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Inhibition of AMPK via phosphorylation at Ser485/491: multiple mechanisms of regulation

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INHIBITION OF AMPK VIA PHOSPHORYLATION AT SER485/491: MULTIPLE MECHANISMS OF REGULATION

by

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DEDICATION

I would like to dedicate this work to my mom and dad, my brother Randy, and my fiancé Jon, all of whom have supported, helped, and inspired me in this work and in life.

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INHIBITION OF AMPK VIA PHOSPHORYLATION

AT SER485/491: MULTIPLE MECHANISMS OF REGULATION

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ABSTRACT

AMP-activated protein kinase (AMPK) is an energy-sensing enzyme that is activated when cellular energy is low and causes muscle and other cells to increase glucose uptake and fat oxidation, diminish lipid synthesis, and alter expression of various genes. AMPK activity is diminished in animals with the metabolic syndrome, though the mechanisms causing this reduction are unknown. To examine nutrient-induced changes in AMPK activity over time and factors that may regulate it, we compared rat muscle incubated with high glucose (HG) (30min-2h) and muscle of glucose-infused rats (3-8h) with appropriate controls. In addition to diminished AMPK activity (measured by the SAMS peptide assay) and phosphorylation of its activation loop at Thr^{172}, we observed increased muscle glycogen, phosphorylation of AMPK's α1/α2 subunit at $Ser^{485/491}$, and PP2A activity, and decreased SIRT1 expression, all of which have been shown to diminish AMPK activity. Dysregulation of one or more of these factors could contribute to pathophysiological changes leading to metabolic syndrome associated disorders.

Since recent studies suggest phosphorylation at $Ser^{485/491}$ may play an important role in AMPK inhibition, we sought to determine how phosphorylation of this site is regulated. We investigated whether insulin or diacylglycerol (DAG) signaling pathways

may be involved, since both are increased in at least one of the HG models. Akt and Protein Kinase (PK)D1 phosphorylated AMPK at $Ser^{485/491}$ and diminished its activity in C2C12 myotubes, downstream of insulin and the DAG-mimetic PMA, respectively. Additionally, p-AMPK $Ser^{485/491}$ was increased in muscle and liver of fed versus fasted mice and liver of diabetic mice. Our results suggest that Akt- and PKD1-mediated inhibition of AMPK via $Ser^{485/491}$ phosphorylation may inhibit energy-metabolizing processes, while favoring energy-storing processes. Our results highlight the fact that phosphorylation of $Ser^{485/491}$ can inhibit AMPK activity independent of changes in p-AMPK Thr^{172}, a measure which is often used as a readout of AMPK activity. We hypothesize that Akt-mediated inhibition of AMPK is an acute, physiological response to insulin, whereas PKD1-mediated inhibition may be associated with more chronic pathophysiological changes. Thus, PKD1 inhibition or prevention of $Ser^{485/491}$ phosphorylation may represent new strategies for therapeutic AMPK activation as treatment for the metabolic syndrome.

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LIST OF ABBREVIATIONS

2-DOG.....2-deoxy-D-glucose

ACC .....Acetyl-CoA Carboxylase

ADP.....Adenosine diphosphate

AMP.....Adenosine monophosphate

AMPK..... AMP-activated Protein Kinase

AS160 ..... Akt substrate of 160 kDa

ATP.....Adenosine triphosphate

BCAA .....Branched chain amino acid

BIM I.....Bisindoylmaleimide I

CaMK.....Ca(2+) /calmodulin-dependent protein kinase

CaMKKβ..... Ca(2+) /calmodulin-dependent protein kinase kinase β

DAG.....Diacylglycerol

ECL.....Enhanced chemiluminescence olution

EDL.....Extensor digitorum longus

ELISA ..... Enzyme-linked immunosorbent assay

ET-1 .....Endothelin-1

FA .....Fatty acid

FFA ..... Free fatty acid

FBS .....Fetal bovine serum

GAPDH.....Glyceraldehyde 3-phosphate dehydrogenase

GLUT4..... Glucose transporter type 4

GS ..... Glycogen synthase

GSK-3 .....Glycogen synthase kinase-3

GTT..... Glucose tolerance test

HDAC .....Histone deacetylase

HG..... High glucose

HS ..... Horse serum

HUVEC..... Human umbilical vein endothelial cell

IGF-1..... Insulin-like growth factor 1

IL-6 .....Interleukin-6

IKKα/β ..... Inhibitor of nuclear factor kappa-B kinase α/β

IR.....Insulin resistance

IRS-1 ..... Insulin receptor substrate 1

JNK ..... C-Jun N-terminal kinase

LKB1.....Liver kinase B1

MAPK.....Mitogen-activated protein kinase

MEF2 .....Myocyte enhancer factor 2

MnSOD.....Manganese superoxide dismutase

mTOR .....Mammalian target of Rapamycin

NAD..... Nicotinamide adenine dinucleotide (oxidized)

NADH..... Nicotinamide adenine dinucleotide (reduced)

NADPH..... Nicotinamide adenine dinucleotide phosphate

NAMPT.....Nicotinamide phosphoribosyltransferase

NEMO ..... NF-κB essential modulator

NF-κB ..... Nuclear factor kappa-light-chain-enhancer of activated B cells

P/S ..... Penicillin/streptomycin

PAP ..... Phosphatidic acid phosphatase

PBS ..... Phosphate buffered saline

PDK-1 ..... 3-phosphoinositide-dependent kinase-1

PI3K ..... Phosphoinositide 3-kinase

PIP3 ..... phosphatidylinositol 3,4,5-trisphosphate

PKA ..... Protein Kinase A

PKB ..... Protein Kinase B

PKC ..... Protein Kinase C

PKD ..... Protein Kinase D

PLD1 ..... Phospholipase D1

PMA ..... Phorbol 12-myristate 13-acetate

PPAR-γ ..... Peroxisome proliferator-activated receptor-γ

ROS ..... Reactive oxygen species

SAMS ..... Substrate for AMPK

Ser ..... Serine

SIRT1 ..... Sirtuin (silent mating type information regulation 2 homolog) 1

TAK1 ..... Transforming growth factor-β activated protein kinase-1

TBC1D1 ..... TBC1 domain family member 1

TBC1D4 ..... TBC1 domain family member 4

T2D ..... Type 2 diabetes

Thr ..... Threonine

TZD ..... Thiazolidinedione

VSMC ..... Vascular smooth muscle cell

WHO ..... World Health Organization

CHAPTER ONE

Introduction and Overview

Overview

The prevalence of worldwide obesity, type 2 diabetes (T2D), and the metabolic syndrome are increasing at an alarming rate. Insulin resistance (IR) in peripheral tissues (muscle, liver, and adipose) is a key pathological defect in T2D. Since skeletal muscle is responsible for 70-80% of insulin-stimulated glucose uptake [1], skeletal muscle IR is considered a critical pathological component of the metabolic syndrome and T2D [2]. Acute IR may be a normal or protective response of the cell to excess nutrients under physiological conditions, possibly a mechanism to reroute unneeded fuel to other parts of the body, or to prevent oxidative stress and glucotoxicity [3]. In this setting, normal insulin signaling resumes when nutrient levels return to normal. In contrast, chronic nutrient excess causes changes which are less easily reversible that prevent normal glucose uptake, thus causing hypergylcemia and hyperinsulinemia in the plasma, but glucose deprivation in the tissue. Additionally, more damaging secondary changes such as pancreatic β-cell apoptosis, inflammation, oxidative stress, and vascular/endothelial dysfunction result [4]. The molecular sequence of events by which chronic exposure to excess nutrients (high glucose, lipids) impairs insulin signaling has been studied in detail, but remains incompletely understood. Hyperinsulinemia, inflammation, oxidative stress, ER stress, and the accumulation of toxic lipid derivatives, such as diacylglycerol (DAG) and ceramides, have all been implicated to contribute to the development of IR [1,5].

Which of these factors is the primary cause, or whether it is a combination of them, remains under debate.

AMP-activated protein kinase (AMPK) is an energy sensing enzyme that plays a central role in nutrient sensing/insulin sensitivity. It is a heterotrimeric protein that is activated when energy levels are low (i.e., exercise or starvation) and signals to increase ATP generating processes and decrease ATP consuming processes. The consequences of its activation (mediated through a high AMP/ATP or ADP/ATP ratio and phosphorylation of αAMPK Thr^{172}) have been studied in detail. Pharmacological agents that indirectly activate AMPK by altering the cellular energy state (AMP/ATP ratio) are currently used in the clinic (i.e., Metformin, TZDs) and are of interest in drug development [6], as they prevent and/or reverse some of the pathologies of T2D. However, a direct modulator of AMPK has yet to make it to the clinic.

In contrast to the plethora of knowledge regarding AMPK activation, the mechanisms and consequences of AMPK downregulation below basal levels, which our lab has shown to occur early on in the setting of high nutrient induced IR, are less understood [7]. Notably, multiple animal models with a metabolic syndrome phenotype have decreased AMPK activity in muscle and liver [6,8-11], and loss of AMPK is detrimental in a number of metabolic challenges [9,12-16], such as diet-induced IR and obesity [17], calorie restriction [18], and exercise [19]. In addition, decreased AMPK activity in skeletal muscle [20] and adipose tissue [21,22] of humans with T2D or obesity has been reported. Although no causal inferences can be established in humans, dysfunction of AMPK may predispose obese individuals to a variety of metabolic

complications, including IR and T2D. The precise mechanism for the suppressed activity of AMPK is unknown; however, prolonged exposure to excess nutrients (glucose, branched chain amino acids (BCAA), and fatty acids (FA)) has been shown to cause diminished AMPK activity [7].

Our lab has previously shown that incubation of rat extensor digitorum longus muscle (EDL) with 25mM glucose for 1h suppresses AMPK activity, as evaluated by decreased phosphorylation of Thr^{172} on the "activation loop" of the α subunit, diminished phosphorylation of its downstream substrate ACC at Ser^{79} (a site only phosphorylated by AMPK), and also a reduction in kinase activity as measured by the SAMS peptide assay [7]. Many factors may contribute to the decrease in AMPK activity, such as reduced phosphorylation of AMPK Thr^{172} (either by reduced activity of upstream kinases or increased phosphatase activity), increased phosphorylation of α1/α2AMPK $Ser^{485/491}$, a site that is presumed to be inhibitory, changes in adenine nucleotide levels (decreased AMP/ATP ratio), a change in redox state, or other factors that may alter aspects of this pathway [23]. The overall aims of this work are (1) to determine the changes in AMPK activity and phosphorylation over time in skeletal muscle exposed to excess glucose (*ex vivo* incubation with glucose and *in vivo* glucose infusion) and factors that may contribute to its inhibition, (2) to investigate the upstream kinases responsible for phosphorylating AMPK at $Ser^{485/491}$ phosphorylation and their contribution to diminishing AMPK activity and insulin sensitivity, and (3) to determine the regulation of AMPK $Ser^{485/491}$ phosphorylation in physiological (fasting/refeeding) and pathological (*db/db* mice) models of nutrient excess.

Type 2 Diabetes

T2D is a metabolic disease characterized by elevated blood glucose levels in the presence of peripheral insulin resistance. According to the International Diabetes Federation, more than 350 million people worldwide had diabetes in 2014, and it is projected that this number will rise to nearly 600 million by 2035 [24]. T2D is associated with a number of complications and co-morbidities, including cardiovascular disease, blindness, kidney failure, and lower limb amputation [24]. The number one risk factor for T2D is obesity, in which chronic nutrient overconsumption leads to hyperglycemia, insulin resistance, and impaired metabolic function.

Insulin resistance

Insulin resistance is defined as the inability of a known quantity of exogenous or endogenous insulin to stimulate glucose uptake and utilization and suppress hepatic glucose production as much as it would in a healthy individual [25]. Since excess intracellular glucose causes cellular toxicity by a variety of mechanisms, insulin resistance is a protective mechanism to avoid this toxicity in environments of over-nutrition. However, this protective mechanism leads to pathological changes in the setting of prolonged exposure to nutrient overload. In this state of chronic insulin resistance, reduced glucose uptake in muscle and adipose tissue, impaired suppression of hepatic gluconeogenesis, and impaired suppression of lipolysis lead to hyperglycemia, hyperinsulinemia, and hyperlipidemia. Initially, pancreatic β-cells compensate by secreting more insulin. Eventually, however, these cells become dysfunctional due to a combination of factors including endoplasmic reticulum (ER) stress, inflammation, and

oxidative stress [26]. This ultimately leads to decreased β-cell mass and inadequate insulin secretion, at which point exogenous insulin becomes necessary.

In a normally functioning muscle cell, insulin, which is a peptide hormone made in pancreatic β-cells and secreted in response to elevated blood glucose, binds to the insulin receptor, which is a transmembrane receptor tyrosine kinase [27]. Binding of insulin to the insulin receptor causes the autophosphorylation of the receptor on several tyrosine residues on the intracellular tyrosine kinase domain. These changes lead to the recruitment and phosphorylation of various substrate proteins, such as insulin receptor substrate 1 (IRS-1). Activated IRS-1 then recruits and activates phosphatidylinositol 3-kinase (PI3K), which catalyzes the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 recruits 3-phosphoinositide-dependent kinase-1 (PDK-1) and Akt (also known as protein kinase B (PKB)) to the plasma membrane [27]. Akt is then activated by being phosphorylated at two sites: Thr^{308} by PDK-1 and Ser^{473} by mTOR complex 2 (mTORC2); phosphorylation at both sites is required for full activation [28]. Akt phosphorylates Akt substrate of 160 kDa (AS160, also known as TBC1 domain family member 4 (TBC1D4)) and TBC1 domain family member 1 (TBC1D1), which inhibit Rab GTPase activity and promotes GTP binding to Rabs, thereby allowing glucose trasporter type 4 (GLUT4) translocation to the membrane and glucose uptake (See Figure 1.1 for a simplified insulin signaling diagram) [27]. Akt also has many other downstream substrates, including glycogen synthase kinase-3 (GSK-3). GSK-3 is inhibited by Akt phosphorylation, which leads to dephosphorylation and activation of glycogen synthase (GS) and a subsequent increase in muscle glycogen content. In an insulin resistant state,

phosphorylation and activation of Akt is often impaired, which prevents normal glucose uptake. Thus, diminished insulin-stimulated Akt phosphorylation is often used as an indicator of insulin resistance. Activity of IRS-1 is often also diminished in IR due to inhibitory phosphorylation on serine residues as a consequence of protein kinase C (PKC) activation.

The molecular mechanisms responsible for insulin resistance are incompletely understood. Many proteins and biological pathways involved in metabolic homeostasis are dysregulated, and many factors, such as inflammation and oxidative and ER stress, are thought to play a role. The enzyme AMPK is of interest because it is not only inhibited in IR states, but its activation also has insulin sensitizing effects.

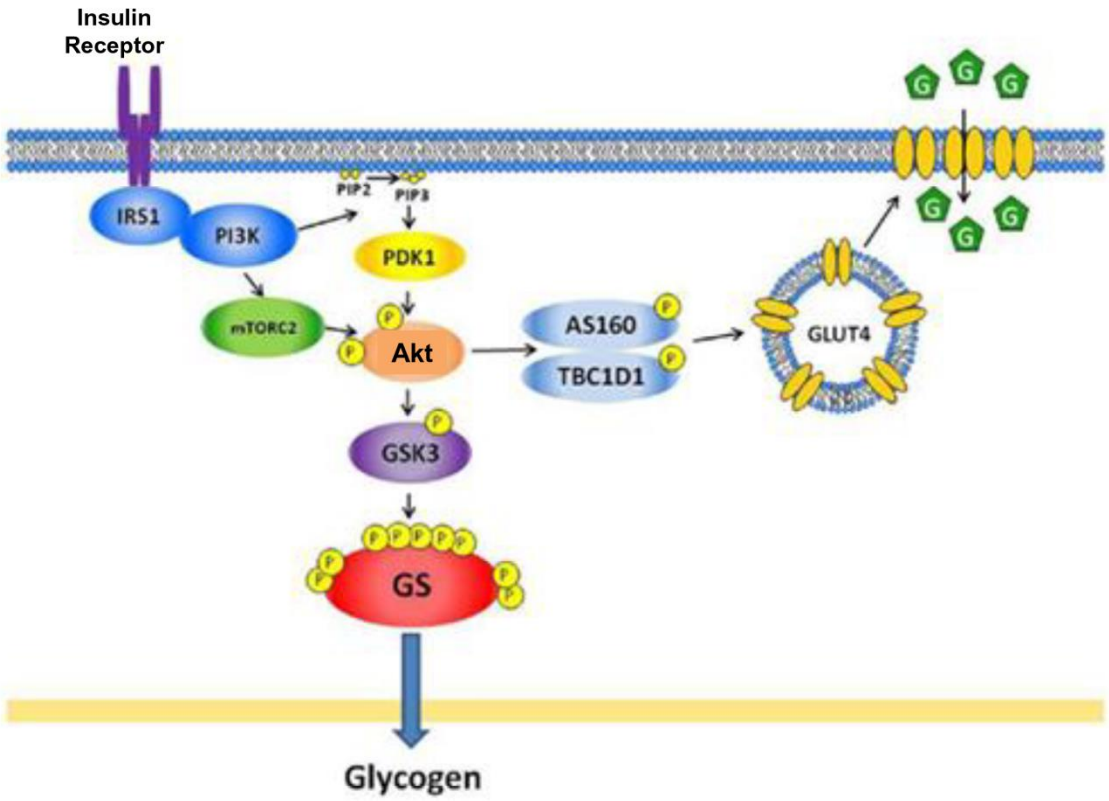


Figure 1.1: Insulin signaling in skeletal muscle.

As described in the text, insulin binds to the insulin receptor, a transmembrane receptor tyrosine kinase, which activates IRS-1 and subsequently PI3K. This leads to activation of Akt by phosphorylation at two sites (Thr^{308} by PDK-1 and Ser^{473} by mTORC2). Akt signals to AS160 and TBC1D1 to stimulate GLUT4 translocation and increase glucose uptake and to GSK3 to increase glycogen synthesis. Adapted from [27].

AMP-activated Protein Kinase (AMPK)

Overview and Structure

AMPK is a phylogenetically conserved serine/threonine kinase that functions as a master metabolic regulator. It exists as a heterotrimer, consisting of a catalytic α-subunit and regulatory β- and γ-subunits. Each subunit has multiple isoforms (α1, α2, β1, β2, γ1, γ2, γ3), making a total of twelve possible heterotrimer combinations. Whether there are functional differences between the different isoforms remains unclear, however, some isoforms are tissue specific. For example, heterotrimers containing the α1 isoform predominate in liver and adipose tissue, whereas those containing α2 predominate in brain, heart, and skeletal muscle [29,30].

Activation of AMPK requires both an increase in the intracellular AMP:ATP ratio and phosphorylation of Thr^{172} on the "activation loop" [31] of the α-subunit by one of its three upstream kinases: the tumor-suppressor liver kinase B1 (LKB1) [32,33], the calcium-dependent calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) [34], or transforming growth factor-β activated protein kinase-1 (TAK1) [35]. Although Thr^{172} on the α-subunit is regarded as the main phosphorylation/activation site, changes in AMPK activity have recently been observed in the absence of altered Thr^{172} phosphorylation. As such, AMPK activity may be impacted by one of several other phosophorylation sites on AMPK with less defined functions [30]. One such site is $Ser^{485/491}$ on the α1/α2 subunit, which will be discussed below in further detail.

The γ subunit contains four CBS domains (each pair is referred to as a Bateman domain) to which adenine nucleotides bind [36]. Three of the four CBS domains bind adenine nucleotides; site three primarily has AMP bound, however this can be replaced by ATP under specific conditions [37]. The other two binding domains can bind AMP, ADP, or ATP depending on their relative concentrations [36]. Under normal conditions, ATP is bound to these domains; however, when the AMP:ATP ratio is increased, AMP replaces ATP at the Bateman domains, causing an allosteric change that contributes to AMPK activation. This allosteric change makes AMPK a better substrate for its upstream kinases to phosphorylate it at Thr^{172} and inhibits dephosphorylation of this site by the protein phosphatases PP2A and PP2C [38,39]. The combination of allosteric activation and phosphorylation at Thr^{172} leads to a greater than 1000-fold increase in kinase activity in cell-free assays [40], although the changes under physiological conditions are likely much smaller [41]. Recently, it has been proposed that ADP, as well as AMP, may be able to activate AMPK by binding to the Bateman domains [42,43]. Whether this occurs under normal physiological conditions remains under debate, as AMP is a much more potent allosteric activator [41]. The structures of AMPK's subunits are shown in Figure 1.2.

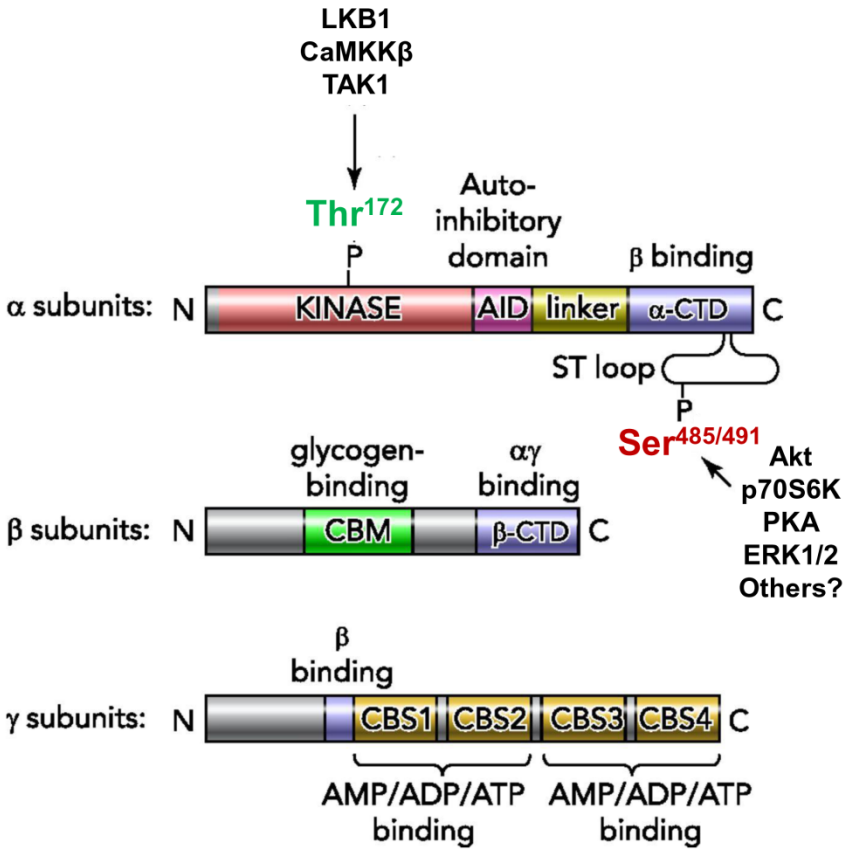


Figure 1.2: AMPK subunits and their structural domains.

AMPK's catalytic α1/α2 subunit consists of a kinase domain, and autoinhibitory domain, a linker domain, and a C terminal domain, which includes a serine/threonine (ST) rich loop. Phosphorylation of Thr^{172} in the kinase domain contributes to enzyme activation, while phosphorylation of $Ser^{485/491}$ of the ST loop is inhibitory.The β1/β2 subunits consist of a glycogen-binding domain (also known as carbohydrate binding motif (CBM)) and a C terminal domain that binds to the α and γ subunits. The γ1/γ2/γ3 subunits contain four CBS domains, which bind AMP, ADP, or ATP depending on their relative concentrations. Adapted from [44]

Upon activation, AMPK phosphorylates its downstream targets, a main one being acetyl-CoA carboxylase (ACC) [45]. AMPK phosphorylates ACC at Ser^{79} (an inhibitory site), preventing the conversion of acetyl-CoA to malonyl CoA, which allows long-chain FA to enter the mitochondria for oxidation. Other downstream targets of AMPK include TSC2, which inhibits mTORC1 and protein synthesis [46]; HMG-CoA reductase, which leads to inhibition of cholesterol synthesis [47]; PGC1α, which stimulates mitochondrial biogenesis [48,49], and many others. See figure 1.3 for a broader list of AMPK substrates and their functions.

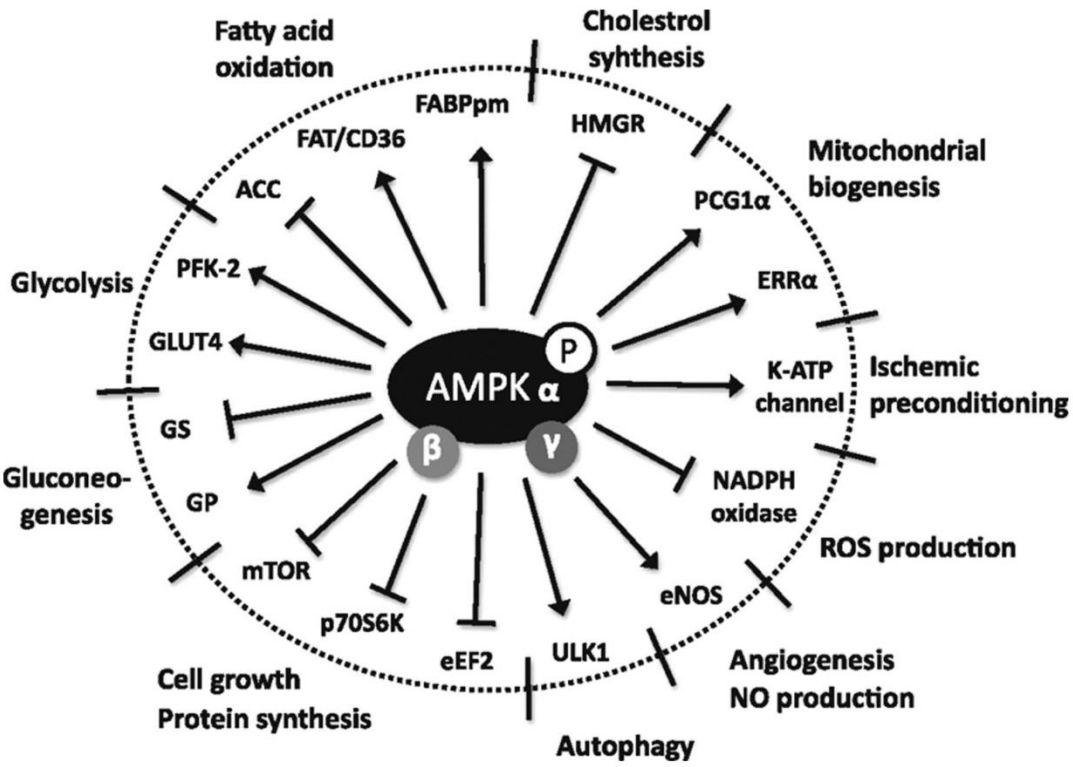


Figure 1.3: Downstream substrates of AMPK and their functions.

AMPK phosphorylates many downstream substrates. As shown, AMPK activation has many effects on cellular functions, such as increasing fatty acid oxidation, mitochondrial biogenesis, autophagy, angiogenesis, and glycolysis, while inhibiting ROS production, cell growth, and protein and cholesterol synthesis. Reproduced from [50]

AMPK activation has effects on a multitude of tissues (see Figure 1.4). In skeletal muscle, its activation stimulates glucose uptake, FA oxidation, GLUT4 translocation, and mitochondrial biogenesis, while inhibiting protein and glycogen synthesis [30]. Similarly, in cardiac muscle, AMPK activation stimulates glucose uptake, FA oxidation, and glycolysis [51]. AMPK stimulates glucose uptake and FA oxidation in liver, while inhibiting gluconeogenesis and cholesterol, FA, and protein synthesis. In adipose tissue, it stimulates FA oxidation and reduces FA synthesis and lipolysis [30]. AMPK inhibits insulin secretion from pancreatic β-cells [30], and it signals to increase food intake in the hypothalamus [52]. Nearly all of the physiological effects of peripheral AMPK activation would be beneficial for a patient with T2D. For this reason, pharmacological activation of AMPK has been a seemingly promising target for drug discovery and development during the past two decades.

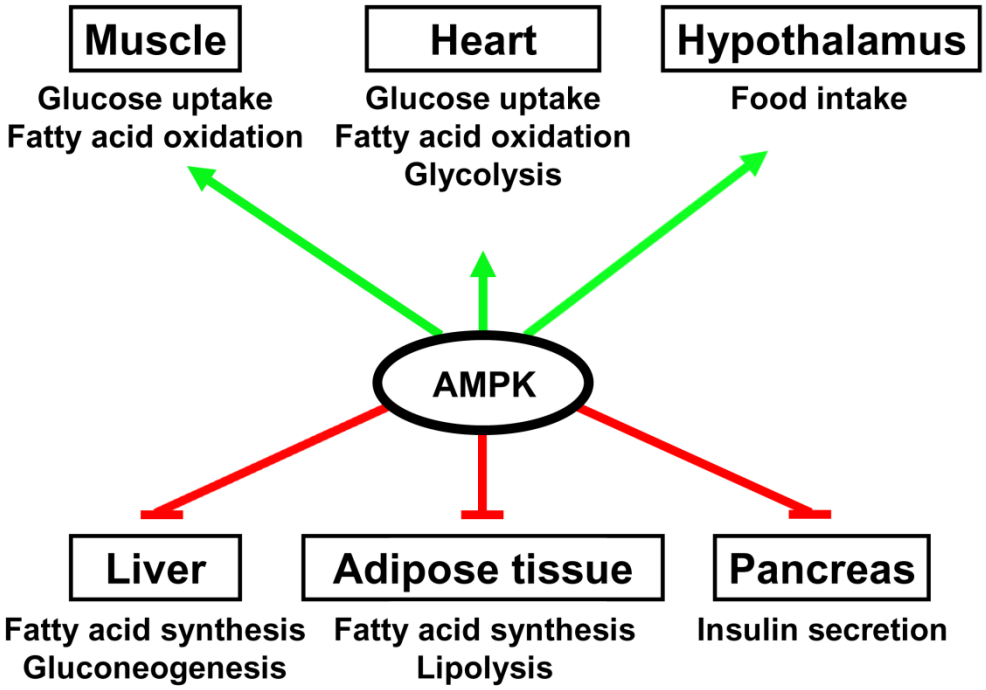


Figure 1.4: Roles of AMP-activated protein kinase (AMPK) in the control of whole-body energy metabolism.

Activation of AMPK (green lines) stimulates energy-generating pathways in several tissues while inhibiting (red lines) energy-consuming pathways. In skeletal muscle and heart, activation of AMPK increases glucose uptake and fatty acid oxidation. In the liver, AMPK activity inhibits fatty acid and cholesterol synthesis. Lipolysis and lipogenesis in adipose tissue are also reduced by AMPK activation. Activation of AMPK in pancreatic -cells is associated with decreased insulin secretion. In the hypothalamus activation of AMPK increases food intake. [53]

AMPK Ser$^{485/491}$

As previously mentioned, recent data suggests that the Ser$^{485/491}$ site on AMPK's α1/α2 subunit may play an important role in modulating AMPK activity. Phosphorylation of this site has been shown to inhibit AMPK in several tissues, though its role in muscle or liver has not been investigated. Ser^{485} on the α1 subunit has been shown to be phosphorylated by Akt [54,55], protein kinase A (PKA) [56,57], or autophosphorylation [56] in various cell types and tissues, such as heart [54], adipocytes [57,58], and vascular smooth muscle cells [55]. Similarly, Ser^{491} of the α2 subunit can be phosphorylated by PKA [56,57], p70S6K [59], or autophosphorylation [60] in tissues such as adipocytes [57], hypothalamus [59], heart [54], and HEK293 cells [60], resulting in reduced AMPK activity. Although previous studies suggested that Ser^{491} is also an Akt phosphorylation site, a recent study by Hawley et al. showed that Akt does not phosphorylate Ser^{491} in a cell-free assay [60]. In heart, it has been shown that insulin and IGF-1 rapidly stimulate phosphorylation of this site through Akt, leading to a decrease in AMPK activity. In the hypothalamus, Dagon et al. [59] showed that p70S6K phosphorylates α2AMPK Ser^{491} to inhibit AMPK and decrease food intake. Some of these changes in serine phosphorylation correlate inversely with that of Thr^{172}; however, in many instances, there seems to be discordance between the two phosphorylation sites.

Whether Ser$^{485/491}$ phosphorylation plays a role in the inhibition of AMPK seen in diabetic models is unknown. Notably, recent studies suggest that phosphorylation of this site may be increased under conditions of excess nutrients. For example, it was shown that when the MIN6 cell line of pancreatic β-cells were switched from low glucose

(3mM) culture medium to high glucose (25mM) for either 1h or overnight, phosphorylation of α1AMPK Ser^{485} was increased [61]. In this experiment, phosphorylation at this site was inversely correlated to phosphorylation of αAMPK Thr^{172}.

AMPK Activators

Numerous physiological, pharmacological, natural, and hormone activators of AMPK are known. Some of these are currently used clinically for the treatment of T2D. The following is a non-comprehensive list of some of the most established and newly identified AMPK activators that may have positive effects in patients with T2D, and their mechanisms of action, if known (Figure 1.5). A more thorough list of AMPK activators can be found in recent reviews by Steinberg et al. [30] and Fogarty et al.[62].

Physiological

Exercise and Calorie Restriction

Exercise and calorie restriction exert beneficial effects on metabolic health and decrease risk for a variety of diseases, including T2D and cardiovascular disease. Both exercise and caloric restriction are metabolic stresses that increase the AMP:ATP ratio in an organism's cells, and thus can activate AMPK. Studies in the past two decades have revealed that AMPK is stimulated by muscle contractions in both rodents [63-67] and humans [68-70] and is a crucial enzyme through which exercise imparts many of its positive effects. ATP turnover in skeletal muscle is elevated over 100-fold during exercise [71], causing a rapid rise in AMP and ADP levels in an intensity-dependent

manner [71]. High-intensity muscle contractions preferentially activate α1-containing heterotrimers; while α2 activity is stimulated by low-intensity exercise and increases progressively with intensity [29].

