# EXAMINATION OF MAJOR TEA CATECHINS ON GLUT4 TRANSLOCATION IN L6 SKELETAL MUSCLE CELLS

by

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ABSTRACT OF THE THESIS

Examination of Major Tea Catechins on GLUT4 Translocation in L6 Skeletal Muscle Cells

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Obesity and its manifested pathophysiological effects (insulin-resistance, metabolic disease, type 2 diabetes, cardiovascular disease, etc.) are reaching epidemic proportions in the developed world. Roughly one-third of adults twenty years and over are considered overweight or obese, therefore interventional approaches have been of interest in the scientific community. Besides its palatable popularity, tea brewed from the leaves of the plant *Camellia sinensis* has become increasingly popular due to its possible effects on human health in alleviating complications that arise from diseases such as metabolic syndrome, diabetes, cardiovascular disease, and various types of cancers. These beneficial qualities have mainly been attributed to catechins, compounds found in tea leaves that account for 30-42% of the water-extractable solids.

In the present study, I investigated major tea catechins epigallocatechin-3-*O*-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-*O*-gallate (ECG), and epicatechin (EC), as well as gallic acid (GA) on L6 rat skeletal muscle cells in order to test their ultimate effects on glucose transporter 4 (GLUT4) translocation via activation

of two distinct signaling pathways implicated in the process: (1) the insulin-mediated phosphoinositide 3-kinase (PI3K) pathway and (2) the insulin-independent AMPK pathway. Treatment of cells with the catechins resulted in Thr308 phosphorylation of Akt (a key protein in the PI3K pathway that facilitates GLUT4 translocation). Activation was stronger with the gallated catechins (EGCG and ECG) than the non-gallated catechins (EGC and EC) and gallic acid. Similar results were observed in AMPK activation. Furthermore, GLUT4 translocation from the cytosol to the plasma membrane was higher in samples treated with gallated catechins than cells treated with other polyphenols. Together, these data suggest that major polyphenols found in tea promote translocation of the GLUT4 protein in skeletal muscle. The effectiveness of the catechins on GLUT4 translocation may be attributable to their abilities to generate reactive oxygen species (ROS), decrease intracellular ATP levels, or their abilities to interact with components on the outer leaflet of the plasma membrane, but these possible mechanisms remain to be investigated.

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# List of Abbreviations

**ACO-1** – Aconitase

**Akt/PKB –** Protein kinase B

**ADP** – Adenosine diphosphate

**AMP** – Adenosine monophosphate

**AMPK –** AMP-activated protein kinase

**AP-1** – Activator Protein 1

**AS160 (TBC1D4)** – Akt substrate of 160 kDa, (TBC1 domain family member 4)

**ATP** – Adenosine triphosphate

**Bax** – Bcl-2-associated X protein

**Bcl-2** – B-cell lymphoma 2

**CAMKK** – Calcium/calmodulin-dependent protein kinase kinase

**Cav-1** – Caveolin-1

**Cav-2** – Caveolin-2

**Cav-3** – Caveolin-3

**CME** – Clathrin-mediated endocytosis

**CPT-1** – Carnitine palmitoyltransferase I

**CRP** – C-reactive protein

**CSD** – Caveolin Scaffold Domain

**CVD** – Cardiovascular disease

**EC –** (-) Epicatechin

**ECG –** (-) Epicatechin-3-*O*-gallate

**EE** – Early endosome

**EGC** – (-) Epigallocatechin

**EGCG –** (-) Epigallocatechin-3-*O*-gallate

**EGF** – Epithelial growth factor

**ER** – Endoplasmic reticulum

**ERC** – Endosomal recycling compartment

**Erk** – Extracellular-signal-regulated kinases

**FOXO** – Forkhead box O class

**G6P** – Glucose-6-phosphate

**GA** – Gallic Acid

**GLUT (1, 2, 3, 4)** – Glucose transporter (1, 2, 3, 4)

**GPI** – Glycophosphatidylinositol

**GSV** – Glucose storage vesicle

**HA-GLUT4-GFP** – Hemagglutinin/green fluorescent protein-tagged construct

**HDL** – High-density lipoprotein

**HMIT** – H+ Myo Inositol symporter

**HNF-1α** – Hepatocyte nuclear factor 1 alpha

**IKK-β** – Inhibitor of nuclear factor kappa-B kinase beta

**IL-6** – Interleukin 6

**IRAP** – Insulin-regulated aminopeptidase

**IRS-1** – Insulin Receptor Substrate 1

**IRS-2** – Insulin Receptor Substrate 2

**IR** – Insulin Receptor

**JNK** – c-Jun N-terminal kinase

**L6** – Rat skeletal muscle cell line

**LDL** – Low-density lipoprotein

**LKB1** – Liver kinase B1

**LRP1** – Low density lipoprotein receptor-related protein 1

**MAPK** – Mitogen-activated protein kinase

**MCP-1** – Monocyte chemotactic protein 1

**MetS** – Metabolic Syndrome

**MO25** – Calcium-binding protein 39

**MODY** – Maturity onset diabetes of the young

**mTOR** – Mammalian target of rapamycin

**mTORC2** – mTOR Complex 2

**NFκB** – Nuclear factor kappa-light-chain-enhancer of activated B cells

**p53** – tumor suppressor p53

**PAI-1** – Plasminogen activator inhibitor-1

**PDGF** – Platelet-derived growth factor

**PDK-1** – Phosphoinositide-dependent kinase-1

**PEPCK** – Phosphoenolpyruvate carboxykinase

**PI3K** – Phosphoinositide 3-kinase

**$PIP_2$** – Phosphatidylinositol 4,5-bisphosphate

**$PIP_3$** – Phosphatidylinositol (3,4,5)-trisphosphate

**PKC** – Protein kinase C

**PM** – Plasma membrane

**ROS** – Reactive oxygen species

**SAPK** – Stress-activated protein kinase

**SGLT** – Sodium-glucose linked transporter

**siRNA** – Small interfering RNA

**SNAP; SNAP23** – Soluble NSF-attachment protein (23)

**SNARE** – Soluble NSF attachment protein receptor

**SOD** – Superoxide dismutase

**Src-family kinases** – Sarcoma-family kinases

**STRAD** – STE20-related kinase adapter protein

**TBC1D1** – TBC1 domain family member 1

**TGN –** Trans-Golgi network

**TIDM** – Type I Diabetes Mellitus

**TIIDM** – Type II Diabetes Mellitus

**TNF-α** – Tumor necrosis factor alpha

**TPA** – Tetradecanoylphorbol acetate

**TUG** – Tether containing a UBX domain for GLUT4

**UV** – Ultraviolet

**VAMP** – Vesicle-associated membrane protein

**VEGF** – Vascular endothelial growth factor

# 1 Introduction

## 1.1 Glucose Metabolism

Glucose is the fundamental metabolic fuel for all eukaryotic cells under physiologic conditions. It acts not only as an energy source, but also as a starting material in many biosynthetic reactions such as glycolysis, lipid synthesis, amino acid synthesis, and nucleotide synthesis [1]. As crucial as glucose is for maintaining homeostatic conditions, blood glucose levels must be kept under strict regulation. The glycemic level in healthy individuals is typically between 3.4 and 6.2 mM before meals (pre-prandially) and rarely exceeds 7.8 mM after meals (postprandially) [2]. Glucose homeostasis is managed primarily via two major hormones secreted from the pancreas: insulin and glucagon. Together, they coordinate the balance between glucose uptake, storage, breakdown, and production [3].

Of all of the glucose that enters the systemic circulation after a meal, 40% is taken up by skeletal muscle where it is initially oxidized via the glycolytic pathway and then later stored as glycogen [4, 5]. Skeletal muscle makes up roughly 55% of total body mass and aside from its other roles such as locomotion and heat production, it also takes part in the regulation of whole-body glucose homeostasis [6]. It is the predominant site for peripheral glucose removal and is responsible for roughly 80-90% of insulin-stimulated glucose disposal [7, 8].

## 1.2 The Pancreas

The adult pancreas is the organ responsible for regulating both blood glucose homeostasis and nutrient digestion [9]. It contains morphologically and functionally distinct endocrine and exocrine elements. The exocrine tissue which makes up 95% of the

pancreatic mass is composed of acini, a lobed cluster of cells, and ducts that produce digestive enzymes such as trypsin and amylase that get shunted to the gut in order to ensure nutrient digestion and absorption in the small intestine [10, 11]. The endocrine tissue dispersed throughout the exocrine tissue is organized into islets of Langerhans which are globular clusters of various cell types responsible for producing distinct peptide hormones that get secreted into the bloodstream [10]. Multiple levels of regulation determine the appropriate level of hormonal release from the islets by sensing plasma glucose levels in the body, including a vast endothelial network innervated by sensory nerves and various paracrine signals released through intra-islet communication [12]. Alpha (α) cells which make up roughly 15% of the islet mass are responsible for producing glucagon. Beta (β) cells (60 to 80% of islet mass), are responsible for producing insulin. The remainder of the islet mass is composed of delta (δ) cells which produce somatostatin and gamma (γ) cells which produce pancreatic polypeptide [11].

The antagonistic secretions of glucagon and insulin predominantly control blood glucose. Insulin is released from β-cells in response to hyperglycemic states and acts mainly in an anabolic effect, stimulating the uptake and subsequent accumulation of glucose in responsive tissues, particularly skeletal muscle, liver, and adipose tissue [12, 13]. It stimulates glycolysis as well as glycogen synthesis in response to excess glucose uptake. In contrast, glucagon is released from α-cells in response to hypoglycemic conditions and induces a catabolic effect mainly by activation of glycogenolysis and gluconeogenesis in the liver, ultimately releasing free glucose into the bloodstream. Glucagon acts primarily on the liver tissue, but also has an effect on the pancreas, heart, kidney, brain, and smooth muscle tissue as well [13]. The irregular regulation of these

hormones causes a failure of blood glucose control and ultimately leads to the onset of metabolic diseases such as diabetes.

### 1.3 Metabolic Syndrome

Metabolic syndrome is collectively recognized as a group of abnormalities dealing with metabolism. Many features contribute to MetS, although not all features appear in individuals with the disease. People diagnosed with the disease are afflicted with at least three of the characteristics [14, 15]. One of the most prevalent features of MetS is insulin resistance which occurs in most people with the disease and is strongly correlated with other features such as hypertension, glucose intolerance, and obesity [14]. Excess circulating fatty acids strongly contribute to the development of insulin resistance by inhibiting the antilipolytic effects of insulin [16]. Perhaps one of the most recognized features of metabolic syndrome worldwide is obesity, as its prevalence has doubled in the past three decades. Obesity, as reported by the World Health Organization, is defined as having a BMI of 30 and over. Roughly one-third of adults twenty years and over is considered overweight or obese [17]. Excess visceral fat and central obesity in particular are believed to be causative factors of MetS.

