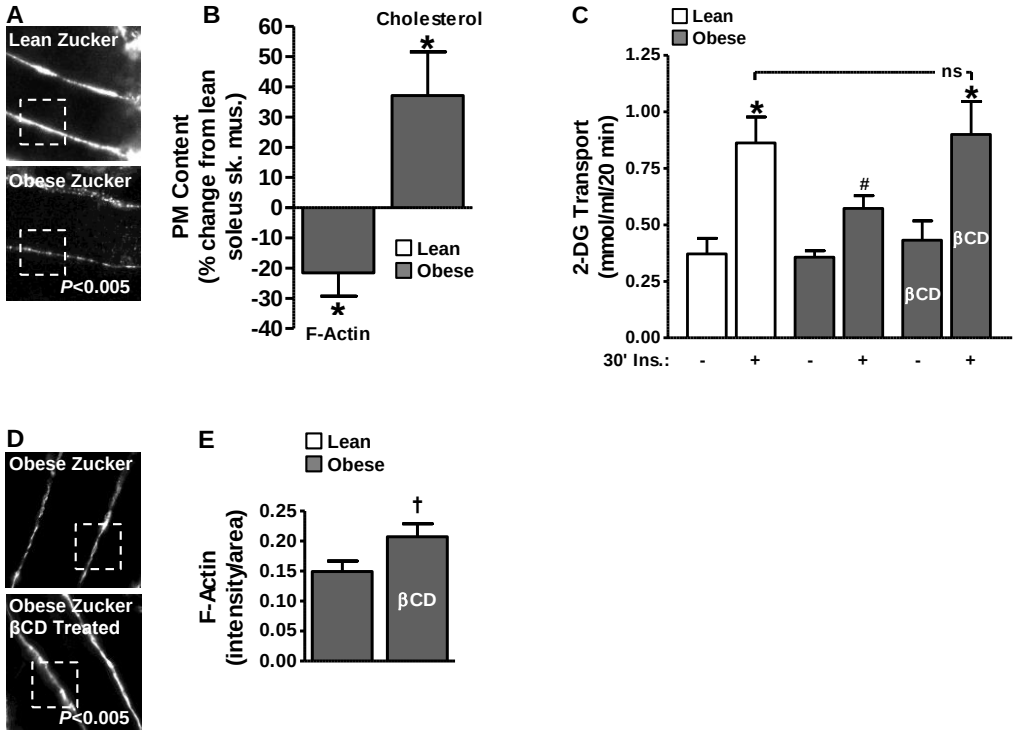

Figure 17. Cholesterol Lowering Normalizes Insulin Sensitivity in Obese Zucker Skeletal Muscle. Rat epitrochlearis muscle (A) and soleus muscle (B, C, D and E) from lean/obese Zucker rats were labeled with antibodies against F-actin, imaged by confocal microscopy (A and D), and digitally quantitated using MetaMorph software (B and E). Remaining soleus muscle was fractionated for membrane cholesterol analyses (B) as described in *Experimental Procedures*. Contralateral soleus muscles were subjected to basal and insulin-stimulated 2-DG uptake measurements (C) as described in *Experimental Procedures*. Subgroups of these muscles were exposed to 2.5 mM βCD for 30 min prior to the 30 min insulin stimulation. Values are means ±SEM from five independent experiments.*, P<0.05 vs. Lean; #, P<0.05 vs. Lean (+Insulin); †, P<0.05 vs. Obese (-) βCD.

cholesterol before the fractionation procedure. Imaging of those samples revealed a reciprocal change in F-actin (**Fig. 17B**). Insulin-stimulated glucose transport measured in contralateral soleus muscle was characteristically lower in obese than lean muscle (**Fig. 17C**, compare bars 2 and 4). When insulin-resistant muscles were exposed to 2.5 mM βCD and then treated with insulin, glucose transport in the obese group was restored to levels that were equal to those achieved by the lean group (**Fig. 17C**, compare bars 4 and 6). Importantly, treatment of soleus muscle obtained from the obese group with βCD resulted in a gain in F-actin immunofluorescent intensity (**Fig. 17, D and E**). As muscle extracellular [^{14}C] mannitol space was not affected, this βCD-induced cholesterol lowering of 25-40% (data not shown) did not compromise membrane integrity, results consistent with use of this low-dose βCD reduction of PM cholesterol in cell culture (230).

In summary, the data presented in Chapter IIB offer several observations that extend our understanding of mechanisms of insulin resistance, in particular those pertaining to cytoskeletal-based mechanisms (7, 8). First, in support of our studies in Chapter IIA, accompanying a hyperinsulinemia-induced decrease in cortical F-actin is an increase in membrane cholesterol content (**Fig. 18A**). Second, the cholesterol-lowering ability of increased AMPK activity effectively lowered the insulin-induced elevation in membrane cholesterol to cholesterol levels observed in control, insulin-sensitive myotubes (**Fig. 18B**). Third, in line with findings in Chapter IIA this membrane cholesterol normalization restored cortical F-actin structure and restored insulin-stimulated GLUT4 translocation

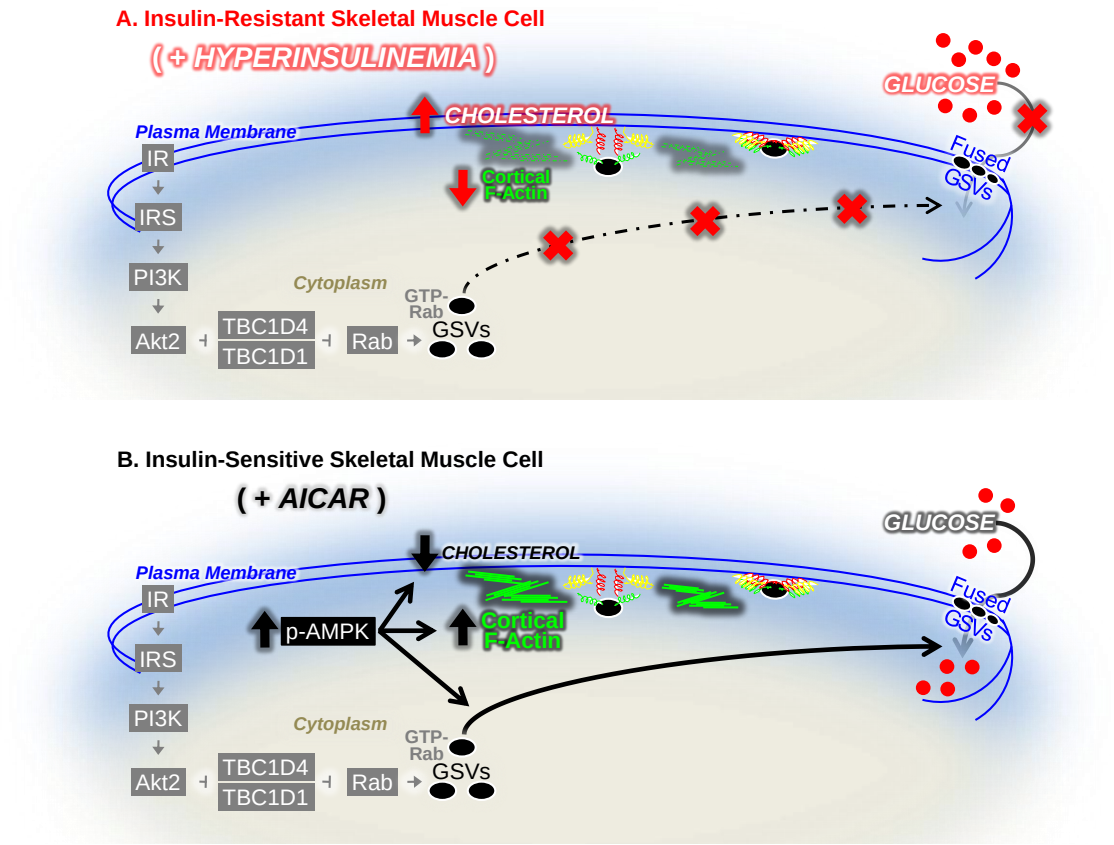

Figure 18. Model of hyperinsulinemia-induced insulin resistance and AMPK action in skeletal muscle cells. Exposure of L6-GLUT4*myc* myotubes to hyperinsulinemia induces insulin resistance. Specifically, exposure to hyperinsulinemia results in membrane cholesterol accrual and a reciprocal loss of F-actin, which induces GLUT4 and glucose transport dysregulation without detectable abnormalities in insulin signaling (A). Treatment of L6-GLUT4*myc* myotubes with AICAR protects against hyperinsulinemia-induced insulin resistance. Activation of AMPK with AICAR prevents hyperinsulinemia-induced membrane cholesterol accrual, F-actin loss and GLUT4/glucose transport dysregulation without enhancing insulin signaling (B).

(**Fig. 18B**). Furthermore, testing whether these cell culture-based findings translate to the whole animal revealed strikingly similar and correctable membrane/cytoskeletal abnormalities in isolated skeletal muscle, a tissue responsible for approximately 80% of postprandial glucose disposal (29) and regarded as a major peripheral site of insulin resistance in T2D (28). To further translate our *in vitro* findings to an animal model of insulin resistance, the following studies in Chapter IIC were performed to determine the effects of CrPic supplementation *in vivo* on skeletal muscle AMPK activity and membrane/cytoskeletal parameters of insulin sensitivity.

II.C. Chromium Improves Skeletal Muscle Membrane/Cytoskeletal Parameters and Insulin Sensitivity in High-Fat Fed C57Bl/6J Mice

II.C.1. Summary

To next test Cr^{3+} action *in vivo*, we utilized obesity-prone C57Bl/6J mice fed either a low fat (LF) or high fat (HF) diet for eight weeks without or with CrPic supplementation administered in the drinking water (8 µg/kg/day) beginning five weeks before dietary intervention. HF feeding increased body weight beginning four weeks post-diet intervention regardless of CrPic supplementation and was independent of changes in food consumption. HF feeding for eight weeks induced glucose intolerance and insulin resistance. As observed in other animal models, isolated muscles from HF-fed mice displayed membrane cholesterol accrual and loss of F-actin. Early CrPic supplementation protected against glucose intolerance and insulin resistance in HF-fed mice. CrPic supplementation also protected against skeletal muscle membrane cholesterol accrual, F-actin loss and selectively increased AMPK activation in HF-fed mice. Together these data identify a mechanism by which Cr^{3+} may positively impact glycemic status, thereby stressing a beneficial action of this micronutrient in glucose homeostasis.

II.C.2. Results

***In vivo* CrPic supplementation study design:** To begin translating our *in vitro* findings to an animal model of insulin resistance and test Cr^{3+} action *in vivo*, we utilized the obesity-prone C57Bl/6J mice fed either a LF or HF diet.