Although AMPK activation has not been proven to be the mechanism by which exercise exerts its positive metabolic effects, several studies have shown that pharmacological AMPK activation mimics the effects of endurance training (eg, increased FA oxidation, mitochondrial biogenesis) in rodents [72], suggesting that AMPK may mediate the effects of exercise. A recent study showed that AMPK β1/β2 skeletal muscle knockout mice not only have a reduced exercise capacity, but also have reduced contraction-stimulated glucose uptake and skeletal muscle mitochondrial content [73]. Similarly, AMPKα1/α2 skeletal muscle knockout mice have reduced exercise tolerance, maximal force production, and fatigue resistance. However, in contrast to the β1/β2 knockout mice, these mice have reduced oxidative capacity, but not mitochondrial number [74]. These findings suggest that AMPK is, at least in part, responsible for exercise-induced stimulation of glucose uptake and mitochondrial biogenesis.

Pharmacological

AICAR

AICAR (5-aminoimidazole-4-carboxamide riboside) was the first compound identified to activate AMPK [75,76]. It is structurally similar to adenosine and, upon entering cells, is phosphorylated by adenosine kinase to become ZMP [75]. ZMP is an AMP analog that can bind to the CBS domains on AMPK's γ-subunit to cause allosteric activation and allow for increased phosphorylation of Thr^{172}. AICAR treatment has been

shown to prevent and/or reverse some aspects of the metabolic syndrome in animal models such as *ob/ob* mice [77], *fa/fa* rats [78,79], and rats fed a high fat diet [80]. For example, AICAR treatment improves glucose tolerance and whole-body glucose disposal and reduces hepatic glucose output and plasma triglyceride and FFA levels [77-80]. AICAR also induces expression of genes involved in oxidative metabolism and improves running endurance [81]. For these reasons, the World Anti-Doping Code banned its use by athletes in 2011 [82].

Despite these promising effects in animal models, AICAR is unlikely to be used in the treatment of human T2D or metabolic syndrome due to poor bioavailability and a short half-life. Additionally, AICAR can mimic other actions of AMP to have AMPK-independent effects, such as inhibition of the enzyme fructose-1,6-bisphosphatase (FBPase) [83] and stimulation of muscle gylcogen phosphorylase [84]. However, AICAR may be useful in treating humans with acute lymphoblastic leukemia [85,86] and cardiac ischemic injury [87,88]. Interestingly, another recently described AMPK activator, Compound 13, is taken up into cells and converted to the AMP analog Compound 2, which is a much more potent and specific activator of AMPK compared to ZMP [89]. Compounds of this series are being optimized for oral bioavailability and pharmacokinetics; however, their clinical utility remains to be seen.

Biguanides

Metformin, which belongs to the biguanide family of insulin-sensitizing drugs, is currently the first-line oral therapy for T2D, according to national and international guidelines [90-93]. The biguanides also include phenformin, buformin, and the

antimalarial agent proguanil. This class of drugs originates from the French lilac plant, which has been used in folk medicine to treat diabetes for centuries, due to its glucose-lowering properties [94,95]. Although phenformin and buformin are more potent insulin senstitizers than metformin, they also have a higher risk for unwanted side effects, namely lactic acidosis. For this reason, they were withdrawn from the market, leaving metformin as the only biguanide available for treatment of T2D. Within 12 weeks of receiving FDA approval in 1994, metformin became the most frequently prescribed oral anti-diabetic drug in the US [96], and it is currently prescribed to over 100 million patients worldwide [90]. Metformin reduces HbA1c by 1-2% in patients with T2D and reduces mortality compared to diet modifications alone [97]. In addition to its hypoglycemic effects, metformin has very few side effects, is weight neutral, and recent epidemiological studies suggest that patients taking metformin may have lower risks of cardiovascular disease [98] and certain types of cancer [99,100].

Since metformin was discovered before the use of targeted drug discovery techniques, for a long time its mechanism of action was not known, and it still is not fully understood. In 2001, Zhou et al. reported that metformin activates AMPK [101], and many studies since then have attributed metformin's insulin-sensitizing actions to AMPK [102]. Metformin does not activate AMPK directly; instead, it has been shown to inhibit complex I of the mitochondrial respiratory chain [103], which promotes a switch from aerobic to anaerobic glycolysis, thus increasing the AMP:ATP ratio and promoting AMPK activation. This indirect mechanism is supported by the fact that metformin fails to activate AMPK in cell-free assays [104] and in cells expressing AMP-insensitive

(R531G) gamma2 variants [105]. Inhibition of hepatic gluconeogenesis is thought to be the primary means by which metformin exerts its effects on glucose homeostasis. It can also stimulate glucose uptake in adipose and skeletal muscle, although times and concentrations needed to stimulate AMPK and glucose uptake were much larger than would be found *in vivo* [101,106]. A recent study using liver-specific AMPKα1/α2 or LKB1 knockout mice brought into question the dependence of metformin's effects on AMPK, since metformin treatment lowered blood glucose levels in both of these mouse models [107]. An even more recent study reported that metformin exerts its effects on the liver by antagonizing glucagon signaling through cAMP and PKA, independent of AMPK [108]. In contrast, mice with mutations in ACC1/2 that prevent phosphorylation and inactivation by AMPK, when made obese by high-fat feeding, are refractory to the lipid-lowering and insulin-sensitizing effects of metformin, suggesting that inhibitory phosphorylation of ACC by AMPK is essential for metformin-induced improvements in insulin sensitivity [109]. Despite these conflicting results, AMPK is likely an effecter of some of metformin's insulin sensitizing effects, though further studies are needed to distinguish the AMPK-dependent from AMPK-independent effects.

Thiazolidinediones

Thiazolidinediones (TZDs) are a class of insulin-sensitizing drugs, including rosiglitazone, pioglitazone, and troglitazone. Although their primary target is the nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPARγ), they are thought to exert some of their anti-diabetic effects through AMPK activation [110]. TZDs have been shown to rapidly stimulate AMPK and ACC phosphorylation in a variety of tissues,

including skeletal muscle [110,111] and liver [112]. Like metformin, they do so indirectly by inhibiting complex 1 of the mitochondrial respiratory chain to increase the cellular AMP:ATP ratio [105,110,113]. Additionally, TZDs may indirectly activate AMPK through the effects of PPARγ to stimulate adiponectin secretion (described in more detail below) [114,115].

In patients with T2D, TZDs improve insulin sensitivity in muscle, liver, and adipose, improve glycemic control (reduce HbA1c), enhance endothelial function, and reduce inflammation [116]. However, the main drawbacks of TZDs are that they cause weight gain (particularly subcutaneous adiposity), may increase risk of bladder cancer [117], and may worsen congestive heart failure, though they are not associated with increased mortality [116,118].

GLP-1 receptor agonists

Glucagon-like peptide-1 (GLP-1) is an incretin that is secreted from intestinal L-cells following ingestion of food. GLP-1 stimulates insulin secretion in a glucose-dependent manner, decreases pancreatic glucagon secretion, increases β-cell mass and insulin gene expression, stimulates satiety in the brain, and increases peripheral insulin sensitivity [119]. Based on these anti-diabetic actions, GLP-1 mimetics, such as exenatide and liraglutide, have been developed for the treatment of T2D. An alternative strategy that has been undertaken to increase GLP-1 levels is the development of DPP-4 inhibitors, which prevent the inactivation of GLP-1 [120]. Recent studies have shown that these compounds, as well as endogenous GLP-1, can activate the AMPK pathway [121]. For example, exenatide treatment was shown to increase AMPK phosphorylation at

Thr172 in hepatocytes [121] and to decrease body weight, serum FFA and triglyceride levels, and reverse hepatic accumulation of lipids and inflammation in high-fat fed mice, while increasing AMPK mRNA and protein expression [122].

A-769662

The first compound to be identified as a direct activator of AMPK was A-769662. This thienopyridone, identified by Abbott laboratories, activates AMPK in a similar manner to AMP; it causes allosteric activation and prevents dephosphorylation of Thr^{172} [123-125]. Unlike AMP, however, A-769662 binds in a cleft between the kinase domain of the α-subunit and the carbohydrate-binding domain of the β-subunit [126]. It is specific for the β1-isoform and requires βSer108 phosphorylation [125,127]. Treatment of *ob/ob* mice with this compound caused improvements in glucose homeostasis and lipid levels [123]. Despite these benefits on metabolic parameters, A-769662 is unlikely to be used to treat human metabolic syndrome due to its poor oral absorption and its reported AMPK-independent effects in which it can inhibit 26S proteasome activity and arrest cell-cycle progression [128]. However, A-769662 has utility as a research tool to further study the effects of AMPK activation. Recently, another compound referred to as 991, which is a cyclic benzimidazole derivative that binds to the same site as A-769662, was shown to be a much more potent AMPK activator [126]. Studies regarding the efficacy of this compound are in their infancy.

Salicylate

Salicylates are natural substances produced by many plants to defend themselves against infections [129]. The medicinal use of salicylate was first described thousands of

years ago, when it was extracted from willow bark [130], making it one of the oldest medicines used by humans. It is often taken in the form of acetyl salicylate (trade name aspirin) or the diester salsalate, both of which are rapidly converted to salicylate *in vivo* [131,132]. It was recently reported that salicylate, but not aspirin, activates AMPK in HEK-293 cells at concentrations found in plasma of patients treated with high doses of aspirin [133]. Salicylate was determined to bind to the same site as A-769662 on the β1-subunit based on findings that the ability of both compounds to activate AMPK is greatly diminished in complexes where the β2 rather than β1-subunit is expressed [133] and that the effects of both compounds are nearly abolished by an S108A mutation in β1 [133]. Further confirmation of a role for salicylate-induced AMPK activation *in vivo* was found when mice treated with salicylate had lower respiratory exchange ratios (RER) following food withdrawal, indicating a switch to fat oxidation [133]. However, these effects were not seen in β1 knockout mice [133].

These findings suggest that although salicylate is a less potent activator than A-769662, it may have some utility in improving metabolic parameters in patients with T2D. Indeed, two randomized controlled trials showed that oral salsalate treatment decreased plasma glucose levels and insulin C-peptide and increased plasma adiponectin levels in obese young adults [134] and patients with impaired fasting glucose and/or impaired glucose tolerance [135]. Although these findings seem promising for the use of salicylate as an AMPK-mediated anti-diabetic treatment, further research is needed to fully define the role of AMPK in these outcomes. High fat-fed wild type and AMPK β1-knockout mice treated with salicylate for two weeks both showed improved glucose

tolerance and reduced fasting glucose and insulin levels, suggesting that some of salicylate's insulin-sensitizing effects are AMPK-independent [133].

PT1 and C24

PT1 is another small molecule compound that has recently been identified as a direct activator of AMPK [136]. Its mechanism of action is thought to be antagonism of the auto-inhibitory (residues 313-335) domain of the α-subunit [136]. Treatment with PT1 dose-dependently increased AMPK activity and ACC phosphorylation in L6 myotubes and HepG2 cells with no significant changes in the AMP:ATP ratios [136]. PT1 is not effective *in vivo* due to a poor pharmacokinetic profile, but structural optimization led to the discovery of the similar, but orally bioavailable compound C24 [137,138]. C24 was shown to reduce glucose production and decrease triglyceride and cholesterol contents in hepatocytes [137]. Chronic oral treatment with C24 lowered blood glucose and lipid levels and improved glucose tolerance in *db/db* mice [137]. Whether C24 or a similar compound will make it to the clinic remains to be seen.

Natural Compounds

Numerous naturally occurring compounds and phytochemicals have been shown to activate AMPK *in vitro* and *in vivo* and elicit metabolic benefits dependent on AMPK activation.

Resveratrol

Resveratrol is a polyphenol found in red wine that has been suggested to mimic some of the effects of calorie restriction to increase lifespan. Treatment of high-fat fed animals with resveratrol causes improvements in insulin sensitivity and decreases

markers associated with aging [139]. Resveratrol has been shown to stimulate AMPK activity in multiple cell types, including hepatocytes [140-142], muscle cells [143,144], and neurons [145]. The mechanism by which resveratrol activates AMPK is thought to be an increase in AMP levels due to inhibition of the mitochondrial F1 ATPase [105,146]. Resveratrol treatment stimulates glucose uptake [144] and mitochondrial biogenesis [143] in muscle cells and stimulates mitochondrial biogenesis [140] and reduces lipid accumulation in liver [142]. The latter effect is blocked by a dominant negative AMPK, suggesting that it is AMPK-mediated. Further studies are required to determine how much of resveratrol's effects are due to AMPK activation as opposed to activation of Sirtuin 1 (SIRT1), a redox-sensitive deacetylase whose activation has been shown to increase longevity. Of note, however, Ruderman et al. have shown that AMPK and SIRT1 can both regulate each other and share many common target molecules [147].

Rooibos

Rooibos (Aspalathus linearis) is a plant grown in South Africa that is popularly used in tea and has been shown to activate AMPK. Treatment of C2C12 myotubes with rooibos extract increases glucose uptake, mitochondrial activity, GLUT4 expression, and ATP production and reverses palmitate-induced insulin resistance [148]. *In vivo*, rooibos extract was reported to reduce serum cholesterol, triglyceride, and FFA concentrations in mice fed a high-fat diet [149]. Adipocyte size and triglyceride content were also reduced and hepatic steatosis was prevented. These metabolic improvements were attributed to AMPK activation in liver and adipose [149]. Similarly, *ob/ob* mice fed a diet containing 0.1% rooibos extract had improved fasting blood glucose levels and improved glucose

tolerance compared to mice fed a control diet [150]. Furthermore, rooibos treatment decreased expression of gluconeogenic and lipogenic hepatic genes in these animals [150].

Berberine

Berberine is an isoquinoline alkaloid found in certain plants and has traditionally been used in Chinese and Korean cultures to treat fungal and bacterial infections, as well as T2D. Berberine has been shown to improve glucose tolerance [151], reduce body weight [151], increase expression of the insulin receptor (IR) and LDL receptor (LDLR) [152], lower total and LDL cholesterol [152], and reduce triglyceride levels [151,152] in several rodent models. Berberine has also been shown to lower blood glucose, triglyceride, and cholesterol levels to nearly the same degree as metformin [153]. It has been shown to potently activate AMPK in skeletal muscle [154], hepatocytes [155], and adipose tissue [156,157], although some of its anti-diabetic effects are likely mediated through AMPK-independent mechanisms, such as DPP-4 inhibition [158] and enhancement of SOD activity [159]. Like metformin and TZDs, berberine is thought to activate AMPK by inhibiting complex I of the mitochondrial respiratory chain, thus increasing the AMP:ATP ratio [105,160,161]. It has also been shown to increase adiponectin expression, which may contribute to both AMPK-dependent and independent effects [162].

α-lipoic acid

The short-chain fatty acid α-lipoic acid is an essential cofactor for mitochondrial respiration and a powerful antioxidant and has been shown to activate AMPK in skeletal

muscle [7,163], heart [164], and endothelium [165]. It also inhibits AMPK signaling in the hypothalamus [166], thus reducing food intake. It has been shown to improve insulin sensitivity in obese rodents [167] and to reduce insulin secretion and β-cell growth [168]. Furthermore, *ex vivo* incubation of rat skeletal muscle with α-lipoic acid prevents high glucose- or leucine-induced impairments in insulin signaling [7], skeletal muscle lipid accumulation, and hepatic steatosis in obesity [167], Shen et al. showed that the mechanism by which α-lipoic acid activates AMPK is through CaMKKβ-mediated phosphorylation of Thr172 [169]. They reported that the selective inhibitor of CaMKKβ STO-609 prevented α-lipoic acid-stimulated AMPK activation and subsequent ACC phosphorylation. Alpha-lipoic acid has also been reported to have beneficial effects on diabetic neuropathy, although whether AMPK is also involved in mediating these effects is unknown [170,171].

Hormones

In addition to the exogenous pharmacological and natural compounds that can activate AMPK, there also exist endogenous hormones that can activate AMPK and elicit many of the same anti-diabetic effects.

Leptin

Leptin is a hormone made and secreted by adipocytes that acts on the brain to regulate food intake and body weight. It can also act directly and indirectly on peripheral tissues, as almost all tissues express the leptin receptor.[172] Leptin increases the AMP:ATP ratio in skeletal muscle, thus activating AMPK and stimulating fatty acid oxidation [173]. This activation occurs only in α2-containing heterotrimers [174],

although the reason for this isoform specificity is not known. In addition to directly acting on skeletal muscle to activate AMPK, leptin can also indirectly stimulate AMPK in muscle via α-adrenergic signaling from the CNS [175]. This activation is more delayed and requires the melanocortin 4 (MC4) receptor, since intracerebroventricular (ICV) administration of an MC4 receptor antagonist prevents CNS-mediated activation of skeletal muscle AMPK by leptin [175]. In contrast to its effects in skeletal muscle, leptin inhibits AMPK in the hypothalamus to inhibit food intake [52,176]. Yang et al. reported that it does so indirectly via a mechanism involving release of an opioid from a cell different from that in which AMPK is located [177]. However,it was recently reported that AMPK inhibition via phosphorylation of Ser^{491} on its α2-subunit by p70S6 kinase is required to mediate leptin's anorectic effects [59].

Adiponectin

This circulating hormone acts through its two receptors (adipoR1 and adipoR2) expressed in tissues such as adipose and skeletal muscle to regulate glucose levels and stimulate fatty acid oxidation [178] [179]. Adiponectin levels are reduced in obese humans and animals [180]. AMPK activation by adiponectin is dependent on signaling through AdipoR1 [181] and requires the adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1 (APPL1) [182]. Purified adiponectin from human plasma potently activates AMPK activity in C2C12 myotubes [183], and adiponectin's ability to suppress hepatic glucose output has been shown to be AMPK-dependent [184]. Adiponectin over-expression has been shown to reduce body weight, improve insulin sensitivity, and increase FA oxidation in various rodent models of genetic and diet-

induced obesity [185-188]. Interestingly, adiponectin trimers and hexamers, but not high-molecular-weight forms, stimulate food intake through AMPK activation in the hypothalamus [189].

Interleukin-6

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that is elevated in obesity [190]. It is also produced and released from muscle during exercise to increase circulating levels almost 100-fold [191]. Interestingly, in obesity, IL-6 is associated with insulin resistance, whereas during exercise, it may enhance glucose uptake via AMPK activation. IL-6 increases muscle glucose uptake through an AMPK-dependent mechanism in cultured cells [192], rodents [192,193], and humans [194]. These effects of IL-6 are additive to those of insulin in stimulating glucose uptake [195]. However, some studies have shown that these effects are only seen at super-physiological concentrations [195]. AMPK activity is diminished in muscle and adipose tissue of IL-6 knockout mice, and exercise-stimulated AMPK activity is diminished in these mice [190,193]. Kelly et al. showed that IL-6 activates AMPK by increasing the concentration of cAMP and, secondarily, the AMP:ATP ratio [192].

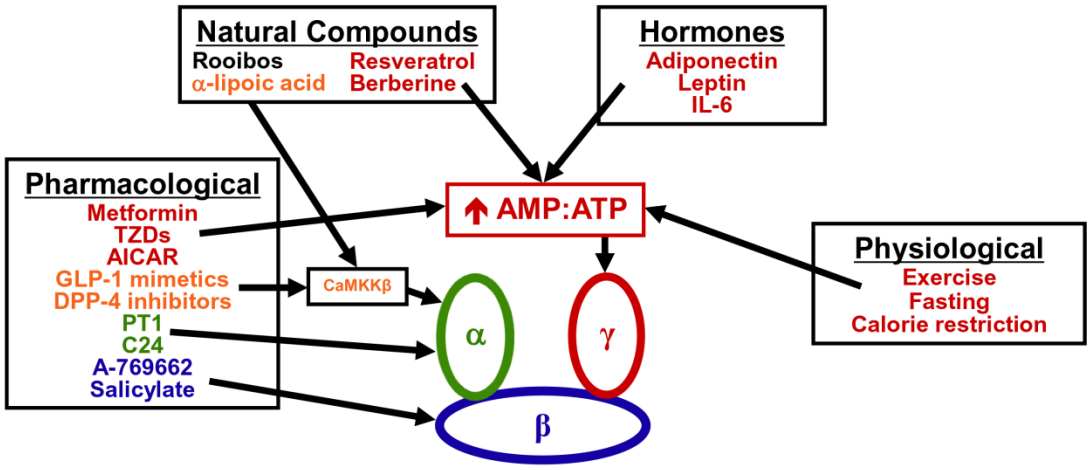


Figure 1.5: Physiological, pharmacological, natural, and hormonal activators of AMPK:

As discussed in the text, there are many known activators of AMPK. This non-comprehensive list highlights many of the well-established and newly discovered AMPK activators that have positive effects on T2D. Many activators activate AMPK via an increased AMP:ATP ratio (shown in red), causing AMP to bind to the γ-subunit. However, a subset of compounds stimulate AMPK activation via other mechanisms, such as binding directly to the α-subunit (shown in green), stimulating phosphorylation of αThr172 through CaMKKβ (orange), or binding directly to the β-subunit (blue). The mechanism by which rooibos activates AMPK is not known. AICAR is phosphorylated to ZMP, an analog of AMP that can activate AMPK via the γ-subunit. [53]

Protein Kinase C

Protein kinase C is a family of lipid-sensitive serine/threonine kinases that are involved in many cellular signaling pathways. The structurally related isoforms are broken into three main categories based on sequence homology and modes of activation. The conventional (cPKC) isoforms (α, βI, βII, and γ) are activated by both DAG and Ca^{2+}; the novel (nPKC) isoforms (δ, ε, θ, and η) are DAG-sensitive but don't require Ca^{2+}; and the atypical (aPKC) isoforms (ζ and ι/λ) are activated by 3-phosphoinositide-dependent kinase-1 (PDK1) or the toxic lipid derivative ceramide [196]. Many studies have reported that increased activation of various conventional and novel PKC isoforms is associated with the IR caused by chronic glucose or lipid oversupply (which causes DAG accumulation). Figure 1.6 shows the structures of the different families of PKC isoforms.

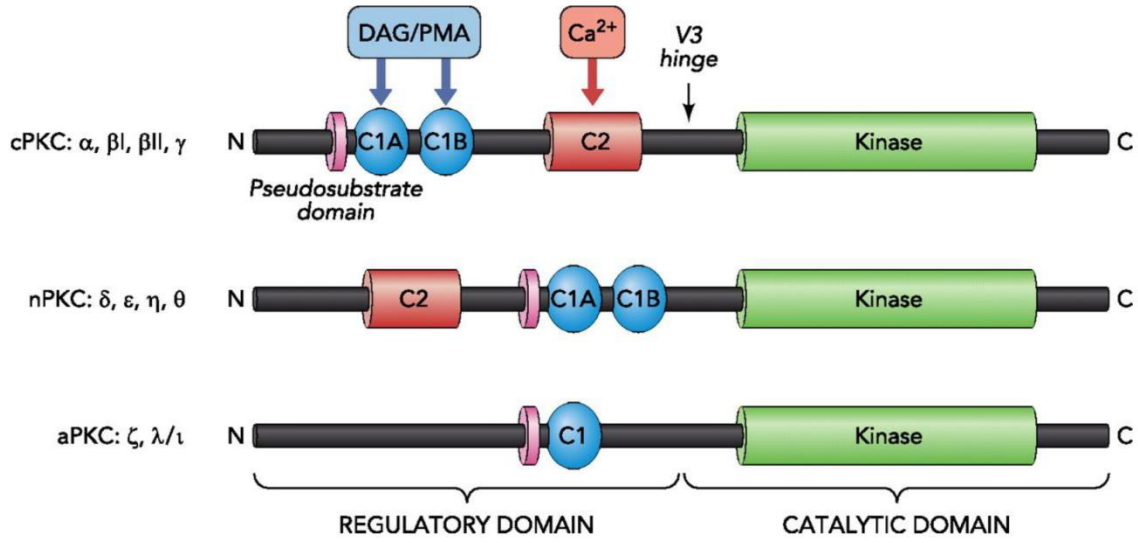


Figure 1.6: Structural domains of conventional, novel, and atypical families of PKC isoforms.

All PKC isoforms consist of an N-terminal regulatory domain and a C-terminal catalytic domain. Conventional and novel isoforms contain C1A/B domains that bind DAG or phorbol esters, such as PMA. The atypical PKCs contain a C1 domain that binds PIP_3 and ceramide, but not DAG or PMA. The conventional and novel isoforms also contain a C2 domain, which binds calcium in the conventional isoforms, but not the novel isoforms since they lack critically positioned calcium-coordinating acidic residues. Reproduced from [197].

Since DAG is located at the plasma membrane or other intracellular membranes, such as the endoplasmic reticulum (ER), PKCs translocate to these membranes during activation. Thus, membrane localization is often used as a measure of PKC activation. Rats fed a high-fat diet have higher proportions of membrane-associated PKCθ, ε, and δ in skeletal muscle, which correlate with increased muscle triglyceride and DAG content and insulin resistance [198]. Obese Zucker rats have increased DAG content, PKC activity, and PKCε and θ membrane localization compared to lean controls [199]. Genetically modified mice lacking certain PKC isoforms have improved glucose homeostasis, supporting a causal role for PKCs in IR [200,201]. For example, PKCβ knockout mice have slightly lower blood glucose and plasma insulin levels and enhanced 2-deoxy-D-glucose (2-DOG) uptake in adipose tissue and muscle [201]. Mice lacking PKCθ are protected from IR following a 5-hour lipid infusion, whereas their wild-type counterparts show reductions in skeletal muscle glucose uptake and diminished IRS-1-associated PI3K activity [200]. In humans, Itani et al. showed that DAG mass and PKCβII and θ membrane association are increased in skeletal muscle following a 6 hour lipid infusion [202]. A reduction in insulin-stimulated glucose disposal was also reported.

In contrast to the conventional and novel PKC isoforms, atypical PKCζ and λ/ι have been shown to play a positive role in insulin-stimulated glucose uptake [203,204]. PDK-1, which is activated by insulin downstream of PI3K and phosphorylates Akt at Thr^{308}, also phosphorylates and activates these PKC isoforms. The atypical PKCs then associate with GLUT4 vesicles and help mediate their translocation to the membrane to increase glucose uptake. To further confirm their importance in mediating glucose

uptake, mice lacking PKCλ in skeletal muscle are insulin resistant and dyslipidemic [205]. Atypical PKC activity is diminished in muscle and adipose tissue of humans with obesity and T2D [206,207]. However, activity of these isoforms is conserved in livers of humans with T2D, where it may have deleterious effects by increasing hepatic lipid and cytokine production [208]. Thus, inhibition of atypical PKC isoforms may represent a potential treatment strategy for IR or T2D if targeted selectively to the liver; whereas activation of atypical PKCs seems to improve insulin sensitivity in skeletal muscle and adipose tissue.

Despite the multitude of studies linking increased conventional and novel PKC activation with IR, the mechanistic relationship is incompletely understood. What is well known is that PKCs can inhibit IRS-1 through phosphorylation on serine residues. This prevents normal tyrosine phosphorylation of IRS-1 in response to insulin and subsequent downstream insulin signaling (See Figure 1.7) [209]. Other proposed mechanisms by which aberrant PKC activation could cause IR include the internalization and degradation of the insulin receptor, which is associated with prolonged PKC activation in NIH 3T3 fibroblasts [210]. Altered PKC expression is also associated with diminished IRS-1 expression in MCF7 breast cancer cells [211], muscle of obese humans [212] and adipose tissue of fat-fed rats [213]. Additional mechanisms could involve inhibition of the insulin receptor, IRS-1 or Akt through downstream effectors of PKCs, such as the mitogen-activated protein kinases (MAPK) or c-Jun N-terminal kinase (JNK).

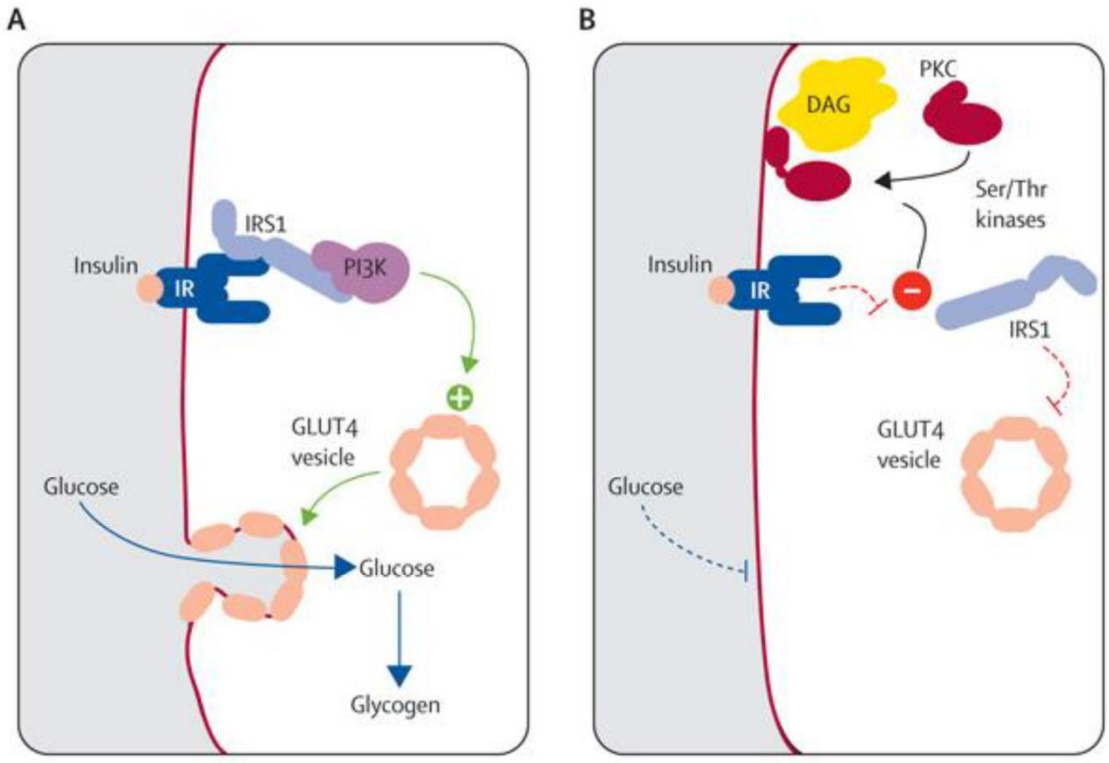


Figure 1.7: Normal and impaired insulin signaling pathways in skeletal muscle.

During normal insulin signaling (A), insulin binds to the insulin receptor, which subsequently activates IRS1 and PI3K. Downstream signaling causes translocation of GLUT4 vesicles to the plasma membrane, which allows for the transport of glucose into the cell. One mechanism by which insulin signaling can be impaired (B) is through the accumulation of DAG, which leads to PKC activation and inhibitory phosphorylation of IRS1 on serine residues. In this case, GLUT4 vesicles do not translocate to the plasma membrane and glucose is not transported into the cell. Adapted from [214].

Ido et al. [215] showed that incubation of human umbilical vein endothelial cells (HUVECs) with the AMPK activator AICAR prevented both high glucose induced DAG accumulation and impairment of insulin-stimulated Akt phosphorylation. This suggests that activation of AMPK may prevent aberrant PKC activation. Whether a reciprocal relationship exists whereby PKC activation directly or indirectly contributes to AMPK inhibition in response to excess nutrients is not known. Interestingly, Tsuchiya et al. recently showed that the PKC activator phorbol 12-myristate 13-acetate (PMA), which is a DAG mimetic, diminishes AMPK α2 activity in cardiac myocytes [216]. The mechanism behind this is not yet known.

Protein Kinase D

Overview and Structure

Protein kinase D (PKD) was first discovered in 1994. It was initially name PKCμ and classified as an atypical PKC [217]; however, it was later determined to be structurally and functionally distinct from the other PKC isoforms and was reclassified as a member of the Ca^{2+}/calmodulin-dependent kinase (CaMK) family of serine/threonine kinases based on sequence and substrate homology [218]. Since then, two other PKD isoforms (PKD2 and PKD3) have been discovered, with the original one now referred to as PKD1. PKD1 is a 918 amino acid protein that is made up of an N-terminal regulatory domain, which consists of cysteine-rich, zinc-finger like motifs and an autoinhibitory pleckstrin-homology domain, and a C-terminal catalytic domain. DAG can bind to the cysteine-rich domains, which activates PKD *in vitro*. When activated, PKD1 undergoes transphosphorylation by novel PKCs at $Ser^{744/748}$ of the activation loop and

autophosphorylation of Ser^{916} at the C terminus (See Figure 1.8). Thus, PKD can be activated either downstream of, or in parallel with PKC. In addition to DAG and PKC, PKD can be activated by other stimuli, such as G protein–coupled receptor agonists (eg, bombesin, vasopressin, bradykinin and endothelin-1 (ET1)) and oxidative stress [218].

Figure 1.8: Structure of PKD1.

PKD1 contains two cysteine-rich domains (C1a and C1b), which bind DAG or PMA, a pleckstrin homology domain, and a kinase domain. During activation, PKD1 is phosphorylated on Ser$^{744/748}$ of the activation loop by novel PKCs and is autophosphorylation at Ser^{916}. Reproduced from [219]

Functions

PKDs are involved in a wide variety of cellular functions, including signal transduction, membrane trafficking, and cell survival, migration, differentiation, and proliferation (See figures 1.9 and 1.10) [220]. PKD is activated by oxidative stress (in which phospholipase D1 (PLD1) and phosphatidic acid phosphatase (PAP)-catalyse DAG synthesis) and promotes cell survival. It does so by activating a cytoplasmic inhibitor of nuclear factor kappa-B kinase (IKK)α/IKKβ/NF-κB essential modulator (NEMO) complex, which causes Iκb degradation and NF-κB nuclear translocation. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) then upregulates expression of the mitochondrial antioxidant MnSOD, which removes toxic reactive oxygen species (ROS).