Other factors identified in MetS are atherogenic dyslipidemia (in which elevated levels of triglycerides and LDL particles in the blood arise while levels of HDL cholesterol are reduced), hypertension, increased prothrombotic (blood clotting) factors such as fibrinogen and PAI-1, proinflammatory cytokines such as TNF-α and CRP, and decreases in anti-inflammatory cytokines such as adiponectin [14, 16]. MetS increases the risk for developing diseases such as cardiovascular disease (by two-fold) and type II

diabetes (by five-fold) [17]. The International Diabetes Federation approximates one-fourth of the world's adult population having MetS.

### 1.4 Diabetes Mellitus

Diabetes mellitus is a disease characterized by high blood glucose. It can be caused by an insufficient release of insulin, insulin resistance, or a combination of both [18]. Diabetes affects roughly 29.1 million people in the United States, accounting for 9.3% of the population and is the seventh leading cause of death in the nation [18]. Most cases can be classified into two main categories: Type I and Type II. TIDM, which accounts for roughly 5-10% of all cases, results from a targeted destruction of β-cells by the immune system [19]. Although the autoimmune-mediated form of this disease is more common during childhood and adolescence, it can occur at any age. People later in life can be diagnosed after never once having symptoms, however, the earlier a person is diagnosed with TIDM, the more devastating are the effects.

The more common form of diabetes is TIIDM which accounts for roughly 90-95% of all cases [18]. This form of the disease is most commonly associated with obesity and can go undiagnosed for years due to the gradual development of hyperglycemia. TIIDM is displayed by a combination of insulin resistance as well as deficient insulin secretion from β-cells [18]. In response to obesity and insulin resistance, the pancreatic islets need to work harder to sustain normal blood glucose levels in a process referred to as β-cell compensation [20]. Conventionally, two actions are carried out to initiate the process. One mechanism is through increasing β-cell mass, and the other is via increasing glucose sensitivity in order to increase both insulin synthesis and secretion [10, 20, 21]. The compensation process allocates a great deal of stress on the ER to synthesize insulin

in order to meet increased demand. Over time, β-cells are exhausted as the production of insulin is no longer sufficient enough, leading to a physical depletion in insulin release [20]. The risk for developing TIIDM increases with age, obesity, and a lack of physical activity, although there has been a cause for concern as this form of the disease has become more common in overweight children and adolescents.

There is a well-recognized link between obesity and an increased risk of developing insulin resistance and TIIDM. Roughly two-thirds to three-quarters of diabetic cases are associated with a history of obesity [22]. The link between obesity and type II diabetes has been credited to a multitude of interrelated factors including aberrant fatty acid metabolism, inflammatory cytokines, beta cell dysfunction, and oxidative stress [22-24]. Excess adipose stores in obese individuals leads to an increase in fatty acid circulation in the body. Insulin normally limits fatty acid supply by suppressing lipolysis, but adipose stores that increase in size become less sensitive to insulin over time which causes an imbalance in the glucose-fatty acid cycle, leading to a favor for fatty acid oxidation rather than glucose uptake [22, 24]. Increased metabolism of glucose and fatty acids through oxidation leads to increased superoxide production [22]. While ROS is important in certain contexts (i.e. inducing apoptosis, cell proliferation, and the expression of some genes), excess amounts can be cytotoxic and cause necrosis of cell and tissues.

Fatty acid metabolites further negatively impact insulin signaling by promoting serine phosphorylation of IRS-1 and IRS-2 rather than tyrosine phosphorylation [22]. Under normal conditions, insulin induces rapid tyrosine phosphorylation of IRS-1 and IRS-2 and is followed by a slower increase in serine and threonine phosphorylation [21,

22, 25]. Phosphorylation at serine and threonine sites results in first, dissociation of IRS-1 from the insulin receptor, and then a subsequent degradation of the IRS-1 protein, serving as a desensitization mechanism to turn off insulin signaling under normal conditions [26]. Increased serine/threonine phosphorylation of IR reduces the activation of the PI3K pathway, a critical component in glucose metabolism. Serine phosphorylation of IRS-1 also increases the activation of the JNK/SAPK, IKK-β, and NF-κB pathways, integral elements linked to stress-activated cell death and inflammatory responses [21].

Excessive intake of macronutrients induces the upregulation of pro-inflammatory cytokines such as TNF-α and IL-6 produced by adipocytes due to an increase in ROS production in response to the decreased accommodation for glucose oxidation [21, 22, 25]. TNF-α and IL-6 are known to cause the increase of serine phosphorylation of IR, the inhibition of tyrosine phosphorylation of the IR, and even cause the suppression of IR expression altogether, promoting insulin resistance [25].

There are a number of other factors attributed to the onset of diabetes besides obesity, the most commonly described influence. Inherited genetic abnormalities in some genes have been discovered to predispose individuals to the disease. In the case of MODY for example, monogenetic defects in β-cell function are inherited in an autosomal dominant manner [27]. The most common defects in MODY cases are the mutations of the hepatic transcription factor HNF-1α located on chromosome 12 and of the glucokinase gene on chromosome 7 [18, 27]. HNF-1α is responsible for maintaining the differentiated β-cell phenotype, while glucokinase is the enzyme responsible for glucose to G6P conversion. Other genetic abnormalities can contribute to the propensity for individuals to develop diabetes such as an inability to convert proinsulin to insulin [18].

Additional factors besides genetic-related causes have been attributed to diabetic cases such as diseases of the exocrine pancreas (pancreatitis, pancreatic carcinoma), dysregulation of several hormones that antagonize insulin (growth hormone, cortisol, glucagon, somatostatin, etc.), infections (cytomegalovirus, congenital rubella), and drugs (nicotinic acid, glucocorticoids) [18].

Hyperglycemia, as a result of diabetes, has long-term and chronic effects on individuals diagnosed with the disease, including the damage (and in severe cases, failure) of organs such as the eyes, kidneys, heart, nerves, and blood vessels [18]. Some compounded complications it can induce are hypertension, cardiovascular disease, stroke, blindness, kidney disease, and the neuropathy that can lead to the amputation of limbs [18].

### 1.5 Polyphenols

Over 8,000 polyphenols have been identified in dietary sources such as fruits, vegetables, legumes, tea, coffee, wine, cocoa, and grains [28]. Naturally, these phytochemical compounds are secondary metabolites that serve primarily to protect the plant against external factors such as UV radiation and different pathogens [28, 29]. In the human diet, they are believed to serve primarily as antioxidants. Polyphenol intake is higher than any other class of antioxidants, with consumption being roughly ten times higher than vitamin C intake and one hundred times higher than vitamin E intake [29]. Polyphenols are known to have profound effects on human health and have been extensively studied in the scientific community for decades.

Polyphenols fall into four distinct categories based on the number of phenol rings and the structural elements that hold these rings together: phenolic acids, flavonoids,

stilbenes, and lignans [29-31]. The phenolic acids contain one or more phenolic rings and the defining feature, carboxylic acid. This class of polyphenols further divides into two classes: benzoic acid derivatives and cinnamic acid derivatives [30, 31]. Flavonoids are the most prevalent in the human diet and are classified primarily by the presence of multiple phenyl rings and a heterocyclic ring. They branch off into subcategories based on different chemical moieties that are found on the phenyl rings, the heterocyclic ring, or both. These categories are flavonols, flavanols, flavones, isoflavones, flavanones, and anthocyanidins [30, 31]. Lignans are polyphenolic structures formed from phenylpropyl units [30, 31]. Stilbenes, which make up a very small part of the human diet, are composed of two polyphenol rings bound together by a double bonded ethene group [30, 31].

### 1.6 Polyphenols in Tea

Tea brewed from the leaves of the plant *Camellia sinensis* is one of the most popular beverages. It is consumed extensively by over two-thirds of the world's population [32]. There are three main types of tea based on their level of oxidation: green which is unfermented, oolong which is partially fermented, and black which is fermented [33]. Of these types, 78% of tea produced and manufactured is black, 20% is green, and 2% is oolong [32]. The predominant form of tea polyphenols known as catechins (flavan-3-ols) vary in content between the types with green tea containing the most, followed by oolong tea, and then black tea [34]. In green tea, catechins constitute 30-42% of the dry weight of water-extractable solids [35]. In the production of black tea, however, the fermentation process causes roughly 75% of catechins in the tea leaves to undergo

oxidative polymerization that lead to a significantly lower catechin content of 10-12% overall [34, 36].

The four most common catechins found in tea are epigallocatechin-3-*O*-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-*O*-gallate (ECG), and epicatechin (EC) with EGCG being the most abundant, accounting for 50-80% of total catechins [36, 37]. The general structure of tea catechins consists of two benzene rings called the "A" and "B" ring which are joined together by a heterocyclic pyran or pyrone called the "C" ring. Catechins have two characteristic hydroxyl groups located on the C-5 and C-7 positions of the A ring, as well as di- or tri-hydroxyl groups on the B ring [33, 35, 36]. In addition, some catechins such as EGCG and ECG possess a gallate moiety linked to the C ring [33]. The different chemical structures of catechins contribute to their special properties.

The hydroxyl groups located on the B ring have strong radical scavenging abilities because they can trap reactive oxygen and nitrogen species. They can also chelate metal ions to inhibit related free radical generation [36, 38, 39]. The gallate moiety at the C-3 position of the C ring also plays a prominent role in antioxidant activity [33]. The number and position of the hydroxyl groups in the molecule contributes to the strength of the antioxidant activity. Because EGCG contains eight hydroxyl groups, it has been suggested to have the highest activity, followed by ECG which has seven groups, EGC which has six groups, and EC which has five groups [33]. Another component found in tea is gallic acid, a phenolic acid with a trihydroxybenzoic composition. It has also been studied for antioxidant and protective effects as well. Its concentration in tea increases during the fermentation process due to its release from catechin gallates [40].

Although polyphenols are renowned for their antioxidant effects, studies have shown that they can also be pro-oxidant and have the ability to generate ROS [41, 42]. Due to the ability of ROS to increase the levels of expression of phase II antioxidant enzymes (such as glutathione peroxidase, catalase, superoxide dismutase, nuclear factor erythroid 2-related factor 2, and glutathione-S-transferase) [43, 44, 45, 46], it may be considered an "indirect antioxidant effect."

In addition to their antioxidant effects, polyphenols are also known to regulate cell metabolism through a variety of events that begin with their interaction with the plasma membrane and subsequent penetration [47]. Like radical-scavenging effectiveness, diffusion through the plasma membrane is related to the number of hydroxyl groups (as well as gallate moieties) located on the molecule. The hydroxyl groups located on the B ring and on the gallate moiety attached to the C ring are thought to interact with the trimethylamine groups of the sphingomyelin present on the outer leaflet of the plasma membrane [47]. Other factors that affect this molecule to membrane interaction are the hydrophobicity of the molecule, planar structures, and electrostatic interactions [47, 48]. It has been suggested that flavanoids might exert modulatory effects in cells through selective actions at different components of a number of protein kinase and lipid kinase signaling cascades [49]. This special characteristic can explain how certain polyphenolic compounds might aid in alleviating complications that arise from various diseases.