Development of obesity and insulin resistance as a result of excessive dietary fat is frequently studied in this model (438-441), which is considered to be representative of diet-induced obesity and insulin resistance in humans compared to other genetically-modified diabetic animal models. Studies have utilized these mice to test how excess dietary fat affects the physiology of liver, muscle and fat metabolism (442-444). Based on the fact commercial Cr^{3+}-containing nutritional supplements generally contain 200 to 600 µg Cr^{3+} and an average human body mass of 70 kg, the supplements provide ~3 to 9 µg Cr^{3+}/kg body weight/day. In these studies we administered 8 µg Cr^{3+}/kg body weight/day (CrPic) dissolved in the drinking water to LF and HF-fed C57Bl/6J mice beginning during the acclimation period (-5 to 0 weeks). The mice in this study were fed *ad libitum* NIH standard chow and water for two weeks, acclimated to the LF diet for three additional weeks to allow acclimation to the increased palm oil content in the diet and then divided into treatment groups (**Fig. 19**). At week 0, mice in this dietary intervention study were randomly divided into one of four groups: LF (-) CrPic, HF (-) CrPic, LF (+) CrPic and HF (+) CrPic. Control (LF) mice received a diet containing 20% kcal from protein, 70% kcal from carbohydrates and 10% kcal from fat. HF-fed mice received a diet containing 20% kcal from protein, 35% kcal from carbohydrates and 45% kcal from fat. These diets were modified with adaptations regarding type of fat (palm oil instead of lard) and carbohydrates to better mimic the FA/carbohydrate composition of the average human diet in Western societies and have been shown to induce significant insulin resistance in C57Bl/6J mice (445). The modified HF diet mimics the ratio of saturated to

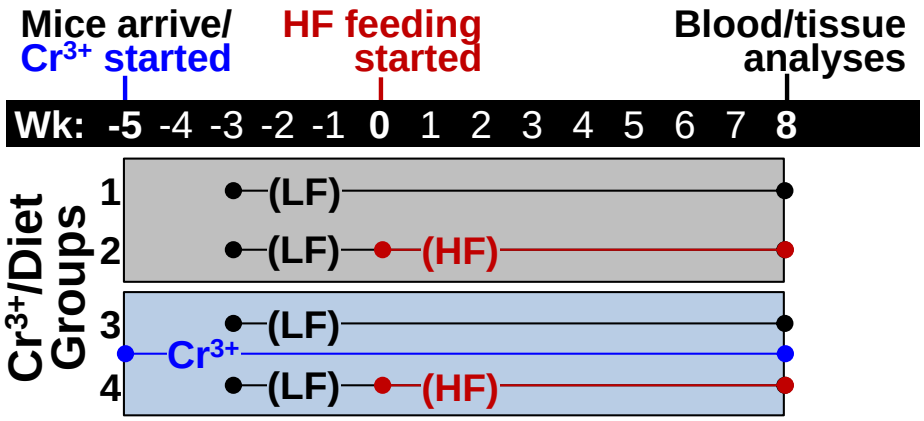

Figure 19. Early CrPic supplementation and eight week diet intervention study design. C57Bl/6J mice were acclimated for 5 weeks with or without CrPic supplementation. Mice were fed *ad libitum* NIH standard chow and water for two weeks, acclimated to the LF diet for three additional weeks and then divided into treatment groups. At 0 weeks, mice were either LF- or HF-fed ± continued CrPic supplementation. All procedures and *ex vivo* tissue analyses were performed after eight weeks of dietary intervention.

monounsaturated to polyunsaturated FAs (40:40:20). In addition, these diets were made with a Cr^{3+}-free mineral mix (S17902) and Avicel PH101 (instead of cellulose, which contains Cr^{3+}). By addition of chromium potassium sulfate, to the Cr^{3+}-free diets, both the LF and HF test diets were controlled to contain an equal amount of this micronutrient. Note the energy density of all nutrients, except fat and starch, is equal. Body weights, food consumption and water consumption were measured once per week. All other procedures and analyses outlined below were performed after eight weeks of dietary intervention to determine the effects of early CrPic supplementation on whole body glucose tolerance and membrane/cytoskeletal parameters of insulin sensitivity in skeletal muscle. We primarily focused on skeletal muscle in these studies since skeletal muscle is responsible for a large majority of postprandial glucose disposal (29) and represents a major site of insulin resistance (28). Therefore, we performed this series of experiments to deepen our insight into the effects of Cr^{3+} in this significant tissue during the development of insulin resistance.

HF feeding increases body weight independently of changes in food consumption and/or CrPic supplementation: Body weight was measured once per week during the acclimation and dietary intervention period. As shown in **Figure 20**, HF feeding significantly increased body weight in C57Bl/6J mice compared to LF feeding beginning four weeks after dietary intervention. CrPic supplementation had no significant effect on body weight in C57Bl/6J mice fed either the LF or HF diet (**Fig. 20**). The observed changes in body weight were

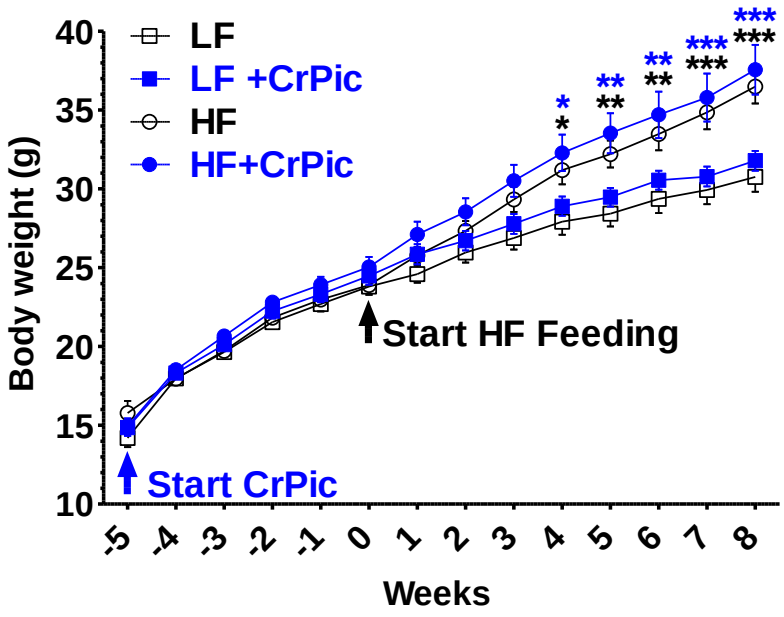

Figure 20. HF feeding increases body weight and is not affected by CrPic supplementation. C57Bl/6J mice were acclimated for 5 weeks with (blue) or without (black) CrPic supplementation. At 0 weeks, mice were LF- (square) or HF- (circle) fed ± continued CrPic supplementation. Body weight was measured once per week throughout the acclimation period and dietary intervention. Body weight values are means ± SEM from 12 mice per group. *, P<0.05 vs. LF (-/+) CrPic; **, P<0.01 vs. LF (-/+) CrPic; ***, P<0.001 vs. LF (-/+ CrPic).

independent of changes in food consumption (*i.e.* neither HF feeding nor CrPic supplementation had a significant effect on food consumption at any point during the dietary intervention) (**Fig. 21**). Water consumption was measured once per week during the acclimation period and dietary interventions. Water consumption averaged between 2.6-2.7 ml/day in CrPic-supplemented mice (**Fig. 22**) similar to levels observed in previous pilot studies. In pilot studies we determined that these water consumption values equate to approximately 8 µg/kg/day CrPic intake, very close to our target amount. For a 70 kg human, this intake equates to ~600 µg/day within the 200-600 µg dose range found in Cr^{3+} supplements.

HF feeding induces glucose intolerance, and CrPic supplementation improves glucose tolerance and insulin sensitivity in HF-fed C57Bl/6J mice: To characterize the effects of HF feeding and CrPic supplementation on whole body glucose tolerance and insulin sensitivity, we performed intraperitoneal glucose and insulin tolerance tests following eight weeks of dietary intervention. C57Bl/6J mice were fasted for six hours (from 8:00 a.m. to 2:00 p.m.) in these studies. In our intraperitoneal glucose tolerance tests (IPGTTs), we injected the mice with 2 g D-glucose/kg body weight (individually-dosed) at time 0 and measured blood glucose via tail blood collection at 15 min, 30 min, 60 min, 90 min and 120 min post-injection to monitor response to the glucose challenge. As shown in **Figure 23A,** HF feeding induced glucose intolerance compared to LF (-/+ CrPic) mice. Area under the curve (AUC) analyses revealed a 54% (P<0.001) increase in HF-fed AUC compared to LF-fed (**Fig. 23B**, compare bars 1 and 3).

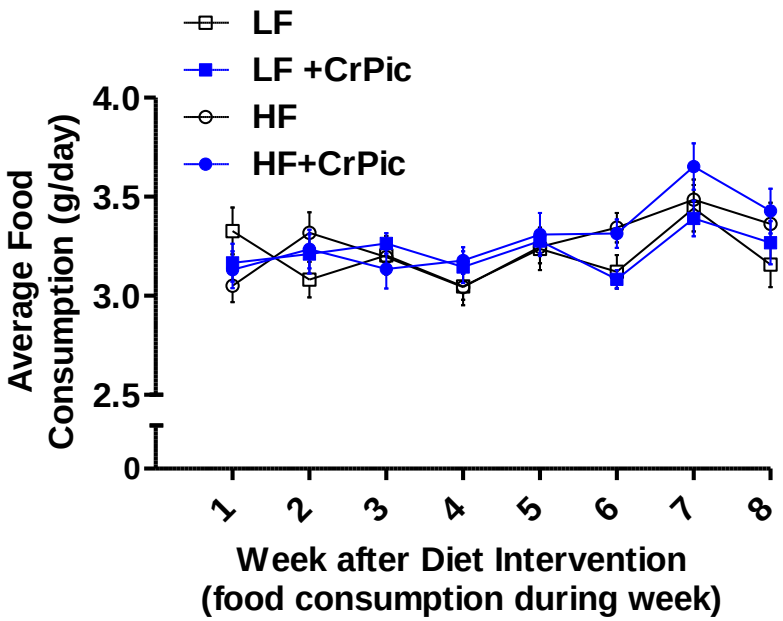

Figure 21. HF feeding and CrPic supplementation do not affect food consumption. Food consumption was measured once per week following dietary intervention for eight weeks. Weekly food consumption values were divided by the number of days between each food consumption determination to obtain average food consumption per day values. Food consumption values are means ± SEM from 12 mice per group.

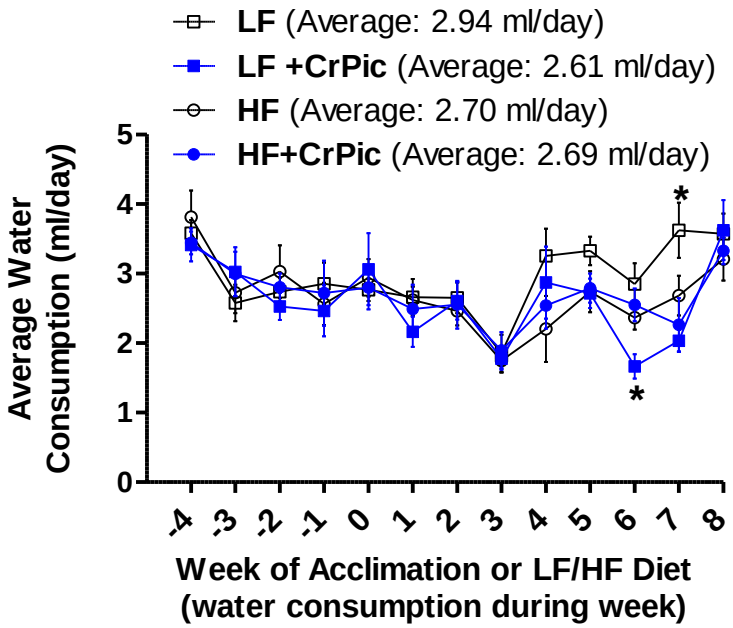

Figure 22. HF feeding and CrPic supplementation do not affect water consumption. Water consumption was measured once per week throughout the 5-week acclimation period and 8-week dietary intervention period. Weekly water consumption values were divided by the number of days between each water consumption determination to obtain average water consumption per day values. While HF feeding and CrPic did not affect water consumption throughout most of the study, we observed statistical differences in the LF (+) CrPic group at 6 weeks post-dietary intervention and the LF (-) CrPic group at 7 weeks post-dietary intervention. Water consumption values are means ± SEM from 10-12 mice per group. *, $P<0.05$ vs. all other groups.