PKD1 can also regulate transcription by acting as a class IIa histone deacetylase (HDAC) kinase. Phosphorylation of HDAC5 causes it to dissociate from the transcription factor myocyte enhancer factor 2 (MEF2), thus allowing MEF2 to upregulate many muscle-specific and metabolic genes. This is particularly relevant in cardiac muscle, since PKD1 has been shown to play a role in cardiac hypertrophy [218,221]. PKD1 expression and activation are increased in humans and animals with heart failure [218,222]. Furthermore, mice with cardiac-specific deletion of PKD1 are protected from cardiac hypertrophy and fibrosis in response to pressure overload (thoracic aortic constriction) and chronic adrenergic and angiotensin II stimulation [223]. Conversely, transgenic mice expressing a constitutively active PKD1 mutant in heart have cardiac hypertrophy, followed by ventricular chamber dilation, wall thinning, and a marked

deterioration of contractile function [224]. These data suggest that PKD1 activation is sufficient to cause pathological cardiac remodeling.

In skeletal muscle, PKD1 activation has been shown to be involved in adaptations to acute exercise through HDAC5 phosphorylation [225]. Interestingly, AMPK also phosphorylates HDAC5 in response to exercise, again leading to derepression of MEF2 [226]. Thus, both of these proteins promote the formation of slow-twitch, oxidative (type I) muscle fibers, which increases the capacity for aerobic exercise. It was recently shown that genetic deletion of PKD1 in type I muscle fibers causes susceptibility to fatigue [225]. However, AMPK activation also upregulates slow-twitch-fiber contractile genes such as peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α), which stimulates mitochondrial biogenesis and oxidative metabolism [227]; PKD1 overexpression does not induce these changes [225].

More recently, McGee et al. [228] found that expression of constitutively active PKD1 in C2C12 myoblasts resulted in increased glycolytic capacity and a modest increase in glucose oxidation. However, they found no effects on basal oxygen consumption rate, extracellular acidification rate, mitochondrial function, or palmitate oxidation. Interestingly, PKD1 has been shown to play a positive role in GLUT4 translocation and contraction-stimulated glucose uptake in cardiac myocytes [229]. In a related study, Steinbusch et al. [230] showed that overexpression of either AMPK or PKD1 could prevent loss of insulin-stimulated glucose uptake due to high palmitate or insulin exposure in cardiac myocytes. However, overexpression of AMPK also restored insulin-signaling through Akt, while PKD1 overexpression did not. These data suggest

that some, but not all, functions of AMK and PKD1 may be redundant. In this case, there may be a need for the two proteins to have mechanisms by which they can negatively regulate eachother to keep their overlapping effects in check.

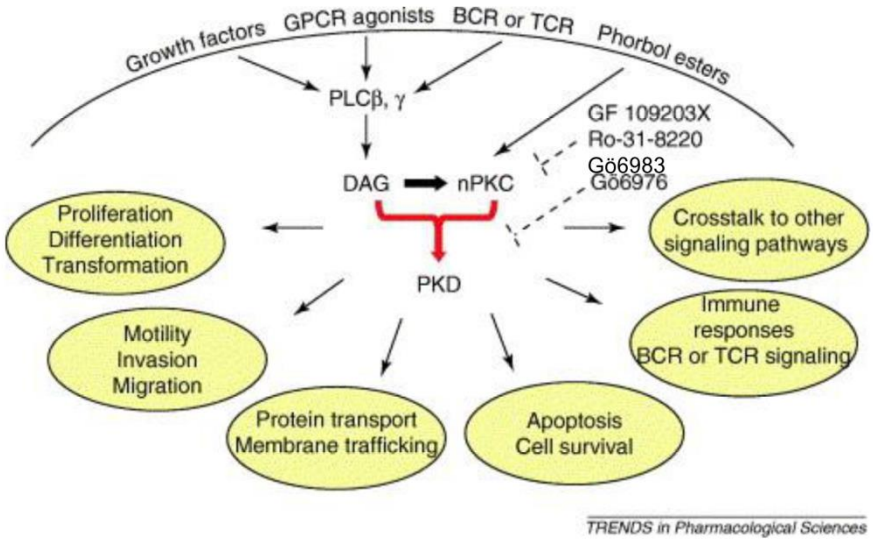


Figure 1.9: Functions of PKD1

PKD1 can be activated by a number of of signaling molecules which lead the the accumulation of DAG and/or activation of novel PKC isoforms. As shown, PKD1 activation leads to many downstream responses including proliferation, differentiation, transformation, motility, invasion, migration, protein transport, membrane trafficking, apoptosis, cell survival, immune responses, and crosstalk to other signaling pathways. Adapted from [231].

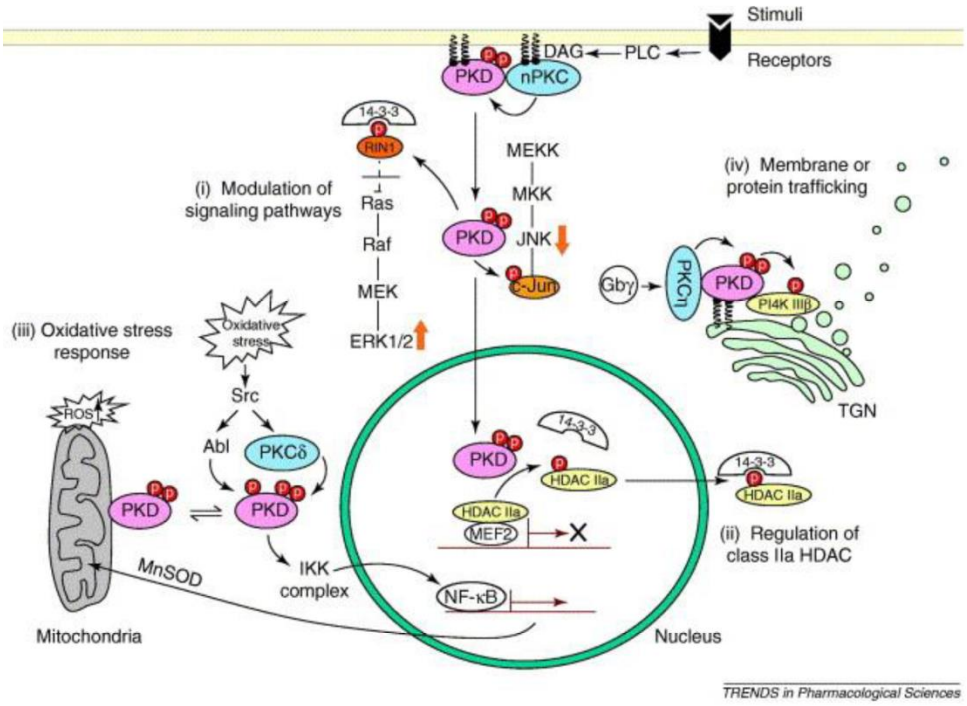


Figure 1.10: PKD1 Signaling Pathways

PKD1 is involved in many signaling pathways in the cell. The diagram shows the involvement of PKD1 signlaing in (i) modulation of signaling pathways, (ii) regulation of class IIa HDAC, (iii) oxidative stress response, and (iv) membrane or protein trafficking. Reproduced from [231]

Overall Aims

The **overall aims** of this work are:

(1) To determine the changes in AMPK activity and phosphorylation over time in skeletal muscle exposed to excess glucose (*ex vivo* incubation with glucose and *in vivo* glucose infusion) and factors that may contribute to its inhibition.

(2) To investigate the upstream kinases responsible for phosphorylating AMPK at $Ser^{485/491}$ phosphorylation and their contribution to diminishing AMPK activity and insulin sensitivity.

(A) To determine whether insulin signaling phosphorylates and inhibits AMPK activity in skeletal muscle.

(B) To determine whether protein kinase C (PKC) signaling phosphorylates and inhibits AMPK activity in skeletal muscle.

(3) To determine the regulation of AMPK $Ser^{485/491}$ phosphorylation in physiological (fasting/refeeding) and pathological (*db/db* mice) models of nutrient excess

CHAPTER TWO: Materials and Methods

Materials

C2C12 cells were purchased from ATCC (Manassas, VA). DMEM, Penicillin-Streptomycin (P/S), fetal bovine serum (FBS) and horse serum (HS) were from Invitrogen (Grand Island, NY). D-(+)-Glucose solution, 45%, and insulin were obtained from Sigma-Aldrich (St. Louis, MO). Primary antibodies for Acetyl-CoA carboxylase (ACC), AMPK, phospho-AMPKα (Thr^{172}), phospho-AMPKα1/α2 ($Ser^{485/491}$), phospho-PKD (Ser^{916}), PKD, phospho-PKC (pan) (βII Ser^{660}), phospho-PKCδ/θ ($Ser^{643/676}$) , phospho-(Ser) PKC Substrate, phosphor-(Ser/Thr) PKD substrate, phospho-mTOR (Ser^{2448}), mTOR, phospho-p70S6K (Thr^{389}), phospho-Akt (Ser^{473}), and Akt antibodies, as well as secondary horseradish peroxidase (HRP)-linked antibodies were purchased from Cell Signaling Technology (Danvers, MA). Phospho-ACC (Ser^{79}) antibody was from Upstate/Millipore (Temecula, CA). Anti-β-actin was purchased from Sigma-Aldrich (St. Louis, MO). AMPKα1 and α2 antibodies used for immunoprecipitation were purchased from Santa Cruz Biotechnology, Inc. SAMS peptide was purchased from Abcam (Cambridge, MA) and [γ-32P] ATP was from Perkin-Elmer (Boston, MA). See Table 2.1 for comprehensive list of antibodies used.

Antibody	Dilution	Source	Predicted Molecular Weight	Incubation	Supplier	Catalog number
Phospho-AMPKα1 (Ser485)/AMPKα2 (Ser491)	1:1000	Rabbit	62 kDa	4°C O/N	Cell Signaling	4185
Phospho-AMPKα (Thr172)	1:1000	Rabbit	62 kDa	4°C O/N	Cell Signaling	2531
AMPKα	1:1000	Rabbit	62 kDa	4°C O/N	Cell Signaling	2532
AMPKα1 (for IP)	1:100	goat	62 kDa	4°C O/N	Santa Cruz	sc-19128
AMPKα2 1 (for IP)	1:100	goat	62 kDa	4°C O/N	Santa Cruz	sc-19131
phospho-ACC (Ser79)	1:1000	Rabbit	280 kDa	4°C O/N	EMD Millipore	07-303
ACC	1:1000	Rabbit	280 kDa	4°C O/N	Cell Signaling	9272
Phospho-Akt (Ser473)	1:1000	Rabbit	60 kDa	4°C O/N	Cell Signaling	9271
Akt	1:1000	Rabbit	60 kDa	4°C O/N	Cell Signaling	9272
Phospho-PKD/PKCμ (Ser916)	1:1000	Rabbit	115 kDa	4°C O/N	Cell Signaling	2051
Phospho-PKD/PKCμ (Ser744/748)	1:1000	Rabbit	115 kDa	4°C O/N	Cell Signaling	2054
PKD/PKCμ	1:1000	Rabbit	115 kDa	4°C O/N	Cell Signaling	2052
Phospho-(Ser/Thr) PKD Substrate	1:1000	Rabbit	Many	4°C O/N	Cell Signaling	4381
Phospho-PKC (pan) (βII Ser660)	1:1000	Rabbit	78, 80, 82, 85 kDa	4°C O/N	Cell Signaling	9371
Phospho-(Ser) PKC Substrate	1:1000	Rabbit	Many	4°C O/N	Cell Signaling	2261
β-actin	1:10,000	Mouse	42 kDa	4°C O/N	Sigma Aldrich	A5441
Phospho-mTOR (Ser2448)	1:1000	Rabbit	289 kDa	4°C O/N	Cell Signaling	2971
mTOR	1:1000	Rabbit	289 kDa	4°C O/N	Cell Signaling	2972
GAPDH	1:1000	Rabbit	37 kDa	4°C O/N	Santa Cruz	Sc-25778
Phospho-PKCδ/θ (Ser643/676)	1:1000	Rabbit	78 kDa	4°C O/N	Cell Signaling	9376
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	1:1000	Rabbit	44, 42 kDa	4°C O/N	Cell Signaling	9101

Phospho-SAPK/JNK (Thr183/Tyr185)	1:1000	Rabbit	54, 46 kDa	4°C O/N	Cell Signaling	9251
Phospho-IRS-1 (Ser636/639)	1:1000	Rabbit	180 kDa	4°C O/N	Cell Signaling	2388
SIRT1 (SIR2)	1:1000	Rabbit	110 kDa	4°C O/N	Millipore	07-131
CaM kinase kinase	1:1000	Mouse	68 kDa	4°C O/N	BD Transductio n	610544

Table 2.1: List of Antibodies used.

Table 2.1 lists the primary antibodies used in these studies, as well as the dilutions, sources, predicted molecular weights, incubation conditions, suppliers, and catalog numbers.

Cell culture

C2C12 myoblasts were cultured in normal glucose (5.5 mM) DMEM supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin/streptomycin (P/S). Media was replaced every 24-48 h and cells were passaged upon reaching 80-90% confluence. At 80-90% confluence they were differentiated into myotubes in DMEM supplemented with 2% horse serum (HS) and 1% P/S. Glucose and FBS-free DMEM, supplemented with 1% P/S and glucose at a final concentration of 5.5 mM, was used for all experimental incubations.

Cell lysate preparation

Cells were washed once on ice with Dulbecco's PBS, lysed in buffer containing 20 mM Tris-HCl - pH 7.5, 150mM NaCl, 1mM Na2EDTA, 1% triton, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na_3VO_4, 1µg/ml leupeptin, 1x phosphatase inhibitor cocktail 3 (Sigma), and 1x protease inhibitor cocktail containing 0.5 mM EDTA (Thermo Fisher Scientici), and removed from wells using a cell scraper with a polyethylene copolymer blade (Fisher Scientific). Cell debris was removed by centrifugation at 13,200 *g* for 10 minutes at 4°C, and the supernatant was removed and stored at –80°C until analysis. Protein concentration was assessed by the bicinchoninic acid method (BCA; Pierce Biotechnology, Inc., Rockford, IL).

SDS-PAGE Western blot analysis

Protein expression and phosphorylation were determined in 10-30µg of protein lysate using SDS-PAGE gel electrophoresis and immunoblotting. Following transfer onto a polyvinylidene difluoride membrane, membranes were blocked in Tris-buffered saline

(pH 7.5) containing 0.05% Tween-20 (v/v;TBST) and 5% non-fat dry milk (w/v) for 1h at room temperature, followed by incubation in primary antibodies (dilution as indicated in table 2.1) at 4˚C overnight. After washing, membranes were incubated in a secondary antibody conjugated to horseradish peroxidase at a 1:5,000 dilution for 1h at room temperature. Bands were visualized using enhanced chemiluminescence solution (ECL; Pierce Biotechnology, Inc., Rockford, IL), and densitometry was performed with Scion Image software.

AMPK activity assay

AMPK activity was assessed as previously described [7,67]. Briefly, AMPK α1 or α2 was immunoprecipitated from 500 μg of protein from cell lysates by incubation at 4˚C overnight on a roller mixer using AMPK α1 or α2-specific antibodies (1:80) and protein A/G agarose beads (1:10; Santa Cruz Biotechnology, Inc). Following several washes, activity was measured in the presence of 200 μM AMP and 80 μM [γ-^{32}P] ATP (2 μCi) using 200 μM SAMS peptide (Abcam) as a substrate. Label incorporation into the SAMS peptide was quantified using a LabLogic (Brandon, FL) scintillation counter.

Cell-free in vitro phosphorylation assay

Recombinant proteins were purchased from EMD Millipore (Billerica, MA) (AMPK) and Enzo Life Sciences (Farmington, NY) (PKD1 and Akt). Recombinant α2AMPK/β1/γ1 complex was incubated with recombinant Akt or PKD1 in 50 mM Na-HEPES, 5 mM MgCl2, 500 μM ATP, 1 mM DTT for 30 min at 30°C.

Animal studies

Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of Boston University Medical Center and were in accordance with National Institutes of Health guidelines. Animals were maintained on a 12:12-h light-dark cycle in a temperature-controlled (19–21°C) room and were fed standard chow and water *ad libitum*, unless otherwise noted.

Experimental animals

Male Sprague-Dawley rats weighing 55–65 g were purchased from Charles River Breeding Laboratories (Wilmington, MA). They were maintained on a 12:12-h light-dark cycle in a temperature-controlled (19-21°C) room and were fed Teklad Global 18% Protein Rodent Diet (Harlan, Madison, WI) and water *ad libitum*. Muscles were removed from rats anesthetized with pentobarbital (6mg/100g BW). For glucose infusion studies, adult male Wistar rats (Animal Resources Centre, Perth, Australia) were communally housed in temperature controlled (22 ± 0.5°C) room on a 12:12-h light–dark cycle. Rats were fed *ad libitum* a standard chow diet (Rat Maintenance Diet; Gordon Specialty Feeds, Sydney, Australia). After a 1 week acclimatization period, cannulae were inserted into both jugular veins.

Muscle incubations

Male Sprague-Dawley rats weighing 55–65 g were purchased from Charles River Breeding Laboratories (Wilmington, MA). Following an overnight fast, they were anesthetized with pentobarbital (60 mg / kg body weight), and extensor digitorum longus (EDL) muscles were removed for incubation. After removal, muscles were first equilibrated for 20 min at 37°C in oxygenated Krebs-Henseleit solution (95% O2/5%

CO2) containing 5.5 mM glucose, then were transferred to 25mM glucose (or other treatment condition, as indicated). Following incubations muscles were snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

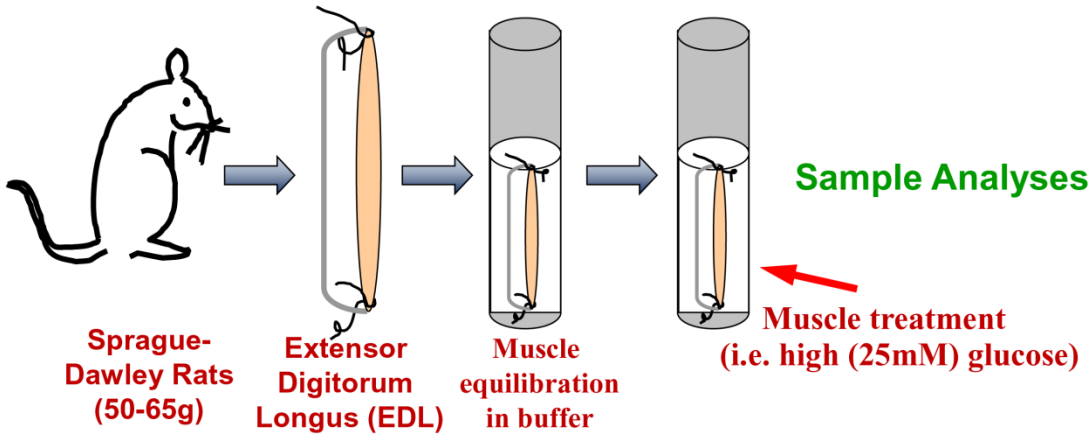


Figure 2.1: Schematic of EDL incubation procedure.

Extensor digitorum longus (EDL) muscles are extracted from anesthetized male Sprague-Dawley rats (50-65g) and tied onto metal clips to keep the muscles extended. Muscles are equilibrated for 20 min at 37°C in oxygenated Krebs-Henseleit solution (95% O2/5% CO2) containing 5.5 mM glucose, and then transferred to another tube containing 25mM glucose (or other treatment, as indicated). Following incubations muscles were snap-frozen in liquid nitrogen, and stored at -80°C until analysis.

Glucose Infusion

Glucose infusion was carried out as described previously [11]. Briefly, seven days after cannulation surgery, rats were randomly divided into treatment groups. After a basal blood sample (600μl) was taken, a 50% (w/v) glucose solution was infused for 0, 3, 5 or 8h using a peristaltic roller pump (101U/R; Watson-Marlow, Falmouth, UK). Blood samples were taken every 30min and the glucose infusion rate was altered to maintain a blood glucose concentration of 11mM (~16-17mM plasma glucose). Following infusion, red gastrocnemius was collected, frozen, and stored at -80°C until homogenization.

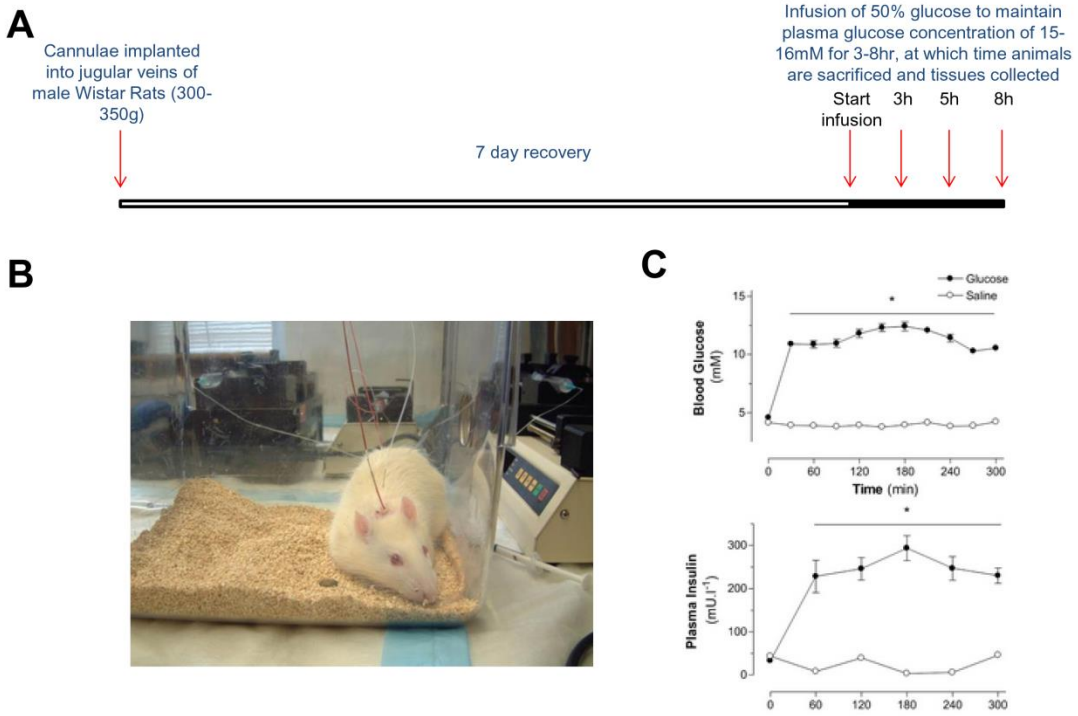


Figure 2.2: Schematic illustrating glucose infusion procedure.

Timing of experimental procedures is shown in (A), starting with cannulation of jugular veins, followed by a 7 day recovery, and the infusion of glucose for 3-8h. Example image showing cannulated rat during glucose infusion (B). Image reproduced from (http://www.tasmc.org.il/sites/en/Research/Tech-Transfer/Pages/Basic-Research-Loboratory.aspx). Representative measurements of blood glucose and plasma insulin levels over time during glucose infusion (C) adapted from [232].

Fast/Re-feed studies

C57BL/6 male mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Food was removed at approximately 4PM, and mice were starved overnight for 18 h. The next morning, half were sacrificed at 10AM by isoflourane and cervical dislocation, while the other group was re-fed for 2h with standard chow before being sacrificed. Muscle and liver were removed, frozen in liquid nitrogen, and stored at -80°C until analysis.

db/db mice

Male *db/db* and *db/+* control mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). Bodyweights and fasting blood glucose were measured weekly beginning at 9 weeks of age. Mice were sacrificed after 4 consecutive weeks of elevated blood glucose in the *db/db* mice compared to *db/+* controls. Mice were sacrificed using isoflourane and cervical dislocation. Muscle and liver were removed, frozen in liquid nitrogen, and stored at -80°C until analysis.

Other analyses

Protein concentrations were determined with the bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL) using bovine serum albumin as the standard. ATP, AMP, ADP, and phosphocreatine were measured spectrophotometrically as described previously [7,233]. Lactate and pyruvate were determined spectrophotometrically using lactate dehydrogenase and NAD [7]. NAD and NADH were measured as described by Fulco et al [234]. Muscle glycogen content was determined using the phenol-sulfuric acid reaction [235].

Statistical analysis

Results are reported as means ± standard error of the mean (SEM). All data were interval/ratio and normally distributed, which allowed for the use of parametric analyses. Statistical significance was determined by a two-tailed unpaired Student's t-tests when comparing two experimental groups or conditions. For comparisons of three or more experimental groups, a one-way ANOVA was performed to determine whether there was a statistical difference in the means of the groups. If $p<0.05$ for the ANOVA, Tukey's post-hoc test was used to determine whether there were differences between individual treatment groups. For repeated measures, such as the measurements of mouse body weight for *db/+* and *db/db* mice over time, a two-way repeated measures ANOVA was used to determine whether the independent variables (genotype and time) affect the dependent variable (body weight), and whether there was an interaction effect of the independent variables. If $p<0.05$ by two-way repeated measures ANOVA, a Bonferroni post-test was performed to determine whether body weight differed between genotypes at each timepoint. A level of $p<0.05$ was considered statistically significant. N values are indicated for individual experiments. Graphpad Prism 5 was used for all statistical analyses.

CHAPTER THREE: Mechanisms of AMPK inhibition in response to high glucose

Introduction

It has long been appreciated that nutrient excess leads to insulin resistance in many tissues [7,215,233,236-238]. In a recent study [7], our lab compared the events associated with insulin resistance in rat extensor digitorum longus (EDL) muscles incubated with a high concentration of glucose (25 vs. 5.5mM) or a normal glucose concentration (5.5mM) with added leucine (100 or 200μM) for 1h. The results strongly suggested that elevated concentrations of glucose or leucine cause insulin resistance by a common mechanism. Thus, both a high concentration of glucose and glucose plus leucine diminished AMPK activity and phosphorylation at Thr^{172} and increased mTOR/p70S6K phosphorylation. Treatment with the mTOR inhibitor rapamycin did not affect AMPK phosphorylation; however, incubation with two distinct AMPK activators, AICAR and alpha lipoic acid, prevented both the insulin resistance (impaired insulin-stimulated Akt phosphorylation) and mTOR/p70S6K phosphorylation. These results suggest that the decrease in AMPK activity is responsible, at least in part, for the glucose- and leucine-induced increases in mTOR activation and insulin resistance. In contrast, the factors responsible for the decrease in AMPK activity and whether AMPK is inhibited further with more prolonged exposure to these nutrients have not been determined.

To examine these questions, we evaluated whether several factors that have been shown to decrease AMPK activity in other settings are altered in the aforementioned EDL model in response to excess glucose for 30min-2h. Additionally, we assessed whether the same factors were changed *in vivo* in rats in which AMPK activity was

diminished by a 3-8h glucose infusion that produced hyperglycemia, hyperinsulinemia, and insulin resistance. One factor examined was phosphorylation of $Ser^{485/491}$ on AMPK's α-subunit, an event that has been linked to the acute inhibition of AMPK by insulin within minutes in various tissues [54,58,239] and to the inhibition of hypothalamic AMPK by leptin [59]. Another was the upregulation of protein phosphatase 2A (PP2A), which has been shown to mediate the deactivation of AMPK in rodent aorta following the infusion of palmitate [240]. We also measured muscle glycogen content, since glycogen has been shown to inhibit AMPK in cell-free conditions by binding to the glycogen-binding domain (GBD) of its β-subunit [241]. Finally, we related diminished AMPK activity in muscle to decreases in the activity of SIRT1 and factors that regulate it. As shown by a number of groups [141,147,242,243], the activation and downregulation of SIRT1, a histone-protein deacetylase, typically parallels that of AMPK.

Intriguingly, the results revealed that all of these putative regulatory factors were altered by hyperglycemia in the incubated EDL and in muscle of the glucose-infused rats. However, the timing of the changes varied with the model, such that the initial decrease in AMPK activity generally preceded the changes in its putative regulators in the incubated muscle but not in muscle of the glucose-infused rat. Increased glycogen content was the only change temporally associated with the initial decrease in AMPK activity in the muscles incubated with high glucose or leucine, suggesting that increased cellular energy in the form of glycogen may be the initiating factor leading to AMPK inhibition by excess nutrients.

Results

Incubation of EDL with 25mM glucose decreased AMPK Thr^{172} and ACC Ser^{79} phosphorylation and AMPK activity over time (30min-2h)

Time-course studies revealed that incubation of the EDL with 25 vs. 5.5mM glucose decreased the phosphorylation of AMPK at Thr^{172} by 40% at 30min, 50% at 60min, and 60% after 2h (Figure 3.1A). An almost identical pattern was observed when the activity of α2 AMPK, which is the dominant isoform in skeletal muscle was measured using the SAMS peptide assay (Figure 3.1D). In contrast, activity of α1 AMPK was unchanged, indicating that the effect was isoform specific. Finally, the decrease in p-ACC Ser^{79}, which is only phosphorylated by AMPK (Figure 3.1B), paralleled that of α2 AMPK activity. The subsequent studies examined changes in factors that may mediate this downregulation of AMPK.

Incubation of EDL with high glucose for 1 or 2h increased phosphorylation of AMPK Ser$^{485/491}$

Incubation of the EDL with a high glucose (25 vs. 5.5mM glucose) medium increased the inhibitory phosphorylation on AMPK at Ser485/491 by 250% and 200% at 1 and 2h, respectively (Figure 3.1C). As already noted, no insulin was added to the medium.

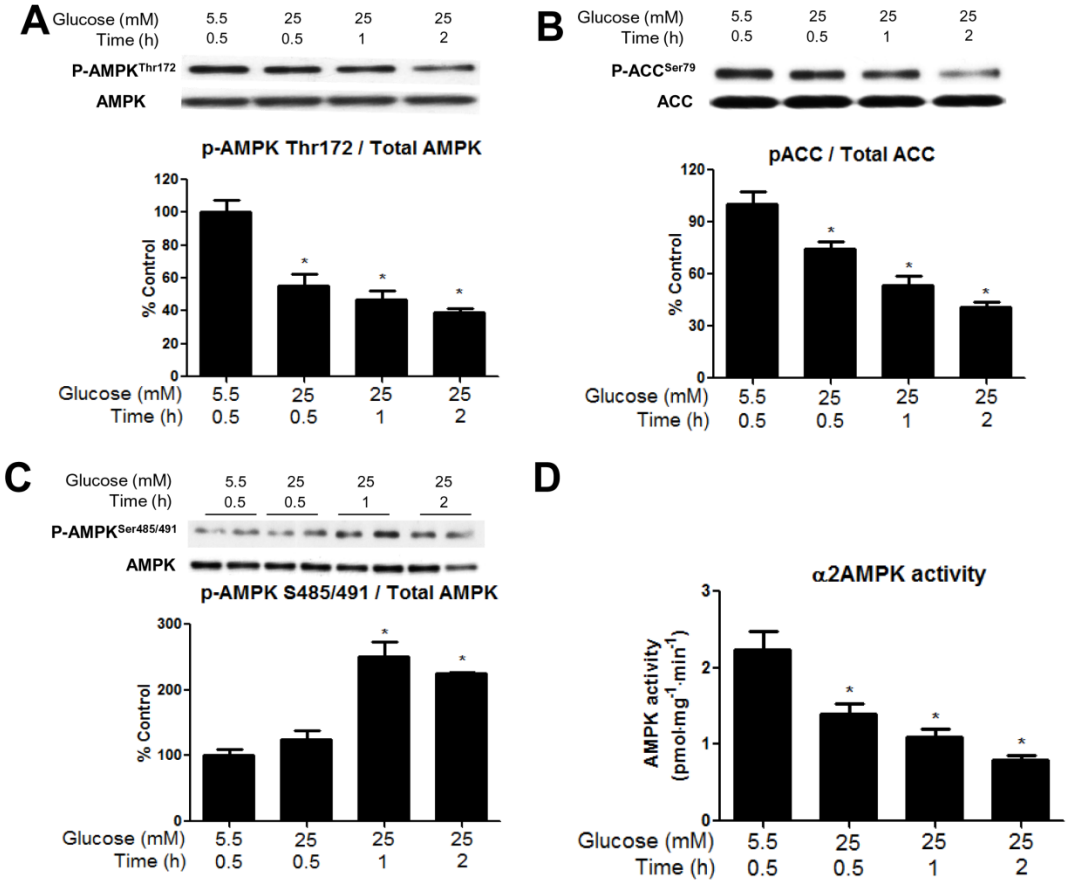


Figure 3.1: Incubation of EDL with 25mM glucose diminishes AMPK Thr^{172} phosphorylation, ACC Ser^{79} phosphorylation, and α2 AMPK activity, and increases AMPK Ser$^{485/491}$ phosphorylation

EDL muscles were incubated in Krebs-Henseleit solution containing 25 mM glucose for 0.5, 1, and 2h. Phosphorylation of AMPK Thr^{172} (A), ACC Ser^{79} (B), AMPK Ser$^{485/491}$ (C) were measured by western blot and AMPK activity (D) was determined using the SAMS peptide assay. Results are means + SE (n = 6). *P < 0.05 relative to 0.5h incubation with 5.5 mM glucose. [244]

SIRT1 and factors that activate it were diminished in EDL following 2h of incubation with 25mM glucose

SIRT1 protein expression (Figure 3.2A) and the ratio of nicotinamide adenine dinucleotide oxidized/reduced forms (NAD/NADH) (Figure 3.2C) were decreased by more than 2-fold at 2h when the concentration of glucose was increased from 5.5 to 25mM. Both tended to decrease at earlier time points, but the differences were not statistically significant. Additionally, the activity of Nicotinamide phosphoribosyltransferase (NAMPT) (Figure 3.2B), a SIRT1 activator, was 50% lower in muscle incubated with 25mM glucose both at 1 and 2h, and the concentration of lactate and the lactate/pyruvate ratio were increased at both 1 and 2h (Table 3.1).