### 1.7 Green Tea and Disease Prevention

Special properties have been attributed to polyphenols that result in alleviating the effects of complications that arise from diseases such as metabolic syndrome, diabetes, cardiovascular disease, and different kinds of cancers. In addition to alleviation of already obtained symptoms, there has been in vitro and in vivo evidence of polyphenols aiding in the prevention of the onset of these diseases.

Studies on the effects of green tea consumption on MetS have been extensive over many years. In one study by Bose et al., mice that were fed a high-fat diet and received EGCG in their diets were found to have a decreased body weight gain, percentage of body fat, and visceral fat weight compared to control mice that were fed a high-fat diet without EGCG supplementation in a 16 week trial. In addition to these effects, an increase in fecal lipid deposits was seen in mice that were administered EGCG, suggesting EGCG's effect on body weight is caused at least partially by an inhibition of lipid absorption [50]. Similar effects were concluded in another study by Hasegawa et al. A daily oral administration of powdered green tea to Zucker rats fed a high sucrose diet resulted in a reduction in body weight gain as well as reduced adipose tissue and liver weight [51]. EGCG has also been found to decrease the effects of insulin resistance [50]. Additionally, a decrease in plasma cholesterol levels and MCP-1 levels accompanied the attenuation of insulin resistance shown in one study [50]. MCP-1 (a key chemokine that regulates the migration and infiltration of monocytes and macrophages) leads to tissue inflammation, a well-known consequence of obesity, and a basic component leading to the development of complications such as insulin resistance, MetS, and diabetes [52].

Catechins have also been used in TIIDM studies for decades due to their anti-hyperglycemic effects in addition to the effects previously listed. In one study, Wistar rats orally supplemented with catechins prior to starch and sucrose administration were discovered to have suppressed blood glucose and insulin concentrations when compared to the catechin-free control group. The suppression was caused by the inhibition of the intestinal enzyme activities of α-amylase and sucrase [53]. Another study using Zucker Diabetic Fatty (ZDF) rats and db/db mice obese and with glucose intolerance both showed improved glucose tolerance and decreased fasting glucose levels after being treated with dietary EGCG for seven weeks [54]. EGCG was also shown to increase the expression of glucokinase, ACO-1, and CPT-1, while decreasing PEPCK expression in the liver and adipose tissue in a dose-dependent manner. Glucokinase is involved in glycolysis and glucose uptake, while PEPCK is involved in gluconeogenesis. ACO-1 and CPT-1 are both required for fatty acid catabolism [54]. A study performed by Cao et al. showed that Wistar rats fed a high-fructose diet and given green tea extract for six weeks results in an increase in the mRNA levels of GLUT4 in the liver by 90% and skeletal muscle by 40% as well as IRS-2 in the liver by 60% and IRS-1 in skeletal muscle by 80% [55]. The expression of other genes involved in glucose uptake and insulin signaling have been under investigation as well to help explain the mechanisms behind the antidiabetic effects of tea catechins.

Many studies have found protective effects of tea catechins against the development of cardiovascular diseases primarily through lipid metabolism regulation, reducing lipid absorption, inducing the production of vasodilating factors, inhibiting vasoconstriction factors, and removing free radicals [28]. In a study performed by

Papparella et al., rats dosed with angiotensin-II (a peptide hormone that causes vasoconstriction) were provided a green tea extract in their drinking water. The green tea extract inhibited NADPH oxidase activity by specifically blocking its gp91phox subunit which led to a decrease in ROS production. The same study also concluded that the metabolites of the green tea polyphenols prevented the activation of the Src/EGFR pathway, the mediators that induce cardiac hypertrophy, showing that green tea has a dual activity in attenuating endothelial oxidative stress and cardiac hypertrophy [56]. Another study explored the effects of EGCG specifically on spontaneously hypertensive rats (SHR) [57]. The rats were separated into groups treated with EGCG, enalapril (a drug used to treat high blood pressure and congestive heart failure), and an untreated control. EGCG was shown to reduce infarct size as well as improve cardiac function of hearts with tissue damage induced by ischemia. In addition, EGCG stimulated the production of nitric oxide via the PI3K pathway which led to subsequent vasodilation and a decrease in systolic blood pressure [57].

Cancer is a leading cause of death worldwide, and the second leading cause of death in the United States, exceeded by cardiovascular disease [58]. Decades of research on the effects of tea on cancer has been conducted. Whereas results on human studies are inconclusive, laboratory studies have shown strong inhibitory effects of EGCG against cancer cells [59]. EGCG, for example, has been shown to induce apoptosis in cancer cells by down-regulating anti-apoptotic proteins such as Bcl-2 and up-regulating pro-apoptotic proteins and their activities such as Bax, p53, caspase 3, and FOXO transcription factors [60-63]. This effect has been seen in many different kinds of cancers such as breast

cancer cells [62], prostate cancer cells [61], pancreatic cancer cells [63], and ovarian cancer cells [60].

The inhibitory activities were further explored by comparing the effects between catechins EGCG, ECG, GCG, CG, EGC, and EC, as well as phenolic acid GA. The presence of a gallate moiety was reported to have higher inhibition of AP-1 activity and cell growth [64]. In addition to inhibition of phosphorylating c-Jun, EGCG also inhibited Erk phosphorylation, a mitogenic stimulus of the MAPK pathway. Cell proliferation was thus inhibited [64]. In another study by Kondo et al., the effects of EGCG, EGC, ECG, and EC on angiogenesis were investigated. The formation of new blood vessels in cancer cells enables the cells to receive a continuous oxygen and nutrient supply, thereby promoting their growth and expansion. VEGF is one of the most effective factors in the induction of angiogenesis and is often upregulated in tumors [65]. All four catechins tested in this experiment appeared to have inhibitory effects on cell growth, migration, and tube formation, however, EGCG was the most effective of the four, as it inhibited VEGF receptor binding. ECG, being the second most effective inhibitor suggested that the gallate moiety may exert the greatest effects on these inhibitions [65], once again indicating that the gallate moiety might hold crucial modulatory effects in cell signaling.

### 1.8 The PI3K-dependent Insulin Signaling Pathway

Insulin-stimulated glucose uptake in target tissues such as fat and skeletal muscle is crucial for reducing blood glucose levels and maintaining whole-body glucose homeostasis [66]. The process is carried out through the activation of a complex signaling cascade following the binding of insulin to its receptor on the cell surface. Insulin's role in glucose disposal was made evident in the 1950s after it was shown to increase the rate

of glucose uptake in rat skeletal muscle [67]. Decades later, it was discovered that the mechanism by which insulin stimulates glucose uptake is through the translocation of the GLUT4 transporter protein from the cytosol to the plasma membrane [68]. Since then, the signaling pathway by which insulin-mediated glucose uptake occurs has been extensively studied and well-characterized.

The classical PI3K-dependent insulin pathway begins with the binding of insulin to the α subunits of the insulin receptor, subsequently activating the kinase activity of one of its β subunits [69]. The activated β subunit then trans-autophosphorylates the second β subunit, making it a platform for docking to by an adaptor protein of the insulin receptor substrate family [69, 70] (Figure 1). There are four isoforms (IRS-1, IRS-2, IRS-3, and IRS-4) found in rodents and three (IRS-1, IRS-2, and IRS-4) in humans [71]. IRS-1 and IRS-2 are the isoforms involved in insulin-stimulated glucose uptake, although knockout experiments in mice demonstrate that the predominant isoform involved is IRS-1 [72]. The activated IRS protein then binds the PI3K protein via its regulatory p85 subunit (Figure 1).

The role of PI3K in the insulin-mediated transport of glucose was first highlighted in studies that used wortmannin, an inhibitor of PI3K. The inhibition of PI3K completely ablated insulin-stimulated glucose transport, emphasizing the PI3K pathway's importance in activating downstream targets succeeding insulin binding [73]. The phosphorylation of PI3K induces its recruitment to the plasma membrane where it catalyzes the conversion of phosphoinositide $PIP_2$ to $PIP_3$ via its p110 subunit [74]. The phosphorylation of $PIP_2$ to $PIP_3$ causes the recruitment of the downstream proteins PDK-

1 and Akt (PKB) (Figure 1). The docking of PDK-1 and Akt to $PIP_3$ brings the two proteins close enough to induce PDK-1 phosphorylation of Akt [75].

Akt has been shown to play a pivotal role in insulin-mediated glucose uptake in many studies. In one study, a constitutively active form of Akt in L6 cells increased GLUT4 translocation and thus glucose uptake [76]. In a study with Akt knockout mice, however, a reduction in glucose uptake is observed even when stimulated with insulin. In addition, the mice developed insulin resistance and high glucose and insulin blood concentrations [77]. Three Akt isoforms have been identified (Akt1, Akt2, and Akt3), but only Akt1 and Akt2 are expressed in adipose and skeletal muscle tissue [78]. PDK-1 phosphorylates Akt on the Thr308 residue, however, Akt is not fully activated until it is also phosphorylated on the Ser473 residue carried out by the mTORC2 protein [79-82] (Figure 1). The mTORC2 protein, a component of the mTOR protein, becomes activated after Thr308 p-Akt activates mTOR [81]. The full activation of Akt not only leads to the mediation of glucose uptake, but of many different cellular pathways as well such as growth, proliferation, survival, protein synthesis, and apoptosis [80].

Perhaps the most important role of Akt phosphorylation in insulin-induced glucose uptake is the phosphorylation of the downstream Rab proteins AS160 and TBC1D1 (Figure 1). Under basal, non-stimulated conditions, AS160 and TBC1D1 bind to the GLUT4 vesicles and act as negative regulators for GLUT4 translocation [83]. Akt thus promotes GLUT4 translocation by inhibiting the Rab proteins. Multiple studies have confirmed this relationship between Akt and AS160 and TBC1D1. Experiments using siRNA against AS160 resulted in an increase in plasma membrane-localized GLUT4 in the absence of insulin [84]. AS160 mutated at the phosphorylation sites where Akt

interaction occurs, however, inhibits insulin-mediated GLUT4 translocation [83]. TBC1D1 shares a 47% sequence similarity to AS160 [85]. A form of TBC1D1 with sites mutated at Akt-binding motifs resulted in a slight inhibition of GLUT4 translocation [86].

### 1.9 The Insulin-Independent Signaling Pathway: the role of AMPK

In skeletal muscle, contraction, exercise, and hypoxia stimulate glucose transport independently of the action of insulin [87]. This was shown to occur when rat skeletal muscle was treated to contract in vitro in the presence or absence of the PI3K inhibitor wortmannin. As expected, wortmannin completely blocked insulin-stimulated glucose transport, but did not inhibit contraction-stimulated transport [88]. Like the insulin-mediated pathway, however, the mechanism by which glucose uptake is increased is also achieved via redistribution of the GLUT4 protein from the cytosol to the plasma membrane. Numerous candidates such as calcium and PKC have been suspected of being important signaling elements in mediating this contraction-induced glucose uptake in muscle, however, several studies propose that it is AMPK that predominantly facilitates the process due to its rapid activation in muscle that is exercised or electrically-stimulated to contract [87, 89] (Figure 2).