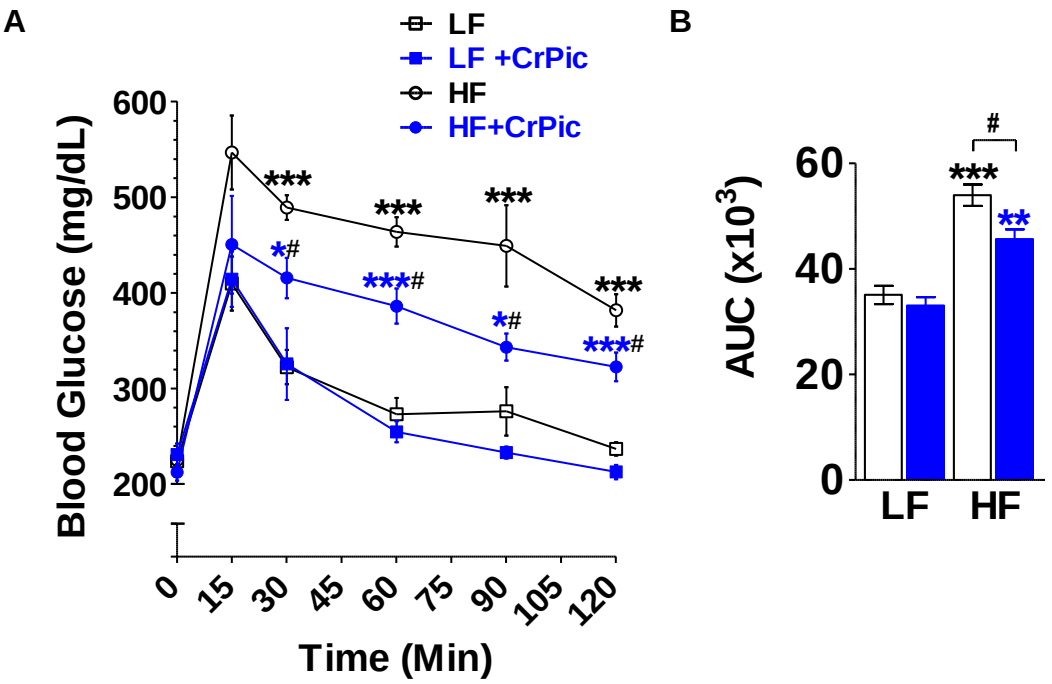

Figure 23. HF feeding for eight weeks induces glucose intolerance, and early CrPic supplementation improves glucose tolerance in HF-fed mice. Intraperitoneal glucose tolerance tests (IPGTT) were performed following eight weeks of dietary intervention. C57Bl/6J mice were fasted for six hours (from 8:00 a.m. to 2:00 p.m.) in all IPGTT studies. Mice were injected with 2 g/kg D-glucose (individually-dosed) at time 0 and blood was obtained for blood glucose analysis via tail blood collection at 15 min, 30 min, 60 min, 90 min and 120 min post-injection. Blood glucose values (A) and AUC values (B) are means ± SEM from 5-6 mice per group. *, P<0.05 vs. LF (-/+) CrPic; **, P<0.01 vs. LF (-/+) CrPic; ***, P<0.001 vs. LF (-/+ CrPic); #, P<0.05 vs. HF (-) CrPic.

CrPic supplementation in LF-fed mice had no significant effect on glucose tolerance (**Fig. 23A**) as determined by AUC analysis (**Fig. 23B**, compare bars 1 and 2). In line with Cr^{3+} action being dependent on the diabetic milieu, CrPic supplementation significantly improved glucose tolerance in HF-fed mice compared to non-supplemented HF-fed mice (**Fig. 23A**) as evidenced by a 15% reduction ($P<0.05$) in IPGTT AUC (**Fig. 23B**, compare bars 3 and 4). However, CrPic supplementation did not completely restore glucose tolerance to levels observed in LF (-) CrPic mice (**Fig. 23B**, compare bars 1 and 4).

To complement our IPGTT analyses, we performed intraperitoneal insulin tolerance tests (IPITTs) following eight weeks of dietary intervention (**Fig. 24A**). C57Bl/6J mice were fasted for six hours (from 8:00 a.m. to 2:00 p.m.) before injection of insulin. In our IPITTs, we injected the mice with 1.0 unit of insulin per kg body weight (individually-dosed) at time 0 and measured blood glucose via tail blood collection at 15 min, 30 min, 60 min, 90 min and 120 min post-injection to observe the response of each mouse to an insulin challenge. In line with IPGTTs, HF feeding reduced insulin sensitivity (*i.e.* 24% decrease in AUC; $P<0.05$) eight weeks after dietary intervention (**Fig. 24B**, compare bars 1 and 3). CrPic had no effect on insulin sensitivity in LF-fed mice (**Fig. 24B**, compare bars 1 and 2); however, CrPic supplementation in HF-fed mice improved insulin sensitivity (**Fig. 24B**, $P<0.05$; compare bars 3 and 4). Together these data strongly suggest an improvement in both glucose intolerance and insulin sensitivity in CrPic-supplemented HF-fed mice compared to non-supplemented HF-fed mice.

To complement our IPGTT and IPITT procedures, blood was collected in the

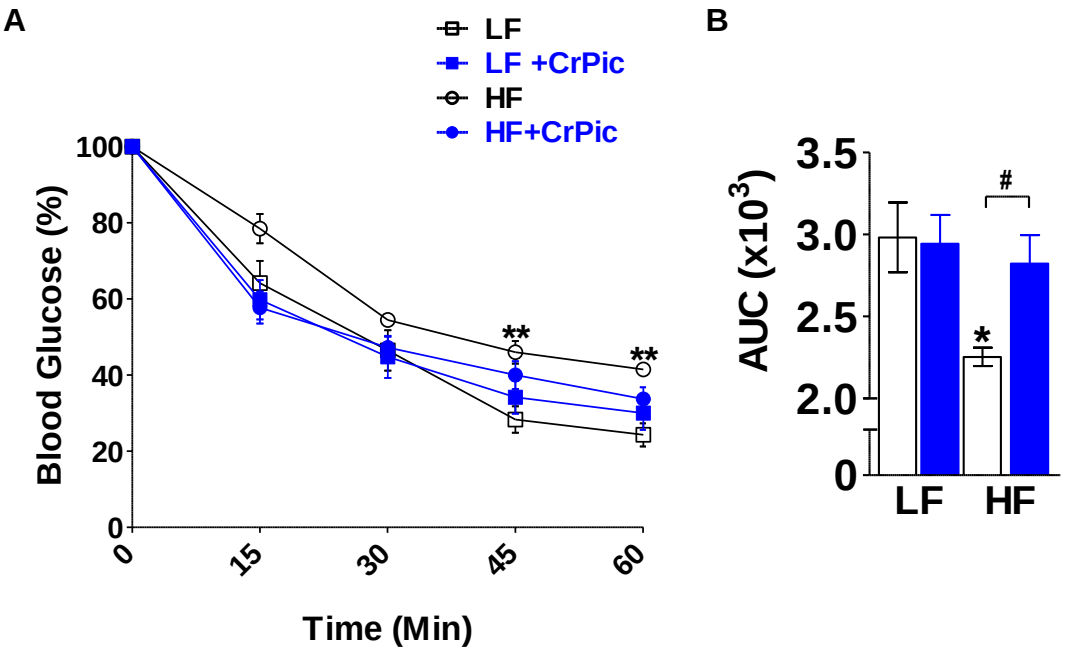

Figure 24. HF feeding for eight weeks induces insulin resistance, and early CrPic supplementation improves insulin sensitivity in HF-fed mice. Intraperitoneal insulin tolerance tests (IPITT) were performed following 8 weeks of dietary intervention. C57Bl/6J mice were fasted for 6 hours (from 8:00 a.m. to 2:00 p.m.) in all IPITT studies. Mice were injected with 1.0 unit of insulin per kg body weight (individually-dosed) at time 0 and blood was obtained for blood glucose analysis via tail blood collection at 15 min, 30 min, 60 min, 90 min and 120 min post-injection. Blood glucose values (A) and AUC values (B) are means ± SEM from 4-6 mice per group. For AUC analyses, the axis was set at 100; therefore, the area *above* the curve was determined for IPITTs as opposed to area *under* the curve for IPGTT analyses. *, P<0.05 vs. LF (-/+) CrPic; **, P<0.01 vs. LF (-) CrPic; #, P<0.05 vs. HF (-) CrPic.

prandial state after eight weeks of dietary/Cr^{3+} intervention to determine the effects of the HF diet and CrPic supplementation on plasma insulin levels to monitor insulin sensitivity. Plasma was obtained by centrifugation, and plasma insulin concentrations were determined using an Ultra Sensitive Mouse ELISA kit. Plasma insulin concentration strongly trended to be increased (75%) in the HF-fed (-) CrPic group compared to LF-fed mice (-/+ CrPic) (**Fig. 25**, compare bars 1, 2 and 3). Importantly, plasma insulin concentrations in CrPic-supplemented HF-fed mice were not as elevated (17%) compared to non-supplemented HF-fed mice (**Fig. 25**, compare bars 2 and 4).

CrPic supplementation protects against HF diet-induced skeletal muscle membrane/cytoskeletal derangements in C57Bl/6J mice: To determine if the effects of CrPic on membrane/cytoskeletal parameters in insulin-resistant skeletal muscle myotubes translate to our animal model of insulin resistance, isolated skeletal muscles from C57Bl/6J mice were analyzed following eight weeks of dietary intervention. To measure membrane cholesterol, mixed hindlimb muscles were dissected in the prandial state and homogenized. Membrane fractions were prepared as described in *Experimental Procedures*, and cholesterol content was determined using the Amplex Red cholesterol assay and normalized to protein levels. As observed *in vitro* using hyperinsulinemia-induced insulin resistance, HF feeding significantly increased membrane cholesterol content (15%, $P<0.05$) compared to the LF-fed (-) CrPic group (**Fig. 26**, compare bars 1 and 2). In support of a cholesterol-based mechanism of Cr^{3+} action in

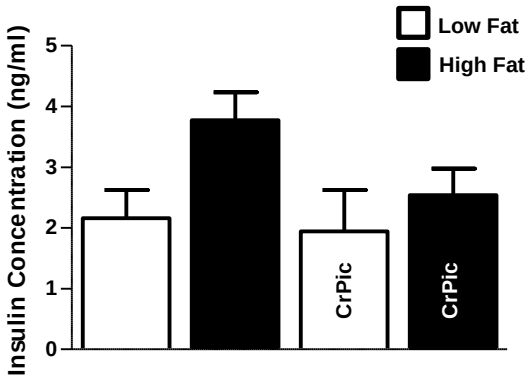

Figure 25. CrPic supplementation protects against the HF diet-induced increase in plasma insulin. Blood was obtained from C57Bl/6J mice in the prandial state following eight weeks of dietary intervention by cardiac puncture. Plasma was obtained by centrifuging blood samples for at 4°C for 15 min at 2000 rpm. Plasma insulin levels were determined using an Ultra Sensitive Mouse Insulin ELISA kit. White bars and black bars represent LF and HF-fed groups, respectively. Insulin values are means ± SEM from 6 mice per treatment group.

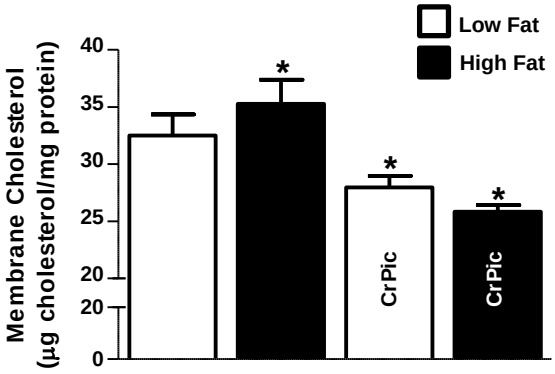

Figure 26. CrPic supplementation protects against HF diet-induced membrane cholesterol accrual in skeletal muscle. Mixed hindlimb muscles were dissected from C57Bl/6J mice in the prandial state following eight weeks of dietary intervention. A plasma membrane-enriched fraction was prepared as described in *Experimental Procedures*. Membrane cholesterol contents were determined by the Amplex Red cholesterol assay and were normalized to protein content as determined by the Bradford method. White bars and black bars represent LF and HF-fed groups, respectively. Values are means ±SEM of 5-6 muscles per treatment group. *, $P<0.05$ vs. Low Fat (-) CrPic.

skeletal muscle, CrPic supplementation significantly decreased membrane cholesterol content in LF-fed mice (14%, $P<0.05$) (**Fig. 26**, compare bars 1 and 3), as well as in HF-fed mice (22%, $P<0.05$) (**Fig. 26**, compare bars 1 and 4).

To analyze F-actin integrity in skeletal muscle, we isolated soleus muscles in the prandial state. Following fixation and permeabilization, soleus muscles were labeled with an F-actin antibody and imaged by confocal microscopy. Cortical F-actin labeling of individual muscle fibers were analyzed using Metamorph software. In line with increased membrane cholesterol disrupting cortical F-actin structure in the HF-fed mice, we observed a reciprocal decrease (21%, $P<0.05$) in F-actin signal in HF-fed mice compared to LF-fed (-) CrPic mice (**Fig. 27**, compare bars/images 1 and 2). CrPic supplementation in LF-fed mice significantly increased (19%, $P<0.05$) skeletal muscle F-actin signal (**Fig. 27**, compare bars/images 1 and 3). This increase in F-actin is accompanied by a decrease in membrane cholesterol, as described above (**Fig. 26**, compare bars 1 and 3). Strikingly, CrPic supplementation in HF-fed mice restored the F-actin signal to a similar level as LF-fed (-) CrPic mice (**Fig. 27**, compare bars 1 and 4).