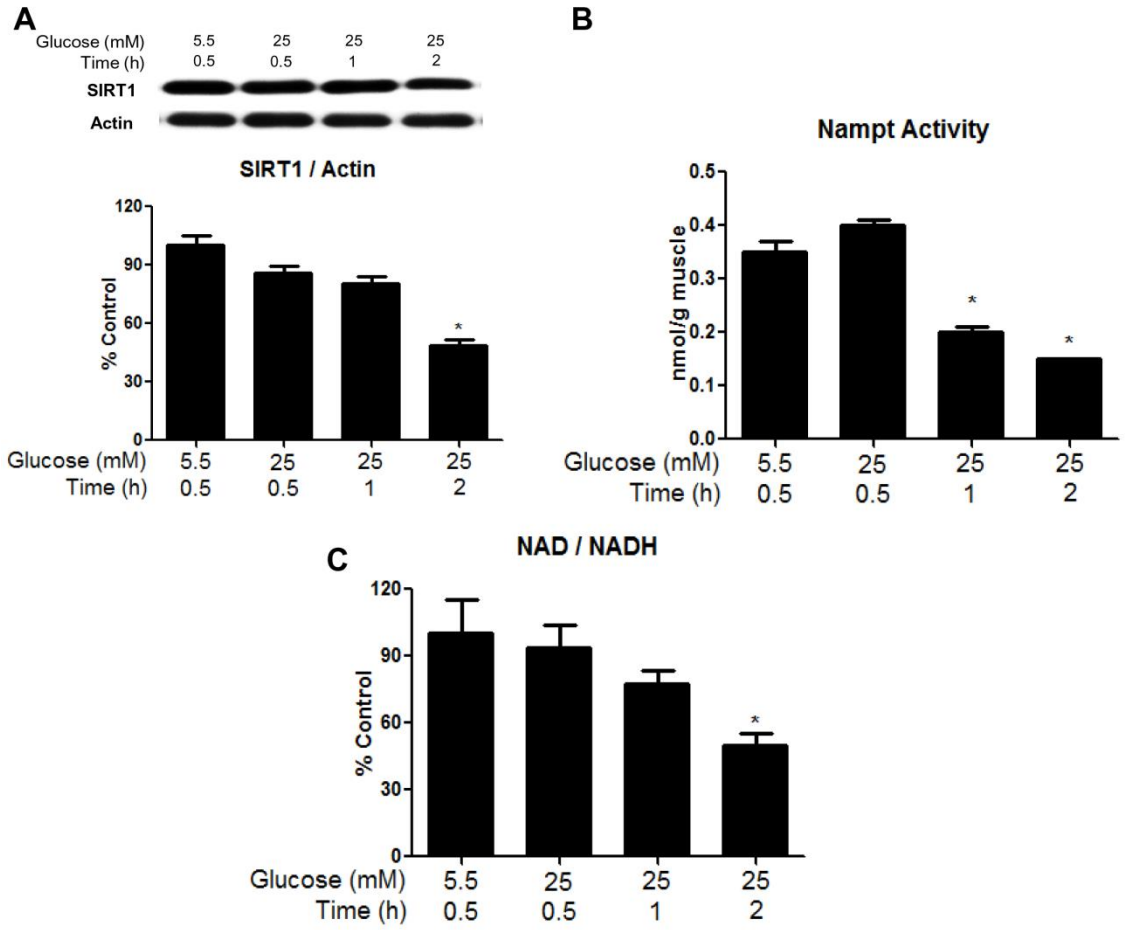


Fig. 3.2: Incubation of muscle in 25mM diminishes SIRT1 protein abundance, NAMPT activity, and NAD/NADH ratio after 1 or 2h.

EDL were incubated with 25 mM glucose for 0.5, 1, or 2h. Western blot analysis and quantification of representative blots are shown. SIRT1 protein expression (A) NAMPT activity (B) and NAD/NADH (C) were determined as described in the methods section. Results are means $\pm$ SE (n=6). *P < 0.05 compared to incubation with 5.5 mM glucose at each timepoint. [244]

	Lactate (µmol/mg)	Pyruvate (µmol/mg)	Lac/pyruv (µmol/mg)
5.5 mM Glucose (1 h)	13±3	1.3±0.1	10.0±2.0
25 mM Glucose (30 min)	17±2	1.6±0.1	10.6±3.0
25 mM Glucose (1 h)	31±4*	2.0±0.3*	15.5±2.0
25 mM Glucose (2 h)	33±5	2.1±0.4	15.8±2.0

Table 3.1: Effects of high glucose on lactate and pyruvate.

Data are means ± SEM (n+4-5/group)**.** Lactate and pyruvate are expressed as µmol/mg muscle. *P < 0.05 relative to 30 min incubation with 5.5mM glucose [244]

Cellular energy state was unchanged in EDL incubated in 25mM glucose for 30min-2h

In search for additional factors responsible for the decrease in AMPK phosphorylation caused by a high glucose concentration, we assessed cellular energy state. In keeping with previous observations [243], we found no differences in tissue concentrations of ATP, ADP, AMP or CrP in muscles incubated with 25 vs. 5.5mM glucose for 30min, 1h, or 2h (Table 3.2).

	ATP (nmol/mg)	AMP (nmol/mg)	ADP (nmol/mg)	CP (nmol/mg)
5.5 mM Glucose (1 h)	3.9±0.04	0.06±0.003	0.60±0.04	15.0±4.0
25 mM Glucose (30 min)	3.7±0.02	0.05±0.001	0.50±0.02	13.0±2.0
25 mM Glucose (1 h)	3.9±0.06	0.04±0.001	0.55±0.04	14.0±1.7
25 mM Glucose (2 h)	3.8±0.05	0.04±0.002	0.60±0.05	13.5±2.0

Table 3.2: Effects of high glucose on adenine nucleotides and creatine phosphate:

Data are means ± SEM (n=4-5/group). Nucleotide values are expressed as nmol/mg muscle. [244]

Muscle glycogen content was increased in EDL incubated with 25mM glucose for 30min and 1h, while phosphorylation of GSK3β was unchanged

Since glycogen has been shown to inhibit AMPK by associating with the CBD of the β-subunit [241], we measured muscle glycogen content following a 30 or 60 min incubation in 25 vs. 5.5mM glucose. We found that muscle glycogen was significantly increased at both timepoints, suggesting that glycogen may be responsible for the early and sustained inhibition of AMPK (Figure 3.3A). Activation of another known regulator, GSK3β, which has been shown to inhibit catabolic activity of AMPK by associating with the β-subunit and phosphorylating it at Thr^{479} of the α-subunit [245], was unchanged (Figure 3.3B) at all timepoints measured.

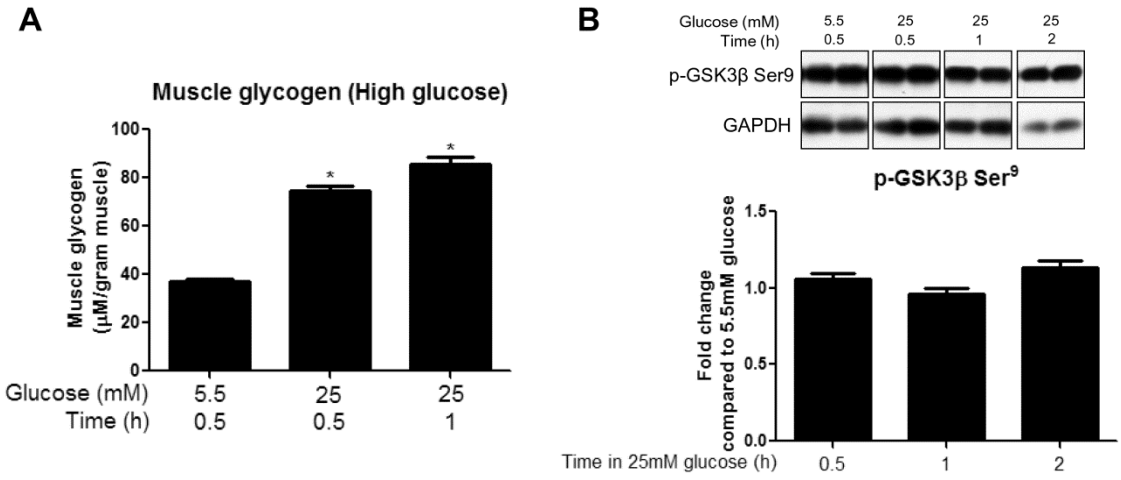


Figure 3.3: Incubation with elevated glucose levels increases muscle glycogen content at 30 min and 1h.

Glycogen content was measured in EDL muscles incubated for 0.5 or 1h (A) or 0.5, 1, or 2h (B) in media containing 5.5 or 25 mM glucose. Muscle lysates were analyzed P-GSK3β Ser^{9} (B) by western blot. Results show quantification of western blots by densitometry. Results are means $\pm$ SE (n = 4-6). *P <0.05 compared to incubation with 5.5 mM glucose at each timepoint. [244]

PP2A activity was increased in EDL incubated in 25mM glucose for 2h

PP2A is a major protein serine/threonine phosphatase that regulates AMPK, among other molecules in mammalian cells [246-248]. As shown in Figure 3.4A, incubation with 25 vs. 5.5mM glucose increased PP2A activity by 1.7-fold; however, as with SIRT1, this change was only observed at the 2h time point.

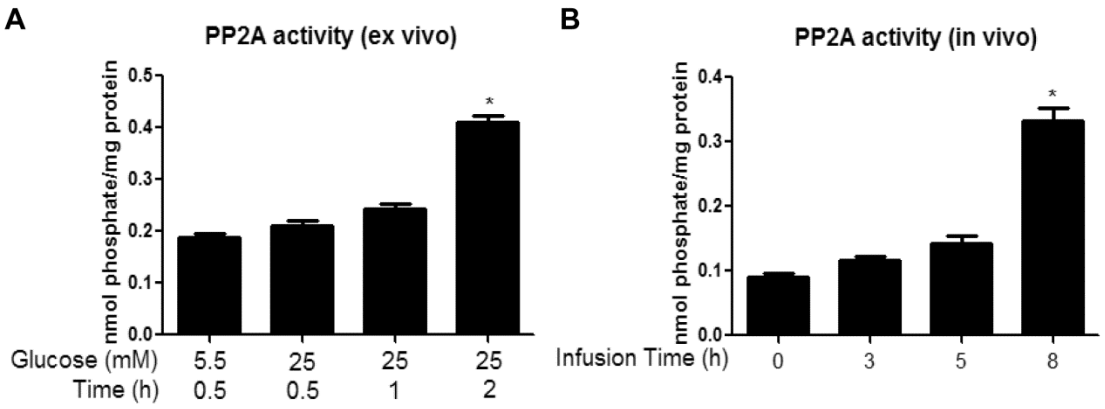


Figure 3.4: Incubation with elevated glucose levels and *in vivo* glucose infusion increases PP2A activity at 2h (*ex vivo*) or 8h (*in vivo*).

EDL muscles incubated for 0.5, 1, or 2h in media containing 5.5 or 25 mM glucose (A) and red gastrocnemius muscles from rats infused with glucose for 0, 3, 5, or 8h (B) were analyzed for PP2A activity as described in the methods section. Results are means $\pm$ SE (n = 4-6). *P <0.05 compared to incubation with 5.5 mM glucose at each timepoint (A) or the 0h group for infusions (B). [244]

Infusion of glucose in vivo caused similar changes in AMPK and its potential regulators as EDL incubation with 25mM glucose, but with differences in timing

To determine whether the effects of glucose observed in the incubated EDL also occurred in vivo and in the presence of insulin, rats were infused with 50% glucose for 0, 3, 5 or 8h at a rate adjusted to maintain plasma glucose concentration at 16-17mM. The plasma insulin level was 250μU/L during the infusion vs. 50μU/L prior to its start. In the red gastrocnemius (RG) muscle, the glucose infusion decreased both AMPK Thr^{172} phosphorylation (Figure 3.5A) and AMPKα2 activity (by the SAMS peptide assay, Figure 3.5C) with a significant change observed at 5h. Interestingly, this was followed by a secondary increase in AMPK activity, something we have not observed in previous studies [232,249]. In keeping with these findings, the phosphorylation of ACC also was only significantly decreased at 5h (Figure 3.5D), although it is somewhat diminished at 3 and 8h of infusion. In contrast, the increase in phosphorylation of AMPK at $Ser^{485/491}$ (Figure 3.5B) and the decreases in SIRT1 protein (Figure 3.6A), NAD/NADH (Figure 3.6B), NAMPT activity (Figure 3.6C) and lactate/pyruvate ratio (Figure 3.6D), which occurred after the decrease in AMPK-Thr^{172} in the incubated EDL, took place at the same time (5h) and were maintained at 8h. Only the increase in PP2A activity (Figure 3.4B) was a later event (observed at 8h), as it was in the EDL incubated with a high glucose medium (Figure 3.4A).

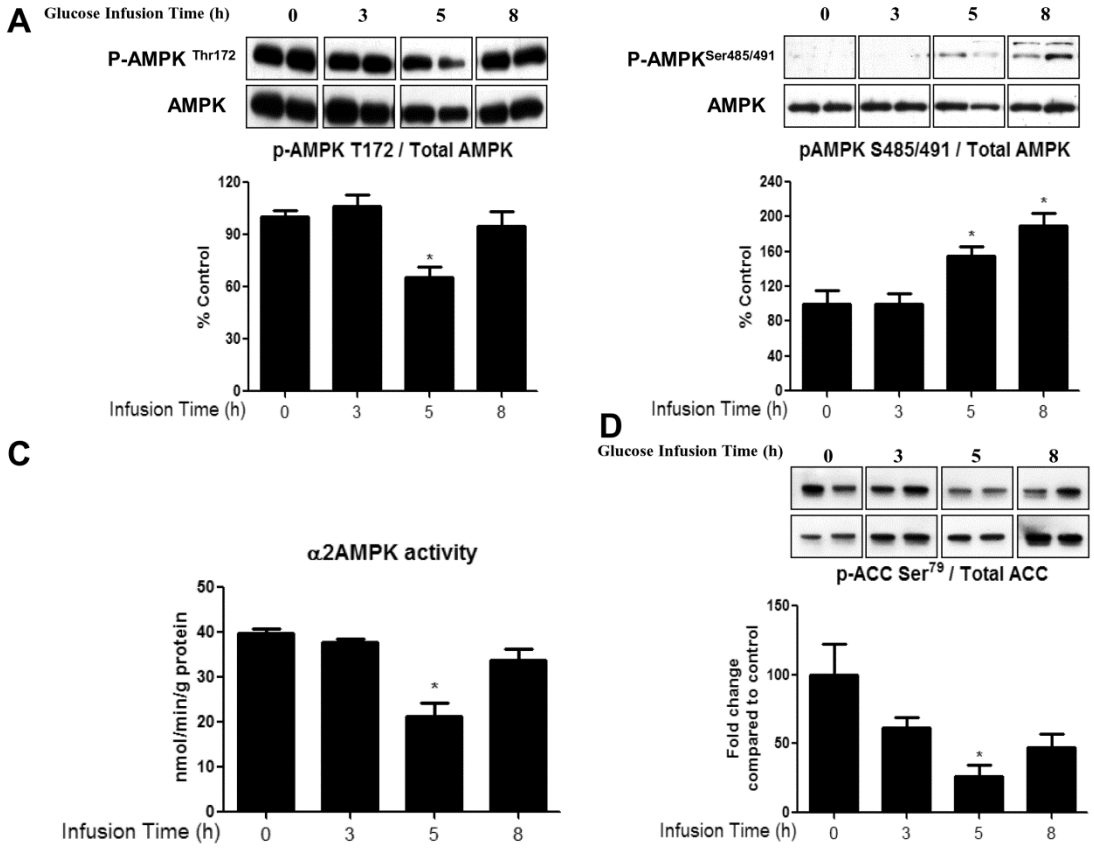


Figure 3.5: Glucose infusion decreases AMPK Thr^{172} phosphorylation and α2 AMPK activity, and increases AMPK Ser$^{485/491}$ phosphorylation in red gastrocnemius muscle.

AMPK Thr^{172} (A) and AMPK Ser$^{485/491}$ (B) and ACC Ser^{79} (D) phosphorylation were analyzed by western blot. α2 AMPK activity (C) was measured using the SAMS peptide assay as described in the methods section. Data are means ± SEM. n = 4–6 rats per group, $P < 0.05$ vs. 0h group. [244]

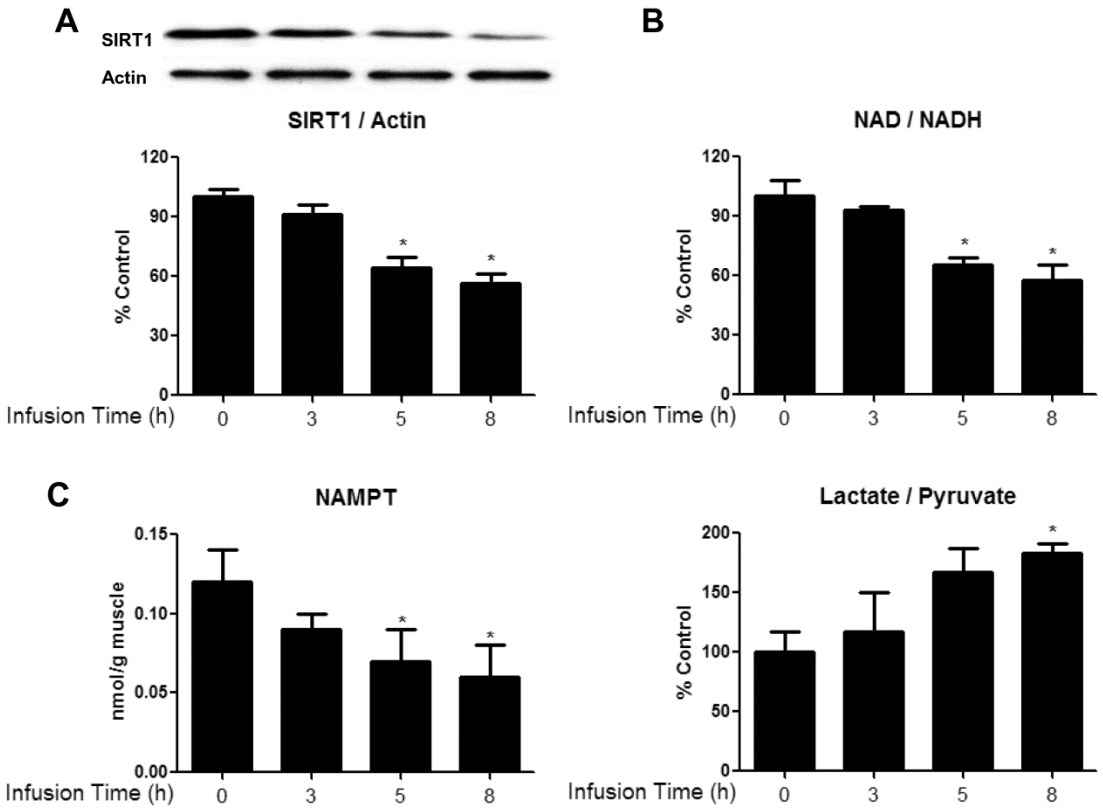


Figure 3.6: Glucose infusion diminishes SIRT1 abundance (A), NAD/NADH ratio (B), and NAMPT activity (C), and increases lactate/pyruvate ratio (D) in RG muscles.

Red gastrocnemius muscles from rats infused with glucose for 0, 3, 5, or 8h were analyzed for SIRT1 protein expression (A) NAD/NADH ratio (B) NAMPT activity (C) and lactate/pyruvate (D). Data are means ± SEM. N = 4–6 rats per group, *P < 0.05 vs. 0h group. [244]

Discussion

The biological relevance of AMPK activation, as well as how such regulation occurs, has been well described [30,62,147,227,250-252]. In contrast, less is known about the factors that contribute to AMPK inhibition and its biological effects. Our lab previously showed that incubation of rat EDL muscle for 1h in a high glucose (25mM) medium decreased AMPK activity and impaired insulin signaling (Akt phosphorylation) compared to EDL incubated in a normoglycemic medium (5.5mM). In addition, we found that all of the changes, including increases in mTOR/p70S6K phosphorylation and protein synthesis, in addition to the insulin resistance, could be prevented by co-incubation with the AMPK activator AICAR. In the present studies, we investigated the effects of high glucose (25mM) over time (30min-2h) on AMPK activation and several factors that could modulate its activity, including serine phosphorylation at $Ser^{485/491}$ and changes in SIRT1 and PP2A. We also sought to determine whether similar changes in these parameters occur in muscle *in vivo*, during a glucose infusion that increased plasma insulin as well as glucose.

We found that incubation of the EDL with 25mM glucose led to a 50% decrease in AMPK activity within 30min and an additional 50% reduction by 2h, as evidenced by decreases in both its phosphorylation at Thr^{172} and SAMS peptide phosphorylation. Interestingly, the later decreases in AMPK activity in these muscles were associated with increased phosphorylation of AMPK at $Ser^{485/491}$ and changes in NAMPT and redox state at 1h, and alterations in SIRT1 expression (decreased) and PP2A activity (increased) at 2h. Similarly, *in vivo*, infusion of glucose at a rate that maintained plasma levels at 16-

17mM and increased plasma insulin 30-fold diminished AMPK activity and its phosphorylation at Thr^{172} at 5h. In contrast to the findings in the incubated EDL, changes in $Ser^{485/491}$ phosphorylation, SIRT1 abundance, NAMPT activity, and redox state were observed concurrently. Only PP2A activation was a later event (8h). These findings raise several fundamental questions: (1) by what mechanism does excess glucose lead to the initial decrease in AMPK activity in the EDL, (2) what is the physiological significance of the later decreases in AMPK activity and the changes in its putative regulators, and (3) why does the temporal sequence of events differ between incubated muscle and muscle from rats infused with glucose *in vivo* despite similar changes in all of the above-mentioned parameters?

To our surprise, in the incubated EDL, increases in most putative downregulators of AMPK activity occurred after, rather than before the initial decrease in AMPK activity (30min). Increased muscle glycogen content was the only regulatory factor we found to be changed at this early timepoint (Figure 3.3). Previous studies have shown that AMPK activation is lower in glycogen loaded versus depleted muscles in response to contraction [253] or AICAR [254] in rodents or in response to exercise in humans [255].

Furthermore, glycogen was recently shown to directly inhibit AMPK in a cell-free system by binding to the GBD of its β-subunit, thus causing an allosteric change that inhibits its phosphorylation at Thr^{172} [241]. Although further studies are required to determine whether glycogen is responsible for the initial inhibition of AMPK in our muscle incubation model, this data is consistent with the hypothesis of Mcbride et al. [241] that AMPK is sensitive not only to changes in immediately available cellular

energy in the form of AMP and ATP (which were unchanged in our studies (Table 3.2)), but also to energy reserves in the form of glycogen. Despite the significant increase in muscle glycogen seen at 30min and 1h, no changes in phosphorylation of GSK3β were observed at any timepoint measured (30min-2h) (Figure 3.3). This is somewhat surprising, since GSK3β contributes to the regulation of glycogen synthesis by phosphorylating and inhibiting glycogen synthase. However, transient changes in p-GSK3β may have preceded the increase in glycogen content and been missed in our studies.

Following these early changes, the next event observed was an increase in AMPK $Ser^{485/491}$ phosphorylation, which was increased by 250% after 1h of incubation in a high glucose medium (Figure 3.1C). Prior studies in other tissues suggest that phosphorylation at this site in response to other stimuli occurs very quickly (within 5-10min) and plays an important role in inhibiting AMPK [54,56,59,239].

In the muscles incubated with high glucose, changes in NAMPT and redox state, as well as decreased SIRT1 expression (Figure 3.2), all of which also have been linked to diminished AMPK activity [234]**,** occurred at various times after the decreases in Thr^{172} and SAMS peptide phosphorylation (both of which were observed at 30min). The reduction in SIRT1 abundance first became evident at 2h, well after the initial decrease in AMPK activity. Previous studies have revealed an interaction between SIRT1 and the AMPK kinase LKB1. For example, Lan et al.[243] demonstrated that overexpression of SIRT1 led to increased AMPK activity by causing translocation of LKB1 from the nucleus to the cytoplasm, where most of the cellular AMPK is located. Whether a

decrease in SIRT1 leads to opposite changes and contributes to a reduction in AMPK activity is not known.

Another finding in the incubated EDL and muscles of glucose infused rats was an increase in PP2A activity (Figure 3.4). The dephosphorylation of AMPK at Thr^{172} has been associated with the actions of various Ser/Thr protein phosphatases [246]. Wu and coworkers [240] found that a decrease in AMPK activity observed in bovine aortic endothelial cells after incubation with palmitate for 40h is associated with an increase in PP2A activation. Surprisingly, the increase in PP2A activity in the present study occurred much later (2h) than the initial decreases in AMPK Thr^{172} phosphorylation (30min). Hyperactivity of PP2A has been shown in muscle and other tissues in models of glucolipotoxicity and diabetes [256]. Since this is the last factor to change of those that were examined, one could speculate that the increase in PP2A activity produces a less reversible inhibition of AMPK by maintaining its dephosphorylation on Thr^{172}, and possibly other sites.

In vivo, the infusion of glucose concurrently decreased AMPK activity (SAMS peptide assay and p-AMPK Thr^{172}) and increased AMPK $Ser^{485/491}$ phosphorylation in the red gastrocnemius (Figure 3.5). The co-occurrence of these changes *in vivo* differed from the delay in phosphorylation at $Ser^{485/491}$ observed in the EDL incubated with high glucose. Although the precise explanation for this difference remains to be determined, one obvious factor could be the presence of insulin *in vivo*. As already noted, in heart, insulin has been shown both to increase AMPK $Ser^{485/491}$ phosphorylation and decrease the phosphorylation of αAMPK at Thr^{172} and AMPK activity within 5-10min [54,239].

Of further note, we observed changes in SIRT1 and related parameters at the same time as the changes in Thr^{172} and $Ser^{485/491}$ phosphorylation in the muscle of glucose-infused rats. However, the increase in PP2A activity occurred much later, as it did in the EDL incubated in a high-glucose medium (without insulin). The presence of insulin and other circulating factors, as well as differences in animal age and weight could be responsible for the differences in the timing and sequence of changes between the two models; however, this remains to be determined.

In summary, we have demonstrated that incubation of EDL with high glucose resulted in diminished AMPK Thr^{172} phosphorylation and activity that continued to decrease over time. An *in vivo* glucose infusion also caused a reduction in AMPK activation along with changes in other putative regulators of the enzyme. In both models, concurrent or sequential changes in αAMPK $Ser^{485/491}$ phosphorylation (increased), SIRT1 protein level (decreased), and PP2A activity (increased) were observed. Though all of these factors may diminish AMPK activity, glycogen content was the only regulatory factor found to be changed at 30 min in the incubated EDL (See Figure 3.7 for summary of observed changes over time). These data suggest that the initial inhibition of AMPK in response to high glucose or leucine may be due to sensing an increase in cellular energy state in the form of glycogen, though further studies are required to confirm this hypothesis. The physiological and/or pathophysiological significance of the later occurring changes that correlate with additional decreases in AMPK activity also requires further investigation. An interesting possibility is that they contribute to more long-lasting changes that could alter gene expression or mitochondrial function and are

less readily reversible. Future studies with an expanded time course in which these factors are individually inhibited or knocked down could address these questions. Subsequent studies in this thesis will focus on the phosphorylation of AMPK at $Ser^{485/491}$ to determine its role in inhibiting AMPK activity and possible regulatory factors.

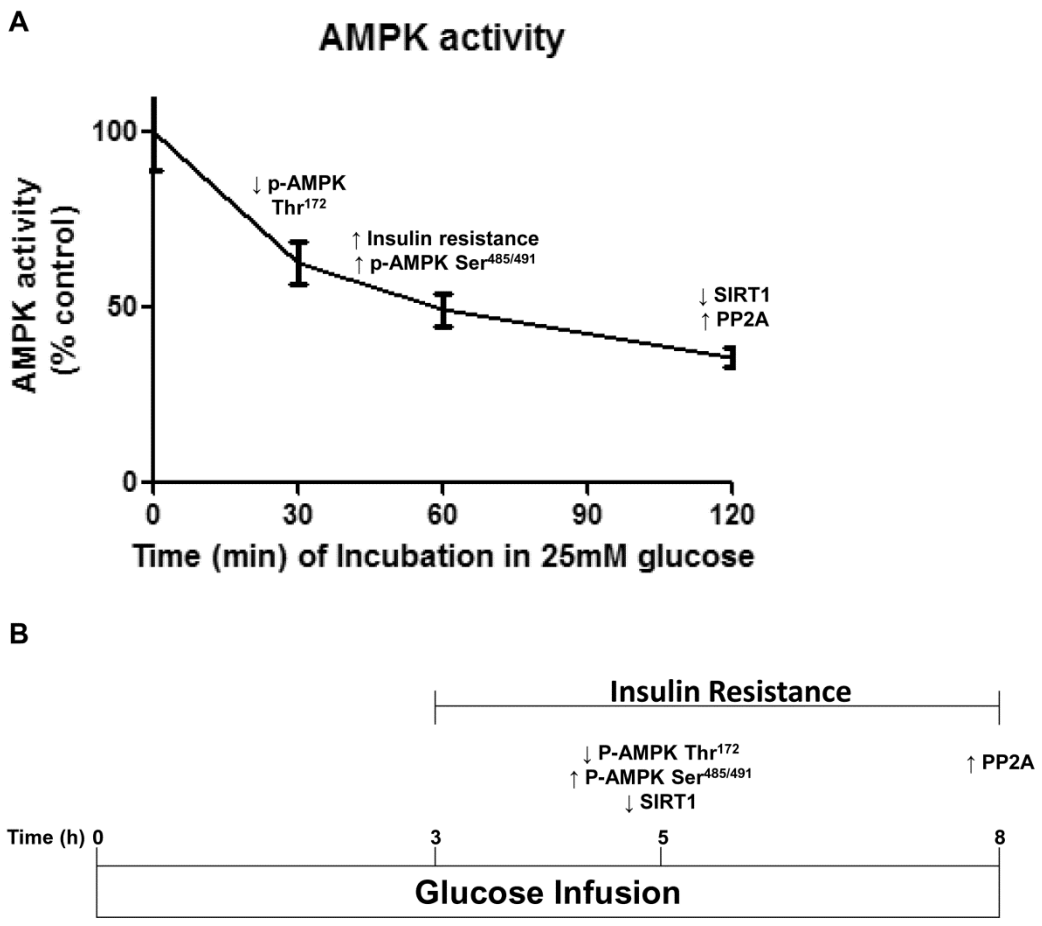


Figure 3.7: Chapter 3 Summary

Effects of high glucose on AMPK phosphorylation and activity as well as factors that may regulate it in EDL incubated in 25mM glucose (A) and in muscle of glucose infused rats (B) are shown in the illustration.

CHAPTER 4: Inhibition of AMPK via insulin-induced phosphorylation at Ser$^{485/491}$

Introduction

As described in the previous chapter, we found that phosphorylation of AMPK at Ser$^{4855/491}$ was increased in EDL incubated with 25 vs 5mM glucose for 1 or 2h and in muscle of rats infused with glucose for 5 or 8h. In the present studies, we sought to determine whether phosphorylation of this site contributes to the inhibition of AMPK activity, as has been reported in other tissues [54,59], and what upstream kinase(s) could be it. In the EDL, the increase in Ser$^{485/491}$ phosphorylation was observed after the initial decrease in AMPK Thr^{172} phosphorylation and enzyme activity, whereas in the muscle of glucose-infused rats, the increase in serine phosphorylation occurred concurrently with the decreases in Thr^{172} phosphorylation and AMPK activity. One notable difference between the models is presence of insulin in the glucose-infusion model, which we hypothesized could be responsible for the difference in timing. It has previously been shown that insulin and insulin-like growth factor-1 (IGF-1), both of which are elevated in T2D, can stimulate phosphorylation of AMPK at Ser$^{485/491}$ in heart [47, 64], white adipocytes [51], vascular smooth muscle [48] and tumor cells [53] through activation of Akt (See Figure 1.1). For these reasons, we sought to investigate whether insulin stimulates AMPK Ser$^{485/491}$ phosphorylation and inhibits enzyme activity in muscle. The C2C12 mouse skeletal muscle cell line was used in these studies to evaluate signaling pathways. These cells differentiate into myotubes that express characteristic muscle proteins and can be stimulated to contract.

Results

Insulin stimulated phosphorylation of AMPK Ser$^{485/491}$ in C2C12 myotubes and rat EDL muscle

To determine whether insulin treatment stimulates phosphorylation of AMPK at Ser$^{485/491}$ in muscle, C2C12 myotubes and rat EDL muscle were treated with insulin for 10-15min. Insulin treatment (100nM) significantly increased p-AMPK Ser$^{485/491}$ in C2C12 myotubes (Figure 4.1A, 4.1B) by >5-fold. Similarly, incubation of rat EDL muscle with insulin increased AMPK Ser$^{485/491}$ phosphorylation within 10 minutes (Figure 4.1C, 4.1D). Although 100nM is supraphysiological, increases in serine phosphorylation were seen in the myotubes with doses in the picomolar range, and timecourse studies revealed that increases were seen as early as 10 minutes and lasted at least 3 hours (See Supplemental Figure 4.7 for dose response).