The enzyme AMP-activated protein kinase is characterized as a cellular "fuel gauge" because it senses a reduction of energy in cells marked by a rise in the AMP:ATP ratio as a result of metabolic stresses that cause accelerated ATP consumption or ATP synthesis inhibition such as hypoxia, nutrient deprivation, and muscle contraction [90, 91]. Activation of AMPK in these energy-starved states changes the cellular metabolism from an anabolic to a catabolic state in order to elevate ATP levels [92]. AMPK is an evolutionarily-conserved αβγ heterotrimer, composed of an alpha catalytic subunit (α1 or

α2), and beta (β1 or β2) and gamma (γ1, γ2, or γ3) regulatory subunits [93-97]. There are twelve AMPK complexes that have been identified. Skeletal muscle mostly expresses the α2 and β2 isoforms and very little α1 and β1. The γ3 isoform is restricted to skeletal muscle and associates with different alpha and beta isoforms depending on the muscle type it is found in [98, 99].

AMPK activity is regulated in a number of ways: allosterically through the binding of AMP or ADP to the gamma subunit, through the phosphorylation of the threonine 172 residue in the alpha subunit by upstream kinases, and through binding of glycogen to the beta subunit [94] (Figure 2). The displacement of ATP with AMP or ADP results in a two to five-fold increase in activation, while phosphorylation by other kinases increases activity over one hundred-fold. While phosphorylation by upstream kinases is the most potent activator of AMPK, ATP displacement acts two-fold in the process by indirectly suppressing dephosphorylation of the T172 site [100]. The binding of glycogen to AMPK inhibits its activity. The upstream kinases identified in the phosphorylation of AMPK in mammalian cells are the tumor suppressor liver kinase B1 (LKB1) and $Ca^{2+}$/calmodulin-dependent protein kinase kinase (CAMKK) [101-105] (Figure 2).

LKB1 is the predominant AMPK kinase in skeletal muscle and phosphorylates AMPK in response to the changes in energy state of the cell during contraction [104]. It becomes activated after forming a complex with two regulatory proteins STRAD and MO25 [102]. Studies have shown that deletion of LKB1 in skeletal muscle results in ablated T172 phosphorylation of the α2 subunit [104, 106]. This drop in AMPK activation subsequently led to a decrease in glucose uptake as well.

CAMKK is activated by an increase in intracellular $Ca^{2+}$ stimulated by its release from the sarcoplasmic reticulum during muscle contraction [107]. Many studies have been performed to determine its effects on AMPK activation and have resulted in conflicting evidence for and against its involvement in AMPK activity. In one study, AMPK activation in LKB1-deficient mouse embryonic fibroblast (MEF) and HeLa cells was shown to be stimulated by $Ca^{2+}$ ionophores. Inhibition of CAMKKβ with siRNA and inhibitor STO-609 blocked its activation [101]. These results suggest that CAMKK promotes AMPK activation, however, CAMKK expression is much higher in the brain than in other tissue such as skeletal muscle meaning possibly that AMPK activation via CAMKKs are physiologically significant in neural tissue rather than other tissue types [101]. In another study, CAMKKα, the only isoform expressed in muscle, was tested for AMPK activation in mouse skeletal muscle. CAMKKα increased AMPK phosphorylation by roughly two-fold. The phosphorylation of Akt was not affected, demonstrating that CAMKKα specifically activates AMPK, not substrates of the PI3K pathway [108].

While CAMKK positively activated AMPK in these studies, it has been shown to act independently of AMPK in other studies. In a study by Witczak et al., for example, muscles from wild-type mice showed increased [3H]-2-deoxyglucose uptake and AMPK activity by 2.5-fold. In muscles of mice with ablated AMPK activity, CAMKKα did not increase its activation, but glucose uptake remained increased by 2.5-fold indicating that CAMKKα does stimulate glucose uptake, but independently of AMPK activation [109]. Additional studies need to be conducted in order to definitively determine if CAMKK affects AMPK activation.

AMPK has been proposed to be a vital intermediate in elevated glucose uptake since a loss in its function eliminates the response, but only under certain conditions such as hypoxia. Glucose uptake is only partially suppressed in the absence of AMPK during muscle contraction, indicating that other AMPK-independent pathways are responsible for the process [110]. The absence of AMPK has no effect on insulin-stimulated glucose uptake. The first evidence of AMPK's role in mediating glucose uptake came from a study done by Merrill et al. using AICAR, a compound that activates AMPK by mimicking the effects of AMP by forming ZMP after it is metabolized in the cell [111]. AICAR-stimulated activation of AMPK leads to glucose uptake in skeletal muscle in the absence of insulin and the PI3K pathway [88].

AMPK-mediated glucose uptake is carried out by its role in stimulating the translocation of the glucose transporter protein GLUT4 and has been reported in numerous studies. In one study using isolated rat epitrochlearis muscle, AMPK activation stimulated by contraction and hypoxia coincided with increased glucose uptake [112, 113]. Fryer et al., 2002a demonstrated that a constitutively-active form of AMPK α leads to both GLUT1 and GLUT4 translocation from the cytosol to the plasma membrane and prompted an increase in glucose uptake in H-2K$^{b}$ skeletal muscle cells similar to the effects of treatment with AICAR and insulin. They also showed that the expression of a dominant-negative mutant of AMPK blocked glucose transport even with treatment of AICAR [114]. Chronic stimulation of AMPK is also found to increase both the expression and activity of hexokinase, while decreasing glycogen synthase activity [115].

Other studies have focused on AMPK activation during exercise and its effects in different systems. In studies in rats and humans, an observed increase in AMPK α1 and

α2 took place during exercise depending on the intensity (oxygen consumption during exercise) of the exercise [116, 117]. AMPK α2 increases normally at low intensity (40-50% $V_{O2max}$), while α1 was increased during high intensity (70-90% $V_{O2max}$) [116, 117]. In addition to AMPK activation, increased GLUT4 translocation and glucose uptake was accompanied. The same effects are seen in individuals with TIIDM and rodent models with metabolic syndrome phenotypes (ob/ob mice, ZDF rats, etc.), therefore exercise is often encouraged as preventative and corrective therapy for insulin resistance [97, 99, 118, 119]. It is interesting to note that in addition to promoting GLUT4 translocation, AMPK has been speculated to promote transcription of the GLUT4 gene. One study concluded that AMPK does so by translocating to the nucleus, subsequently phosphorylating the transcriptional repressor HDAC5, and dissociating it from MEF2, the pivotal regulator of GLUT4 transcription [120]. Such evidence provides even further insight for potential therapeutic targets for insulin resistance.

There has been increasing evidence supporting the idea that the contraction and insulin-stimulated pathways for glucose uptake in skeletal muscle converge downstream of Akt. Proteins members of the TBC1 family AS160 and TBC1D1 are the suspected points of signaling convergence [6, 121]. In fact, studies in rat skeletal muscle have shown that AICAR and contraction are capable of phosphorylating both AS160 and TBC1D1. TBC1D1 actually contains an AMPK phosphomotif, and both TBC1D1 and AS160 have calmodulin binding domains [122, 123]. Multiple AMPK knock out experiments have shown a correlative decrease in phosphorylation of these proteins and slight inhibition of glucose uptake. An α2 knockout, γ3 knockout, and α2 kinase-dead forms of AMPK severely blunt AS160 phosphorylation even when stimulated with

AICAR and contraction [124]. When treating rat epitrochlearis muscle with compound C, an inhibitor of AMPK, phosphorylation of TBC1D1 and glucose uptake is partially blocked after stimulating contraction [125]. These data indicate that AMPK does play some role in contraction-stimulated activation of AS160 and TBC1D1, however, future studies in this line of work are being conducted to uncover the mechanism of action of AMPK in this stimulation.

### 1.10 The Glucose Transport Proteins

Glucose obtained from the diet that enters the circulation must be transported across the plasma membrane into target cells. Integral glucose transport proteins are responsible for carrying out this process [126]. The glucose transporters fall into two distinct groups: the $Na^+$-dependent glucose co-transporters (SGLT family), and the $Na^+$-independent transporters (GLUT family) [126-128]. Members of the SGLT family use the $Na^+$ electrochemical gradient established by the $Na^+/K^+$-ATPase pump to transport glucose against its concentration gradient into target cells [128]. This process occurs across the lumen of cells that line the small intestine [126, 127]. The members of the GLUT family of proteins are ATP-independent facilitative transporters and are subdivided into three classes [126, 129, 130]. All isoforms share a characteristic twelve transmembrane helix structure and form aqueous pores through the membrane to allow glucose entry [126, 129, 130].

The class I GLUT transporters (GLUT1-4) are the best characterized of the family. GLUT1 is highly expressed in erythrocytes, and moderately in the cells that compose the blood-brain barrier, muscle, and adipose tissues [126-128]. GLUT2 is expressed in the liver, kidney, and pancreatic beta cells [126-128]. GLUT3, which has a

high affinity for glucose, is found mainly in neurons since the brain has such a high demand for glucose [126-128]. GLUT1-3 are highly expressed on the plasma membrane due to their high exocytotic rates where they constitutively transport glucose into the cell [128].

The GLUT4 protein is found in adipose tissue, cardiac muscle, and skeletal muscle. It is the only insulin-responsive transporter and is responsible for reducing plasma glucose levels postprandially and during times of metabolic demand such as exercise [126, 128-131]. Unlike the other class I transporters, GLUT4 is not highly located at the plasma membrane, but is rather slowly recycled to and from via intracellular membrane fractions during basal conditions due to its low exocytotic rate [131, 132]. As a highly stable protein, GLUT4 has a half-life of about 48 hours, meaning one molecule can undergo multiple rounds of recycling before it gets targeted for degradation [132, 133]. During steady-state conditions in the absence of any stimulating factors such as insulin or exercise, GLUT4 mostly remains in intracellular vesicles in the cytosol with less than five percent expressed on the cell surface [66]. This intracellular trafficking property is much different than the trafficking of GLUT1 despite their shared 65% amino acid sequence identity [134].

Class II isoforms consist of GLUT5, GLUT7, GLUT9, and GLUT11, transporters with the ability to transport fructose across the plasma membrane [126, 127, 129]. Class III isoforms GLUT6, GLUT8, GLUT10, GLUT12, and HMIT share structural differences that set them apart from classes I and II such as the presence of an internalization signal that retain them in the cytosol at steady-state conditions [126, 127, 129, 130].

It is believed that muscle and adipose tissue developed a uniquely specific system for glucose uptake, as insulin has an insignificant effect on GLUT1 trafficking, the constituent type that is responsible for glucose transport at rest, but rapidly upregulates the rate of GLUT4 translocation when needed [131]. This highly specialized system put in place is important during times when blood glucose levels are high (such as after a meal), when metabolic demand increases (such as during exercise), and even during steady-state conditions: a high retention of GLUT4 within the cytosol is necessary when blood glucose levels are not elevated in order to prevent hypoglycemic conditions [131]. GLUT4 translocation via insulin stimulation is the rate-limiting step of glucose uptake in both skeletal muscle and adipose tissue, therefore an event that causes diminished movement of GLUT4 is linked to insulin resistance [127].