CrPic supplementation increases skeletal muscle AMPK activation in HF-fed mice: Based on the role of AMPK in Cr^{3+} action we observed *in vitro*, we isolated skeletal muscles to analyze AMPK activation and downstream signaling to ACC and HMGR. Gastrocnemius muscles were isolated in the prandial state following 8 weeks of dietary intervention. Muscles were freeze-clamped immediately following dissection in an effort to preserve AMPK activation. Whole-

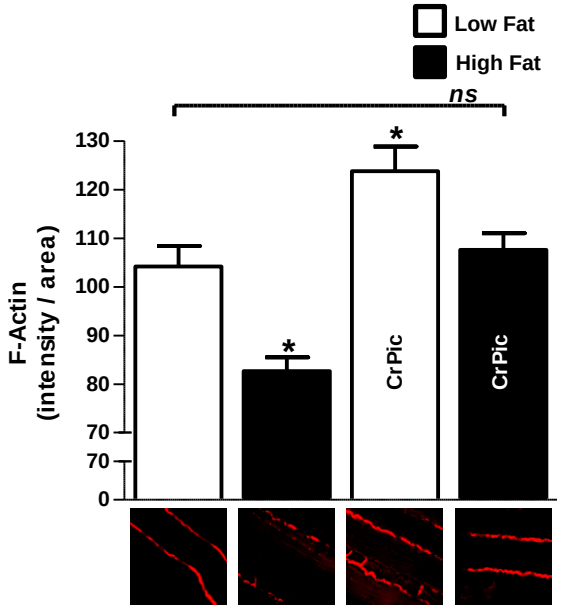

Figure 27. CrPic supplementation protects against HF diet-induced F-actin loss in skeletal muscle. Soleus muscles were dissected from C57Bl/6J mice in the prandial state following eight weeks of dietary intervention. Following fixation and permeabilization, muscles were labeled with antibodies against F-actin, imaged by confocal microscopy, and digitally quantitated using MetaMorph software. White bars and black bars represent LF and HF-fed groups, respectively. F-actin intensities from isolated soleus muscles are means ± SEM from 3-6 muscles (20-40 fibers) analyzed per treatment group, and representative muscle fibers are shown below respective treatment groups. *, $P<0.05$ vs. Low Fat (-) CrPic.

cell lysates were prepared and subjected to Western blot analyses to assess AMPK Thr172 phosphorylation, ACC Ser79 phosphorylation and HMGR Ser872 phosphorylation. As observed *in vitro* following CrPic treatment of skeletal muscle myotubes, CrPic supplementation selectively increased phosphorylation of AMPK Thr172 in muscles from HF-fed mice (45%, $P<0.05$), with no effect observed in muscles obtained from LF-mice (**Fig. 28A**). To confirm that this increase in phosphorylation was associated with an increase in AMPK activity, we analyzed downstream AMPK signaling to ACC and HMGR. CrPic supplementation robustly increased downstream phosphorylation of ACC Ser79 in muscles from HF-fed mice (99%, $P<0.05$) (**Fig. 28B**) in line with a selective increase in AMPK activity in the insulin-resistant myotubes and skeletal muscle. Finally, we analyzed HMGR phosphorylation at Ser872, which is a known downstream target of AMPK that inactivates HMGR. These analyses were based on the observed effects of Cr^{3+} on cellular cholesterol levels, as HMGR is the rate-limiting enzyme in cholesterol synthesis. Preliminary analyses suggests that phosphorylation at HMGR Ser872 is increased by CrPic supplementation in HF-fed mice (**Fig. 28C**), further supporting a role for AMPK in Cr^{3+}'s modulation of membrane cholesterol content and F-actin integrity in insulin-resistant skeletal muscle.

Taken together, these *in vivo* findings are consistent with early CrPic supplementation improving glucose tolerance and insulin sensitivity in C57Bl/6J mice fed a HF diet for eight weeks. In summary, the beneficial effects of Cr^{3+} in this study on whole body glucose tolerance and insulin sensitivity were

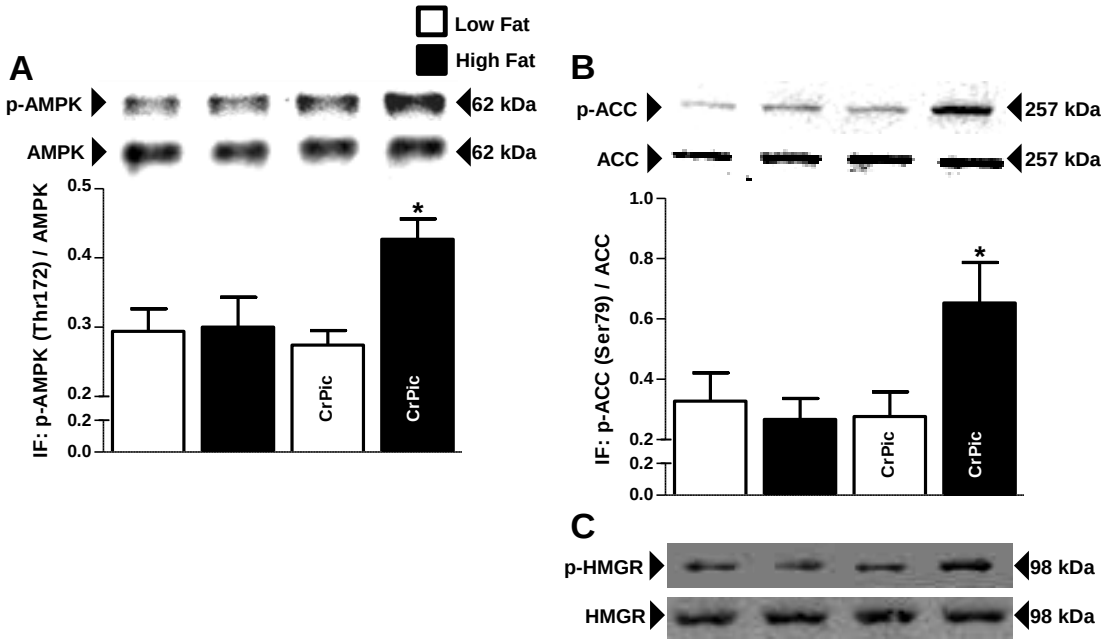

Figure 28. CrPic supplementation increases skeletal muscle AMPK activation in HF-fed mice. Mixed hindlimb muscles were dissected from C57Bl/6J mice in the prandial state following eight weeks of dietary intervention and snap-frozen in liquid nitrogen. Whole-cell lysates were prepared and subjected to Western blot analyses to assess AMPK(Thr172) phosphorylation (A), ACC(Ser79) phosphorylation (B) and HMGR(Ser872) phosphorylation (C). White bars and black bars represent LF and HF-fed groups, respectively. Immunoblots shown in A, B and C are representative of 5-11 mice per treatment group. All quantitated values shown in A and B are presented as means ± SEM from 5-11 mice and were determined by densitometry and normalized to total protein (AMPK and ACC, respectively). *, $P<0.05$ vs. Low Fat (-) CrPic.

independent of changes in food intake and/or body weight. Mechanistically, we observed membrane cholesterol and F-actin loss induced by the HF feeding (**Fig. 29A**) similarly to studies from our group in other animal models of insulin resistance (8, 10). The levels of hyperinsulinemia and glucose intolerance we observed in HF-fed mice, indicative of insulin resistance, were not observed in HF-fed mice supplemented with CrPic in the drinking water for the acclimation period and eight week diet intervention (**Fig. 29B**). Consistent with the idea that Cr^{3+} has no beneficial effect on glucose metabolism in lean, healthy animals, we did not observe an effect of CrPic supplementation on glycemic status in the LF-fed mice. Importantly, CrPic supplementation protected against membrane cholesterol accrual and F-actin loss in skeletal muscles obtained from HF-fed mice similarly to the effects of CrPic treatment in skeletal muscle myotubes *in vitro* that have been shown to positively impact insulin sensitivity (**Fig. 29B**). Our isolated skeletal muscle analyses support a mechanism of Cr^{3+} action similar to what we observe in cell culture model systems. We determined that CrPic supplementation selectively increased AMPK activation in HF-fed mice (**Fig. 29B**), consistent with CrPic activating AMPK more robustly in insulin-resistant myotubes in our *in vitro* studies. In conclusion, our *in vitro* data presented in Chapter IIA and IIB and *in vivo* data presented in Chapter IIC suggest that AMPK activation by CrPic may mediate the beneficial effects of CrPic on membrane/cytoskeletal parameters of insulin sensitivity. Furthermore, these data provide novel mechanistic insight into the mechanisms by which Cr^{3+} and AMPK activation benefit GLUT4 regulation and glucose/lipid homeostasis.

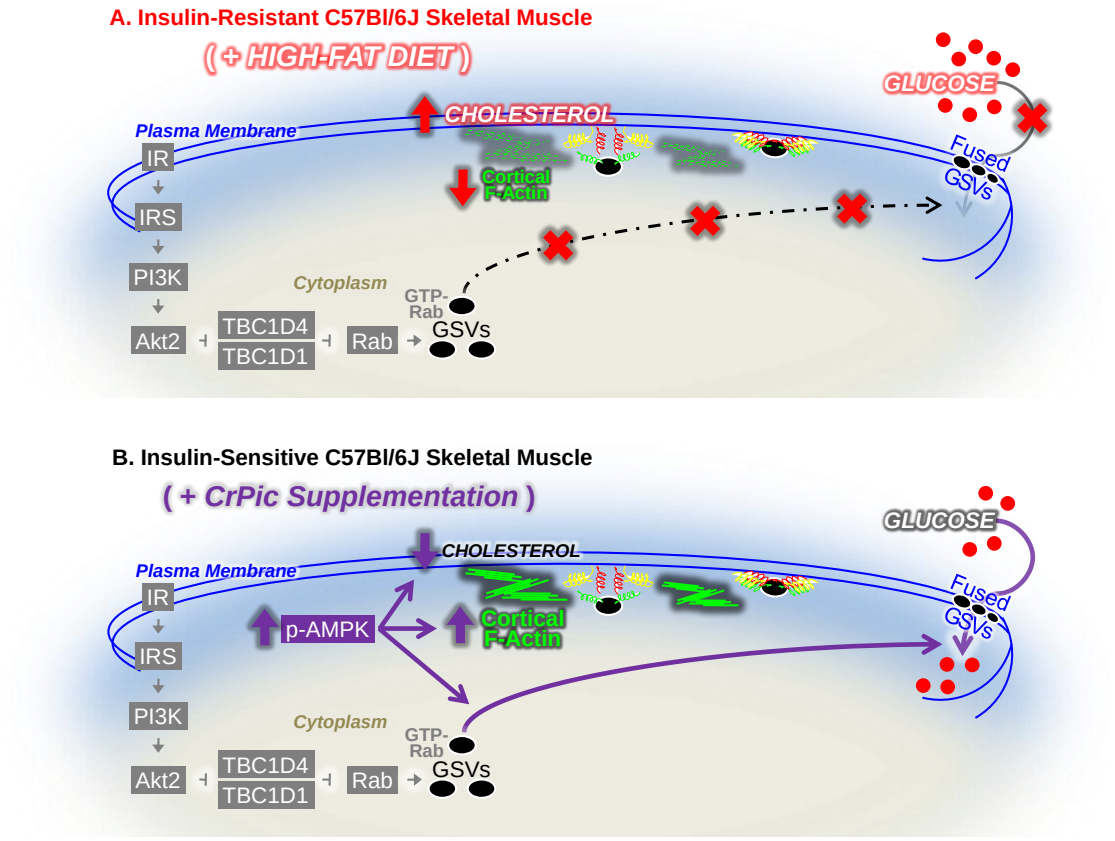

Figure 29. Model of high-fat diet-induced insulin resistance and effects of CrPic supplementation in C57Bl/6J skeletal muscle. HF feeding for 8 weeks induces glucose intolerance and insulin resistance in C57Bl/6J mice. Specifically, HF feeding results in skeletal muscle membrane cholesterol accrual and a reciprocal loss in F-actin loss, which is accompanied by whole body glucose intolerance and reduced insulin sensitivity (A). Early CrPic supplementation in HF-fed mice during the 5-week acclimation period and during the 8-week feeding period improves glucose tolerance and insulin sensitivity. CrPic supplementation in HF-fed mice also increases activation of AMPK and protects against membrane cholesterol accrual and F-actin loss in isolated skeletal muscles (B).