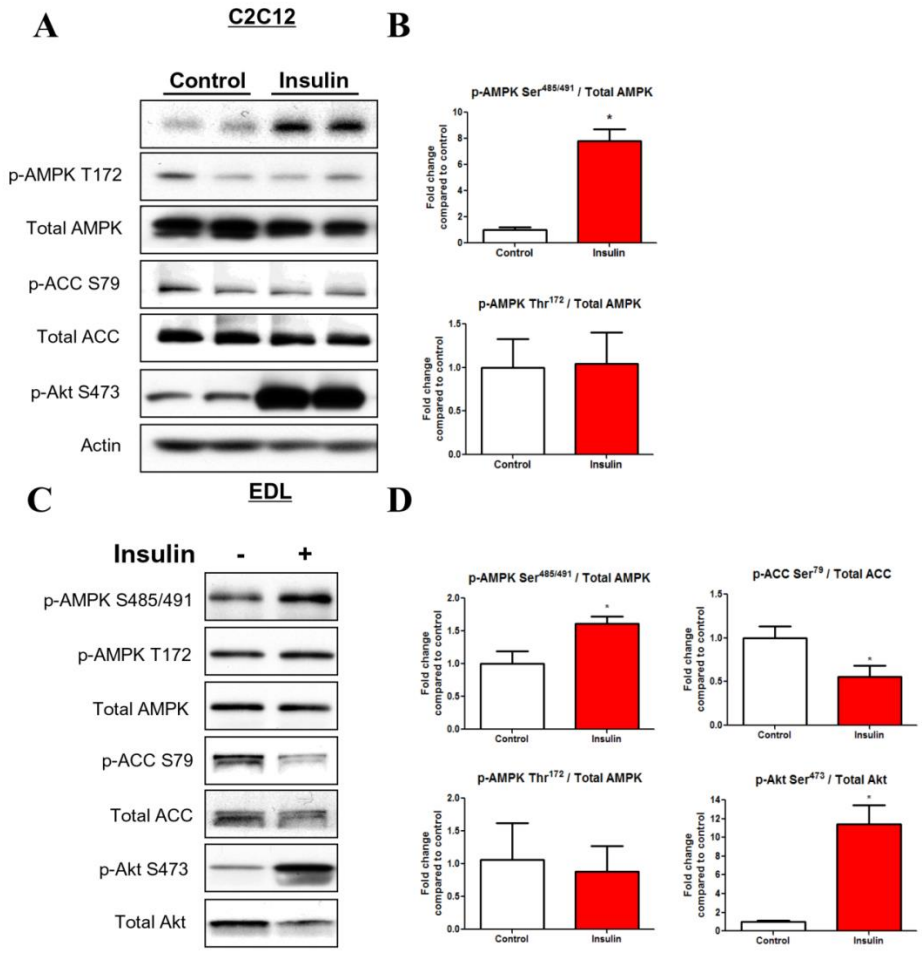


Figure 4.1: Insulin stimulates phosphorylation of AMPK Ser$^{485/491}$ in C2C12 myotubes and incubated EDL muscle.

C2C12 myotubes (A) were cultured in normal glucose (5.5 mM), serum starved overnight, and stimulated with insulin (100 nM) for 15 min. Rat EDL muscles were removed and equilibrated in Krebs–Henseleit buffer for 20min, then stimulated with insulin (10 mU/ml) for 10min (C). Western blot analyses were performed, and representative blots are shown. Blots were quantified by densitometry. Phosphorylation of AMPK was normalized to total AMPK, and normalized data are displayed for myotubes (B) and EDL (D). Results are means ± SE (n = 3–6 per treatment). All experiments were performed in triplicate. $^{*}p < 0.05$ vs. control. [239]

Insulin reduced AMPK activity in C2C12 myotubes and rat EDL muscle

Our results also showed that insulin significantly diminished AMPK activity, as measured by the SAMS peptide assay, in C2C12 myotubes and incubated EDL muscles (Figure 4.3), despite no change in p-AMPK Thr^{172} (Figure 4.1C, 4.1D). Phosphorylation of ACC Ser^{79} was also unchanged in response to insulin treatment in the myotubes; however, insulin diminished p-ACC Ser^{79} in the incubated EDL muscle (Figure 4.1C). The reason for this discrepancy is not yet known.

Inhibition of Akt attenuated insulin-induced p-AMPK Ser$^{485/491}$ and partially prevented the reduction in AMPK activity

The enzyme Akt has been shown by others to be responsible for the phosphorylation of Ser$^{485/491}$ in other tissues. Since Akt plays an important role in the insulin signaling pathway, we used an inhibitor of Akt, Akt inhibitor VIII, to test the role of Akt on insulin's stimulation of p-AMPK Ser$^{485/491}$ in myotubes. Pretreatment of myotubes for 2h with this inhibitor attenuated the insulin-stimulated increase in phosphorylation of AMPK at Ser$^{485/491}$ (Figure 4.2A, 4.2B). No changes were observed in p-AMPK Thr^{172} (Figure 4.2A, 4.2B). We measured AMPK α1 and α2 activity (α2 is the predominant isoform in skeletal muscle) and found that Akt inhibition significantly attenuated insulin's effect on kinase activity in both C2C12 myotubes and EDL (Figure 4.3A and 4.3C, respectively). Notably, a significant inverse correlation was observed between insulin-induced phosphorylation of Ser$^{485/491}$ of AMPK and inhibition of AMPK activity (Figure 4.3B), despite no changes in p-AMPK Thr^{172}, which is often assumed to be a surrogate readout for AMPK enzyme activity.

Wortmannin prevented insulin-induced phosphorylation of AMPK Ser$^{485/491}$

To further confirm that Akt is responsible for insulin-stimulated phosphorylation of AMPK Ser$^{485/491}$, we used the PI3K inhibitor wortmannin. Since PI3K is upstream of Akt in the insulin-signaling pathway, wortmannin should prevent Akt activation in response to insulin. We found that wortmannin had the same effect as Akt inhibitor VIII in C2C12 cells. Specifically, pre-treatment with wortmannin (1h) prevented the insulin-induced increase in p-AMPK Ser$^{485/491}$, with no effect on p-AMPK Thr^{172} (Figure. 4.2C, 4.2D).

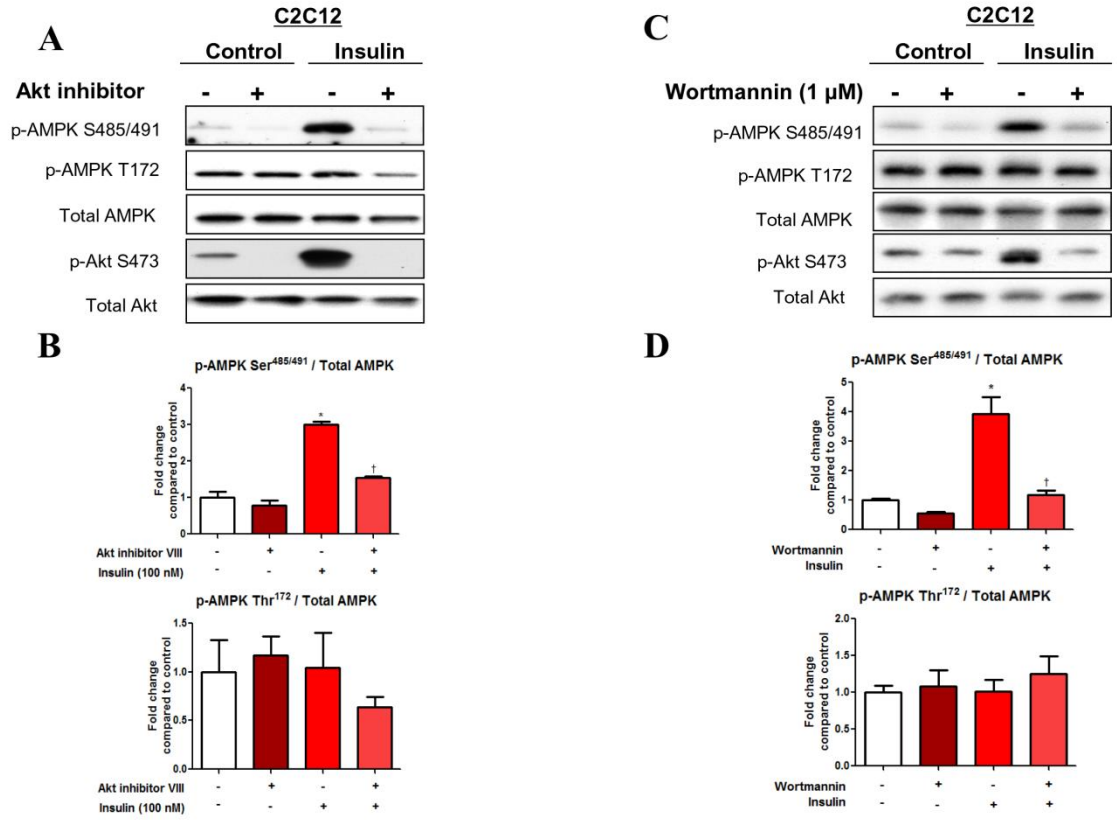


Figure 4.2: Inhibition of Akt attenuates insulin stimulated phosphorylation of AMPK Ser$^{485/491}$.

C2C12 cells were treated with or without Akt inhibitor VIII (250 μM) for 2 h, then stimulated with insulin (100 nM) for 15 min. Cells were lysed and subjected to western blot analysis. Representative western blots are shown (A). Quantification of western blots was performed using densitometry, and AMPK phosphorylation was normalized to total AMPK (B). In Figure C, C2C12 cells were pre-treated with the PI3-kinase inhibitor wortmannin (10 nM) for 1 h, then stimulated with insulin (100 nM) for 15 min. Cells were processed and protein expression and phosphorylation were quantified as described in (A) and (B) and quantifications are shown in (D). Results are means ± SE (n = 3–6 per treatment). All experiments were performed in triplicate. $^{*}p < 0.05$ indicates a significant effect of insulin vs. control, $^{\dagger}p < 0.05$ vs. insulin treatment alone. [239]

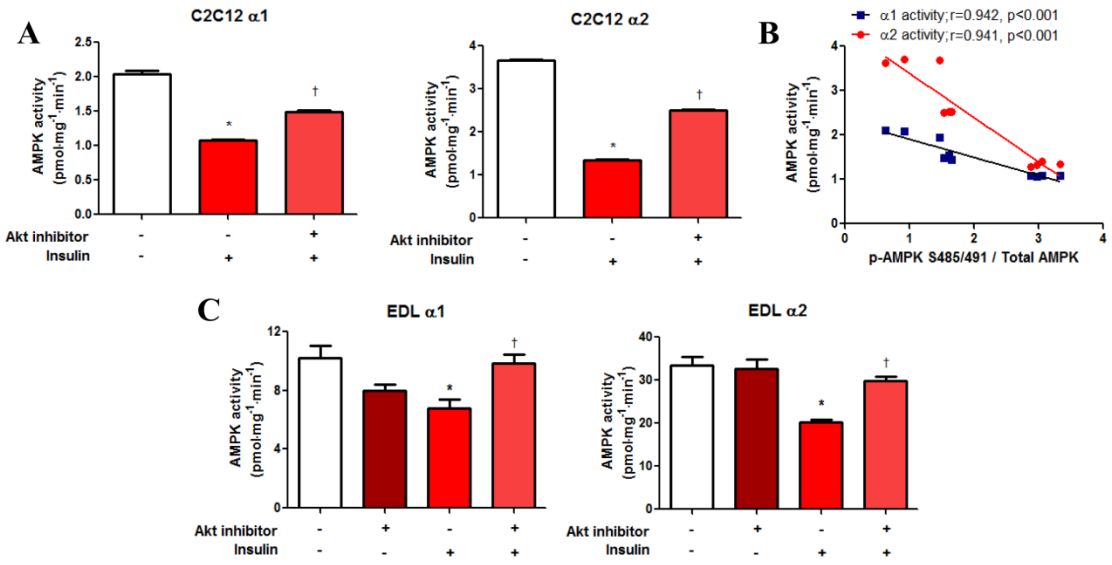


Figure 4.3: Insulin-induced inhibition of AMPK activity is partially prevented by Akt inhibition.

AMPK α1 and α2 activities were assessed using the SAMS peptide assay, as described in the materials and methods section. C2C12 (A) and EDL (C) AMPK activity data are presented. Correlations between $Ser^{485/491}$ AMPK phosphorylation data from western blot analysis (Fig. 4.1) and AMPK activity are shown (B). [239]

IGF-1 mimicked the effect of insulin on p-AMPK Ser$^{485/491}$ and was attenuated by Akt inhibition

Insulin-like growth factor 1 (IGF-1) is an anabolic factor that, like insulin, signals through the PI3K-Akt pathway. It was recently published that IGF-1 dose-dependently stimulates phosphorylation of AMPK at Ser^{485} on the α1 subunit in vascular smooth muscle cells (VSMCs) [48], and the authors determined that Akt was the upstream kinase responsible for this serine phosphorylation. Additionally, since IGF-1 is upregulated in T2D, we examined whether IGF-1 could produce the same effect as insulin in C2C12 cells. We found that IGF-1 treatment does increase the phosphorylation of AMPK Ser$^{485/491}$ in a dose-dependent manner. Furthermore, we found that pre-treatment with Akt inhibitor VIII prevented IGF-1 stimulation of this phosphorylation (Figure 4.4A, 4.4B), just as it did with insulin.

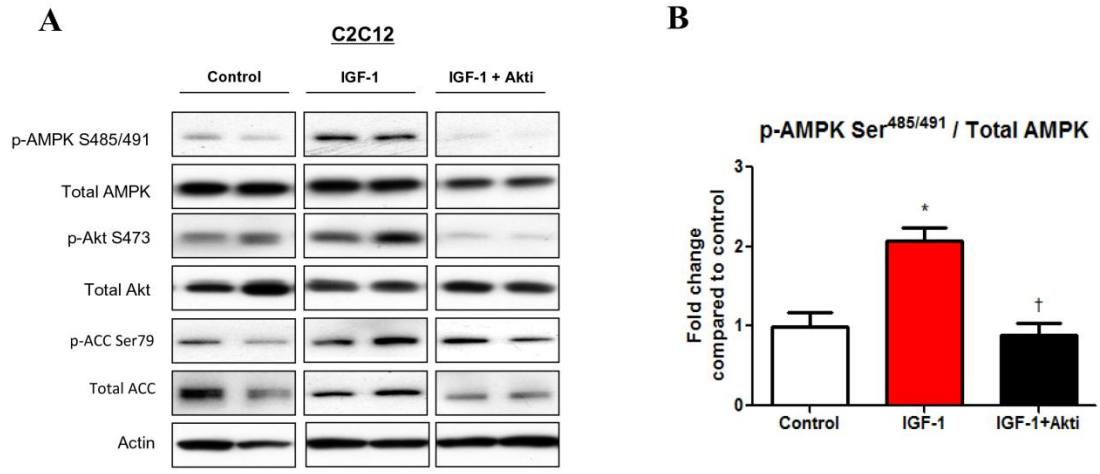


Figure 4.4: IGF-1 phosphorylation of AMPK Ser$^{485/491}$ is prevented by Akt inhibition.

C2C12 cells were serum starved overnight and treated with or without Akt inhibitor VIII (Akti; 250 μM) for 2 h, then stimulated with IGF-1 (20 ng/ml) for 15 min followed by whole cell lysis and analysis by western blot. Representative western blots are shown (A). Densitometric analysis of western blots was used to quantify protein phosphorylation and expression. Phosphorylation of proteins of interest were normalized to the corresponding total protein, and results are presented as fold-change compared to control (B). Results are means ± SE (n = 3–6 per treatment). All experiments were performed in triplicate. $^{*}p < 0.05$ indicates a significant effect of IGF-1, $^{\dagger}p < 0.05$ for the effect of Akti VIII. [239]

Inhibition of mTOR by rapamycin did not affect insulin-stimulated p-AMPK Ser$^{485/491}$

As previously noted, Dagon et al. recently showed that leptin can stimulate phosphorylation of α2AMPK Ser^{491} in the hypothalamus, and that this is mediated by the mTOR/p70S6Kinase (p70S6K) pathway [52]. Since insulin treatment is also known to activate p70S6K activity in myotubes [65, 66], we sought to determine whether this pathway might be involved in insulin-sitmulation of p-AMPK Ser$^{485/491}$. To examine this question, myotubes were pretreated with the mTOR inhibitor rapamycin for 2 h prior to insulin-stimulation. As expected, insulin treatment increased the phosphorylation of mTOR Ser^{2448} and p70S6K Thr^{398}, both of which were prevented by rapamycin (50nM). Interestingly, rapamycin had no effect on p-AMPK Ser$^{485/491}$ stimulation caused by insulin (Figure 4.5) or IGF-1. These results suggest that the mTOR/p70S6K pathway is not involved in insulin- or IGF-1-induced phosphorylation of AMPK Ser$^{485/491}$ in C2C12 myotubes.

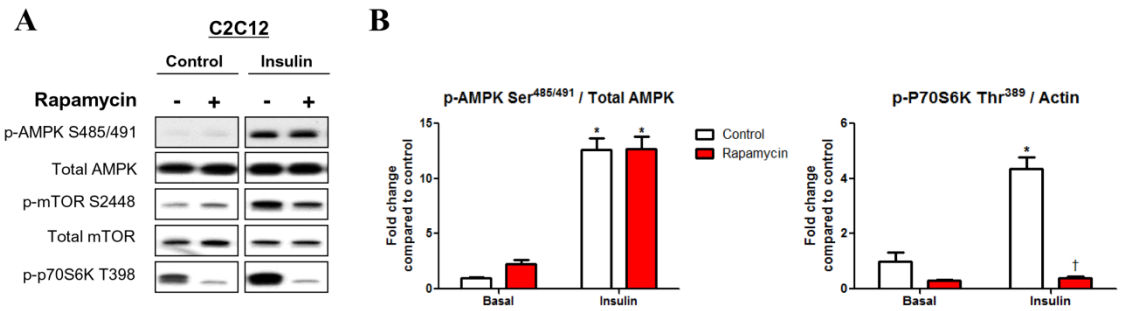


Figure 4.5: Insulin-stimulation of p-AMPK Ser$^{485/491}$ is not affected by the mTOR inhibitor rapamycin.

C2C12 cells were treated with or without rapamycin (50 nM) for 2 h, then stimulated with insulin for 15 min. Cell lysates were run on western blots and protein expression and phosphorylation were quantified using densitometry. Representative western blots are shown (A). Densitometry results are presented in graphs (B). Results are means ± SE (n = 3–6 per treatment). All experiments were performed in triplicate. $^{*}p < 0.05$ indicates a significant effect of insulin, $^{\dagger}p < 0.05$ for the effect of rapamycin treatment. [239]

Discussion

The multitude of functions attributed to AMPK activation highlights the importance of understanding how its activity is regulated at a molecular level. Although the enzyme has many post-translational modifications, phosphorylation of αThr^{172} has received the most attention, by far. Phosphorylation of this site is required to achieve maximal activity [40,41] and often corresponds with enzyme activity. Thus, it is often used as a readout of AMPK enzyme activity irrespective of kinase activity toward its downstream substrate, ACC, or its target sequence on ACC, known as SAMS (substrate for AMPK) peptide. In these studies, we measured the propensity of AMPK's catalytic α-subunit to phosphorylate SAMS peptide *in vitro* in settings where phosphorylation of AMPK at Ser$^{485/491}$ was increased. We found decreases in AMPK activity with no changes in phosphorylation at Thr^{172}. These results, along with those of others [59], contribute to a growing body of literature showing discordance between phosphorylation at Thr^{172} and activity towards SAMS peptide, but an inverse correlation between Ser$^{485/491}$ phosphorylation and activity towards SAMS peptide.

In these studies, we show, for the first time, that insulin and IGF-1 caused phosphorylation of AMPK at Ser$^{485/491}$ in skeletal muscle. This has previously been shown in other tissues, such as heart, where Horman et al. [54] demonstrated in rat heart that at a normal glucose concentration, insulin increases αAMPK Ser$^{485/491}$ phosphorylation within 5 min, an effect mediated by Akt activation. In another series of investigations, Dagon et al [59] demonstrated that phosphorylation of α2 AMPK at Ser^{491} in response to leptin inhibits its activity in the hypothalamus. They demonstrated that this

increase in AMPK Ser^{491} phosphorylation is mediated by mTOR/p70S6 kinase and can be inhibited by the mTOR inhibitor rapamycin. Since the mTOR/p70S6K is also activated by insulin, we used rapamycin to determine whether p70S6K is involved in Ser$^{485/491}$ phosphorylation in muscle cells. Rapamycin had no effect on insulin-induced serine phosphorylation of AMPK, suggesting that p70S6K is not responsible for the inhibition of AMPK in this setting. Inhibition of Akt, however, did prevent insulin- and IGF-1-stimulated phosphorylation of AMPK at Ser$^{485/491}$. Both the Akt inhibitor Akt VIII and the PI3K inhibitor Wortmannin prevented insulin-stimulated serine phosphorylation of AMPK, suggesting that Akt is the kinase responsible for phosphorylating and inhibiting AMPK in response to insulin (See Figure 4.6).

The physiological significance of insulin mediated inhibition of AMPK through Ser$^{485/491}$ phosphorylation remains to be determined. Since AMPK stimulates catabolic processes aimed to restore normal ATP levels when cellular energy levels are low, whereas Akt is activated when cellular energy is high, the Akt mediated inhibition of AMPK likely represents a switch from a catabolic to anabolic state. In a healthy individual, this would allow for synthesis of proteins and glycogen in order to maintain muscle health and store energy for when it is needed. However, in states of chronic nutrient excess, sustained hyperinsulinemia and elevated IGF-1 levels, both of which are present in obesity, prediabetes, and T2D, could cause chronic inhibition of AMPK through this mechanism. As discussed in the introduction, this inhibition may contribute to the pathologies of insulin resistance by inhibiting glucose uptake and FA oxidation,

while prevention of AMPK through this mechanism could represent a novel prevention or treatment strategy for T2D.

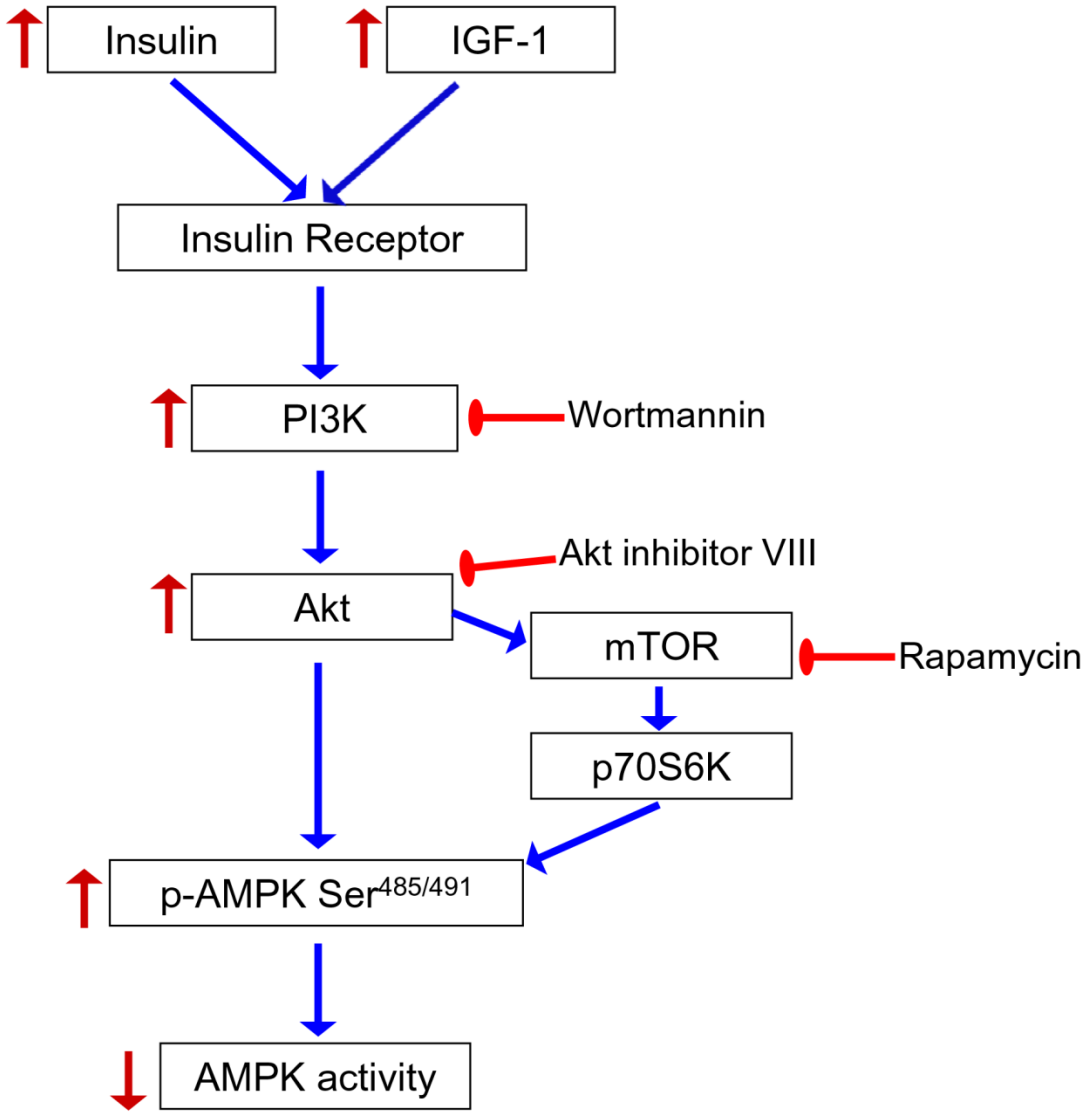
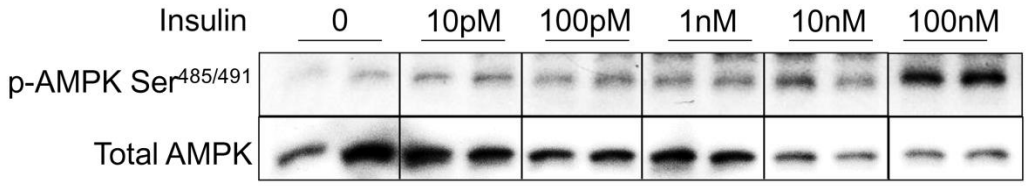


Figure 4.6: Summary of Chapter 4 results.

We found that both insulin and IGF-1 stimulate phosphorylation of AMPK at $Ser^{485/491}$ and diminish its activity in skeletal muscle. These events can be prevented by inhibition of PI3K and Akt, but not mTOR/p70S6K, suggesting that Akt is the kinase responsible for this inhibitory phosphorylation.



Supplemental Figure 4.7: Insulin dose-dependently stimulates phosphorylation of AMPK Ser$^{485/491}$ in C2C12 myotubes.

C2C12 myotubes cultured in 5.5mM glucose were serum starved overnight, then treated with 10pM-100nM insulin for 15 min. Cells were lysed an analysed by western blot for phosphorylation of AMPK Ser$^{485/491}$.

CHAPTER 5: Inhibition of AMPK via PKD1-mediated phosphorylation at Ser$^{485/491}$

Introduction

The studies presented in the previous chapter showed that insulin-mediated activation of Akt caused phosphorylation of AMPK at Ser$^{485/491}$, resulting in inhibition of enzyme activity. In studies described in chapter 3, we showed that incubation of rat EDL muscle with elevated glucose levels (25mM) for 1 or 2h decreased AMPK activity (Figure 3.1A, 3.1D) and increased phosphorylation of AMPK at Ser$^{485/491}$ (Figure 3.1C) in the absence of insulin, suggesting that a different mechanism may be involved. Similarly, it was reported that when MIN6 pancreatic beta cells were switched from low glucose (3mM) culture medium to high glucose (25mM) without insulin, for either 1h or overnight, phosphorylation of α1AMPK at Ser^{485} was increased [61]. This serine phosphorylation was inversely correlated to phosphorylation of AMPK Thr^{172}.

Several upstream kinases have been identified that phosphorylate AMPK at Ser$^{485/491}$ and inhibit its activity in various tissues. As previously mentioned, in heart, skeletal muscle, and liver, insulin and IGF-1 stimulate AMPK phosphorylation at this site by activating Akt [54,55,58,239]. Also, in the hypothalamus, Dagon et al. [59] showed that p70S6K phosphorylates α2AMPK Ser^{491} to inhibit AMPK activity and decrease food intake. Others have shown that in murine macrophage-like RAW 264.7 cells, IKKβ phosphorylates AMPK at Ser^{485} in response to LPS treatment [257], while ERK1/2 can inhibit AMPK by this mechanism in mature dendritic cells in response to CCR7 signaling [258]. Finally, protein kinase A (PKA) has been reported to phosphorylate this site in INS-1 cells in response to forskolin or GIP stimulation [56] and in human diploid

fibroblasts in response to lysophosphatidic acid [259]. Inhibition of AMPK through this mechanism by multiple kinases suggests a biological need to maintain tight control over AMPK enzyme activity. Whether other kinases can also phosphorylate AMPK at $Ser^{485/491}$ to modulate its activity and their biological functions remain to be determined.

Protein Kinase C (PKC) is a family of serine/threonine kinases that can be activated by diacylglycerol (DAG), a phospholipid, and Ca^{2+} (depending on the isoform). Several of the 10 known PKC isoforms have long been implicated in the development of insulin resistance, but beyond inhibitory serine phosphorylation of IRS-1 [209], the mechanisms by which they do so have not been fully clarified. Protein Kinase D1 (PKD1) (the mouse ortholog of human PKCμ) is a related kinase that can be activated either directly by DAG or due to phosphorylation by novel PKC isoforms [218]. When activated, PKD1 undergoes transphosphorylation by novel PKCs at $Ser^{744/748}$ of its activation loop and autophosphorylation at Ser^{916} at its C terminus. This more recently discovered enzyme was initially classified as an atypical PKC isoform, but was later determined to be more similar structurally to the calcium/calmodulin-dependent protein kinase (CaMK) group of serine/threonine kinases with unique substrate specificity [217,218]. Pathological overactivation of PKD1 has been linked to invasiveness of certain cancers [260] and cardiac hypertrophy [222,224], the latter of which is often present in patients with T2D. Interestingly, both PKD1 and AMPK can act as class IIa histone deacetylase (HDAC) kinases [261]. Phosphorylation of HDAC5 by these kinases allows the transcription factor myocyte enhancer factor 2 (MEF2) to dissociate and upregulate many muscle-specific and metabolic genes. This pathway is responsible for

adaptations to acute exercise and may mediate the hypertrophic response to PKD1 overactivation. Recently, McGee and colleagues [228] reported that expression of a dominant negative AMPK in the muscle of mice resulted in a compensatory increase in PKD1 activation during exercise. Whether alterations in PKD1 expression or activity affect AMPK activation is not yet known. What is known is that the DAG mimetic phorbol 12-myristate 13-acetate (PMA), which activates PKC and PKD, as well as many other signaling molecules, decreases AMPK activity in cardiac myocytes [216], although the mechanism by which it does so is unknown.

In the present studies, we sought to determine whether PKC or PKD may be involved in inhibition of AMPK through phosphorylation at $Ser^{485/491}$ in skeletal muscle cells. We used the phorbol ester PMA to determine the effects of broad PKC/PKD activation on AMPK phosphorylation and activity. Through the use of non-specific and specific PKC and PKD inhibitors, we show for the first time that PKD1 phosphorylates AMPK at $Ser^{485/491}$, thus diminishing AMPK activity.

Results

The PKC activator PMA dose- and time-dependently stimulated AMPK $Ser^{485/491}$ phosphorylation in C2C12 myotubes

Treatment with the PKC activator phorbol 12-myristate 13-acetate (PMA) has previously been shown to decrease AMPK activity in cardiac myocytes, as measured by the SAMS peptide assay [216]. We sought to determine whether PMA had the same effect in C2C12 myotubes, and if so, whether it is mediated by an inhibitory

phosphorylation on AMPK's α1/α2 subunit at $Ser^{485/491}$. All doses of PMA (10-100nM) significantly increased serine phosphorylation of AMPK (Figure 1A, 1B), as did PMA treatments of 30min-3h (50nM) (Figure 5.1C, 5.1D). Increases in overall PKC activity, as measured by serine phosphorylation of PKC substrates (data not shown), mirrored the increases in phosphorylation of AMPK $Ser^{485/491}$ (Figure 5.1D). Phosphorylation of PKD at Ser^{916} and $Ser^{744/748}$ of its activation loop were also significantly increased by PMA at doses of 25nM or higher and treatments of 30min-3h (Figure 5.1A-D). Since phosphorylation of these two sites changed similarly in response to all treatments, only Ser^{916} phosphorylation will be shown in subsequent experiments. Notably, no significant changes in phosphorylation of AMPK Thr^{172} (Figure 5.1A, 5.1C) or its downstream substrate ACC Ser^{79} were observed. Phosphorylation of conventional PKC isoforms, as measured by p-PKC (pan) (βII Ser660), was increased in a dose-response manner; however, activation of the atypical PKCι was not increased by PMA treatment (Supplemental Figure 5.12).