Trafficking of the GLUT4 protein has been studied extensively for decades, yet parts of the process remain elusive and need further investigating. Most knowledge of the trafficking routes comes from subcellular fractionation, GLUT4 fusion proteins (HA-GLUT4-GFP), and fluorescence microscopy examinations on cell systems; mainly primary rat adipocytes, 3T3-L1 mouse adipocytes, and L6 rat skeletal muscle cells [132]. Internalization of the GLUT4 transporter protein is carried out by one of two processes: through clathrin-mediated endocytosis (CME) and a cholesterol-dependent pathway, both of which occur in adipocytes, but only the former (CME) in skeletal muscle [132, 135, 136]. Internally, the GLUT4 protein is retained in a number of different types of compartments such as early endosomes (EE), the trans-Golgi network (TGN), a constituent of the TGN called the endosomal recycling compartment (ERC), and specialized compartments that are 50 nm in diameter called GLUT4 storage vesicles

(GSVs) [131, 137]. Roughly 40-50% of internalized GLUT4 resides in the ERC and the remaining 50-60% is retained in the GSV and TGN with recurrent cycling between compartments [132, 138].

GSVs have been identified as major targets of insulin-induced translocation of GLUT4. GSVs have been hard to characterize due to a lack of specific identifying markers, however, by subcellular fractionation and immunofluorescence analyses, a number of proteins accompanied with them have been distinguished: IRAP, VAMP2, sortilin, LRP1, and TUG [131, 132, 139, 140]. In addition to these GSV proteins, numerous other proteins are involved in the regulation of GLUT4 transit. Multiple Rab GTPases (Rab4, 5, 8, 10, 11, 13, 14, and 31) play a role in vesicle sorting. Rab5 and Rab11 help mediate GLUT4 endocytosis while the latter are involved in exocytosis upon insulin stimulation [132, 141]. The Akt substrate AS160 is another Rab protein that participates in GLUT4 vesicle trafficking as a negative regulator of exocytosis under basal conditions . After the system is stimulated with insulin, however, the phosphorylation of AS160 causes its dissociation from the GSV, thereby promoting GLUT4 translocation to the plasma membrane [66, 83]. The docking and fusion of the GSVs to plasma membrane after insulin stimulation is carried out by SNARE proteins, VAMP2, Syntaxin 4, and SNAP23 [132, 142, 143].

The movement of GLUT4 between internal compartments is likened to the transferrin receptor, although parts of the mechanism such as proteins involved in the process remain elusive and need further investigation [144]. After its internalization through CME, GLUT4 subsequently fuses to early endosomes. Beyond early endosomes, GLUT4 is sorted into the ERC where it can slowly be recycled to the PM, be retained in

the TGN, or be sorted into GSVs until it is targeted for degradation in the lysosomes [132, 144]. This branch of GLUT4 trafficking is known as the recycling pathway [70, 131, 132]. The TGN also participates in sorting newly synthesized GLUT4 transporters directly into GSVs through the biosynthetic pathway of GLUT4 trafficking [132, 145]. Understanding the full mechanism behind GLUT4 trafficking could bring about important therapeutic targets to combat insulin resistance in the future.

### 1.11 Lipid Rafts and IR Trafficking

Microdomains of the plasma membrane known as lipid rafts have been speculated to act as regulators in receptor-mediated processes such as signal transduction, potocytosis (transport of small molecules across the PM), transcytosis (macromolecule transport), and endocytosis by assembling signaling proteins into complexes that are easily accessible to activation by different stimulators [146-148]. Lipid rafts are organized subdomains of glycosphingolipids and cholesterol that serve as platforms to distinct classes of proteins such as flotillins, GPI-linked proteins, src family kinases, EGF receptors, PDGF receptors, endothelin receptors, MAPK, PKC, and the p85 subunit of PI3K with many more still being discovered [147, 149]. Caveolae are a specialized subset of rafts characterized as small flask-shaped invaginations of the cell membrane [149, 150]. Like lipid rafts, they too are composed highly of cholesterol and glycosphingolipids, however, unique to caveolae are the presence of cholesterol-binding proteins called caveolins [150]. Caveolins are responsible for stabilizing the caveolae structure and are found in three isoforms: caveolin-1, caveolin-2, and caveolin-3. Cav-1 and Cav-2 are expressed in nearly all tissue types, while Cav-3 is strictly muscle-specific [151, 152]. In addition to caveolins, caveolae are also composed of proteins that

participate in intracellular membrane fusion and vesicular trafficking such as members of the VAMP, GTPase, annexin, and SNAPs families [146].

In order to reduce the responsiveness of insulin-sensitive tissues after continuous stimulation, the insulin receptor becomes endocytosed into the cell upon insulin binding, leading to the downregulation of the receptor [153]. After insulin stimulation, the IR co-localizes with caveolin proteins as confirmed by subcellular fractionation and co-immunocapture of both IR and caveolin antibodies in previous studies [153-156]. The insulin receptor binds to caveolin via a caveolin binding motif that is present within all known caveolin-associated proteins. The CSD can exist as two similar sequences, φXXXXφXXφ and φXφXXXXφ, where φ represents an aromatic amino acid [157]. The insulin receptor beta subunit contains the binding motif WSFGVVLW in its sequence [156]. In one study, the scaffolding domains of cav-1 and cav-3 were shown to directly stimulate insulin receptor kinase activity and served to amplify its activity as well as target the receptor to caveolae [156]. In addition to these factors, the CSD also functionally stabilizes the insulin receptor.

Cav-3 is known to regulate the insulin receptor's expression in the plasma membrane of muscle cells as an enhancer and preventing its rapid internalization and subsequent degradation [158]. In fact, its inhibition leads to diminished insulin signaling [159]. In addition, mice with cav-3 knockouts developed increased whole body adiposity, insulin resistance, hyperglycemia, and enlarged beta islets, all indicative of a pre-diabetic state [158, 160]. In one study, caveolin-3 was also shown to be required for activation of the PI3K pathway as knockouts led to decreased activation of both PI3K and Akt [161].

Little is known about the interplay between microdomains and catechins, but many studies have been performed to test the roles they have at the cell surface and the characteristics that both contribute to and affect their interactions. As mentioned earlier, polyphenols are not only known as antioxidants, but as cell metabolism regulators as well [47, 48]. All of these functions propagate from their initial interaction with the cell surface and subsequent penetration through the plasma membrane into the cytosol. Like the effect of their antioxidant properties, penetration of the cell surface also correlates with the number of hydroxyl groups present on the molecule as well as the hydrophobicity of the molecule which contributes to their abilities to form electrostatic and hydrophobic interactions, hydrogen bonding, and even covalent bonding at the water-lipid interface [47, 48, 162]. This was supported in a study that measured the surface interaction of different flavonoids with phosphatidylcholine through the retention time in an HPLC column and concluded that higher retention time was positively correlated with the number of hydroxyl groups in the compounds [163].

Polyphenols also affect some physical properties of membrane lipids such as fluidity, permeability to hydrophilic compounds, and membrane fusion [47, 164]. Gallated tea catechins that contain a gallic moiety (EGCG and ECG) and therefore more hydroxyl groups are believed to have a greater influence on membrane lipid properties than non-gallated catechins (EGC and EC). Through their attachment to the surface and alterations of various physical properties of the membrane, they are thought to take some part in initiating lipid-protein clusters (similar to that of lipid raft formations) which are important in cell signaling [47].

### 1.12 The L6 Cell Line

L6 cells are an immortalized cell line derived from rat skeletal muscle [165]. They propagate as mononucleated myoblasts, but have a high capacity to differentiate and fuse into multinucleated primary myotubes with myotube muscle cell phenotypes, such as the expression of the GLUT4 glucose transporter protein and myosin [166]. They readily respond to insulin stimulation and at maximal responsiveness when differentiated into myotubes [166, 167]. L6 cells have been used extensively in studies that investigate insulin resistance, diabetes, and novel compounds used to treat insulin resistance and diabetes.

# 2 Goals and Specific Aims

## 2.1 Rationale

The main goal of this thesis research is to compare the effects of common polyphenols found in tea (EGCG, ECG, EGC, EC, and GA) on GLUT4 translocation in L6 myocytes in order to determine the possible mechanisms for alleviating symptoms in individuals that suffer from insulin resistance. This was accomplished by exploring the signaling pathways by which these compounds stimulate the process. The two key pathways that promote GLUT4 translocation are the insulin-mediated (PI3K) pathway and the insulin-independent (AMPK) pathway. The phosphorylation of key proteins in the pathway would indicate their activations. The phosphorylation of downstream Akt (Thr308), a key promoter of GLUT4 translocation, would indicate activation of the PI3K pathway. The stimulation of the AMPK α subunit phosphorylation (Thr172) would implicate its activation.

Most current studies in this area focus on the effects of EGCG, the most abundant catechin found in tea. In one study, GLUT4 translocation and glucose uptake activity in both rat soleus muscle and L6 myotubes were found to increase after being administered doses of EGCG [168]. Similar results were observed in other experiments using dietary EGCG treatment on mice fed a high-fat western diet in which body weight gain, insulin resistance, and blood glucose levels all decreased in addition to decreased cholesterol levels, hepatic steatosis, and inflammatory cytokines [50, 169]. Previous comparative studies on tea polyphenols have investigated their antioxidant properties which indicated that the antioxidant activity depended substantially on the number and positions of hydroxyl groups within the molecule [33].

In this study, we hope to gain a better understanding of the underlying mechanisms of polyphenol action on GLUT4 translocation in skeletal muscle and to find a relationship between compound structure and strength of activity. We therefore hypothesize that polyphenols that are similar in structure to EGCG, a well-studied and well-known promoter of Akt activation, AMPK activation, and GLUT4 translocation, will share similar properties, while those that differ (non-gallated polyphenols and gallic acid) will have lesser effects.

### 2.2 Specific Aims

1. To assess whether major tea polyphenols induce activation of the insulin-mediated PI3K pathway by evaluating the phosphorylation state of its downstream target Akt.
2. To determine whether tea polyphenols promote activation of the insulin-independent AMPK pathway.
3. To determine whether polyphenols promote GLUT4 translocation in skeletal muscle cells.

# 3 Materials and Methods

## 3.1 Cell Culture

The L6 rat skeletal muscle cell line for all experiments performed was obtained from ATCC® (Manassas, VA). Cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) growth media with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning, Manassas, VA) and supplemented with 10% sterile-filtered fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO) and 1% penicillin-streptomycin solution (Corning, Manassas, VA). Cells were seeded in 100 x 20 mm TC-treated petri dishes (Greiner Bio-One, Monroe, NC) at 37°C in 5% $CO_2$. Cells were grown to 90% confluence and passaged every 3 days using 1X Trypsin-EDTA solution (Corning, Manassas, VA). For differentiation in myotubes, myoblasts were introduced to DMEM supplemented with 2% FBS (to stop growth and promote fusion) and 1% penicillin-streptomycin for 5 additional days. Any cells that were collected and saved for storage in -80°C were put in a freezing media containing DMEM, 20% FBS, and 10% sterile-filtered dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO). This culturing technique was used for all experiments performed during this project.