Chapter III. Perspectives

The findings presented above in Chapter II have provided insight into the mechanisms by which Cr^{3+} affects membrane/cytoskeletal aspects of insulin sensitivity in skeletal muscle. The following section considers future research questions stemming from the current research and puts these findings into perspective regarding the fundamental findings in the field described in the introduction above. As previously discussed in detail, the importance of key distal GLUT4 regulatory events beyond the insulin signaling cascade have been demonstrated by multiple groups (reviewed in (17)). It is appreciated that states of insulin resistance involve defects in distal GLUT4 regulatory parameters while insulin signaling remains intact (7, 8, 22). Findings from the present studies further add to this concept by highlighting reversible distal derangements in the PM and cortical actin cytoskeleton that occur in both a hyperinsulinemia-induced insulin-resistant cell culture model and HF-fed mouse model.

To dissect the cholesterol-based mechanism(s) of Cr^{3+} action and determine the role of the AMPK-HMGR signaling axis in the studies in Chapter IIA, we first utilized the HMGR inhibitor atorvastatin. We performed pilot studies using atorvastatin (ATV); however, we observed that ATV increased AMPK Thr172 phosphorylation independent of its inhibition of HMGR, consistent with previous reports (433). Since this off-target effect of ATV on AMPK complicated our data interpretation, we next tried to identify another method of inhibiting cholesterol synthesis without increasing AMPK activation. We tested the squalene synthase inhibitor, zaragozic acid A (ZA), which inhibits cholesterol synthesis downstream

of HMGR (432). This inhibitor has been utilized to decrease cholesterol content primarily in cultured neurons (446, 447) and has been shown to have no detrimental effect on cell viability in rat and human myotubes at the dose (10 μM) we used in these studies (448). We did not observe increased AMPK Thr172 phosphorylation using this method of cholesterol synthesis inhibition, and ZA effectively recapitulated the protective effects of CrPic against hyperinsulinemia-induced membrane/cytoskeletal derangements and insulin resistance. Importantly, this data suggests that F-actin integrity is regulated by membrane cholesterol levels, as evidenced by the observation that inhibition of cholesterol synthesis by ZA protected against F-actin dysregulation induced by hyperinsulinemia. The observed effects of ZA on membrane/cytoskeletal aspects of GLUT4 regulation suggest interplay between the PM lipid environment and cortical F-actin cytoskeleton and warrant further mechanistic studies to determine the cholesterol-dependent mechanisms underlying the cytoskeletal-based actions of Cr^{3+}.

Our studies utilizing siRNA-targeted knockdown of AMPK catalytic activity are consistent with AMPK mediating the protective effect of Cr^{3+} against GLUT4/glucose transport dysregulation induced by hyperinsulinemia. Findings from AMPK knockdown experiments revealed that the antidiabetic actions of CrPic are absent in cells with diminished AMPKα protein. A recent report utilizing pharmacological inhibition of AMPK by compound C has also demonstrated a causal role for AMPK in Cr^{3+}'s inhibition of the secretion of an adipokine implicated in insulin resistance, resistin, in insulin-resistant 3T3-L1 adipocytes

(311). Together these studies begin to establish beneficial roles for AMPK in Cr^{3+} action in the context of insulin resistance. While our findings suggest that AMPK mediates CrPic action in our cell system, it is possible that CrPic action relies on a potential upstream and/or downstream signaling event in the AMPK signaling pathway. Further dissection of the involvement of proximal and distal signaling events is currently underway to determine the role(s) of AMPK activation in Cr^{3+} action. The studies in Chapter IIA implicate HMGR, and subsequent regulation of cholesterol synthesis, as an important aspect of how CrPic positively impacts GLUT4 regulation. However, other mechanisms downstream of AMPK such as interactions with the HBP, potentially through inhibition of GFAT (449), may also be involved. As HBP activation has been demonstrated by our group to be increased in L6 myotubes exposed to palmitate (10) and 3T3-L1 adipocytes exposed to hyperinsulinemia (11), this also represents a potential node of cholesterol synthesis regulation impacted by Cr^{3+} action.

Interestingly, recent data suggest that the antidiabetic drug metformin enhances insulin action by increasing membrane fluidity (307, 308). As we have observed after CrPic treatment, metformin has also been reported to increase GLUT4 translocation to the PM (309, 310, 450-453). It has also been observed that the relative enhancing effect of metformin is higher in 3T3-L1 adipocytes incubated in 25 mM glucose rather than in 5 mM glucose, consistent with its selective action in hyperglycemic conditions *in vivo* (454). We have observed that the relative enhancing effect of CrPic on GLUT4 translocation is also higher in adipocytes incubated in 25 mM glucose rather than 5 mM glucose (23). An

increase in PM cholesterol was noted in the cells cultured in 25 mM glucose, and the beneficial action of CrPic was attributed to lowering PM cholesterol content to that measured in control, 5 mM glucose, insulin-sensitive cells (23). As reported for metformin (455-458), the effects of CrPic are not attributed to increased expression of GLUT4 protein but rather its translocation and/or activation state (262, 459, 460). In line with these observations, our studies herein support a shared mechanism for the beneficial effects of metformin and CrPic on cellular insulin sensitivity, with AMPK activation and the regulation of PM cholesterol being a central component of this mechanism.

The data presented in Chapter IIA suggest that AMPK activation is an important aspect of the mechanism of action of Cr^{3+} in insulin-resistant skeletal muscle cells. Furthermore, this study demonstrates that PM cholesterol influences cytoskeletal structure essential for insulin-regulated GLUT4 translocation and glucose transport. Although intermittent study has coupled membrane fluidity to insulin sensitivity (228, 306, 461), a mechanistic understanding has remained elusive. It is an interesting thought that PM cholesterol lowering may be a common and key action of CrPic and metformin, as well as several other antidiabetic agents known to display AMPK-stimulating and cholesterol-lowering properties (*e.g.*, berberine (342, 462-464), cryptotanshinone (465) and fibrates (466, 467)). Further *in vivo* studies in Chapter IIC discussed below were performed to characterize the effects of CrPic supplementation on glucose metabolism and skeletal muscle membrane/cytoskeletal parameters of insulin sensitivity in an animal model of

insulin resistance. Because cortical F-actin integrity is required for proper GLUT4 regulation by insulin, we hypothesized that the protective effects of CrPic against membrane/cytoskeletal derangements associated with insulin resistance and activation of AMPK observed *in vitro* to translate to an improvement in skeletal muscle insulin sensitivity *in vivo*.

The studies presented in Chapter IIB were performed in direct collaboration with and in continuation of studies performed by a fellow graduate student in our laboratory, Kirk Habegger. In Chapter IIB studies we examined *in vitro* effects of increased AMPK activity on membrane cholesterol and GLUT4 regulation in L6 myotubes. Our group has previously reported that experimental lowering of membrane cholesterol (*i.e.*, with sphingomyelinase or cholesterol binding agents such as βCD, nystatin and filipin increased GLUT4 exocytosis in 3T3-L1 adipocytes (230)). We also found a mechanistic aspect of the antidiabetic activity of Cr^{3+} appeared to entail membrane cholesterol lowering (22, 23). In addition, we observed that AMPK activity was increased in cells treated with Cr^{3+}. These later results led us to speculate that increased AMPK activity may lower membrane cholesterol in insulin resistant cells, which could reverse a portion of the defects in insulin-regulated GLUT4 trafficking.

Therefore, in Chapter IIB we tested if stimulation of AMPK with AICAR or DNP affected membrane cholesterol and whether membrane cholesterol accrual explained, at least in part, defects in GLUT4 regulation in insulin-resistant cell and animal models. Consistent with increased AMPK activity lowering membrane cholesterol, we found both AICAR and DNP lowered membrane cholesterol.

Moreover, the effect of these agents on enhancing insulin-stimulated GLUT4 translocation was eliminated by adding back exogenous cholesterol. Intriguingly, cholesterol add-back did not suppress the insulin mimetic action of AICAR or DNP; whereas siAMPK knockdown did, suggesting AMPK regulates basal and insulin-stimulated GLUT4 by divergent mechanisms. Although in the present study we attempted to delineate this further, which we provide expanded information on below, an important preliminary question we asked was if membrane cholesterol accrual could represent an unappreciated aspect of insulin resistance and whether AMPK stimulation countered membrane cholesterol toxicity.

Based on additional tests we conducted, we were surprised that the insulin mimetic AICAR- and DNP-stimulated GLUT4 translocation was independent of membrane cholesterol lowering. For example, as we depict in **Figure 30**, if AMPK activity regulates basal GLUT4 translocation by a cholesterol-independent mechanism; whereas AMPK enhances insulin-stimulated GLUT4 translocation via lowering membrane cholesterol, a prediction would be lowering membrane cholesterol with βCD would only affect insulin-stimulated, not basal, GLUT4 translocation. Contrary to this prediction, we found that βCD increased both basal and insulin-stimulated GLUT4 translocation in L6 myotubes (**Fig. 30B**). One possible explanation for this observation could be that the βCD-induced 34% loss of membrane cholesterol (**Fig. 30B, inset**) that was more than that induced by AMPK activation (i.e., 19.8% and 24.1% for AICAR and DNP, respectively)

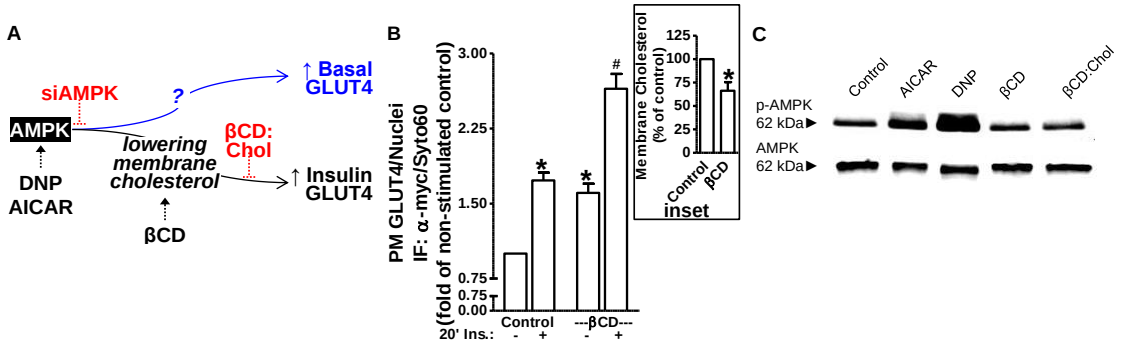

Figure 30. Proposed model of divergent membrane cholesterol-independent and dependent AMPK pathways regulating basal and insulin-stimulated PM GLUT4 content. Proposed model of divergent AMPK pathways highlighting that the insulin-like action of AICAR and DNP on mobilizing GLUT4 to the cell surface results from a cholesterol-independent mechanism; whereas membrane cholesterol lowering by AICAR and DNP contributes to the enhancement of insulin regulation of the transporter (A). Basal and insulin-stimulated (100 nM, 20 min) PM GLUT4 contents (B) were determined in myotubes treated as previously described in the absence or presence of βCD (2.5 mM, 30 min). Membrane cholesterol contents (panel B inset) were determined as previously described. Myotubes were left untreated or treated with AICAR (1 mM, 45 min), DNP (200 μM, 30 min), βCD (2.5 mM, 30 min), or βCD:Chol (1 mM 8:1 molar ratio, 45 min). Cell lysates were subjected to SDS/PAGE and immunoblot analyses using anti-phospho AMPK and anti-pan AMPK antibodies (C). Representative immunoblots are shown from 3 independent experiments. Values are means ±SE of 4-5 independent experiments. *, P<0.05 vs. control (or control-basal) groups; #, P<0.05 vs. control-insulin group.

impaired the endocytic retrieval of cell surface GLUT4, as we and others have found occurs with greater reduction of membrane cholesterol (229, 230). Another possible explanation could be that βCD and/or βCD:Chol may have an "off-target" effect on AMPK activity. For example, if βCD increased AMPK activity then a predicted outcome would be a gain in both basal and insulin-stimulated GLUT4 translocation with the use of βCD and βCD:Chol. However, we did not detect an increase in AMPK Thr172 phosphorylation with either βCD or βCD:Chol (**Fig. 30C**). In this context, the specific inhibition of enhanced insulin-stimulated, not basal, GLUT4 translocation with βCD:Chol seems to implicate membrane cholesterol lowering as a mechanism by which AMPK enhances insulin action, but not basal GLUT4 regulation. We currently hold, and will continue to test the idea, that a divergent AMPK-mediated, cholesterol-independent pathway can regulate basal GLUT4 levels. As previously mentioned, although we considered employing statins to inhibit cholesterol synthesis to avoid the complexities of βCD, multiple reports and our own findings showing increased AMPK activity in cells treated with statins also render their use as a tool in testing this model ineffective (433, 468).