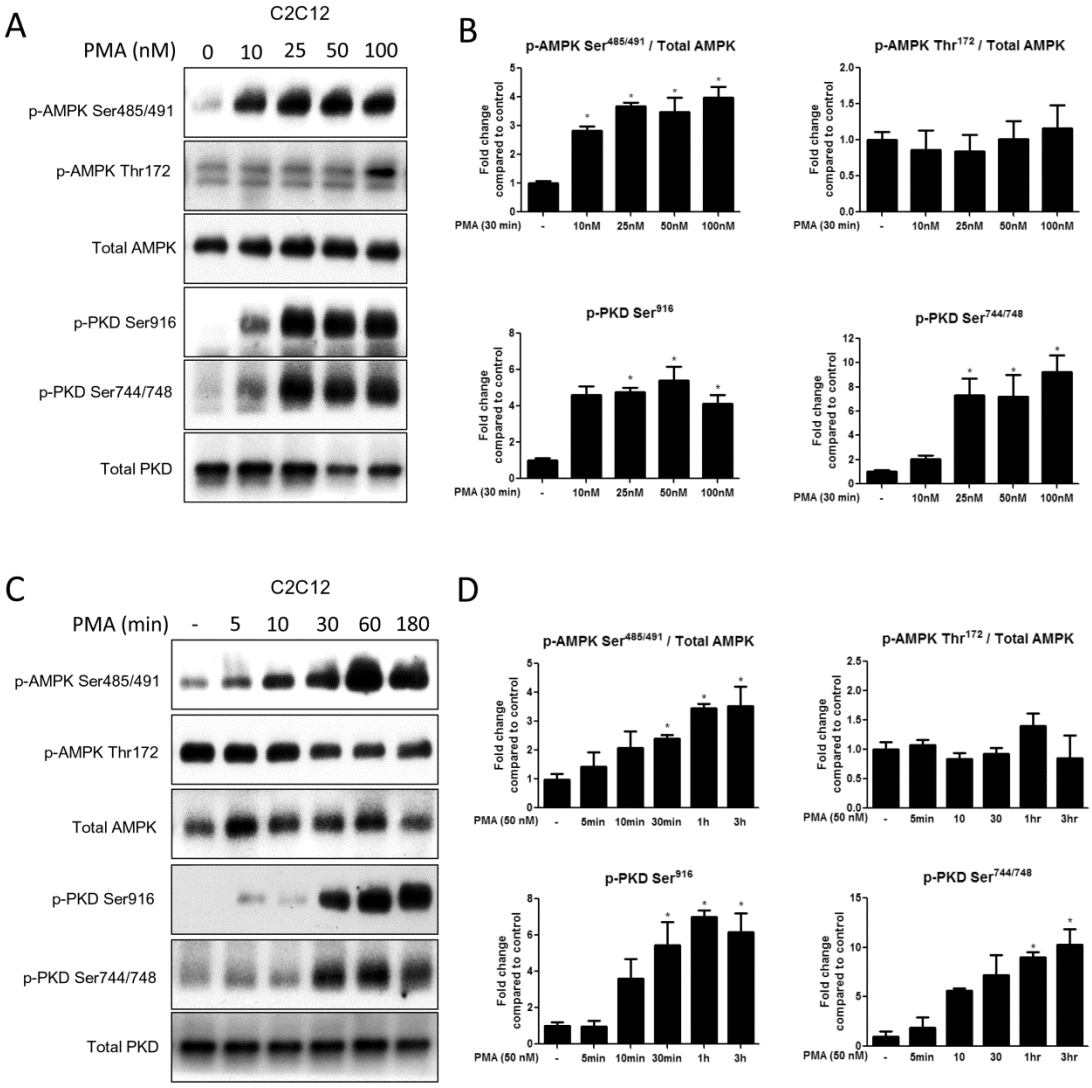


Figure 5.1: PMA treatment time- and dose-dependently stimulates phosphorylation of AMPK at Ser$^{485/491}$ in C2C12 myotubes.

Myotubes were treated with 10-100nM PMA for 30min (A, B) or 50nM PMA for 5-180min (C, D). Cells were lysed and protein expression and phosphorylation were analyzed by western blot. Representative western blots (A, C) and their densitometric analyses (B, D) are shown. Results are means ± SE (*n* = 3–6 per treatment). All experiments were performed in triplicate. *P<0.05 compared to control.

The non-selective PKC inhibitor Gö6983 prevented PMA-induced phosphorylation of AMPK Ser$^{485/491}$

To determine whether the increase in phosphorylation of AMPK at Ser$^{485/491}$ was attributable to PKC activation, we used the non-selective PKC inhibitor Gö6983. Treatment of C2C12 myotubes with Gö6983 (5μM) for 1h prior to PMA treatment (50nM, 30min) significantly attenuated phosphorylation of AMPK at Ser$^{485/491}$ (Figure 5.2A, 5.2B). Phosphorylation of AMPK Thr^{172} (Figure 5.2A) was not affected by PMA treatment, but it was increased by the inhibitor. We found that overall PKC activity was decreased by the inhibitor (Figure 5.2A), as was phosphorylation of conventional PKC isoforms, as evaluated by p-PKC (pan) (βII Ser660), and p-PKD Ser^{916} (Figure 5.2A, 5.2B), but not p-PKC δ/θ (Ser643/676).

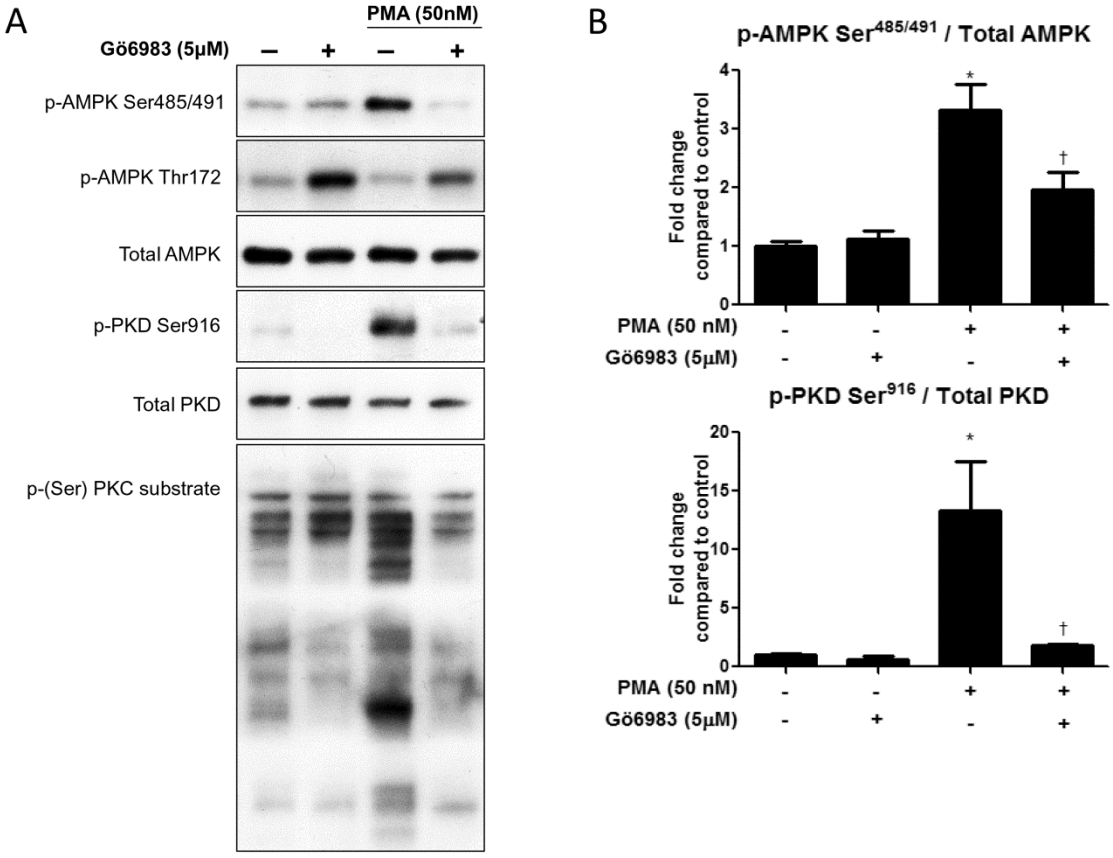


Figure 5.2: PKC inhibition prevents PMA-induced phosphorylation of AMPK Ser$^{485/491}$ in C2C12 myotubes.

Myotubes were treated with the non-specific PKC inhibitor Gö6983 (5μM) for 1h prior to PMA treatment (50nM, 30min). Cells were lysed and protein expression and phosphorylation were analyzed by western blot. Representative western blots (A) and their densitometric analyses (B) are shown. Results are means ± SE (*n* = 3–6 per treatment). All experiments were performed in triplicate. *P<0.05 compared to control, † p<0.05 compared to PMA treatment.

The selective PKD-1 inhibitor CRT0066101 prevented PMA-induced phosphorylation of AMPK Ser$^{485/491}$

Since phosphorylation of the activation loop of PKD at Ser$^{744/748}$ and at Ser^{916} (Figures 5.1, 5.2, Supplemental Figure 5.10) corresponded with changes in phosphorylation of AMPK at Ser$^{485/491}$, we investigated whether specific inhibition of PKD could attenuate PMA-induced serine phosphorylation of AMPK. We found that pre-treatment for 1h with the specific PKD inhibitor CRT0066101 (10μM) prevented phosphorylation of AMPK Ser$^{485/491}$ in C2C12 cells (Figure 5.3A, 5.3B, see Supplemental Figure 5.11 for dose response). Phosphorylation of AMPK at Thr^{172} was unchanged by treatment with this inhibitor (Figure 5.3A). As expected, PKD Ser^{916} phosphorylation was ablated by CRT0066101 treatment, and probing with a phospho-(Ser/Thr) PKD substrate antibody revealed an overall increase in number of bands and band intensity with PMA treatment, which was prevented by pretreatment with CRT0066101 (Figure 5.3A). Bands detected by this antibody at 110kDa and 62kDa may represent PKD (which is autophosphorylated at Ser^{916}) and AMPK, respectively.

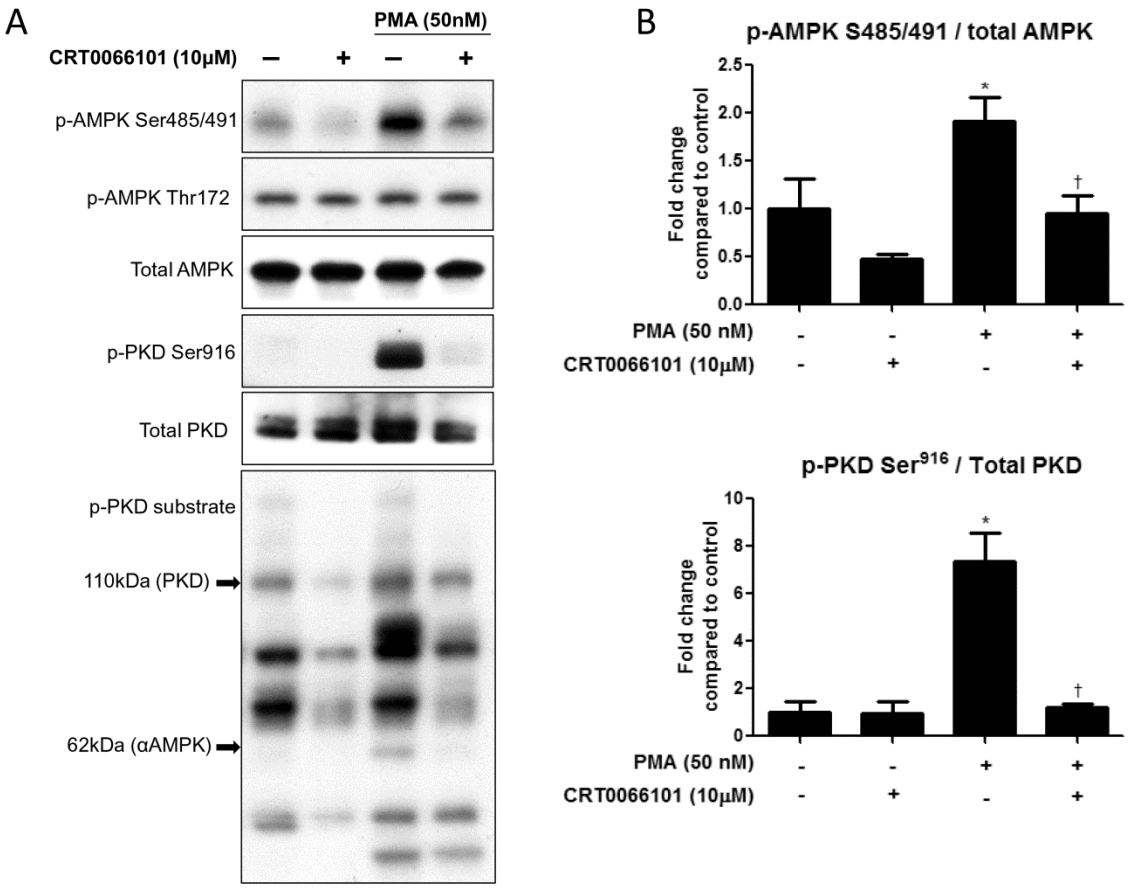


Figure 5.3: PKD inhibition prevents PMA-induced phosphorylation of AMPK Ser$^{485/491}$ in C2C12 myotubes.

Myotubes were treated with the specific PKD inhibitor CRT0066101 (10μM) for 1h prior to PMA treatment (50nM, 30min). Cells were lysed and protein expression and phosphorylation were analyzed by western blot. Representative western blots (A) and their densitometric analyses (B) are shown. Results are means ± SE (*n* = 3–6 per treatment). All experiments were performed in triplicate. *P<0.05 compared to control, † p<0.05 compared to PMA treatment.

PMA treatment inhibited AMPK activity, which was attenuated by pretreatment with Gö6983 or CRT0066101

Recent studies have shown that phosphorylation of AMPK $Ser^{485/491}$ can inhibit its activity, even in the absence of diminished Thr^{172} phosphorylation, which is often used as a surrogate readout for enzyme activity [59,239]. We sought to determine whether the changes in phosphorylation of AMPK $Ser^{485/491}$ caused by PMA and the PKC inhibitors Gö6983 and CRT0066101 corresponded to changes in AMPK activity. Using the SAMS peptide assay described in the methods section, we found that PMA treatment significantly decreased α2 AMPK activity in C2C12 myotubes (Figure 5.4A, 5.5A). Furthermore, we found that pretreatment with the non-selective PKC inhibitor Gö6983 significantly attenuated the reduction in enzyme activity (Figure 5.4A), while the specific PKD inhibitor CRT0066101 completely prevented the PMA-induced decrease in AMPK activity (Figure 5.5A). Despite the lack of changes in phosphorylation of AMPK Thr^{172} or ACC Ser^{79}, we found a significant inverse correlation between AMPK activity and phosphorylation at $Ser^{485/491}$ (Figure 5.4B, 5.5B).

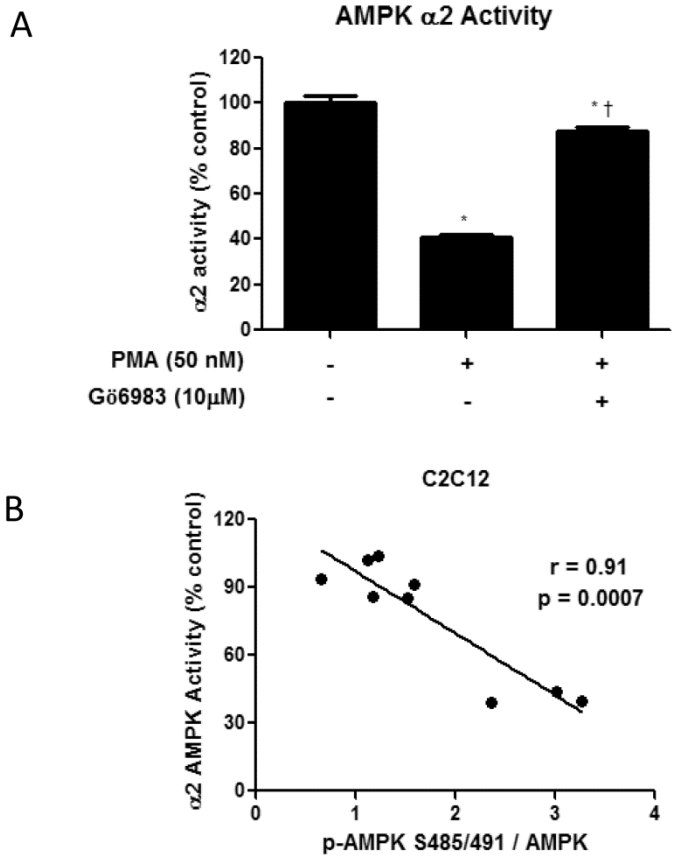


Figure 5.4: PMA treatment diminishes AMPK activity in C2C12 myotubes, while PKC inhibition prevents this decrease.

C2C12 myotubes were treated with the non-specific PKC inhibitor Gö6983 (5μM) for 1h prior to PMA treatment (50nM, 30min). AMPKα2 activity was measured by the SAMS peptide assay (A). (B) shows the correlation between AMPKα2 activity and $Ser^{485/491}$ phosphorylation. Results are means ± SE (*n* = 3–6 per treatment). *P<0.05 compared to control, † p<0.05 compared to PMA treatment.

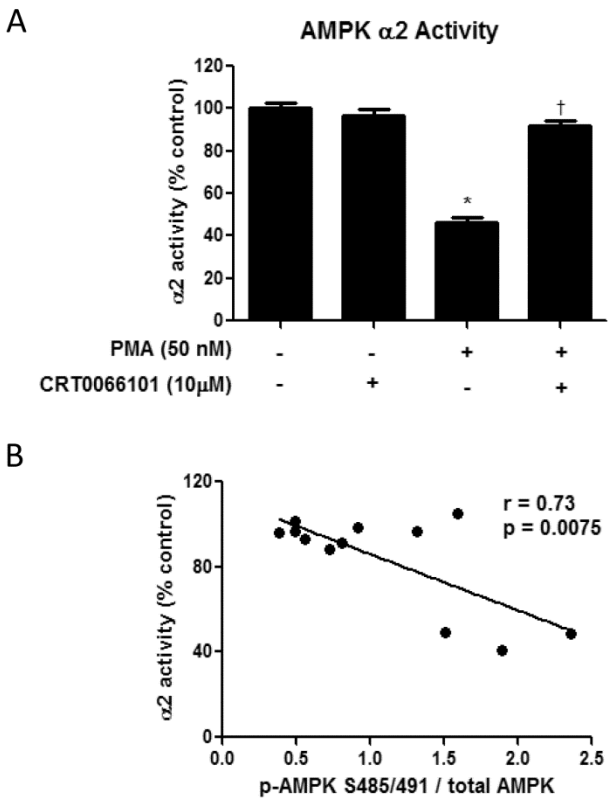


Figure 5.5: PMA treatment diminishes AMPK activity in C2C12 myotubes, while PKD inhibition prevent this decrease.

C2C12 myotubes were treated with the specific PKD inhibitor CRT0066101 (10μM) for 1h prior to PMA treatment (50nM, 30min). AMPKα2 activity was measured by the SAMS peptide assay (A). (B) shows the correlation between AMPKα2 activity and $Ser^{485/491}$ phosphorylation. Results are means ± SE (*n* = 3–6 per treatment). *P<0.05 compared to control, † p<0.05 compared to PMA treatment.

Inhibition of Akt, P70S6K, or ERK did not prevent PMA-induced phosphorylation of AMPK Ser$^{485/491}$

As previously mentioned, the kinases Akt, P70S6K, and ERK1/2 have been shown to phosphorylate AMPK at Ser$^{485/491}$ in response to various stimuli in other cell types. To rule out involvement of these kinases in PMA-induced phosphorylation of AMPK Ser$^{485/491}$, we used inhibitors of each of these kinases. Pretreatment of C2C12 myotubes with the PI3K/Akt inhibitor wortmannin (100nM) did not affect PMA-stimulated serine phosphorylation of AMPK (Figure 5.6A). As expected, PMA treatment did not increase Akt phosphorylation, but p-Akt Ser^{473} phosphorylation was diminished by wortmannin treatment. Next, we used the mTOR inhibitor rapamycin to test whether P70S6K was involved. Although P70S6K Thr^{389} phoshphorylation was increased by PMA treatment, rapamycin (100nM) treatment did not did not affect PMA-induced phosphorylation of AMPK Ser$^{485/491}$ (Figure 5.6B). Similarly, pretreatment with the ERK inhibitor U0126 (5μM) had no effect on AMPK serine phosphorylation (Figure 5.6C), despite inhibiting ERK phosphorylation, which can occur downstream of PKD.

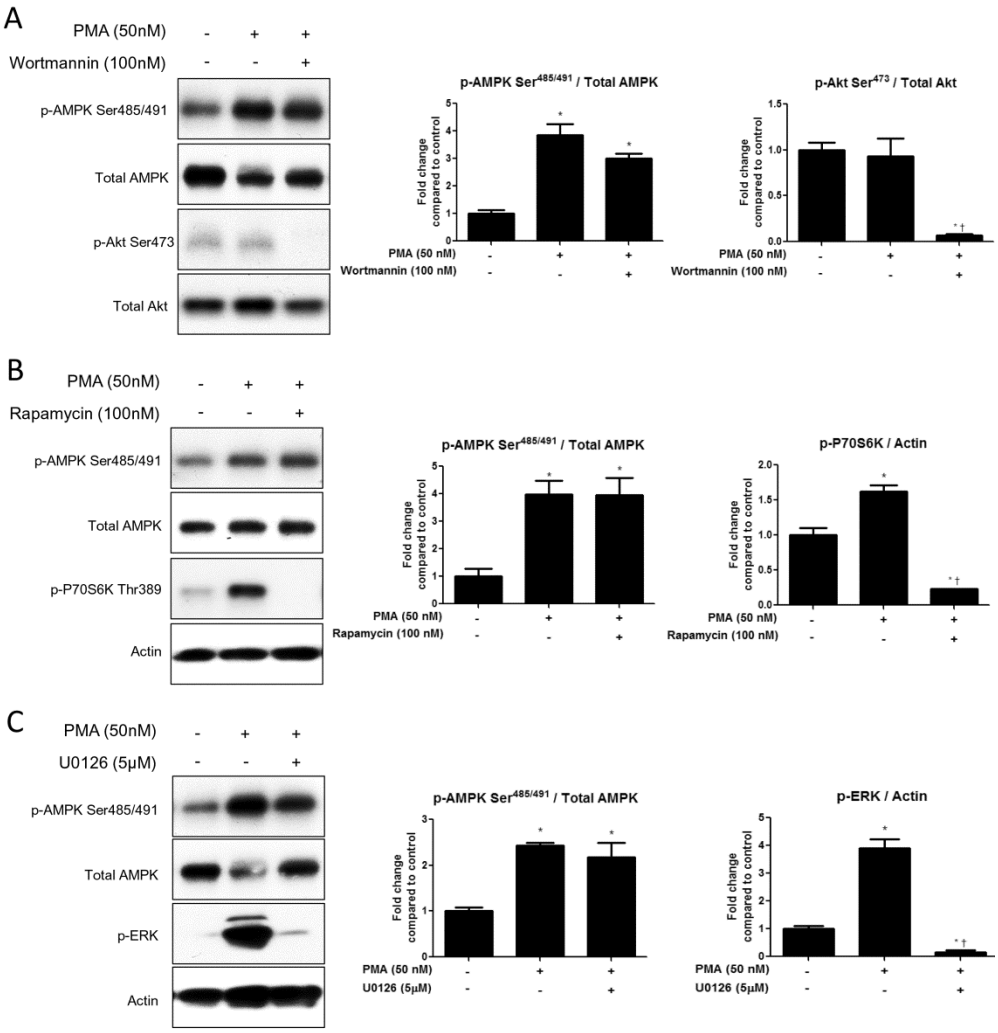


Figure 5.6: Inhibition of Akt, S6K, or ERK does not prevent PMA-induced phosphorylation of AMPK Ser$^{485/491}$. Myotubes were treated with the PI3K/Akt inhibitor wortmannin (100nM) (A), the mTOR/P70S6K inhibitor rapamycin (100nM) (B), or the ERK inhibitor U0126 (5μM) (C) for 2h prior to PMA treatment (50nM, 30min). Cells were lysed and protein expression and phosphorylation were analyzed by western blot. Representative western blots and their densitometric analyses are shown. Results are means ± SE (n = 3–6 per treatment). All experiments were performed in triplicate. *P<0.05 compared to control, † p<0.05 compared to PMA treatment.

PMA treatment inhibited insulin signaling through Akt, which was attenuated by pretreatment with Gö6983 or CRT0066101

In order to test whether the PMA-induced changes we were seeing are associated with impaired insulin signaling, we measured insulin-stimulated Akt phosphorylation. PMA treatment (50nM) for 30min diminished insulin-stimulated Akt Ser^{473} phosphorylation (Figure 5.7A, 5.7B). Pretreatment with Gö6983 or CRT0066101 restored insulin signaling through Akt. Since both insulin and PMA independently stimulate AMPK $Ser^{485/491}$ phosphorylation through Akt and PKD, respectively, all combinations of insulin and PMA with and without inhibitors significantly increased serine phosphorylation of AMPK (Figure 5.7A, 5.7B). PMA treatment with insulin did not further increase $Ser^{485/491}$ phosphorylation beyond that of insulin alone, suggesting that the dose (100nM) and time (15min) of insulin treatment stimulates maximal serine phosphorylation of AMPK in C2C12 cells.

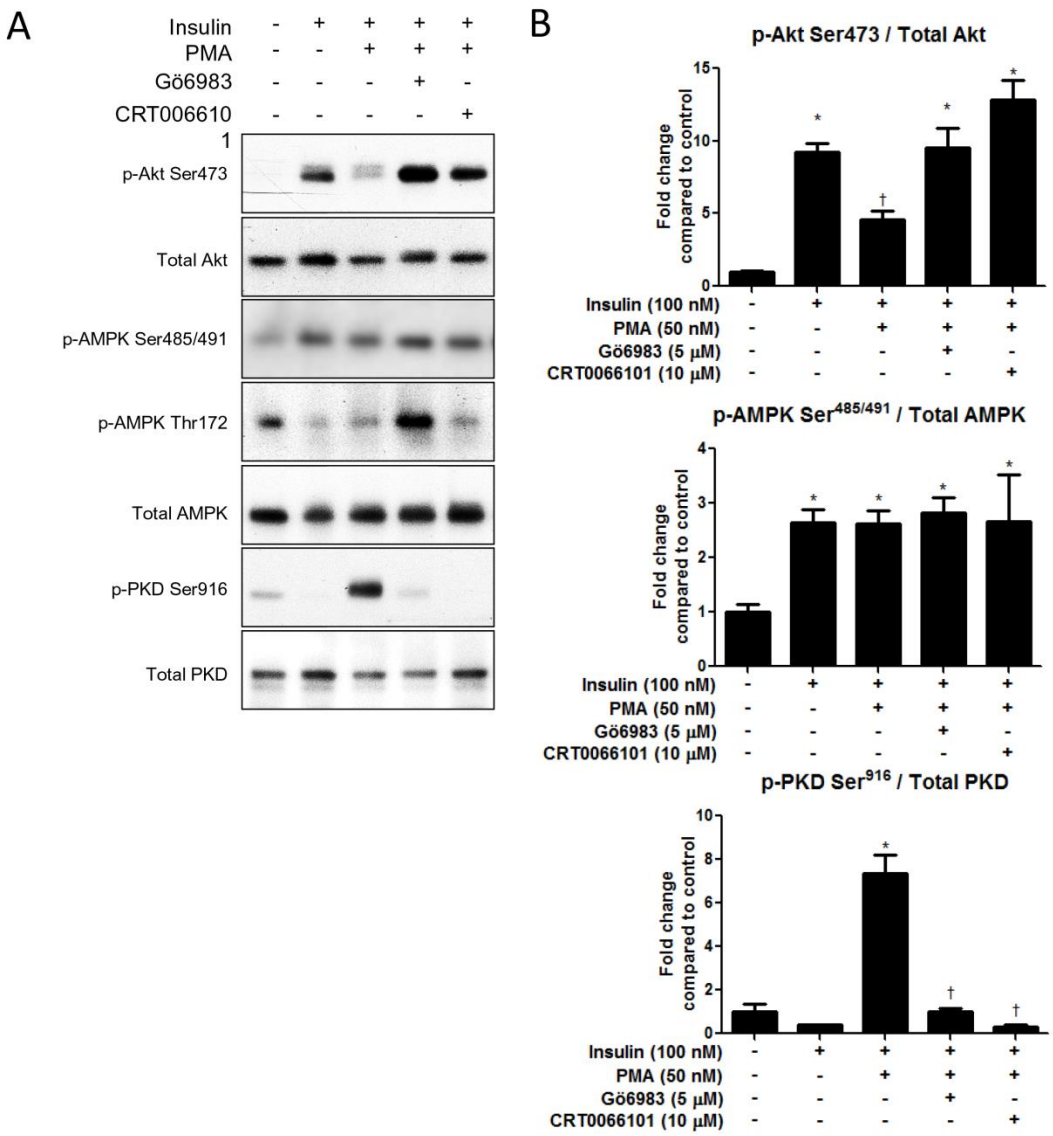


Figure 5.7: PMA treatment impairs insulin signaling through Akt, which is prevented by PKC and PKD inhibition.

C2C12 myotubes were treated with or without Gö6983 (5μM) or CRT0066101 (10 μM) for 1h prior to PMA treatment (30min), followed by insulin stimulation (100nM, 15min). Cells were lysed and Akt, AMPK, and PKD phosphorylation were analyzed by western blot. Representative blots (A) and quantifications (B) are shown. *P<0.05 compared to control, † p<0.05 compared to insulin treatment

Recombinant PKD phosphorylated AMPKα2 at Ser^{491} in cell-free conditions

To determine whether PKD phosphorylates AMPK directly or if a molecule downstream of PKD is required, we tested whether recombinant PKD phosphorylates AMPK in a cell-free system. Compared to AMPKα2 alone, co-incubation of recombinant PKD with AMPKα2 caused phosporylation of AMPKα2 at Ser^{491} (Figure 5.8A). Incubation of Akt, used as a positive control, with AMPK also caused an obvious increase in Ser^{491} phosphorylation. This data suggests that PKD phosphorylates AMPK directly. To evaluate whether AMPKα2 Ser^{491} is a good substrate for PKD, we lined up the PKD substrate consensus sequence with the sequence of residues preceding Ser^{491} on AMPKα2. Although AMPKα2 has a proline rather than PKD's preferred leucine at the -5 position, the arginine at the -3 position does line up (Figure 5.8B). This is similar to Akt, which prefers arginine at both the -5 and -3 positions.

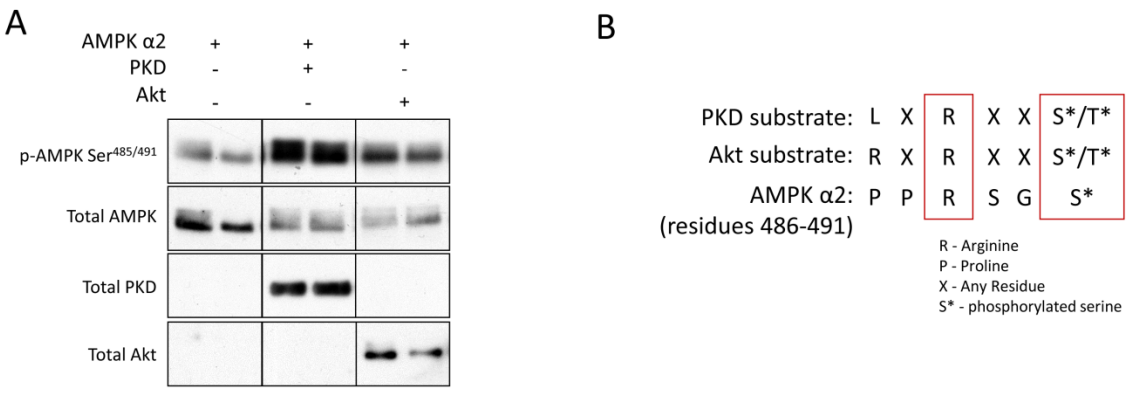


Figure 5.8: Recombinant PKD1 phosphorylates AMPK at Ser$^{485/491}$ in a cell-free assay.

Recombinant AMPKα2/β1/ϒ1 was incubated with recombinant PKD1 or recombinant Akt as described in the methods section. Phosphorylation of AMPK at Ser^{491} was evaluated by western blot (A). The consensus substrate motifs of PKD and Akt were lined up with residues 486-491 of AMPKα2 (B). n=3 per group. All experiments were performed in triplicate.

Discussion

A better understanding of how AMPK is regulated at a molecular level is important for improving drug-targeting strategies for T2D. Phosphorylation of AMPK at Thr^{172} of the activation loop is essential for the activation of AMPK and is often used as readout for activity. However, our data adds to a growing number of studies showing that phosphorylation of AMPK at $Ser^{485/491}$ can inhibit AMPK activity, even in the absence of changes in p-AMPK Thr^{172}. Although p-AMPK Thr^{172} is often used as a measure of AMPK activity, these data suggest that phosphorylation of AMPK at $Ser^{485/491}$ can inhibit AMPK activity independent of changes in p-AMPK Thr^{172} and that inhibition of p-AMPK Thr^{172} is not required for $Ser^{485/491}$ phosphorylation to induce inhibition of AMPK activity. We show, for the first time, that PKD1 activation can stimulate phosphorylation of AMPK $Ser^{485/491}$ in skeletal muscle cells, resulting in impaired insulin signaling. Not only does this work identify a novel kinase that can inhibit AMPK by serine phosphorylation at this site, but it also presents a possible mechanism by which AMPK may be inhibited by excess nutrients since DAG (which activates PKD) is known to be activated by high glucose and lipids.