## 3.2 Experiment 1: Akt Activation Time Course

L6 cells were differentiated into myotubes using the protocol above. A 10 nM solution of recombinant human insulin was prepared from a 5 mg/ml stock (Lonza, Walkersville, MD). 50 mM solutions of (-) EGCG, (-) ECG, (-) EGC, (-) EC, and GA (provided by Dr. Marlon Lee, Rutgers University) were also prepared. On the days when cells were being treated, the myotubes were given fresh 10% media. The 50 mM polyphenol stock solutions were each diluted to 30 μM concentrations. The L6 myotubes

were incubated in either 10 nM insulin, which served as a positive control whenever supplemented, 30 µM EGCG, 30 µM ECG, 30 µM EGC, 30 µM EC, 30 µM GA, or 30 µM EGC + 30 µM GA (the hydrolyzed forms of EGCG) for specific times (0, 5, 15, 30, and 60 min).

### 3.2.1 Production of whole cell lysates

Myotubes were washed in 2 mL of 1X Dulbecco's Phosphate-Buffered Saline (D-PBS) (Corning, Manassas, VA) and lysed with 500 µL of lysis buffer for 30 minutes on ice. Cells were lysed with RIPA buffer with ethylenediaminetetraacetic acid (EDTA) (Boston BioProducts, Ashland, Md) containing protease inhibitors (1 mM Phenylmethylsulfonyl fluoride [PMSF] from Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitors (1 mM phosphatase inhibitor cocktail 1, 1 mM phosphatase inhibitor cocktail 2, and 1 mM phosphatase inhibitor cocktail 3 from Sigma-Aldrich, St. Louis, MO). Lysed samples of each treatment were collected and centrifuged at 12,000 x g for 15 minutes at 4°C. The supernatants were collected and protein concentration was determined by BCA assay.

### 3.2.2 BCA Protein Assay

A Pierce bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Scientific (Rockford, IL) to determine protein concentrations of cell lysates. A 96-well microplate was used to analyze lysate samples (TPP, Trasadingen, Switzerland). A standard curve of bovine serum albumin (BSA) (0 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL, and 2.0 mg/mL BSA in deionized water) was prepared. The working solution was made with Reagents A and B in a 50:1 ratio, respectively. Each standard (25 µl) was added to separate wells of the microplate. Each

lysate sample (25 µl) was also added to separate wells of the microplate. Working solution (200 µl) was then added in addition to all wells containing standards and samples. The plate was incubated for 30 minutes at 37°C. Final concentrations were measured by the Infinite m200 microplate reader at 562 nm (Tecan, Männedorf, Switzerland).

### 3.2.3 Western Blotting

Gels for electrophoresis were prepared using 1.5 mm glass plates from Bio-Rad (Hercules, CA). A 10% gel resolving gel was made using deionized water, (w/v) BioAcryl-P (30%; acrylamide: bis-acrylamide 39:1), (w/v) 1X separating/resolving buffer (1.5 M Tris Buffer, pH 8.8) (Boston BioProducts, Ashland, MD), 10% (w/v) sodium dodecyl sulfate (SDS), 10% (w/v) ammonium persulphate (APS) (Bio-Rad, Hercules, CA), and N,N,N,N'-tetramethylethylenediamine (TEMED) (Sigma-Aldrich, St. Louis, MO) to polymerize the gel. The resolving gel was poured between the glass plates. Bubbles were removed using ethanol. The gel was left to solidify for 30 minutes. A 5% stacking gel was made using the same ingredients listed above, but using (w/v) 1X stacking buffer (1M Tris Buffer, pH 6.8) in place of the separating/resolving buffer (Boston BioProducts, Ashland, MD). After pouring the stacking gel to the glass plates, plastic combs were added. The gel was left to polymerize for another 30 minutes before being loaded to the electrophoretic cells.

Samples containing 20 µg of protein were mixed with 1X Laemmli's SDS-sample buffer (Boston BioProducts, Ashland, MD) and then heated at 95°C for 10 minutes. The proteins were loaded onto the gel alongside the Precision Plus Protein Dual Color Standards marker from Bio-Rad (Hercules, CA) and run in 1X Running Buffer (Boston

BioProducts, Ashland, MD) at 110V for 90 minutes using the Power Pac Basic from Bio-Rad (Hercules, CA). After the proteins had been separated on the gel, they were transferred to a 0.45 μm PVDF membrane pre-activated in methanol (Life Sciences, Grand Island, NY) at 100V for 60 minutes in 1X Transfer Buffer (Boston BioProducts, Ashland, MD). Transfer of the proteins was verified by Ponceau S staining (Sigma-Aldrich, St. Louis, MO). The membranes were cut into strips at the predicted weights of each protein being detected (60 kDa for Akt and p-Akt; 42 kDa for β-actin). Blots were blocked for 1 hour at room temperature in Odyssey Infrared Imaging System Blocking Buffer (LI-COR Biosciences, Lincoln, NE).

Blots were incubated overnight at 4°C in either rabbit phospho-Akt Thr308 antibody, Akt, or mouse β-actin (Cell Signaling Technology, Danvers, MA). The primary antibodies were diluted 1:1000 in 5% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and 1X Tris Buffer Saline (TBS) (Boston BioProducts, Ashland, MD). Following primary incubation, blots were washed with 2% (w/v) Non-fat dry milk (Lab Scientific, Highlands, NJ) in TBS for 10 minutes three times. The blots were then incubated in 1:5000 anti-rabbit Hrp-linked IgG or anti-mouse Hrp-linked IgG (Cell Signaling Technology, Danvers, MA) in 5% BSA (w/v) and 1X TBS for one hour at room temperature. The same washing procedure was followed after the secondary antibody incubation. The membrane was developed by using SuperSignal West Femto Maximum Sensitivity Substrate from Thermo Scientific (Rockford, IL). Substrates A and B in the kit were mixed in a 1:1 ratio. The blots were imaged using the enhanced chemiluminiscence detection system from Amersham Pharmacia (Piscataway, NJ) and

Quantity One software from Bio-Rad (Hercules, CA). Quantification of protein bands was done by using ImageJ version 1.48.

### 3.3 Experiment 2: AMPK Activation Time Course

The protocol for dosing L6 myotubes with the different 30 µm polyphenols was the same as I described earlier, however, insulin was not used in this experiment. The incubation times used were the same from Experiment 1. Cell lysates were produced by using 1% NP-40 lysis buffer (25 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and deionized water) supplemented with protease inhibitors from Sigma-Aldrich (St Louis, MO). Lysed samples were collected and centrifuged at 12,000 x g for 15 minutes at 4°C.

Protein samples (45 µg) were loaded to the gels for Western blot analysis due to there being a lower expression of AMPK in the cell line (as determined when 20 µg of protein yielded a weak signal). The membranes were incubated overnight at 4°C in 1:1000 rabbit phospho-AMPKα Thr172 (62 kDa), 1:1000 rabbit AMPKα, and 1:1000 mouse β-actin antibodies (Cell Signaling Technology, Danvers, MA). Washing steps, and secondary antibody incubations, and membrane development were performed using the same protocol described earlier. Imaging was performed using the Syngene GeneGnome chemiluminescence imaging from Imgen Technologies (New City, NY). Quantification was performed using ImageJ version 1.48.

### 3.4 Experiment 3: Effect of SOD/catalase on Akt and AMPK activation

L6 cells were cultured using the same protocol above. On the day of protein extraction, cell medium was aspirated and cells were re-supplemented with DMEM containing 0.1% superoxide dismutase (SOD) and 0.1% catalase (Sigma-Aldrich, St.

Louis, MO) in addition to 10% FBS. Cells were incubated in 30 µM EGCG or 30 µM ECG for 15 minutes. An untreated sample ([+] FBS, (+) SOD/catalase, (-) EGCG, (-) ECG) served as a control. Protein extraction and Western blot protocols were performed the same as experiment 3. Blots were detected for Akt, p-Akt (Thr308), AMPK, p-AMPK (Thr172), and β-actin. Imaging and quantifications were the same as previously described. The results of this experiment were compared to the results from experiments 2 and 3 ([+] FBS, (-) SOD, (-) catalase) to identify differences in Akt and AMPK activations.

### 3.5 Experiment 4: GLUT4 Translocation Time Course

The protocol for dosing L6 myotubes with 10 nM insulin, 30 µm EGCG, 30 µm ECG, 30 µm EGC, 30 µm GA, and 30 µm EGC + 30 µm GA at different times were the same as I described earlier in Experiment 1. The incubation times in this experiment, however, were 0, 30, 60, and 90 minutes.

The collection of cell lysates in this experiment was carried out by using the Plasma Membrane Protein Extraction Kit obtained from Abcam (Cambridge, MA). The protease inhibitor cocktail in the kit was dissolved in DMSO. The protease inhibitor (0.2% volume of the lysate) was then added to the homogenize buffer. Myotubes were washed in 2 mL of 1X D-PBS following incubation times then scraped off of and spun down at 2,000 x g for 10 minutes. The pellet was re-suspended in 500 µl of homogenize buffer and homogenized with a dounce homogenizer. The homogenate was centrifuged at 1,000 x g for 10 minutes at 4°C. The supernatant was saved and centrifuged again at 14,000 x g for 30 minutes at 4°C. The pellet (the plasma membrane fraction) was labeled

and saved, as well as the supernatant (the cytosolic fraction). The plasma membrane proteins were then purified following the manufacturer's instructions.

The protocols for preparing electrophoresis gels and Western blotting were performed as described above. The membranes with the cytosolic proteins and the membranes with the plasma membrane proteins were all incubated overnight at 4°C in 1:2000 mouse GLUT4 (54 kDa) antibody (Abcam, Cambridge, MA). In order to verify there was a clean separation between cytosolic and plasma membrane fractions, blots were also incubated in 1:1000 rabbit GAPDH (37 kDa), a house-keeping protein expressed intracellularly, and 1:1000 rabbit $Na^+/K^+$-ATPase (100 kDa), a transmembrane protein located in the plasma membrane (Cell Signaling Technology, Danvers, MA). Washing steps, secondary antibody incubation, detection, and quantification were all performed using the same protocol from Experiment 1.

### 3.6 Statistical Analysis

Data were analyzed by mixed-design ANOVA, paired t-tests, and one-way ANOVA where necessary by using SPSS21. Microsoft Excel 2010 was used for all graphical analysis. All numeric values were presented as means ± standard deviation. ImageJ version 1.48 was used for densitometry analysis of Western blots.