Regardless of whether or not divergent pathways mediate the action of AMPK on basal and insulin-stimulated GLUT4, our data suggest a novel mechanism of AMPK action. Whether membrane cholesterol toxicity is a component of insulin resistance certainly requires further testing; however, this study found membrane cholesterol accrual in two well-described *in vitro* and *in vivo* models of insulin resistance. Furthermore, these data support the concept that the accumulation of

membrane cholesterol compromises cortical F-actin structure, essential for insulin-regulated GLUT4 translocation and glucose transport. It is possible that the F-actin changes are localized in cholesterol-enriched caveolae microdomain membrane regions. *In vitro* and *in vivo* imaging analyses from this study seem to intriguingly support the observed reciprocal changes in membrane cholesterol and F-actin. Notably, the F-actin labeling has been documented in electron micrographs to be localized in caveolae regions (469). While caveolae have been postulated to contribute to many functions in insulin and GLUT4 action through the years (17), these findings must be cautiously interpreted. Concerns regarding the study of caveolae are associated with each of the numerous strategic approaches used to study these structures. In spite of these caveats, fluorescence confocal labeling of caveolae and cortical F-actin have revealed actin filaments emanating from caveolae microdomains (74).

Moreover, quantitative electron microscopy and freeze-fracture analyses have revealed that cytoskeletal components, including actin, are highly enriched in the membrane area underlying the neck part of caveolae (75). Together, these findings assign caveolae a critical role in the functionality of cortical F-actin organization. Given the unequivocal importance of cortical F-actin in insulin-regulated GLUT4 translocation, these findings also emphasize the importance of caveolae in GLUT4 regulation. Of interest to our understanding of caveolae-associated actin regulation are new electron microscopic data showing high concentrations of PIP_2 at the rim of caveolae (76). This localization of PIP_2 is consistent with its regulation of the cytoskeleton where this lipid's availability is

recognized to modulate membrane/cytoskeleton interaction, the stability of cortical F-actin and the turnover of cytoplasmic stress fibers (77). Interestingly, reduced PM PIP_2 and cortical F-actin structure are observed in hyperinsulinemia-induced insulin-resistant 3T3-L1 adipocytes and L6 myotubes. In these cell model systems insulin-stimulated GLUT4 translocation is impaired, but can be corrected with exogenous PIP_2 addition to the PM that mediates a restoration of cortical F-actin structure (7, 78).

Although speculative, we view the findings presented in Chapter IIA and IIB, in light of fundamental findings from others, as a possible indication that membrane/cytoskeletal defects could negatively impinge on GLUT4 translocation. Furthermore, these data suggest that the membrane/cytoskeletal defects are induced prior to those in insulin signaling. Supporting this view are studies using various cell model systems of insulin resistance that demonstrate insulin signaling to Akt2/TBC1D4 is not impaired (7, 13, 16, 19, 78, 232). Collectively these data point to the notion that some insulin-resistant states may result from a membrane/cytoskeletal-based mechanical defect in GLUT4 vesicle/PM arrival, tethering, docking, and/or fusion. In summary, these *in vitro* studies suggest that membrane cholesterol influences cytoskeletal structure essential for insulin-regulated GLUT4 translocation and glucose transport. While intermittent study has coupled membrane fluidity to insulin sensitivity (228, 306, 461), a mechanistic understanding has remained elusive. It is an interesting concept that countering membrane cholesterol toxicity may be a common mechanism of action of several antidiabetic agents known to activate AMPK.

In Chapter IIC we aimed to begin translating our *in vitro* studies to an *in vivo* model of insulin resistance. Moreover, we sought to test whether early Cr^{3+} supplementation during a normal glucose tolerant state could reduce or prevent obesity-associated glycemic deterioration. In this regard, obesity-prone C57BL/6J mice provided an extremely useful model to study the development of obesity and insulin resistance as a result of excessive dietary fat (438-440). The results presented in Chapter IIC provide *in vivo* evidence that supports our *in vitro* findings regarding Cr^{3+} action in skeletal muscle. Specifically, these studies using a HF-fed model of insulin resistance provide important evidence that suggests early Cr^{3+} supplementation during a five week acclimation period prevents the negative impact of a subsequent bout of HF feeding for eight weeks.

While these *in vivo* findings improve our understanding of membrane/cytoskeletal aspects of insulin sensitivity and Cr^{3+} action in skeletal muscle, future studies will be required to address several key unanswered questions regarding these mechanisms. For example, what is the therapeutic window of the most efficacious Cr^{3+} action during the progression of insulin resistance? The data presented in Chapter IIC suggest that early CrPic supplementation prior to HF feeding can improve glucose tolerance and key parameters of skeletal muscle insulin sensitivity. These data provide suggestive evidence that CrPic may prevent skeletal muscle membrane/cytoskeletal derangements associated with the progression of insulin resistance. In future studies we will add to our eight week study by obtaining earlier (2-, 4-, and 6-week) and later (12-, 16-, 20-, and 24-week) diet/intervention data and then test

the idea that Cr^{3+}'s effectiveness depends on the state of disease progression. It is well established that there are multiple mechanisms of insulin resistance and that insulin resistance likely gives rise to other abnormalities, including dyslipidemia. Consequently, we reason that beneficial effects of Cr^{3+} are time dependent. Although we find Cr^{3+} mitigates HF-induced membrane cholesterol increases in skeletal muscle after eight weeks of HF feeding, we do not yet know how early membrane cholesterol begins to accumulate, how Cr^{3+} acts during this early period and if Cr^{3+} effectiveness persists during longer diet durations.

Our working hypothesis for these studies is that membrane cholesterol toxicity occurs early and persists throughout the pathogenesis of disease and that Cr^{3+} is efficacious early but its efficacy is progressively lost when other factors (*e.g.,* inflammatory stress, signaling defects) contribute to insulin resistance. We propose that secondary signaling pathways such as inflammation exacerbate insulin resistance through different mechanisms as compared to the initial membrane cholesterol insult and that these pathways are not amenable to Cr^{3+} action. Studies have suggested that HF-induced inflammatory responses are likely to be observed between eight and fourteen weeks in these mice (470). As proof of principle, we plan to test the effect of Cr^{3+} on established cell models of insulin resistance induced by adipokines (471) or cytokines (472) characteristic of inflammation observed in later stages of insulin resistance. Perhaps the partial protective effect of Cr^{3+} on glucose tolerance we see after eight weeks of HF feeding reflects skeletal muscle inflammation. In addition, our *in vitro* and *in vivo* data would predict that this beneficial effect of Cr^{3+} may be more pronounced in

patients treated with Cr^{3+} at early stages of their disease. By testing the prediction that later mechanisms fueling metabolic disorder decrease Cr^{3+} efficacy, we will address the limitations in the current clinical evidence and better define the therapeutic window during which Cr^{3+} supplementation is most effective.

With regard to our expectation of late stage intervention being less effective, this observation could explain the conclusion from the recent review of randomized control trials that Cr^{3+} supplementation in patients with existing T2D may offer only a slight improvement in glycemic status (289). We predict the slight effect is likely a consequence of an inflammatory response and subsequent signaling defects that occur after membrane cholesterol accrual/toxicity. This expectation is from the measurement of inflammation markers presenting later in skeletal muscle, liver and adipose tissue (470), and that insulin resistance measured in terms of *in vivo* tissue glucose handling (evident within three weeks) in these HF-fed mice precedes the onset of impaired insulin signaling in these tissues that became evident between four to eight weeks of HF feeding (473). Interestingly, this study found detectable changes in systemic glucose handling within one and a half weeks of HF feeding (473). The basis for this impairment is unknown, yet we predict our two-week Cr^{3+}/diet intervention will show membrane/cytoskeletal derangement as a basis and that Cr^{3+} offers protection against.

In addition, in future studies we will build upon preliminary siRNA knockdown data suggesting AMPK mediates Cr^{3+} action. We will complement our *in vitro*

analyses by testing if AMPK mediates the *in vivo* action of Cr^{3+}. We have found that Cr^{3+} increases AMPK activity in 3T3-L1 adipocytes (22-24) and L6 myotubes, and increased AMPK activity was also seen in fat and muscle from Cr^{3+}-supplemented HF-fed C57Bl/6J mice. Interestingly, the most potent Cr^{3+}-mediated stimulation of AMPK *in vitro* and *in vivo* occurs in the insulin-resistant state, a clinically-advantageous aspect of Cr^{3+} action. To supplement our *in vitro* studies, we will validate whether our cell-based observations explain the *in vivo* benefits of Cr^{3+} action. To confirm that activation of muscle AMPK is required for the antidiabetic activity of Cr^{3+} *in vivo*, we will examine Cr^{3+}'s activity in mice expressing a muscle-specific inactive AMPK. If muscle AMPK is required for Cr^{3+}'s actions, we would predict that Cr^{3+} will be ineffective in protecting against insulin resistance in these mice. Mice lacking the α1 isoform present no defect in glucose homoeostasis (426), as assessed by the glucose tolerance test. In contrast, mice lacking the α2 isoform clearly exhibit reduced insulin sensitivity (426). More recently the role of AMPKα2 in insulin resistance development was studied using muscle-specific transgenic mice on a B6 background expressing an inactive form of the AMPKα2 catalytic subunit (427). In this study it was found that ablation of muscle AMPKα2 activity worsens the glucose intolerance induced by HF feeding. This exacerbated glucose intolerance was associated with a decrease in muscle glucose transport in response to insulin, measured *in vitro* in isolated muscles (427). These results demonstrate that AMPKα2 activity is an important factor contributing to insulin action on glucose transport in muscle. Using these mice we will perform future studies to determine the importance of

AMPK in skeletal muscle Cr^{3+} action. A single diet/Cr^{3+} treatment duration (*i.e.*, either 2-, 4-, 8-, 12-, 16-, 20-, 24-weeks) will be used in these studies based on when we observe cholesterol accrual, insulin resistance and Cr^{3+} action in the diet intervention studies described above.

Finally, to characterize the distal mechanism(s) involved in Cr^{3+} action future study will assess if inhibition of the HBP and/or HMGR is a mechanism of Cr^{3+} and/or AMPK action. As described in detail above, AMPK can directly inactivate HMGR via phosphorylation at Ser872. We have also recently shown that hyperglycemia-, hyperinsulinemia-, and hyperlipidemia-induced increases in HBP activity lead to increased cholesterol synthesis, membrane cholesterol accrual, F-actin loss and GLUT4 dysregulation (10, 11). Moreover, our group has determined that blockade of the HBP inhibited membrane cholesterol accrual, F-actin loss and glucose transport dysfunction induced by these derangements (10, 11), suggesting a role of *O*-linked glycosylation in engaging cholesterol synthesis. Of interest is that a recent study has demonstrated that AMPK phosphorylates/inhibits GFAT (449), a rate-limiting enzyme of the HBP, implicating the HBP as a potential downstream pathway inhibited by Cr^{3+} action via AMPK. Conversely, it has also been shown that chronic HBP activation can increase fatty acid oxidation by increasing phosphorylation/activation of AMPK in part through O-linked glycosylation of AMPK. However, this transgenic model of HBP activity involved overexpression of GFAT, which does not efficiently represent the transient physiologically-relevant nature of nutrient flux through the HBP (474). The various putative interactions between the HBP and AMPK

suggest a complex interplay exists between these nutrient sensing pathways to regulate nutrient flux and energy balance. Therefore, we will assess if inhibition of the HBP and/or HMGR is a mechanism of Cr^{3+} and/or AMPK action. These future studies will be designed to delineate the specific targets of Cr^{3+} action; *i.e.*, although it is expected Cr^{3+}-dependent activation of AMPK suppresses cholesterol synthesis via phosphorylation/inactivation of GFAT and/or HMGR, it is possible that Cr^{3+} could target these enzymes independently of AMPK. Therefore, we will also test the action of Cr^{3+} in mice expressing an inactive form of AMPK described above. Our working hypothesis is that Cr^{3+} suppresses pathological cholesterol synthesis by activating AMPK that either suppresses GFAT and/or HMGR activity driving cholesterol synthesis. Studying the status of the HBP and HMGR at different time periods of Cr^{3+}/dietary intervention, as well as performing confirmatory studies *in vitro*, will importantly show whether *in vivo* Cr^{3+} action is associated with suppression of HBP activity and whether the mechanism requires AMPK.