In 2012, Tsuchiya et al. [216] demonstrated that 60min PMA treatment (1μM) inhibits AMPK activity by approximately 33% in cardiac myocytes, as measured by the SAMS peptide assay. They found that this inhibition could be prevented by the non-specific PKC inhibitor bisindoylmaleimide I (BIM I) or the ERK inhibitor U0126, which led them to conclude that PMA inhibits AMPK by a mechanism involving the PKC and ERK pathways. There was no apparent change in energy state (AMP/ATP ratio)

accompanying this decrease in AMPK activity. To our knowledge, this was the first report of PMA treatment causing inhibition of AMPK; however, AMPK phosphorylation at Thr^{172} or $Ser^{485/491}$ was not measured. In concert with these results, we found that PMA treatment significantly diminished AMPK activity in C2C12 myotubes (Figure 3A, 3C). However, we found that a dose of only 50nM for 30min resulted in a 50-60% decrease in AMPK activity, nearly twice as much as was seen in the cardiac myocytes. This discrepancy could be due to differences in cell type or methodologies.

Several kinases have been reported to phosphorylate AMPK at $Ser^{485/491}$. We [239] and others [54] have shown that Akt phosphorylates and inhibits AMPK in muscle, liver, and heart in response to insulin. In the hypothalamus, the mTOR/p70S6 kinase pathway has been reported to cause this inhibitory event in response to leptin [59], and this signaling mechanism was shown to be essential for mediating the anorectic effects of leptin. In murine macrophage-like RAW 264.7 cells, Park and colleagues [257] showed that IKKβ phosphorylates AMPK at Ser^{485} in response to LPS treatment. ERK1/2 was recently shown to phosphorylate AMPK on this serine residue in mature dendritic cells [258]; this may be important mechanism by which CCR7 signaling promotes cell survival. Furthermore, protein kinase A (PKA) has been reported to act as an upstream kinase for AMPK $Ser^{485/491}$ in INS-1 cells in response to forskolin or GIP stimulation [56] and in human diploid fibroblasts in response to lysophosphatidic acid [259]. In our studies, we ruled out Akt, S6K and ERK as upstream kinases responsible for AMPK $Ser^{485/491}$ phosphorylation in response to PMA through the use of specific inhibitors of each enzyme (Figure 4). However, we found that pretreatment with the non-selective

PKC inhibitor Gö6983 significantly attenuated the decrease in AMPK activity, while the PKD inhibitor CRT0066101 completely prevented this inhibition (Figure 3A, 3C). Furthermore, we found a significant inverse correlation between AMPK activity and phosphorylation at $Ser^{485/491}$ under these conditions, suggesting that PKD1 is responsible for the serine phosphorylation and inhibition of AMPK in this setting (Figure 3B, 3D). The fact that recombinant PKD1 phosphorylates AMPKα2 at Ser^{491} in cell-free conditions supports our hypothesis that PKD1 is a direct upstream kinase of this site (Figure 5.7).

Many of the aforementioned kinases that can mediate AMPK inhibition through $Ser^{485/491}$ phosphorylation are associated with anabolic or pro-hypertrophic signaling pathways (Akt, mTOR/P70S6K). This is not surprising, since AMPK is generally activated under low energy conditions that favor catabolic processes, and thus inhibited under conditions of energy overload that favor anabolic processes. Consistent with this, PKD has recently been identified as having pro-hypertrophic functions. For example, Harrison et al. [224] reported that transgenic mice expressing a cardiac-specific, constitutively active PKD mutant develop cardiac hypertrophy, followed by ventricular chamber dilation, wall thinning, and contractile dysfunction. They also showed that PKD1 expression and activity are significantly increased in the myocardium of spontaneously hypertensive heart failure rats, an effect which is further exaggerated by thoracic aortic banding. Furthermore, PKD1 activation was found to be elevated in hearts of humans with idiopathic dilated cardiomyopathy [222]. While these results suggest that PKD1 activation may be sufficient to induce pathological cardiac remodeling, the role of

PKD1 in skeletal muscle is less clear. McGee et al. [228] reported that loss of AMPK in muscle resulted in no change in HDAC5 phosphorylation during exercise, but a ~33% compensatory increase in PKD activation, suggesting potential redundancies in the functions of these two proteins in response to exercise. Although they did not investigate the effects of PKD loss or activation on AMPK activation, these data, together with our results, suggest that PKD and AMPK may negatively regulate each other in skeletal muscle.

Further evidence supporting potential redundancy of functions between AMPK and PKD1, at least in regards to HDAC5 phosphorylation in response to exercise, is the fact that mice overexpressing constitutively active PKD1 in skeletal muscle show resistance to fatigue in response to repetitive contraction and a shift to Type I and Type IIa muscle fibers [225], while expression of a dominant negative kinase dead PKD1 in skeletal muscle results in impaired running performance and prevents fiber type switching [262]. Similarly, it has long been known that AMPK mediates many adaptations of muscle to exercise [72], and loss of AMPK in muscle causes impairments in running capacity [73].

Our studies showed that PKD1 activation impaired insulin-signaling through Akt in C2C12 myotubes, which was prevented by PKD inhibition. Though we have not shown a direct role for AMPK inhibition being responsible this impairment, it is consistent with the fact that AMPK activation is known to promote insulin sensitivity [263]. This is the first report of PKD1 activation causing impaired insulin-signaling, and the mechanism by which it does so requires further investigation.

We report a novel inhibitory relationship between PKD1 and AMPK in skeletal muscle cells whereby PKD inhibits AMPK directly by phosphorylating it at $Ser^{485/491}$ of the $\alpha1/\alpha2$ subunit. Since PKD1 may be activated directly by DAG (or the DAG mimetic PMA) or by novel PKC isoforms, we have not ruled out the possibility that novel PKC isoforms may also be involved upstream of PKD1 in these processes. Similarly, though we have shown that PKD1 activation is necessary for the inhibition of AMPK and the impairment of insulin signaling, further studies are required to determine whether $Ser^{485/491}$ phosphorylation of AMPK is essential for mediating these impairments in insulin signaling. Our data suggest that PKD1 inhibition may be a novel strategy for preventing AMPK inhibition and impaired insulin sensitivity in muscle; however, based on the growing list of PKD1 functions in a variety of tissues being reported in the literature, this strategy would have to be approached with caution, and likely in a highly controlled, tissue specific manner.

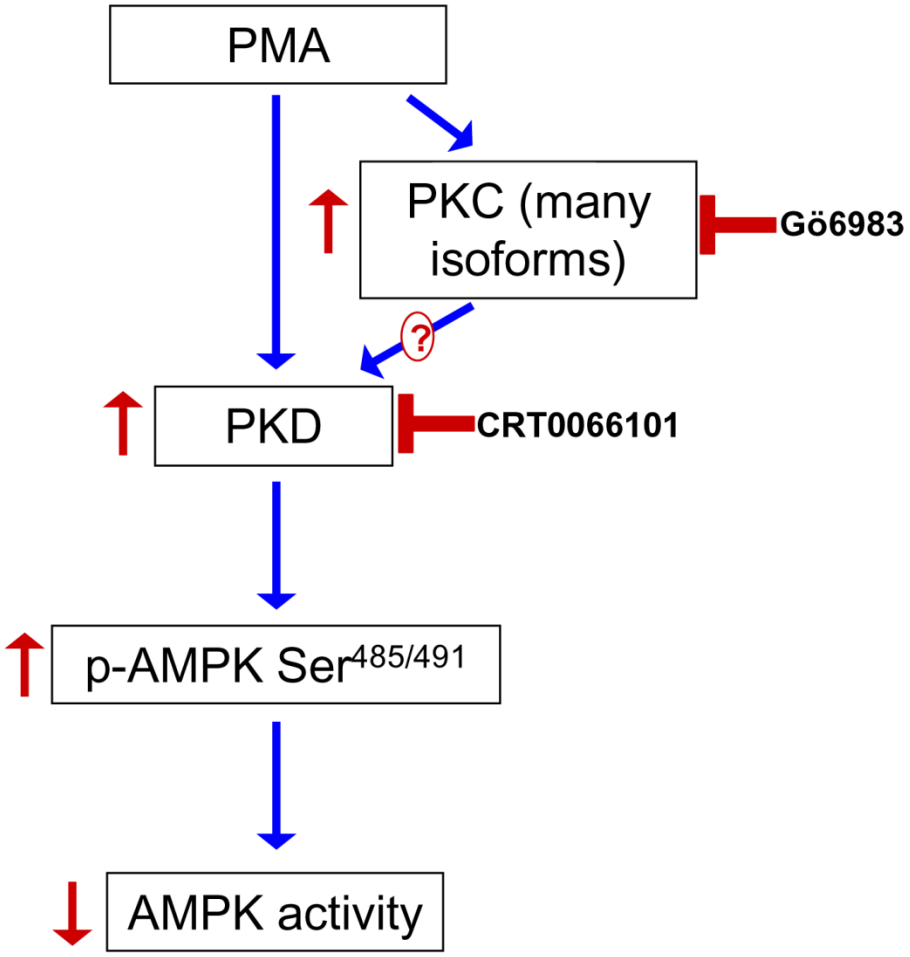
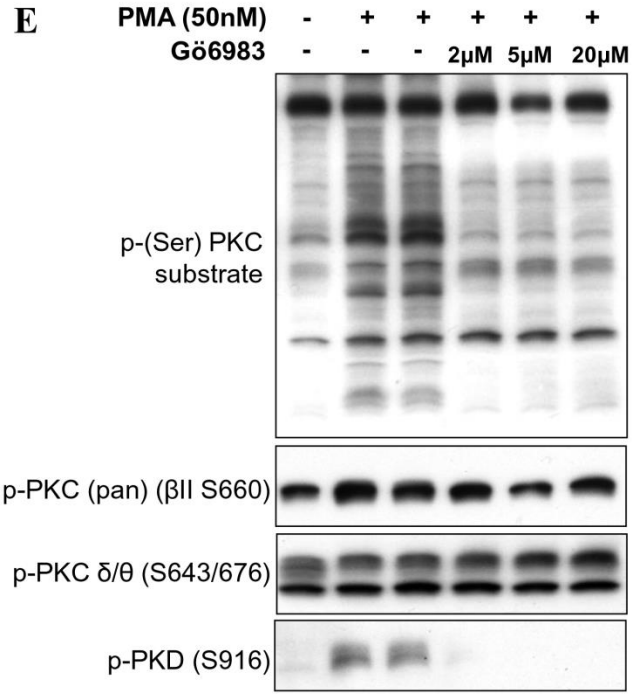


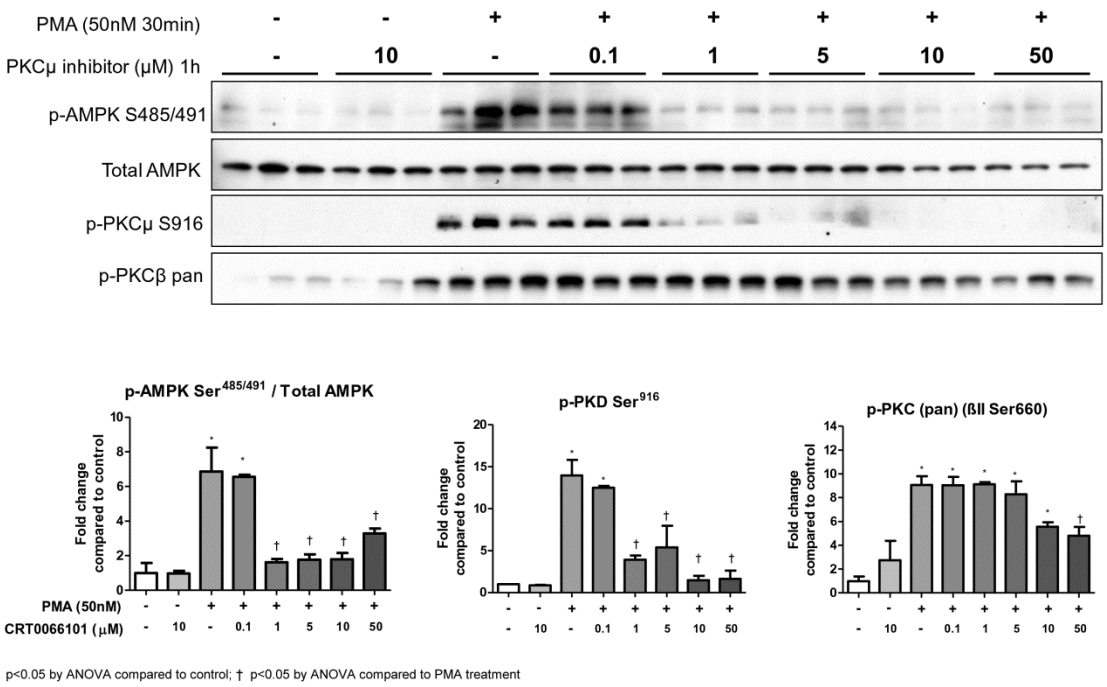
Figure 5.9: Summary of Chapter 5

In C2C12 myotubes, the DAG mimetic PMA stimulated phosphorylation of AMPK at $Ser^{485/491}$ and reduced its activity, as measured by the SAMS peptide assay. This phosphorylation and inhibition were prevented by non-specific PKC inhibition and specific PKD inhibition, suggesting that PKD is responsible for phosphorylation and inhibition through $Ser^{485/491}$ phosphorylation.



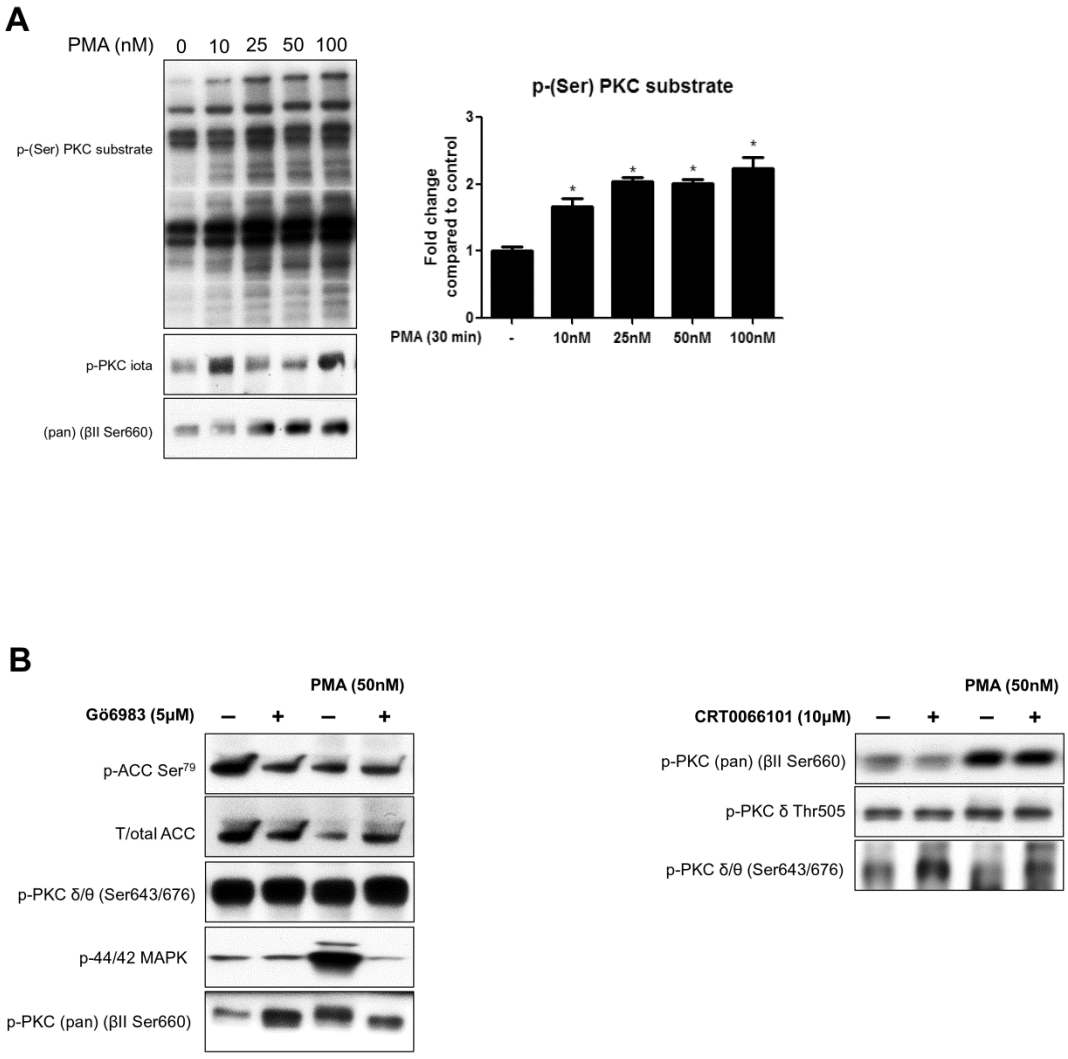
Supplemental Figure 5.10

C2C12 myotubes were treated with 2-20μM Gö6983 for 1h prior to stimulation with 50nM PMA (30min). Cells were lysed and analyzed by western blot.



Supplemental Figure 5.11

C2C12 myotubes were treated with 0.1-50μM CRT0066101 for 1h prior to stimulation with 50nM PMA (30min). Cells were lysed and analyzed by western blot. *P<0.05 compared to control, † p<0.05 compared to PMA treatment.



Supplemental Figure 5.12

Myotubes were treated with 10-100nM PMA for 30min. Serine phosphorylation of PKC substrates, p-PKC (pan) (βII Ser660), and p-PKCι Thr555/p-PKCλ Thr563 were measured by western blot (A). Phosphorylation of PKCδ/θ Ser643/676, ACC Ser79, PKC (pan) (βII Ser660) were measured in myotubes treated with PMA (50nM, 30min) and the non-specific PKC inhibitor Gö6983 (5μM) or the specific PKD inhibitor CRT0066101, as indicated (B). Representative western blots are shown. Results are means ± SE (*n* = 3–6 per treatment). All experiments were performed in triplicate.

CHAPTER 6: Physiological Regulation of AMPK Ser$^{485/491}$

Introduction

The previous chapters have shown that both incubation of rat EDL muscle and infusion of glucose *in vivo* inhibited AMPK activity and caused inhibitory phosphorylation of AMPK at Ser$^{485/491}$. We have also shown that phosphorylation of this site inversely correlates with AMPK activity and that Akt and PKD1 can act as upstream kinases of this inhibitory post-translational modification. Our next aim was to evaluate the regulation of AMPK Ser$^{485/491}$ in the insulin-responsive tissues muscle and liver in response to physiological (fasting/refeeding) and pathological (obesity, T2D) changes in nutrition status. Phosphorylation of AMPK's activation site Thr^{172} has been studied in detail in these settings; however, changes in inhibitory serine phosphorylation of AMPK and potential upstream mediators have not been studied. Since we have shown in the previous chapters that AMPK phosphorylation at Ser$^{485/491}$ can inhibit AMPK activity independently of changes in p-AMPK Thr^{172} and that inhibition of p-AMPK Thr^{172} is not required for Ser$^{485/491}$ phosphorylation to induce inhibition of AMPK activity, a better understanding of how Ser$^{485/491}$ is regulated in response to a variety of stimuli could provide important insight into novel strategies and conditions for therapeutic AMPK activation. In the present studies, we investigated the activation of Akt and PKD1 in EDL incubated in high glucose and in muscle of glucose infused rats to determine whether these kinases may be playing a role in the phosphorylation of AMPK Ser$^{485/491}$ in these settings. We also investigate the regulation of AMPK Ser$^{485/491}$, Akt, and PKD1 in mice fasted overnight vs refed for 2h and in db/db mice, which are obese and insulin resistant.

Results

DAG content and PKD1 activity, but not Akt, were increased in EDL incubated with 25mM glucose

In order to determine whether Akt or PKD1 were responsible for the phosphorylation of AMPK $Ser^{485/491}$ observed in EDL incubated with high glucose for 1 and 2h, we measured activation of these proteins by western blot. There were no significant changes in p-Akt Ser^{473} at any timepoint, but there was a trend toward increased Akt phosphorylation at 1h (p=0.12) (Figure 6.1A, 6.1C) As previously noted, no insulin was added to the incubation medium; however, the PI3K/Akt pathway can be activated by other signaling molecules, such as growth or survival factors. We also found that DAG content was significantly increased in the EDL following 1h incubation with 25mM glucose (Figure 6.1B), suggesting activation of PKC/PKD signaling. Since the antibodies directed toward p-PKD Ser^{916} and $Ser^{744/748}$ did not work in rat tissue, we measured phosphorylation of PKD serine substrates as a readout of PKD activity. Phosphorylation of PKD substrates showed a band at 110kDa, likely representing the autophosphorylation of PKD1 at Ser^{916}, which was initially diminished at 0.5h in 25mM glucose, returned to basal levels at 1h, and was significantly increased following 2h in high glucose.

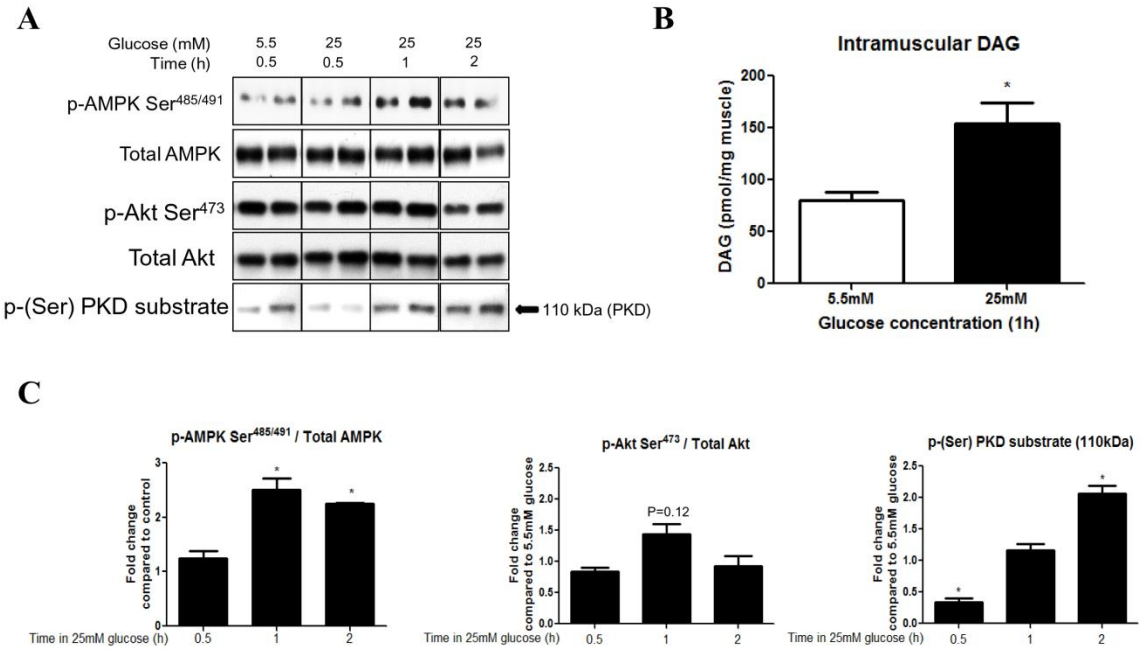


Figure 6.1: DAG content and PKD activation are increased in EDL incubated in high glucose

EDL muscles were incubated in 25mM glucose (no insulin) for 0.5-2h. Muscles were analyzed by Western blot. Representative blots (A) were analyzed by densitometry (C). Intramuscular DAG content was also measured (B). *p<0.05 compared to 5.5mM glucose at each time point.

mTOR inhibition did not affect AMPK phosphorylation in response to high glucose, but PKD1 inhibition did prevent the phosphorylation of AMPK at Ser$^{485/491}$

To confirm that the mTOR/P70S6K pathway was not responsible for the high glucose-induced phosphorylation of Ser$^{485/491}$, we incubated EDL with the mTOR inhibitor rapamycin for 0.5h prior to incubation with 25mM glucose for 1h. Rapamycin prevented neither the decrease in AMPK Thr^{172} nor the increase in Ser$^{485/491}$ phosphorylation, though it did prevent the high glucose-induced increase in mTOR Ser^{2448} phosphorylation (Figure 6.2). However, when the EDL was pretreated with the PKD1 inhibitor CRT0066101 for 1h prior to incubation with high glucose for 2h, the phosphorylation of AMPK at Ser$^{485/491}$ was prevented (Figure 6.3).

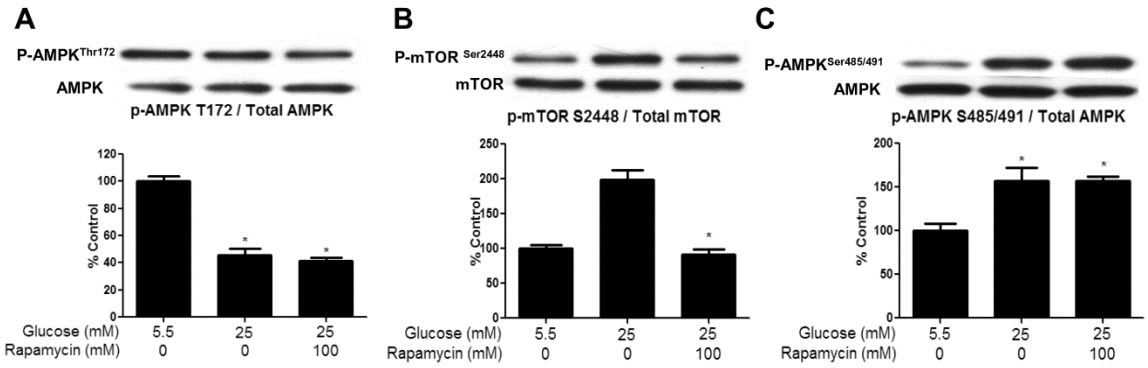


Figure 6.2: Inhibition of glucose-induced mTOR/p70S6K phosphorylation does not affect AMPK phosphorylation.

EDL were preincubated in the presence of rapamycin (100μM) for 0.5h and then with 5.5 or 25 mM glucose for 1hr. Muscle lysates were analyzed for P-AMPK Thr^{172} (A), P-mTOR Ser^{2448} (B) and AMPK Ser$^{485/491}$ (C) by western blot. Results show quantification of western blots by densitometry. Results are means ± SE (n = 5). *, $p<0.05$ compared to values for 5.5 mM glucose.

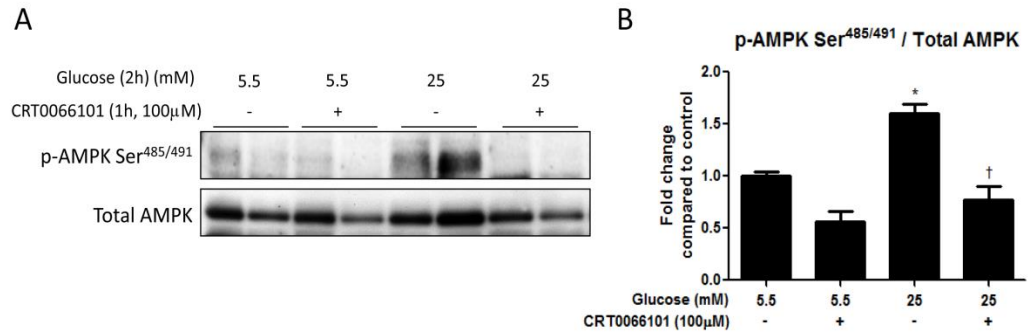


Figure 6.3: PKD1 inhibition prevented high glucose-induced phosphorylation of AMPK at Ser$^{485/491}$ in rat EDL.

EDL muscle was incubated with 5.5mM glucose with or without 100uM CRT0066101 prior to incubation with 5.5mM or 25mM glucose for 2h. Phosphorylation of AMPK Ser485/491 was measured by western blot. Representative blot (A) and densitometric analysis (B) are shown. Graph shows mean +/- SEM. *$p<0.05$ compared to incubation with 5.5mM glucose. N=4/group

Akt and PKD1 were increased by glucose infusion in vivo

Next, we sought to determine whether Akt and PKD1 were activated in the muscle of glucose infused rats, where we had previously showed that AMPK Ser$^{485/491}$ phosphorylation was increased at 5 and 8h (Figure 3.5). We found that Akt Ser^{473} phosphorylation was significantly increased at all timepoints (Figure 6.4). Interestingly, we found that phosphorylation of PKD substrates at 110kDa, likely representing the autophosphorylation of PKD1 at Ser^{916} was initially unchanged, although there was a trend towards being decreased at 3h, and subsequently increased and was significantly elevated following 8h of glucose infusion.

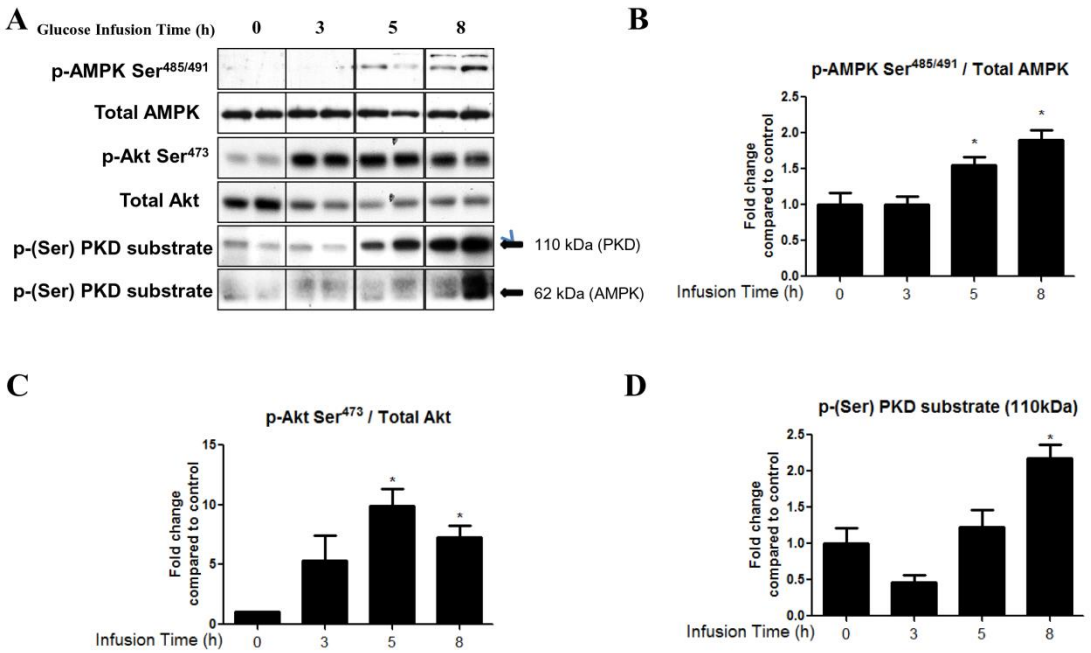


Figure 6.4: Akt and PKD1 activation are increased in muscle of glucose infused rats.

Red gastrocnemius muscles from glucose-infused rats (3-8h) were analyzed by western blot for p-AMPK $^{Ser485/491}$, p-Akt Ser^{473}, and p-(Ser) PKD substrate. Blots were analyzed by densitometry. Data are means ± SEM. n = 4–6 rats per group, $P < 0.05$ vs. 0h group.

Refeeding increased phosphorylation of AMPK at Ser$^{485/491}$following an 18h fast in muscle and liver

To determine the physiological regulation of AMPK Ser$^{485/491}$ phosphorylation in response to feeding, we measured serine phosphorylation as well as Akt and PKD1 activation in response to fasting and refeeding. C57Bl/6 mice were fasted for 18h and either sacrificed immediately or after a 2h refeeding period (Figure 6.5). Phosphorylation of AMPK at Ser$^{485/491}$ was increased in both gastrocnemius muscle and liver of fed compared to fasted mice (Figure 6.6). As expected, p-Akt Ser^{473} was increased in both tissues following feeding, which would stimulate insulin secretion. Interestingly, p-PKD Ser^{916} was elevated in livers of fed mice compared to fasted, but there was no change in muscle of fasted vs. fed mice.

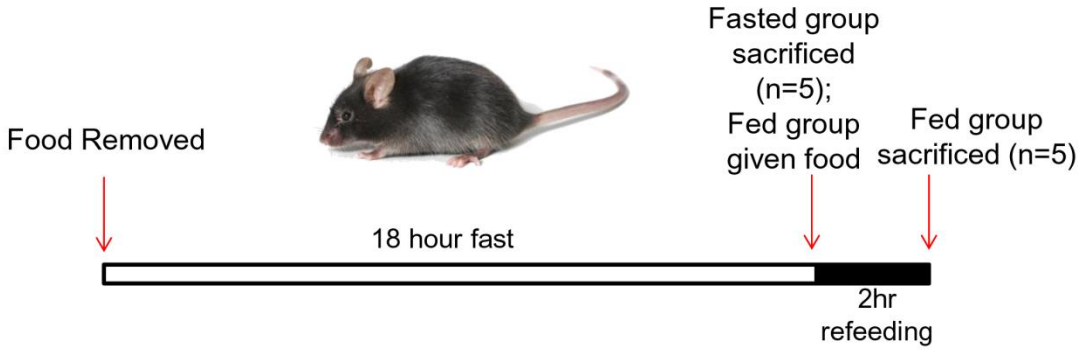


Figure 6.5: Schematic showing protocol for fast/refeed study.

C57BL/6 mice were fasted overnight for 18h. Half were sacrificed immediately, and half were sacrificed after re-feeding of a normal chow diet for 2h.