# 4 Results

## 4.1 Akt Activation Time Course

Polyphenols have been shown to promote glucose uptake in studies using both animal and cellular models [53, 168]. Akt is the key kinase that facilitates glucose uptake via the PI3K-dependent insulin signaling pathway in skeletal muscle [76, 77], therefore, the effects of insulin and various polyphenols on Akt activation (p-Akt Thr308) were determined by Western blot analysis (Figure 6). There was a statistically significant interaction between the effects of different treatments and time on Akt activation ($F = 85.44$, $p = < 0.05$). Mauchly's Test of Sphericity indicated that the assumption of sphericity had not been violated ($\chi 2\ (9) = 12.02$, $p = 0.216$).

A common trend seen between treatments with insulin, EGCG, ECG, EC, and GA was the ratio of p-Akt to Akt being the highest after cells were incubated for 15 minutes (Figures 6, 7). The p-Akt to Akt ratio was slightly higher after 5 minute incubations in cells treated with EGC or EGC + G. A paired t-test, however, showed that there is no significant difference in Akt activation between 5 and 15 minutes for both EGC ($p = 0.43$) and EGC + G ($p = 0.10$).

A post-hoc Tukey HSD test showed a significant difference in Akt activation between treatments. The greatest increase in p-Akt expression was observed in cells treated with insulin compared to the rest of the treatments and the untreated control. Insulin-stimulated Akt activation was 20% higher than EGCG and 520% higher than GA at the highest point of activation (Figure 7). The strongest effects in Akt activation between polyphenols were found in EGCG and ECG (Figure 7). Akt activation in cells treated with EGC and EGC + G were less effective than insulin, EGCG, and ECG, but

were significantly different from EC, GA, and the control ($p = < 0.05$). EC and GA were ineffective on Akt activation, as they were not significantly different from the control (Figure 7).

The fifteen minute time points for insulin, EGCG, ECG, EGC, and GA were reanalyzed for a more quantitative analysis of p-Akt expression. They were all compared to an untreated control. The fifteen minute incubation was chosen because the time course analysis showed Akt activation was the most prominent at this point (Figure 7). A Tukey HSD test showed that insulin had the strongest effect on p-Akt expression and was significantly higher than all other treatments and the untreated control ($p = < 0.05$) (Figure 8). EGCG and ECG were not significantly different from each other ($p = 0.057$), but also resulted in significant Akt activation compared to the control ($p = < 0.05$) and other treatments (Figure 8). EGC and GA were not significantly different from each other ($p = 0.051$) or the control ([EGC] $p = 0.52$; [GA] $p = 0.96$), and thus ineffective in promoting Akt activation (Figure 8).

**4.2 AMPK Activation Time Course**

AMPK plays an important role as a sensor in cellular energy homeostasis [90, 91]. In many studies it has been shown that activation through its α subunit results in an increase of glucose uptake in both normal cells and insulin-resistant cells [117-119]. EGCG itself has been shown to increase AMPK activation in cells [170, 171], therefore its effects on activation (phosphorylation of Thr172 α subunit) along with the effects of other major tea polyphenols were determined via Western blot analysis (Figure 9).

There was a statistically significant interaction between the effects of different polyphenols and time on AMPK activation ($F = 54.09$, $p = < 0.05$). Mauchly's Test of

Sphericity indicated that the assumption of sphericity had not been violated ($\chi 2$ (9) = 13.23, p = 0.16). As shown in Figures 9 and 10, the highest point of AMPK activation for EGCG, ECG, and EGC + G occurred after 30 minutes of incubation, while the highest point for EGC, EC, and G occurred after 15 minutes of incubation. A paired t-test showed that there is no significant difference in AMPK activation between 15 and 30 minutes for EGC (p = 0.10), EC (p = 0.25), and G (p = 0.20).

A post-hoc Tukey HSD test showed a significant difference in AMPK activation between treatments. The greatest increase in p-AMPK expression was observed in cells treated with EGCG and ECG compared to the rest of the treatments and the untreated control (Figure 10). AMPK activation in cells treated with EGC, EGC + G, EC, and GA were ineffective, as they were not significantly different from the control ([EGC] p = 0.52; [EGC + G] p = 0.51; [EC] p = 0.73; [GA] p = 0.75), although EGC + G and EGC were significantly different from EC and GA (p = < 0.05) (Figure 10).

An additional more quantitative measurement of p-AMPK expression between EGCG, ECG, EGC, and GA compared to an untreated control was also performed. Similar to the Akt phosphorylation experiment, the fifteen minute incubation was also used for this experiment. EGCG and ECG (p = 0.332) had the strongest effect on p-AMPK expression and were significant from the untreated control (p = < 0.05) (Figure 11). ECG and EGC were not significantly different from each other (p = 0.36). EGC and GA were not significantly different from each other (p = 0.55) or the control ([EGC] p = 0.42; [GA] p = 0.99), and thus ineffective in promoting AMPK activation (Figure 11).

### 4.3 Effect of superoxide dismutase/catalase on Akt and AMPK activation

Reactive oxygen species are byproducts produced during the aerobic energy metabolism of oxygen gas [172]. Typically ROS is regarded as toxic to biomolecules, causing oxidative stress when their production exceeds that of the antioxidant capacity of enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Despite them being implicated in a large number of diseases, cellular damage, and aging, increasing evidence suggests that low levels of ROS play essential roles in cell signaling processes [173]. Polyphenols, while well-known for their antioxidant effects, can also be pro-oxidant, as they are unstable and undergo auto-oxidative reactions that result in the production of ROS [174]. The auto-oxidation which is catalyzed by superoxide radicals in a chain reaction would be inhibited by superoxide dismutase (SOD). The hydrogen peroxide produced from superoxide would be decomposed by catalase. In my experiments, the antioxidant enzymes SOD and catalase were supplemented to L6 cell cultures during the treatment with EGCG or ECG in order to see if there is an effect on Akt and AMPK activation (Figure 12).

A paired t-test was run on samples treated in the presence and absence of SOD and catalase to determine whether there would be a difference in Akt and AMPK activation. The ratio of p-Akt to Akt in cells treated with EGCG in the presence of SOD/catalase was $0.85 \pm 0.09$, while the ratio in the absence of the enzymes was $0.95 \pm 0.11$ ($p = 0.14$). Similarly, there was no significant difference in Akt activation in cells treated with ECG with the addition of SOD/catalase ($0.65 \pm 0.02$) and without the addition of SOD/catalase ($0.73 \pm 0.06$); ($p = 0.1$).

Unlike Akt activation, AMPK activation in cells supplemented with SOD and catalase showed a marked decrease (Figure 12). Cells treated with EGCG in the absences of SOD/catalase had a p-AMPK/AMPK ratio of 1.3 ± 0.05, while the addition of SOD and catalase resulted in a significantly lower ratio of 0.93 ± 0.03 ($p = < 0.05$). Cells treated with ECG and no SOD/catalase had a p-AMPK/AMPK ratio of 1.1 ± 0.06, while the addition of SOD and catalase had a significantly lower ratio of 0.68 ± 0.03 ($p = < 0.05$). These results suggest that ROS generated through EGCG and ECG auto-oxidation contributes significantly to AMPK activation, but not Akt activation.

### 4.4 GLUT4 Translocation Time Course

The presence of GLUT4 in the plasma membrane is the rate-limiting step for glucose uptake in skeletal muscle tissue [127]. Its activation is carried out by two distinct pathways: the first being the insulin-mediated PI3K-dependent pathway in which Akt phosphorylates downstream targets that promote GLUT4 trafficking toward the plasma membrane [66], and the second being the insulin-independent activation of AMPK through muscle contraction and other cellular stresses [87, 88]. As previously reported, polyphenols have been shown to reduce blood glucose levels postprandially in rodents [53, 54], and EGCG itself has been shown to promote the translocation of GLUT4 in skeletal muscle, thus a comparative analysis of GLUT4 translocation between insulin (positive control) and EGCG, ECG, EGC, GA, and EGC + G was conducted via Western blot analysis. EC was excluded from this study due to its poor effects on Akt and AMPK activation. GA was included, as it is one of the hydrolyzed forms of EGCG (EGC + G). In the experiments on Akt and AMPK activation, the effects of EC and GA followed

similar trends (Figures 6-11). It will therefore be assumed that the effects of GA on GLUT4 translocation are similar to the effects of EC.

GLUT4 expression on the plasma membrane was the highest after cells were incubated for 60 minutes with 10nM insulin, 30 µM EGCG, 30 µM ECG, 30 µM EGC, 30 µM GA, and 30 µM EGC + 30 µM G (Figures 13, 14). After 90 minutes of incubation, the GLUT4 protein expression at the surface had begun to decrease slightly which may be attributable to some degradation or decreased availability of the substrates overtime. It could be due to an increase in the activity of negative-feedback mechanisms such as the increased serine phosphorylation of the IRS-1 protein, or a number of phosphatases tapering downstream signals. Because GLUT4 expression was found to be the highest after 60 minutes on the plasma membrane, a one-way ANOVA comparing the means of each treatment was performed using this time point.

There was a significant difference between the effects of insulin and the various polyphenols on GLUT4 mobility to the cell surface ($F = 49.87$, $p = < 0.05$). Insulin had the highest effect on GLUT4 translocation, increasing its levels on the plasma membrane to 64% compared to the untreated control (Figure 14). Out of the polyphenols used, EGCG was the most effective at inducing GLUT4 translocation with 53% expressed in the plasma membrane of cells compared to the control (Figure 14). ECG increased GLUT4 levels with 48% expression in the plasma membrane, EGC with 35%, GA with 21%, and EGC + G with 37% in the plasma membrane (Figure 14).

A post-hoc Tukey HSD test showed a significant difference in GLUT4 translocation between treatments. The greatest increase in GLUT4 expression in the plasma membrane after treatment was found among cells treated with insulin, EGCG,

and ECG compared to the rest of the compounds and the untreated control ($p = < 0.05$). EGC, EGC + G, and GA were less effective than the other treatments and not significantly different from the untreated control ([EGC] $p = 0.28$; [EGC + G] $p = 0.25$; [GA] $p = 0.11$), although EGC and EGC + G were both significantly different from GA ($p = < 0.05$).

# 5 Discussion and Future Direction

## 5.1 Akt Activation Time Course

The results shown in this experiment collectively provide evidence that polyphenols do possess the ability to activate Akt in skeletal muscle cells in a time-dependent manner via the PI3K pathway similar to the effects of the positive control by insulin. The effects of polyphenols on Akt activation were also found to occur in a dose-dependent manner, as treatment of L6 cells with 5 µM and 10 µM concentrations yielded a weaker p-Akt signal (data not included). Furthermore, some polyphenols had a much more dramatic effect on Akt activation compared to the others. EGCG had the strongest effect, followed by ECG, EGC, EGC + G, EC, and lastly GA. It is worth noting that EGCG and ECG are both gallated catechins and possess more hydroxyl groups in their structures compared to the non-gallated catechins (EGC and EC) and gallic acid.