Since skeletal muscle is responsible for most postprandial glucose disposal and is regarded as a major site of insulin resistance, deepening insight into the effect of Cr^{3+} on insulin sensitivity in this tissue during health and disease will be significant. Identification of AMPK as a mediator of Cr^{3+} action may provide an explanation for the existing controversies over the efficacy of Cr^{3+} in the amelioration of the symptoms and complications of T2D. For example, several agents that normalize blood glucose concentrations and/or improve insulin action, including metformin (333), phenformin (475), rosiglitazone (476) and

troglitazone (477), have been shown to activate AMPK. Our data would predict that the nutritive value of Cr^{3+} would be masked in patients receiving metformin or one of the other therapies listed above. Similarly, our model would explain why exercise, which also activates AMPK, can improve glycemia and dyslipidemia.

Our model in which altered glucose homeostasis and lipoprotein status is a consequence of elevated peripheral tissue membrane cholesterol levels represents a major paradigm shift in our thinking of mechanisms leading to insulin resistance and dyslipidemia. Discovery that Cr^{3+} effectively targets this abnormality stresses a plausible beneficial action of this micronutrient in health and disease. We propose the innovative concept that Cr^{3+} protects against membrane cholesterol accrual/toxicity that represents an early contributing feature of insulin resistance and dyslipidemia. However, in this thesis research we did not test if CrPic supplementation would be protective if initiated after these derangements were already induced. Therefore, future studies will be performed to define where (*e.g.*, adipose tissue, skeletal muscle, liver, etc.) and when during HF induced pathology Cr^{3+} has a significant impact on glucose/lipoprotein disorders. We aim to unambiguously determine the nutritive and pharmacological value of Cr^{3+} supplementation in managing and/or preventing disorders in glucose and lipoprotein metabolism. This information is absolutely essential for a clinically meaningful understanding of the antidiabetic and cardioprotective values of this micronutrient. Moreover, by broadening our basic scientific understanding of the mechanism of Cr^{3+} action, the findings will provide novel avenues for intervention.

Conclusion

In conclusion, together the data presented in Chapter II provide novel mechanistic insight into the mechanisms by which Cr^{3+} improves skeletal muscle insulin sensitivity. These data have demonstrated that CrPic protects against hyperinsulinemia-induced GLUT4 and glucose transport regulation, as well as improves glucose tolerance and insulin sensitivity in a diet-induced mouse model of insulin resistance. *In vitro* and *in vivo* data suggest that CrPic protects against membrane cholesterol accrual that disrupts F-actin integrity required for efficient GLUT4 regulation in skeletal muscle. Mechanistically, data have shown that AMPK plays a causal role in CrPic's protective effects on GLUT4 and glucose transport dysfunction, rather than CrPic enhancing insulin signaling mediators. Importantly, AMPK activation and downstream inhibition of FA and cholesterol synthesis machinery was also observed in skeletal muscles obtained from HF-fed mice supplemented with CrPic. Furthermore, data presented in Chapters IIA and IIB show that membrane cholesterol lowering is an unappreciated aspect of the mechanism by which AMPK activation enhances insulin-stimulated GLUT4 translocation to the PM in both insulin-sensitive and insulin-resistant skeletal muscle myotubes. Overall, these data suggest that Cr^{3+} has beneficial effects on skeletal muscle insulin sensitivity and whole body glucose tolerance by activating AMPK and positively impacting membrane/cytoskeletal aspects of GLUT4 regulation. We propose that Cr^{3+}, via AMPK activation, suppresses cholesterol synthesis and thereby lowers membrane cholesterol accrual that disrupts F-actin and induces insulin resistance in skeletal muscle.

Chapter IV. Experimental procedures

Cell culture and treatments

Rat L6 muscle cells stably expressing GLUT4 that carries an exofacial *myc*-epitope (L6-GLUT4*myc*; generously obtained from Dr. Amira Klip, (The Hospital for Sick Children, Toronto, Canada) were cultured as previously described. Myoblasts were maintained in α-Minimum essential medium (α-MEM) containing 5.5 mM glucose (Gibco, Grand Island, NY) 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. Cells were serum starved for 30 min before all experiments. Note longer serum starvation periods we tested did not enhance the characteristic low response (1.5-2.0 fold) of this cell line to insulin. Cells were then left untreated or treated with Cr^{3+} (CrPic, 16 h, 100 nM, Nutrition 21) or zaragozic acid A (16 h, 10 μM, Sigma-Aldrich). In a separate set of experiments, cells were left untreated or treated with 1 mM AICAR for 45 min or 200 μM DNP for 30 min. During the final 20 min (or 5 min for insulin signaling analyses) of these incubations, cells were left in either the basal state or were acutely stimulated with 100 nM insulin. In a subset of experiments, insulin induction of insulin resistance was performed as previously described (8) by treating the cells with 5 nM insulin for 12 h.

Mice

Male C57BL/6J mice (age 4 weeks) were obtained from Jackson Labs and housed in a light/temperature-controlled animal room maintained on a 12-h light, 12-h dark cycle. The mice were fed *ad libitum* NIH standard chow and water for one week, fed the LF diet for three weeks to acclimate to the increased palm oil in the diet, and then divided into treatment groups. Based on the fact commercial Cr^{3+}-containing nutritional supplements generally contain 200 to 600 µg Cr^{3+} and an average human body mass of 70 kg, the supplements provide ~3 to 9 µg Cr^{3+}/kg body weight/day. In these studies we administered 8 µg Cr^{3+}/kg body weight/day (CrPic; Nutrition 21, Purchase, NY) dissolved in the drinking water to control and high-fat fed C57Bl/6J mice. Control mice received a diet containing 20% kcal from protein, 70% kcal from carbohydrates, and 10% kcal from fat (D01030107, Research Diets Inc., New Brunswick, NJ). High-fat fed mice received a diet containing 20% kcal from protein, 35% kcal from carbohydrates, and 45% kcal from fat (D01030108, Research Diets Inc., New Brunswick, NJ). These diets were modified forms of D12450B and D12451 diets, with adaptations regarding type of fat (palm oil instead of lard) and carbohydrates to better mimic the FA/carbohydrate composition of the average human diet in Western societies, and induce significant insulin resistance in C57Bl/6J mice (445). The modified HF diet mimics the ratio of saturated to monounsaturated to polyunsaturated FAs (40:40:20). [*For simplicity I refer to these diets as LF and HF*]. In addition, these diets were made with a Cr^{3+}-free mineral mix (S17902) and Avicel PH101 (instead of cellulose, which contains Cr^{3+}). By addition of

chromium potassium sulfate, to the Cr^{3+}-free diets, both the LF and HF test diets were controlled to contain an equal amount of Cr^{3+}. Note the energy density of all nutrients, except fat and starch, is equal. All *in vivo* procedures performed using mice were based on protocols approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Rats

Specific pathogen-free obese (fa/fa) and lean (Fa/?) female Zucker rats were obtained from Harlan Sprague-Dawley at 6 weeks of age. Upon arrival, rats were housed individually in a temperature-controlled animal room maintained on a 12:12-h light-dark cycle. The rats were fed ad libitum NIH standard chow and water. All in vivo procedures performed were based on protocols approved by the Eli Lilly Institutional Animal Care and Use Committee.

IPGTT

C57Bl/6J mice were fasted for 6 hours (from 8:00 a.m. to 2:00 p.m.) in all IPGTT studies. Mice were injected intraperitoneally with 2 g/kg D-glucose (individually-dosed) at time 0 and blood was obtained via tail blood collection at 15 min, 30 min, 60 min, 90 min and 120 min post-injection of glucose. Blood glucose was analyzed using the Abbott Animal Health AlphaTRAK blood glucose meter (Abbott Laboratories, Abbott Park, IL).

IPITT

C57Bl/6J mice were fasted for 6 hours (from 8:00 a.m. to 2:00 p.m.) before all IPITT studies. Mice were injected intraperitoneally with 1.0 unit of insulin per kg body weight (individually-dosed) at time 0 and blood was obtained via tail blood collection at 15 min, 30 min, 60 min, 90 min and 120 min post-injection of insulin. Blood glucose was analyzed using the Abbott Animal Health AlphaTRAK blood glucose meter (Abbott Laboratories, Abbott Park, IL).

Immunocytochemistry

Myotube GLUT4*myc* and F-actin labeling was performed as previously described (8). Briefly, myotubes were fixed with 2% paraformaldehyde/phosphate buffered saline (PBS). After fixation, cells were either left unpermeabilized (GLUT4*myc*) or were permeabilized (F-actin) for 15 min at room temperature in 0.2% Triton X-100/PBS (actin for Li-Cor). Cells were then blocked for 1 h at room temperature in Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE). The samples were then incubated in primary antibody overnight, washed and incubated with an infrared-conjugated secondary antibody (Li-Cor) or with 1:50 FITC-conjugated anti-mouse (confocal) for 1 h. Images were collected and quantified with the Odyssey system as previously described (8). Immunofluorescent intensity was normalized to intensity from Syto60, a fluorescent nucleic acid stain (Molecular Probes).

C57BL/6J soleus muscle was prepared and labeled as previously described. Briefly, following an overnight fast soleus muscles were dissected out, blotted on

gauze, quickly rinsed with PBS, and immersed in 4% paraformaldehyde/PBS. Following fixation for 2 h, tissues were washed with PBS then permeabilized for 20 min at room temperature in 0.2% Triton X-100/0.05% Tween 20/PBS. Tissues were rinsed three times in PBS then blocked in 5% donkey serum for 60 min at room temperature. Tissues were then incubated overnight at 4°C in anti-F-actin antibody diluted 1:50 in blocking buffer. Samples were washed extensively in PBS before incubation for 60 min at room temperature in 1:50 rhodamine red-X-conjugated donkey anti-mouse IgM. Secondary antibody incubations were followed by extensive washing in PBS then a quick ddH_2O rinse. Tissues were mounted in slides with Vectashield and were analyzed via confocal microscopy (model LSM 510 NLO; Zeiss, Thornwood, NY).

Rat skeletal muscle was prepared and labeled as previously described (8, 18). Briefly, after 2 weeks of acclimation, rats in the postprandial state were anesthetized with 5 mg/100 g body weight sodium pentobarbital and the epitrochlearis muscles were dissected out, blotted on gauze, quickly rinsed in saline and immersed in 4% paraformaldehyde/PBS. Both epitrochlearis muscles from five lean and 5 obese rats were used, for a total of ten muscles. Myotube and skeletal muscle images were obtained using the Zeiss LSM 510 NLO Confocal Microscope. For all confocal imaging, all microscopic and camera settings were identical within experiments, and representative images are shown.

Insulin ELISA

Blood was collected by cardiac puncture in the prandial state. Plasma was obtained by centrifuging blood samples at 4°C for 15 min at 2000 rpm using a table top centrifuge. Plasma insulin concentrations were determined using the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL).

ATP measurement

Intracellular ATP content was measured using a luminescence ATP detection assay system (ATPlite^{TM}, PerkinElmer Inc.). Briefly, following experimental treatments, L6 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.

Membrane and cholesterol analyses

A plasma membrane-enriched fraction was prepared as described by Khayat *et al.* (437). Briefly, myotube monolayers grown on 10-cm-diameter dishes were gently scraped with a rubber policeman in 5 ml of ice-cold HES homogenization buffer (in mM: 250 sucrose, 20 HEPES, 2 EGTA, and 3 NaN_3, pH 7.4) containing freshly added protease inhibitors (in µM: 200 PMSF, 1 leupeptin and 1 pepstatin A) and homogenized through a 22-gauge needle 10 times. The homogenate was centrifuged at 760 *g* for 5 min at 4°C, and the resultant supernatant was

centrifuged at 31,000 *g* for 20 min to separate a plasma membrane-enriched pellet from an intracellular microsome supernatant. The plasma membrane pellet was resuspended in HES buffer and assayed for protein (Bradford) and cholesterol (Amplex Red) content as previously described (22, 23). Several unpublished analyses we have performed revealed that measured changes in plasma membrane cholesterol were similarly reflected in total cellular membrane fractions prepared by centrifuging the original cellular homogenate at 5,000 *g* for 20 min at 4°C and then subjecting the supernatant to centrifugation at 100,000 *g* for 30 min. As the number of 10-cm-diameter dishes required to obtain this total membrane fraction was half that required to prepare plasma membrane-enriched fractions we used total membrane fractions in the current study.