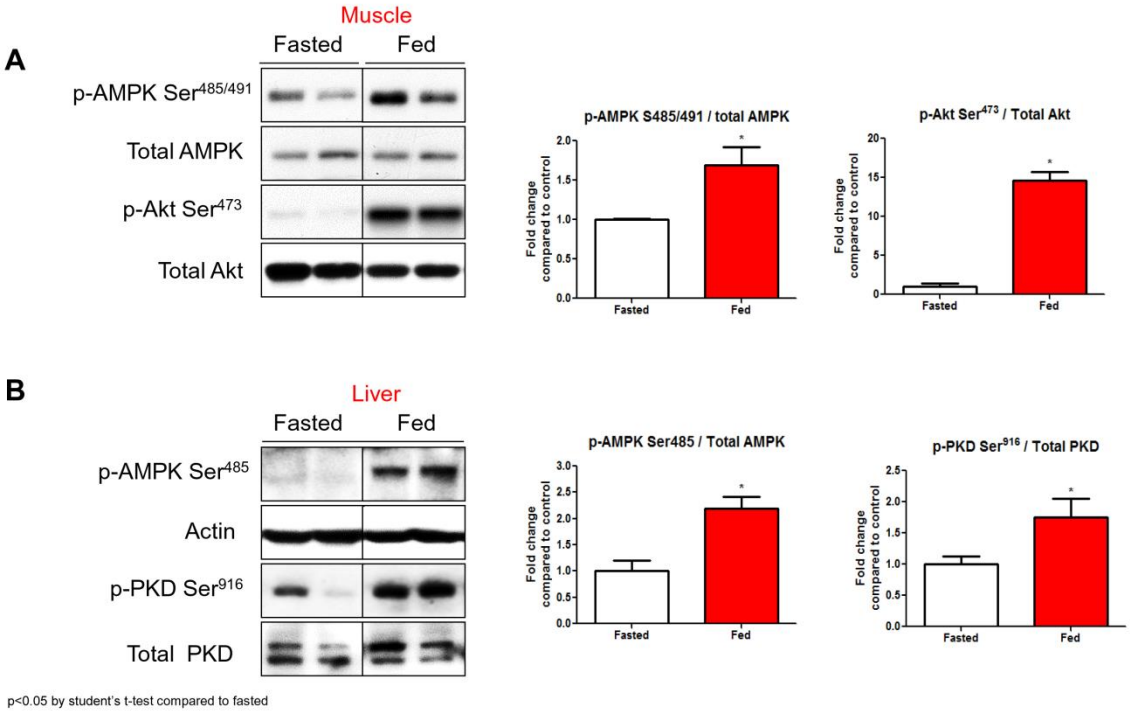


Figure 6.6: Refeeding (2h) increased phosphorylation of AMPK Ser$^{485/491}$ in skeletal muscle, and of Ser^{485} and PKD Ser^{916} in the liver of mice following an 18h fast.

Gastrocnemius muscle (A) and liver (B) from C57BL/6 mice fasted overnight (18h) or refed (2h) were analyzed by western blot. Representative blots are shown. Blots were quantified by densitometry.

Phosphorylation of AMPK Ser$^{485/491}$ was unchanged in muscle but increased in liver of db/db mice compared to db/+ mice

Next, we sought to determine whether phosphorylation of AMPK Ser$^{485/491}$ and PKD1 are altered in a pathological setting of T2D. To do so, we studied the *db/db* mouse model, which lacks the leptin receptor. To model the early stages of T2D, before many other complications are apparent, we sacrificed the mice after 4 consecutive weeks of significantly elevated fasting blood glucose levels (See Fig 6.7A) compared to *db/+* controls, which was at 16 weeks of age. Body weights of *db/db* mice were significantly higher than *db/+* controls at all timepoints measured (9-16 weeks of age) (Figure 6.7A). Phosphorylation of AMPK Ser$^{485/491}$ was increased in liver but not gastrocnemius muscle of *db/db* mice compared to *db/+* controls (Figure 6.7B, 6.7C). Similarly, p-PKD Ser^{916} and phosphorylation of PKD substrates at 110kDa were increased in livers but not muscle of *db/db* mice. Somewhat surprisingly, phosphorylation of PKD substrates at 110kDa was diminished in the muscle of *db/db* mice, suggesting a decrease in PKD1 activity.

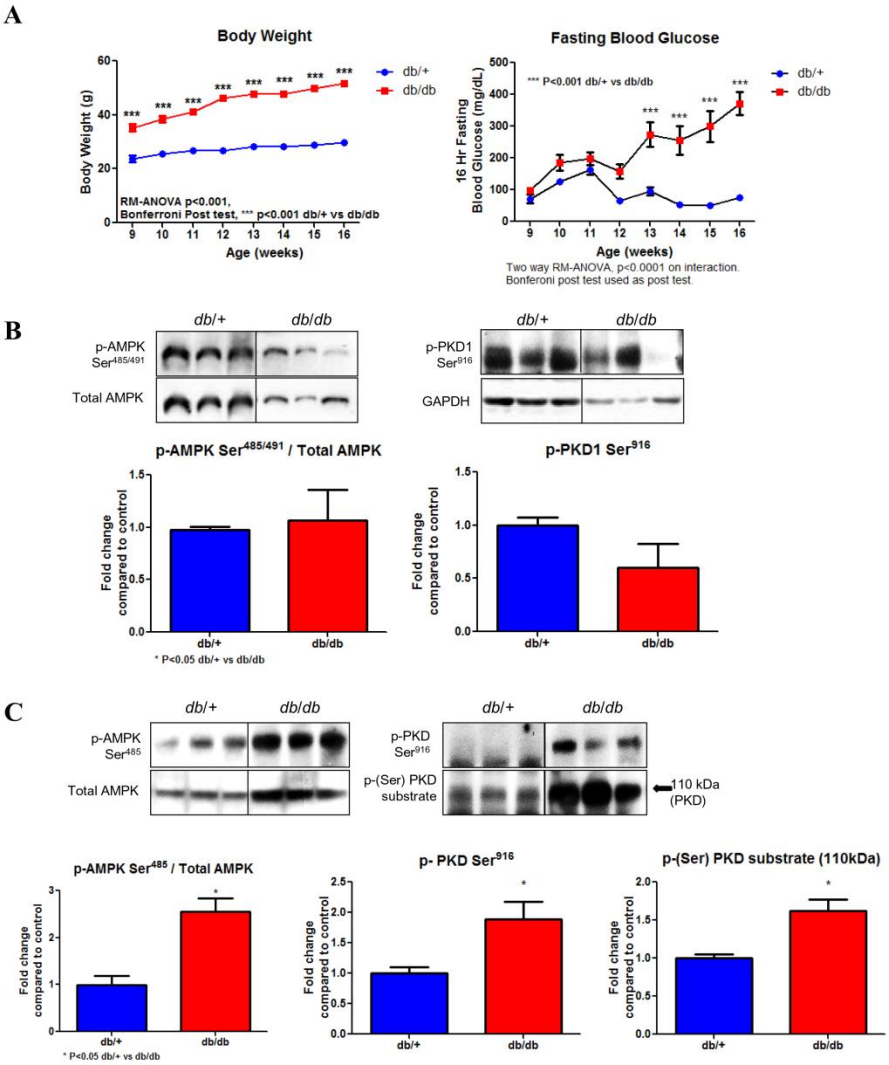


Figure 6.7: db/db mice have elevated p-AMPK Ser485/491 in liver compared to db/+ mice.

Body weights and blood glucose levels of *db/db* and *db/+* mice were measured every week starting at 9 weeks of age (A). Mice were sacrificed after 4 weeks of significantly elevated blood glucose levels between *db/db* and *db/+*. Gastrocnemius muscle and livers were homogenized and analyzed by western blot (B and C, respectively). AMPK serine phosphorylation was quantified by densitometry. Results are means ± SE (n = 5-7 per group). $^{*}p < 0.05$ and $^{**}p < 0.001$ indicates a significant effect of HF vs Control diet. (In collaboration with Jose Cacicedo and Rudy Valentine)

Discussion

The present studies investigated the physiological and pathological regulation of AMPK Ser$^{485/491}$ phosphorylation as well as the regulation of the upstream kinases identified in chapters 4 and 5 to determine whether they are likely involved in the inhibition of AMPK in these settings. We showed in the previous chapter that PKD1 activation and subsequent AMPK Ser$^{485/491}$ phosphorylation impaired insulin signaling through Akt. Here, we investigate whether these mechanisms may be relevant in physiological and pathological settings of nutrient excess.

In the EDL incubated with high glucose, there were no significant increases in Akt phosphorylation, which is not surprising since the incubation medium did not contain insulin. However, there was a non-significant trend towards an increase in p-Akt Ser^{473} at 1h, which could have contributed to the increase in serine phosphorylation of AMPK (Figure 6.1). In the muscle of glucose infused rats, which were hyperinsulinemic in addition to hyperglycemic, Akt phosphorylation was increased at all timepoints. In both models, activation of PKD1 was initially diminished before subsequently increasing to higher than control levels (Figure 6.4). The reason for the initial decrease requires further investigation, but the subsequent increase suggests that PKD1 may be responsible for the phosphorylation of AMPK Ser$^{485/491}$ at later timepoints. Further evidence that PKD1 is involved is that pretreatment with the PKD1 inhibitor CRT0066101 prevented high glucose-induced phosphorylation of AMPK at Ser$^{485/491}$ in the EDL (Figure 6.3).

Although Akt and PKD1 are likely involved in the phosphorylation and inhibition of AMPK in EDL incubated with high glucose, this does not rule out the possibility that

other kinases maybe also play a role. Since mTOR is known to be activated by high glucose [7] and its downstream effector P70S6K has been shown to phosphorylate AMPK at $Ser^{485/491}$ [59], we evaluated whether the mTOR inhibitor rapamycin would prevent changes in AMPK phosphorylation in response to high glucose. Rapamycin did not prevent the decrease in p-AMPK Thr^{172}, nor did it prevent the increase in p-AMPK $Ser^{485/491}$, suggesting that mTOR/P70S6K is not acting upstream of AMPK in this setting (Figure 6.2).

Next, we sought to determine whether AMPK $Ser^{485/491}$ and its upstream kinases were altered in physiological settings in response to feeding and pathological settings of obesity and T2D. Following an 18h fast, we found that p-AMPK $Ser^{485/491}$ was increased in both gastrocnemius muscle and liver (Figure 6.6). We found that Akt phosphorylation was increased in both tissues, while PKD1 phosphorylation was only increased in liver. Why it was not also activated in muscle is not known, but perhaps it would be activated after a longer refeeding period. Interestingly, it was recently shown that PKD1 is activated in adipose tissue of mice fasted for 18h and refed for 2h [264]. Whether serine phosphorylation of AMPK is increased was not measured.

In the diabetic *db/db* mice, it was initially surprising that p-AMPK $Ser^{485/491}$ was not increased, and PKD1 activation was also unchanged in the gastrocnemius muscle (Figure 6.7). However, these data fall in line with the hypothesis that PKD1 activation and, subsequently, AMPK $Ser^{485/491}$ phosphorylation represent an anabolic cellular state. The muscles of *db/db* mice show atrophy since they are insulin resistant and thus, their uptake of glucose is impaired. On the other hand, the surplus of glucose and lipids

accumulates in the livers of *db/db* mice [265], which would lead to elevated DAG levels. As expected in this situation, we found increases in p-AMPK $Ser^{485/491}$ and both p-PKD1 Ser^{916} and phosphorylated PKD substrate at 110kDa. Based on these data, it is no surprise that PKD1 activation and p-AMPK $Ser^{485/491}$ would be increased in states of nutrient excess (intracellularly), where DAG would signal through these molecules to inhibit catabolic processes in favor of anabolic processes such as hypertrophy. This mechanism seems particularly relevant to cardiac hypertrophy. PKD1 expression and phosphorylation are increased in rabbits and humans with heart failure (ischemic cardiomyopathy and idiopathic dilated cardiomyopathy) [222]. Mice lacking PKD1 in heart are protected from cardiac hypertrophy and fibrosis in response to pressure overload [223]. Furthermore, cardiac-specific overexpression of PKD1 triggers pathological hypertrophy in mice, suggesting that PKD1 is sufficient to cause cardiac remodeling [224]. Some studies have shown that AMPK activity is diminished in cardiac hypertrophy [266,267]; however, phosphorylation of $Ser^{485/491}$ was not measured in these studies. Future studies should measure whether AMPK activity is diminished in cardiac hypertrophy via PKD1 phosphorylation of $Ser^{485/491}$. If so, AMPK activators and/or PKD1 inhibitors could be novel strategies for the prevention or treatment of cardiac hypertrophy.

CHAPTER 7: Summary, Conclusions, and Future Directions

Summary

In the present studies, we examined the effects of high glucose over time on AMPK activity and found that there are many mechanisms by which it is inhibited. In these studies, we compared rat muscle incubated with high glucose for 30min-2h and muscle of rats infused with glucose (3-8h) with appropriate controls. Incubation in high glucose (25mM) significantly diminished AMPK activity and phosphorylation at Thr^{172} by 50% within 30min, with further decreases occurring at 1 and 2h. The initial decrease in activity at 30min coincided with a significant increase in muscle glycogen. The subsequent decreases at 1h were accompanied by phosphorylation of αAMPK at $Ser^{485/491}$, and at 2h by decreased SIRT1 expression and increased PP2A activity, all of which have previously been shown to diminish AMPK activity. Glucose infusion *in vivo*, which caused several fold increases in plasma glucose and insulin, produced similar changes but with different timing. Thus, the initial decrease in AMPK activity observed at 3h was associated with changes in $Ser^{485/491}$ phosphorylation and SIRT1 expression and increased PP2A activity was a later event. These findings suggest that both *ex vivo* and *in vivo*, multiple factors contribute to fuel-induced decreases in AMPK activity in skeletal muscle and the insulin resistance that accompanies it.

Recent studies suggest phosphorylation at $Ser^{485/491}$ may play an important role in AMPK inhibition, although this has not been well studied in insulin-responsive tissues. Since it has previously been shown that insulin can inhibit AMPK via phosphorylation of $Ser^{485/491}$ in heart and adipose tissue and because plasma insulin levels were significantly

increased in the glucose-infused rats, we investigated whether the insulin signaling pathway may be involved in regulating phosphorylation of this site in muscle. We found that insulin treatment significantly increased p-AMPK $Ser^{485/491}$ in C2C12 myotubes and in *ex vivo* EDL incubation, resulting in a subsequent reduction in AMPK activity, despite no change in phosphorylation of AMPK at Thr^{172}. Akt inhibition both attenuated the insulin-stimulated increase in p-AMPK $Ser^{485/491}$, and prevented the decrease in AMPK activity. Similarly, the growth factor IGF-1 stimulated AMPK phosphorylation at $Ser^{485/491}$, and this too was blunted by inhibition of Akt. Inhibition of the mTOR pathway with rapamycin, however, had no effect on insulin-stimulated phosphorylation of AMPK at $Ser^{485/491}$. These data suggest that insulin and IGF-1 diminish AMPK activity in muscle through Akt-mediated phosphorylation at $Ser^{485/491}$.

Since insulin was not present in the EDL incubation model in which we observed that high glucose stimulated phosphorylation of AMPK at $Ser^{485/491}$, we investigated whether another kinase may be involved. Diacylglycerol (DAG), which is also increased in muscle exposed to a high glucose concentration, activates a number of signaling molecules including PKC and PKD. Thus, we sought to determine whether PKC or PKD is involved in inhibition of AMPK by causing $Ser^{485/491}$ phosphorylation in skeletal muscle cells. C2C12 myotubes were treated with the PKC/D activator phorbol 12-myristate 13-acetate (PMA), which acts as a DAG mimetic. This caused a dose- and time-dependent increase in AMPK $Ser^{485/491}$ phosphorylation, which was associated with a ~60% decrease in AMPK activity. Under these conditions, serine phosphorylation and inhibition of AMPK activity were partially prevented by the non-specific PKC inhibitor

Gö6983 and fully prevented by specific PKD inhibition with CRT0066101. Inhibition of previously identified kinases that phosphorylate AMPK at this site (Akt, S6K, and ERK) did not prevent these events. PMA treatment also led to impairments in insulin signaling through Akt which were prevented by PKD inhibition. Recombinant PKD phosphorylated AMPK at $Ser^{485/491}$ in cell-free conditions, suggesting that PKD phosphorylates and inhibits AMPK directly. We also found that PKD inhibition prevented AMPK $Ser^{485/491}$ phosphorylation following incubation of EDL in high glucose for 2h. These results identify PKD as a novel upstream inhibitory kinase of AMPK $Ser^{485/491}$ that plays a negative role in insulin signaling in muscle cells and intact muscle.

We next investigated how phosphorylation of AMPK at $Ser^{485/491}$ is regulated under conditions of physiological and pathological nutrient excess. We found that p-AMPK $Ser^{485/491}$ was increased in muscle and liver of fed versus fasted mice. Additionally, we found that p-AMPK $Ser^{485/491}$ was increased in the liver, but not muscle, of *db/db* diabetic mice. Our results suggest that Akt- and PKD1-mediated inhibition of AMPK via $Ser^{485/491}$ phosphorylation may inhibit energy-metabolizing processes, while favoring energy-storing processes.

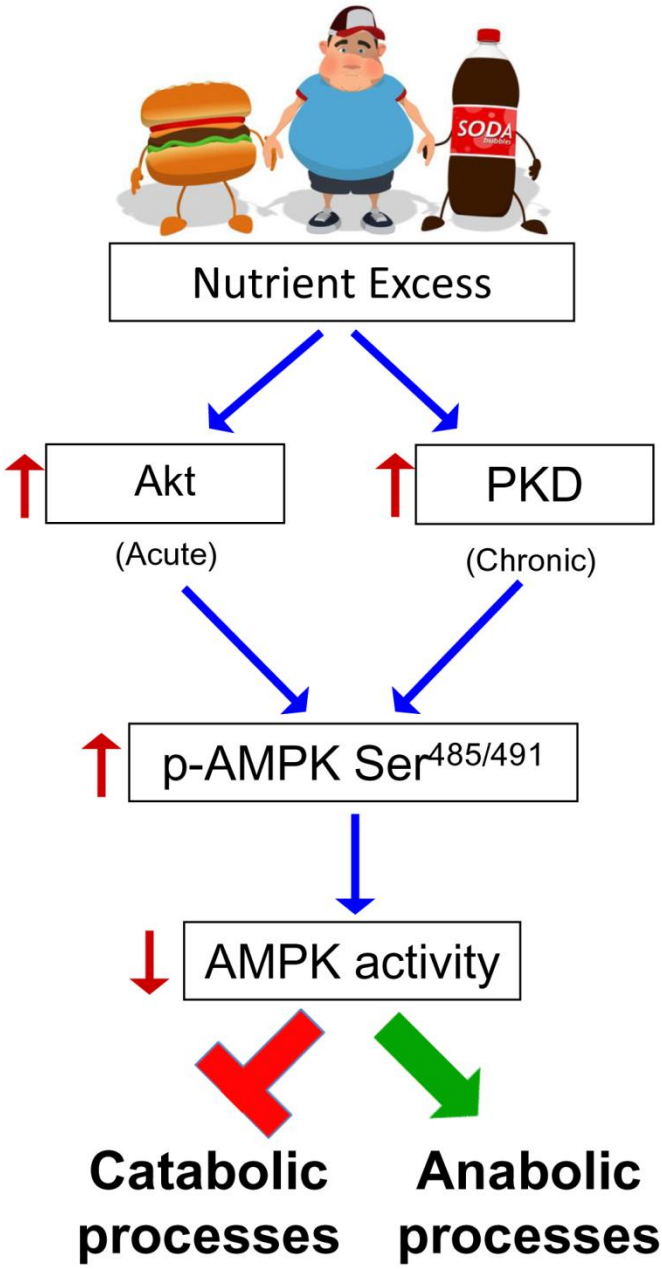


Figure 7.1: Overall proposed model

We have shown that Akt and PKD, which are activated by insulin (and IGF-1) and DAG, respectively, can phosphorylate AMPK at Ser$^{485/491}$ and subsequently inhibit its activity. When activated, AMPK stimulates catabolic processes, such as fatty acid oxidation, in order to restore cellular energy. Thus, we hypothesize that the phosphorylation of Ser$^{485/491}$ is a regulatory mechanism to prevent AMPK-mediated catabolic processes, while allowing anabolic processes, such as growth and hypertrophy, to occur. Since insulin, IGF-1, and DAG are all increased in rodents and humans with the metabolic syndrome, phosphorylation of Ser$^{485/491}$ may contribute to pathological inhibition of AMPK.

Conclusions

In the present studies, we have shown that exposure of muscle to high glucose, both *ex vivo* and *in vivo*, caused a progressive decrease in AMPK activity that was associated with changes in several factors that have all been shown to inhibit AMPK by various mechanism. An increase in muscle glycogen, which has been shown to inhibit AMPK by binding to its β-subunit, was the earliest observed change, which coincided with the diminished AMPK activity and phosphorylation at Thr^{172} at 30min. This was followed by subsequent changes in phosphorylation of $Ser^{485/491}$ at 1h and diminished SIRT1 expression and increased PP2A activity at 2h, which were accompanied by further decreases in AMPK activity. These data suggest that glycogen may be responsible for the initial decrease in AMPK activity, while the other factors may contribute to its sustained inhibition, which may lead to more pathological changes in cellular metabolism. Furthermore, the changes in multiple factors that may regulate AMPK activity over time highlight a biological need to maintain tight control over AMPK enzyme activity.

In subsequent studies, we focused on the role and regulation of phosphorylation of AMPK at $Ser^{485/491}$. Previous studies had shown it to be an important inhibitor of AMPK activity in various tissues, but its significance in insulin sensitive tissues, such as skeletal muscle, had not been investigated. Our findings add to a growing body of literature showing that phosphorylation at $Ser^{485/491}$ is an important negative modulator of AMPK activity. Importantly, they highlight the fact that phosphorylation of $Ser^{485/491}$ can inhibit AMPK activity independent of changes in p-AMPK Thr^{172}, a measure which is often used as a readout of AMPK activity.

We have shown that AMPK Ser$^{485/491}$ was phosphorylated by Akt in response to insulin signaling and by PKD1 downstream of DAG. We also found that PKD1 mediated phosphorylation of Ser$^{485/491}$ was associated with impaired insulin signaling, which was restored by PKD1 inhibition. We hypothesize that Akt-mediated inhibition of AMPK is an acute, physiological response to insulin, whereas PKD1-mediated inhibition may be associated with more chronic pathophysiological changes. Thus, PKD1 inhibition or prevention of Ser$^{485/491}$ phosphorylation may represent new strategies for therapeutic AMPK activation as treatment for the metabolic syndrome.

We also found that p-AMPK Ser$^{485/491}$ was increased in muscle and liver of fed vs fasted mice, and in liver, but not muscle, of *db/db* mice. Although the reason for the absence of changes in Ser$^{485/491}$ phosphorylation in the muscle of *db/db* mice is unknown, it may be due to the timing which was used in this study. All other models in which we observed changes in Ser$^{485/491}$ phosphorylation in muscle (ex vivo incubation, in vivo glucose infusion, and fasting/refeeding) represent early timepoints in the development of insulin resistance, whereas the *db/db* mice represent a later timepoint at which full on diabetes has developed. Thus, increased AMPK Ser$^{485/491}$ phosphorylation may be an early event associated with the development of insulin resistance, but is not present later in diabetes when severe insulin resistance prevents the accumulation of DAG in muscle. In this case, it may not be a viable treatment strategy for insulin resistance in skeletal muscle, but could potentially hold promise as a prevention strategy. In contrast, the fact that we saw increased p-AMPK Ser$^{485/491}$ and PKD1 activation in the liver of *db/db* mice

suggests that these may viable targets for treatment of insulin resistance in liver, which is another important insulin-responsive tissue.

As the T2D epidemic continues to grow, the need for safe and efficacious anti-diabetic medications also increases. Current therapies leave much room for improvement, as most patients require multiple medications to get their blood glucose levels under control, and T2D is the 7th leading cause of death in the US [268]. AMPK is an attractive target for T2D therapies because its activation stimulates a number of catabolic processes that are beneficial in the setting of diabetes, such as glucose uptake and fatty acid oxidation. The present studies have further elucidated the mechanisms by which AMPK is inhbited in response to excess nutrients. We have shown, for the first time, that phosphorylation of AMPK at Ser$^{485/491}$ is an important negative modulator of AMPK activity in skeletal muscle and that Akt and PKD1 can inhibit AMPK by phosphorylation at this site. Further characterization of these and other upstream kinases of this site is required to determine whether inhibition of these kinases could be viable strategies for the treatment or prevention of insulin resistance. Due to other known functions of these proteins, this would likely have to be approached in a highly controlled, tissue specific manner. A treatment that could prevent the phosphorylation of AMPK at Ser$^{485/491}$ directly could potentially hold more promise as a therapeutic strategy.

Future Directions

We have shown that exposure to excess glucose causes decreases in AMPK activity over time, along with sequential changes in a number of factors that may regulate

its activity. We hypothesize that the early changes, such as increased muscle glycogen content, may be more easily reversible, while later changes, such as the decrease in SIRT1 expression, may represent more pathological changes that are less reversible and may be associated with alterations in gene expression. To assess these hypotheses, studies should be carried out to evaluate the reversibility of the decrease in AMPK activity and the changes in its regulatory factors. This could be done in both the *ex vivo* muscle incubation model, where EDL could be incubated in 25mM glucose for various time periods followed by incubation in 5.5mM glucose, and the *in vivo* model of glucose infusion, where rats could be infused with glucose for various periods of time followed by restoration of normal glucose levels. These experiments would determine whether AMPK activity and its putative regulators return to control levels, and if so, how long it takes for this to occur. Additionally, studies could be done in which changes in each of the various factors associated with the decrease in AMPK activity are inhibited or prevented individually to determine the contribution of each factor to AMPK inhibition. For example, glycogen synthesis could be prevented by knocking down glycogen synthase, and PP2A activation could be prevented by treatment with okadaic acid.

We have shown that phosphorylation of AMPK at $Ser^{485/491}$ is a negative modulator of AMPK activity. However, further studies are required to elucidate the effects of this inhibitory event on cellular functions, such as glucose uptake. Also still unanswered is whether inhibition of AMPK via phosphorylation at $Ser^{485/491}$ differs from inhibition via the dephosphorylation at Thr^{172}. Studies using phosphomimetic (S485/491A) and phosphodefective (S485/491D) mutants of AMPK could help

determine the specific effects of serine phosphorylation of AMPK on cellular functions. Measurements of glucose uptake, glycogen synthesis, and fatty acid oxidation are some cellular activities that may be affected. To build upon the studies performed here, in which pharmacological inhibitors of Akt and PKD1 were used to elucidate their role in the inhibition of AMPK, genetic methods should also be used to further confirm these findings. Specifically, siRNA knockdown of Akt and PKD1 should be done to determine whether phosphorylation of AMPK at $Ser^{485/491}$ is prevented in response to insulin, PMA, and high glucose, and whether this completely prevents the inhibition of AMPK activity. Studies in which these enzymes are overexpressed would also be beneficial to determine whether this causes constitutive inhibition of AMPK and whether it mimics the effects of insulin, PMA, and high glucose.

Another future direction of this work would be to test whether currently available diabetes drugs and AMPK activators affect the phosphorylation of AMPK at $Ser^{485/491}$. Although currently used therapies, such as metformin and thiazolidinediones (TZDs) are known to activate AMPK, whether AMPK activation is crucial to their insulin-sensitizing and glucose-lowering effects remains under debate. Determining whether these compounds affect AMPK phosphorylation at $Ser^{485/491}$ could help define their mechanisms of action. Furthermore, if inhibition of $Ser^{485/491}$ phosphorylation is determined to play an important role in the efficacy of these compounds, then compounds with this same property could be sought as novel therapeutics but perhaps with less side effects.

Further characterization of the regulation of AMPK Ser$^{485/491}$ phosphorylation in various physiological and pathological settings should be done to evaluate it as a target for pharmacological intervention. Here, we have examined its regulation in the muscle and liver of *db/db* mice at 16 weeks of age. At this age, these mice had significantly elevated fasting blood glucose levels for four consecutive weeks. However, at different ages and earlier or later stages in diabetes, the phosphorylation status of AMPK at Ser$^{485/491}$ may be different and should be investigated. Additionally, other models of obesity and diabetes may show differences in the regulation of AMPK phosphorylation and activity. For example, rodents fed a high fat diet for 20 weeks have diminished phosphorylation of AMPK at Thr^{172} in muscle [269]; however, the effects of high fat feeding on the inhibitory phosphorylation of AMPK at Ser$^{485/491}$ has not yet been studied. Whether AMPK activity is altered due to more or less inhibitory phosphorylation in PKD1 and Akt knockout and transgenic animals should also be evaluated.

In addition to insulin resistance and diabetes, the results of our studies may be relevant in other disease areas. PKD1 activity and expression have been shown to be increased in numerous models of cardiac hypertrophy and in some cancers, such as pancreatic cancer. Interestingly, some studies have shown that AMPK activity is diminished in cardiac hypertrophy and certain cancers and that AMPK activation could be a beneficial treatment for these conditions. Future studies should investigate whether AMPK Ser$^{485/491}$ phosphorylation is increased in these conditions. These may represent new therapeutic indications where AMPK activation can be used as a treatment strategy.

JOURNAL ABBREVIATIONS

Acta Physiol Scand ..... Acta Physiologica Scandinavica

Am J Pathol..... The American Journal of Pathology

Am J Physiol..... The American Journal of Physiology

Am J Physiol Cell Physiol ..... The American Journal of Physiology - Cell Physiology

Am J Physiol Endocrinol Metab..... The American Journal of Physiology - Endocrinology and Metabolism

Am J Physiol Regul Integr Comp Physiol ..... The American Journal of Physiology – Regulatory, Integrative and Comparative Physiology

Arterioscler Thromb Vasc Biol..... Arteriosclerosis, Thrombosis, and Vascular Biology

Biochim Biophys Acta.....Biochimica et Biophysica Acta

Biochem Biophys Res Commun Biochemical and Biophysical Research Communications

Biochem J.....Biochemical Journal

Biochem Soc Trans.....Biochemical Society Transactions

Biol Pharm Bull .....Biological and Pharmaceutical Bulletin

Biosci Rep.....Bioscience Reports

BMJ.....British Medical Journal

Br J Clin Pharmacol.....British Journal of Clinical Pharmacology

Can J Physiol Pharmacol ..... Canadian Journal of Physiology and Pharmacology

Cancer Res .....Cancer Research

Cardiovasc Res.....Cardiovascular Research

Cell Metab.....Cell Metabolism

Chem Biol ..... Chemistry & Biology

Circ Arrhythm Electrophysiol ..... Circulation: Arrhythmia and Electrophysiology

Circ Res ..... Circulation Research

Clin Evid (Online) ..... Clinical Evidence

Clin Exp Pharmacol Physiol ..... Clinical and Experimental Pharmacology and Physiology

Curr Biol ..... Current Biology

Curr Diab Rep ..... Current Diabetes Reports

Curr Opin Clin Nutr Metab ..... Current Opinion in Clinical Nutrition and Metabolic Care

Curr Opin Genet Dev ..... Current Opinion in Genetics & Development

Curr Opin Plant Biol ..... Current Opinion in Plant Biology

Diabetes Metab ..... Diabetes & Metabolism

Diabetes Metab Res Rev ..... Diabetes/Metabolism Research and Reviews

EMBO J ..... The EMBO Journal

EMBO Rep ..... EMBO Reports

Endocr Pract ..... Endocrine Practice

Eur J Biochem ..... European Journal of Biochemistry

Eur J Nutr ..... European Journal of Nutrition

Exp Clin Endocrinol Diabetes ..... Experimental and Clinical Endocrinology & Diabetes

Exp Mol Med ..... Experimental & Molecular Medicine

Expert Opin Pharmacother ..... Expert Opinion on Pharmacotherapy

FASEB J ..... The FASEB Journal

FEBS J ..... FEBS Journal

FEBS Lett.....FEBS Letters

Front Physiol.....Frontiers in Physiology

Int J Cardiol.....International Journal of Cardiology

JAMA.....Journal of the American Medical Association

J Biol Chem.....The Journal of Biological Chemistry

J Clin Invest .....Journal of Clinical Investigation

J Endocrinol Diabetes Obes.....Journal of Endocrinology, Diabetes & Obesity

J Enzyme Inhib Med Chem.....Journal of Enzyme Inhibition and Medicinal Chemistry

J Lipid Res .....The Journal of Lipid Research

J Mol Endocrinol..... Journal of Molecular Endocrinology

J Mol Cell Cardiol..... Journal of Molecular and Cellular Cardiology

J Mol Signal .....Journal of Molecular Signaling

J Physiol..... The Journal of Physiology

Kidney Int ..... Kidney International

Liver Int ..... Liver International

Mol Cancer.....Molecular Cancer

Mol Cell ..... Molecular Cell

Mol Cell Biol .....Molecular and Cellular Biology

Mol Endocrinol ..... Molecular Endocrinology

N Engl J Med ..... The New England Journal of Medicine

Nat Cell Biol .....Nature Cell Biology

Nat Commun..... Nature Communications

Nat Med .....Nature Medicine

Nat Rev Drug Discov..... Nature Reviews Drug Discovery

Nat Struct Mol Biol..... Nature Structural & Molecular Biology

Nutr Res Pract .....Nutrition Research and Practice

Obes Rev..... Obesity Reviews

Physiol Rev .....Physiological Reviews

Proc Natl Acad Sci USA..... Proceedings of the National Academy of Sciences

Rejuvenation Res .....Rejuvenation Research

Toxicol Appl Pharmacol .....Toxicol Appl Pharmacol

Tumour Biol.....Tumour Biology

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CURRICULUM VITAE

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