The preparation of βCD-cholesterol complex was performed essentially by the method of Christian *et al.* (478), with minor modifications. Briefly, 96.7 µl of cholesterol from 5 mg/ml stock in chloroform-methanol (1:1, vol:vol) was added to a glass tube. The solvent was evaporated under a gentle stream of nitrogen gas, and a dried cholesterol film was formed on the bottom of the tube. Next, 10 ml of 1 mM βCD was added, vortexed and sonicated (bath sonicator), resulting in a solution containing βCD to cholesterol at a ratio of 8:1. This 100% saturated βCD-cholesterol solution was incubated in a 37°C water bath for 48 h with vigorous shaking. This mixed solution was then filtered through a 0.45-µm syringe filter (Millipore) before use. In the cholesterol replenishment experiments, during the 30-min serum starvation period cells were pre-incubated with this

solution prior to treatments with AICAR, DNP, and/or insulin. All treatments were performed during continual exposure to the βCD-cholesterol solution.

2-Deoxyglucose uptake assays

Following treatments, 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. Glucose uptake was initiated with the addition of 2-deoxy-[1,2-^{3}H]-glucose (0.055 µCi/µl). Nonspecific uptake was quantitated via cell-associated radioactivity in the presence of 20 µM cytochalasin B. After 10 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.

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

Total cell extracts were prepared from 10-cm-diameter 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 separated by 7.5% SDS-PAGE. 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 AMPK and AS160 (Cell Signaling Technology, Danvers, MA) and to HMGR and ACC (Millipore, Temecula, CA). Equal protein loading was confirmed by Ponceau staining and by immunoblot analysis with anti-ACC and anti-AMPK (Cell Signaling Technology, Danvers, MA), and anti-actin antibody (Cytoskeleton, Denver, CO). All immunoblots were labeled with IR-conjugated secondary antibodies and analyzed via the Odyssey system (Li-Cor, Lincoln, NE).

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. The oligonucleotides with the highest knockdown efficiency for α1 and α2 were respectively: CGA GUU GAC UGG ACA UAA Att (siRNA ID#: 194424) and GCA ACU AUC AAA GAC AUA Ctt (siRNA ID#: 194794). As these cells express both alpha isoforms, the combination of the two nucleotides led to the greatest knockdown efficiency. 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 Dulbecco's modified Eagle's medium (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

All values are presented as means ±SE. The significance of differences between means was evaluated by one-way repeated measures analysis of variance (ANOVA). Where differences among groups were indicated, the Newman-Keuls test was used for post hoc comparison between groups. Differences between two groups were analyzed by the Student's *t*-test for independent samples. GraphPad Prism 4 software was used for all analyses. $P < 0.05$ was considered significant.

Chapter V. References

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Diabetes and Obesity Research Training Program
T32-DK064466

Indiana University Center for Diabetes Research
July 2008-July 2010
Diabetes and Obesity Research Training Program
DeVault Diabetes Fellowship

PUBLICATIONS

MANUSCRIPTS

Hoffman NJ and Elmendorf JS: Signaling, cytoskeletal and membrane mechanisms regulating GLUT4 exocytosis. (2011) Trends in Endocrinology and Metabolism 22(3): p. 110-116.

Habegger KM*****, **Hoffman NJ***, Ridenour CM, Brozinick JT, Elmendorf JS: AMPK Enhances Insulin-Stimulated GLUT4 Regulation via Lowering Membrane Cholesterol: Evidence for AMPK Activity Countering Membrane Cholesterol-Induced Insulin Resistance. Endocrinology (*Accepted*) ***Authors contributed equally**

Hoffman NJ, Penque BA, Sealls W, Tackett L, Elmendorf JS: AMPK is involved in a membrane/cytoskeletal pathway of chromium action that improves glucose transport regulation in insulin-resistant skeletal muscle cells. (*In Preparation*)

ABSTRACTS

Hoffman NJ and Elmendorf JS: Mechanistic insight into the essentiality of chromium in glucose homeostasis. 2011 Keystone Symposium: Type 2 Diabetes, Insulin Resistance and Metabolic Dysfunction

Hoffman NJ, Habegger KM, Elmendorf JS: (2010) Chromium protects against hyperinsulinemia-induced membrane/cytoskeletal derangements and insulin resistance in cultured skeletal muscle myotubes. Diabetes 59, Supplement 1

Hoffman NJ, Habegger KM, Elmendorf JS: (2009) Identification of an actin-based antidiabetic action of chromium in skeletal muscle. 49th Annual ASCB Meeting, Late-Breaking Abstracts On-Site Addendum

PROFESSIONAL CONFERENCES ATTENDED

- Indiana Life Sciences Collaboration Conference Series
  May 2011
  Health Information Technology: Indiana's Role in the Development of a National Model
  Indianapolis, IN
- Indiana Life Sciences Collaboration Conference Series
  February 2011
  Update on Regulatory Compliance
  Indianapolis, IN
- 1st Annual Indiana Physiological Society Meeting
  February 2011
  Indianapolis, IN
- 2011 Keystone Symposia Conference Series
  January 2011
  Type 2 Diabetes, Insulin Resistance and Metabolic Dysfunction
  Keystone, CO

- 70th Scientific Sessions of the American Diabetes Association
  June 2010
  Orlando, FL
- Indiana Life Sciences Collaboration Conference Series
  February 2010
  Effective Collaboration: A Study in Three Acts
  Indianapolis, IN
- 49th Annual Meeting of the American Society for Cell Biology
  December 2009
  San Diego, CA
- Indiana Life Sciences Collaboration Conference Series
  November 2009
  Comparative Effectiveness: The Dollars and Sense
  Bloomington, IN

PROFESSIONAL AFFILIATIONS

- Indiana Physiological Society, Student Member
  2011-2012
- American Association for the Advancement of Science, Student Member
  2010-2012
- American Society for Cell Biology, Student Member
  2010-2012
- Indiana University Kelley School of Business
  2009-2011
  Center for the Business of Life Sciences, Graduate Student Associate
- American Physiological Society, Student Member
  2008-2012

PRESENTATIONS

- Oral Presentation
  August 2011
  2011 Sigma Xi Biomedical Research Competition
- Oral Presentation
  July 2011
  Baker IDI Heart & Diabetes Institute
  Melbourne, Australia
- Oral Presentation
  July 2011
  Garvan Institute of Medical Research
  Sydney, Australia
- Oral Presentation
  April 2011
  Indiana University Center for Diabetes Research Seminar Series

- Thesis Proposal Oral Presentation
  March 2011
  Department of Cellular & Integrative Physiology Seminar Series
- Research Poster Presentation
  February 2011
  Indiana Biomedical Gateway Program Recruitment Poster Session
- Research Poster Presentation
  February 2011
  1st Annual Indiana Physiological Society Meeting
  Indianapolis, IN
- Research Poster Presentation
  January 2011
  Keystone Symposium: Type 2 Diabetes, Insulin Resistance and Metabolic Dysfunction
  Keystone, CO
- Oral Presentation
  December 2010
  IUPUI Center for Membrane Biosciences Meeting
- Research Poster Presentation
  June 2010
  70th Scientific Sessions of the American Diabetes Association
  Orlando, FL
- Oral Presentation
  May 2010
  2010 Sigma Xi Biomedical Research Competition
- Research Poster Presentation
  April 2010
  2010 IUPUI Research Day
- Research Poster Presentation
  February 2010
  Indiana Biomedical Gateway Program Recruitment Poster Session
- Oral Presentation
  February 2010
  Department of Cellular & Integrative Physiology Seminar Series
- Research Poster Presentation
  January 2010
  Indiana Biomedical Gateway Program Recruitment Poster Session
- Research Poster Presentation
  49th Annual Meeting of the American Society for Cell Biology
  December 2009
  San Diego, CA
- Oral Presentation
  October 2009
  2009 Indiana Physiology Statewide Departmental Retreat

- Oral Presentation
  August 2009
  Department of Cellular & Integrative Physiology Seminar Series
- Research Poster Presentation
  February 2009
  Indiana Biomedical Gateway Program Recruitment Poster Session
- Research Poster Presentation
  January 2009
  Indiana Biomedical Gateway Program Recruitment Poster Session
- Research Poster Presentation
  October 2008
  2008 Indiana Physiology Statewide Departmental Retreat

AWARDS

- Co-3rd Place Presentation: 2011 Sigma Xi Biomedical Research Competition
  2011
- IUPUI Graduate and Professional Student Government
  2011
  Educational Enhancement Grant
- 1st Annual Indiana Physiological Society Meeting Student Abstract Award
  2011
- IUPUI Center for Membrane Biosciences Student Travel Award
  2011
- 2nd Place Presentation: 2010 Sigma Xi Biomedical Research Competition
  2010
- 1st Place Presentation: Center for the Business of Life Sciences Competition
  2010
  “Global Events and Trends Impacting the Life Sciences Industry”
- Butler University Biology Departmental Honors
  2003-2007
- Hendricks Regional Health Scholarship
  2003-2007
- Butler University College of Liberal Arts and Sciences Scholarship
  2003-2007
- National Society of Collegiate Scholars
  2004-2007

RESEARCH INTERESTS

- Cellular and molecular mechanisms of insulin resistance
- Glucose transporter biology and regulation
- Nutritional aspects of health and disease

RESEARCH EXPERTISE

- Cell culture (L6 myotubes and 3T3-L1 adipocytes)
- Preparation and analysis of protein: SDS-PAGE
- Western blot analysis
- Whole cell immunofluorescence
- Subcellular fractionation
- Fluorescent and confocal microscopy in cultured cells and intact tissue
- Animal care and maintenance (mice and swine)
- Dissection: rodent brain, heart, liver, kidney, skeletal muscle, and fat pads
- Intraperitoneal glucose tolerance testing (mice)
- Intraperitoneal insulin tolerance testing (mice)
- Intravenous glucose tolerance testing (swine)
- Blood collection (mice and swine)
- 2-deoxyglucose uptake assay
- Amplex Red cholesterol assay
- Electroporation and plasmid transfection
- Luciferase and renilla assays
- siRNA knockdown
- Preparation and analysis of DNA and RNA: PCR, sequencing, agarose and acrylamide gel electrophoresis, plasmid isolation, restriction digest, ligation, vector construction, isolation of DNA and RNA

TRAINING EXPERIENCES

Indiana University School of Medicine
May 2008-February 2012
Department of Cellular and Integrative Physiology
Mentor: Jeffrey S. Elmendorf, Ph.D. (Associate Professor)

University of Tasmania, Department of Zoology
February 2006-July 2006

Butler University, Biology Department
January 2005-December 2005
Undergraduate Research in Behavioral Ecology
Mentor: Stephen Perrill, Ph.D. (Professor)

Clarian Health Emergency Medical Education Program
May 2004-August 2004
State of Indiana, Emergency Medical Technician Training

Larue D. Carter Memorial Hospital
April 2003-August 2003
Teaching Research Treatment Program

TEACHING EXPERIENCE

F782: Physiology and Pathophysiology of Lipid Rafts
March 2011
Lecture to graduate students: “Bilayering GLUT4: Lipid Rafts and Insulin Resistance”

Indy Parks: Holliday Park
May 2007-August 2007
Youth Nature Education Programs Coordinator

PROFESSIONAL AND UNIVERSITY SERVICE

- IUPUI Responsible Conduct of Research Workshop
  2011
  Panel Member
- Department of Cellular & Integrative Physiology Retreat
  2011
  Moderator for Graduate Student Session
- American Society for Cell Biology Ambassador
  2010-present
- Indiana University School of Medicine
  2010
  Department of Cellular & Integrative Physiology 2010 Summer Seminar Series
  Student Coordinator
- Indiana University School of Medicine
  2010
  Science Olympiad Volunteer
- Indiana University School of Medicine Graduate Committee
  2009-2011
  Student Representative
- Indiana University School of Medicine Second Look Program Student Panel
  2009
- Indiana University School of Medicine Student Mentor
  2008-2011
- Indiana University School of Medicine Student Ambassador
  2008-2011
  Student Coordinator for Campus Visits
- Butler University Biology Department Student Advisory Board
  2006-2007
- Butler University Biology Department Student Mentor
  2005-2007
- Butler University Biology Club Field Trip and Social Chair
  2006-2007

COMMUNITY SERVICE

- Indy Parks: Holliday Park
  2006-2007
  Volunteer Naturalist
- Wishard Memorial Hospital Pediatric Primary Care Center
  2005
  Founder and coordinator, Childhood Obesity Prevention Health Fair
- Larue D. Carter Memorial Hospital
  2003
  Volunteer, Health Education Programs and Recreational Therapy