Previous studies have found a correlation between the number of hydroxyl groups on polyphenols and the effect of certain characteristics and interactions they have on cellular components. For example, their abilities to interact with the plasma membrane, proteins, and polynucleotides via hydrophobic, electrostatic, and covalent binding are related to the number of hydroxyl groups [47, 48]. The presence of gallate moieties establishes binding of the molecule to the plasma membrane surface through interaction with the lipid headgroups. In addition, the interactions of pi electrons in the phenolic ring to the trimethylamine groups of the phosphatidylcholine and sphingomyelin play a role [47]. Together, they cause a reduction in membrane dipole potential, inter-bilayer spacing, and membrane fluidity [47, 164]. In addition, polyphenols rich in gallate moieties can form lipid and protein clusters similar to the effects of lipid raft formations

which is essential for cell signaling [47]. On the inside of the cell, they may bind to a variety of molecules and cause the activation of the PI3K signaling pathway, however, further studies are required in order to give further insight on this idea.

The results of this experiment are consistent with other studies that tested the effect of polyphenols on Akt activation in skeletal muscle. In one study, EGCG and ECG as well as curcumin were found to increase Akt phosphorylation in free fatty acid-induced insulin resistant mouse C2C12 skeletal muscle cells, although ECG was reported to have a greater effect on Akt activation than EGCG [175] unlike the present study. EGCG also improved insulin-stimulated glucose uptake in dexamethasone-induced insulin resistant L6 cells through increased Akt phosphorylation (Ser473) as well [171]. Another study reported Akt activation in mouse soleus muscle after a week of being administered various fermented teas, however it is unclear which specific compounds (polyphenols or theaflavins) played key roles in the phosphorylation event [176]. In the current study, the detection of Akt phosphorylation at Thr308 was performed rather than Ser473. Phosphorylation of Akt at residue Thr308 occurs via PDK1 and is indicative of activation of the PI3K pathway. The results of this study show that major tea polyphenols can activate the insulin-mediated PI3K pathway in L6 rat skeletal muscle cells.

**5.2 AMPK Activation Time Course**

The present study shows the stimulatory effects of polyphenols on AMPK activation in skeletal muscle cells in a time-dependent manner. Similar to the results seen in Akt activation, EGCG and ECG had the highest effects compared to the other polyphenols, although EGCG was significantly more effective than ECG in addition to all other treatments. EGC and EGC + G, which were not significantly different from each

other, were more effective than EC and GA which appeared to have very minimal effects. The effect of EGCG on AMPK activation is consistent with that of other studies investigating its effects.

In L6 cells as well as 3T3-L1 cells, EGCG was found to increase AMPK activity by activating its upstream kinase LKB1 [170]. Activation of AMPK also subsequently attenuated the effects of insulin resistance by reducing IRS-1 serine phosphorylation. The same results were shown in another study testing the effects of EGCG on dexamethasone-induced insulin resistant L6 cells. EGCG was able to reverse the effects of insulin resistance through activation of AMPK which mediated the inhibition of IRS-1 serine phosphorylation [171]. An analysis of eight different catechins further revealed that those with gallate moieties acted as strong AMPK activators unlike the non-gallated catechins, which is consistent with the results in this finding. In this study, the non-gallated catechins EGC and EC had lesser effects on AMPK activation than EGCG and ECG. The phenolic acid GA had the least influence on AMPK activation, suggesting that there is a relationship between the structure of the polyphenol used and its effectiveness as an activator.

**5.3 Effect of SOD/catalase on Akt and AMPK activation**

This experiment demonstrated the role of reactive oxygen species in the activation of both Akt and AMPK. While Akt activation appeared to be unaffected by the addition of SOD and catalase to the system, AMPK activation was visibly hindered compared to the controls and previous experiments which were SOD and catalase-free. These results suggest that (1) polyphenols such as EGCG and ECG generate exogenous reactive oxygen species in vitro and (2) AMPK activation is significantly affected by this ROS

generation. The impact of exogenous ROS on Akt and AMPK activity has many contradicting results.

Similar results were seen in another study using soleus and epitrochlearis skeletal muscles. Exposure to low doses of $H_2O_2$ resulted in an increase in the phosphorylation of AMPK [177]. In another study, rat epitrochlearis muscles incubated in $H_2O_2$ and superoxide resulted in an increase in AMPK activity in a time and dose-dependent manner [178]. Other studies, however, had contradicting results. For example, one study found $H_2O_2$ increased the phosphorylation of Akt at Ser473 and Thr308 in isolated extensor digitorum longus (EDL) muscles, while it had no effect on AMPK phosphorylation [179]. Another study reported that neither Akt nor AMPK activity was effected by exogenous ROS, but rather p38 MAPK signaling [180]. The discrepancies in these results may be due to the different types of muscles used (slow versus fast twitch) or between species used (rats or mice) in the different studies. Future studies should be performed in order to get a better, concise understanding of the role of reactive oxygen species in cell signaling.

**5.4 GLUT4 Translocation Time Course**

The results of this test demonstrate that polyphenols promote GLUT4 translocation in skeletal muscle, as shown by the increase in its expression in the plasma membrane and gradual decrease in expression over time in the cytosol. The point where GLUT4 expression reached its maximum in the plasma membrane was after sixty minutes of incubation with the test compounds and slowly began to decrease after ninety minutes. This pattern is consistent with another study that reports GLUT4 internalization occurring within an hour of insulin stimulation which may be explained by an increase in

negative-feedback mechanisms such as increased phosphatase activity [181]. As expected, insulin had the greatest impact on GLUT4 translocation.

Out of the polyphenols, the gallated catechins EGCG and ECG had the highest effects on GLUT4 movement, while the non-gallated EGC, phenolic acid GA, and combined EGC + G had lesser effects. Several other studies showed similar findings. In one study, the soleus muscle of both Sprague-Dawlay rats and C57BL/6 mice showed increased translocation of the GLUT4 protein after being administered oral doses of EGCG. The same study reported a similar finding in L6 cells treated with EGCG [168]. A similar study using fermented tea (black, oolong, and pu-erh) supplements for a week in ICR mice also revealed enhanced GLUT4 translocation to the plasma membrane of soleus muscle [182]. Another study using C57BL/6J mice fed a high-fat diet demonstrated that those given green tea or black tea in addition to the high-fat diet showed increased GLUT4 translocation in muscle tissue after fourteen weeks compared to the control group [176].

Contradictory evidence was concluded in one study on 3T3-L1 adipocytes, however. It was found that the non-gallated catechins EGC and EC were responsible for promoting GLUT4 translocation while the gallated catechins EGCG and CG had inhibitory effects on translocation [183]. The same group previously used L6 myotubes and found that EGCG increased the translocation of GLUT4 and suggest that the structure-activity relationship of catechins on glucose transport in adipocytes is different from that of skeletal muscle whereby the gallate moiety of gallated catechins causes strong binding to the plasma membrane of adipose cells, and somehow in turn decreases their functioning on glucose transport [183]. More studies would need to be performed in

order to explain this observed difference. Overall, the results in this study show that polyphenols promote GLUT4 translocation in skeletal muscle.

**5.5 Concluding Remarks**

To our knowledge, this is the first study that uses multiple polyphenols in a comparative investigation on their effects on GLUT4 translocation as well as Akt and AMPK activation. The results from these experiments suggest that tea polyphenols do have an effect on GLUT4 translocation operating through both the insulin-mediated (Akt activation) and AMPK-mediated routes. The results also suggest that gallated catechins (EGCG and ECG) generally have a more potent effect than non-gallated catechins (EGC and EC) and gallic acid. The addition of this gallate moiety has been found to be important in other biological activities such as antioxidant and anti-cancer effects [33].

The gallated catechins were found to be effective over the non-gallated catechins, suggesting a relationship between catechin structure and the activation of protein targets involved in GLUT4 translocation. The number of hydroxyl groups of catechins have been proposed of contributing to antioxidant effects [33] as well as interactions with components on the plasma membrane [47, 48]. The gallated catechins (which contain more hydroxyl groups than the non-gallated catechins) would therefore lead to more hydrogen bonding and likely lead to stronger binding to outer leaflet moieties of the plasma membrane such as phosphatidylcholine or trimethylamine groups of sphingomyelin [47, 164]. Future studies testing the retention times of EGCG, ECG, EGC, EC, and GA in lipid rafts via HPLC would provide better insight on how these polyphenols interact with cells at the surface. Polyphenols have been suspected of playing a role in initiating lipid-protein clusters, an important event that initiates a lot of

cell signaling at the cell surface [47]. Testing this hypothesis would be of interest in trying to identify the mechanisms behind polyphenol mode of action in signaling pathways. Whether EGCG and ECG are more effective than non-gallated catechins due to their ability to penetrate the cell surface better or through altering physical properties of the plasma membrane to initiate cell signaling remains to be studied.

There are several limitations to this study. First, it should be noted that the bioavailabilities of tea polyphenols in both rodents and humans are rather low [184, 185]. Whereas the 30 μM of EGCG used in these experiments induced Akt phosphorylation, AMPK phosphorylation, and GLUT4 translocation in the L6 cell system, this concentration is not achievable in the plasma through consumption of tea beverages [186]. However, this should not be used to judge the relevance of the present results because of the different natures of these two types of experiments. Administration of EGCG and green tea extract has been shown to decrease body-weight gain, percentage of body fat [50, 51], plasma cholesterol levels [50, 187], blood insulin and blood glucose, and enhance GLUT4 translocation and glucose uptake in vivo. Secondly, although Western blotting was an effective way to determine the activation states of the key enzymes Akt and AMPK, a high-throughput method such as a protein microarray would both confirm my conclusion and provide a more detailed view of other proteins and pathways that become activated upon catechin treatment. Thirdly, this study was performed in only one immortalized skeletal muscle cell line. The metabolic interactions between cells in vivo are more intricately integrated and more complicated, and therefore difficult to mimic in vitro. Moving this study to an in vivo system with animals (namely rats since the effects were tested in rat skeletal muscle cells) is thus a necessary step

forward in uncovering the effects of different polyphenols on GLUT4 translocation and glucose uptake in skeletal muscle. The effects on other insulin-sensitive tissues such as the liver, kidneys, and adipose could also be investigated in an animal model. Testing the effects of catechins in other skeletal muscle cell lines (such as mouse C2C12 cells or human HSMM cells) would also provide information on whether or not the same effects (Akt and AMPK activation, GLUT4 translocation) can be reproduced in other species for future in vivo studies.

It is important to understand the mechanisms behind glucose transport in skeletal muscle since it is the primary tissue for peripheral blood glucose removal. In metabolic disease and TIIDM, desensitization of insulin-sensitive tissues to insulin is one of the major pathophysiological traits, subsequently causing hyperglycemia. From this work, we conclude that major tea catechins have the ability to stimulate GLUT4 translocation in skeletal muscle, independent of both insulin and exercise. In addition, EGCG and ECG may be promising potential therapeutic agents in individuals that are either insulin-resistant, or lack the ability to produce insulin. It is therefore advantageous to put greater emphasis on this area of research in the future.

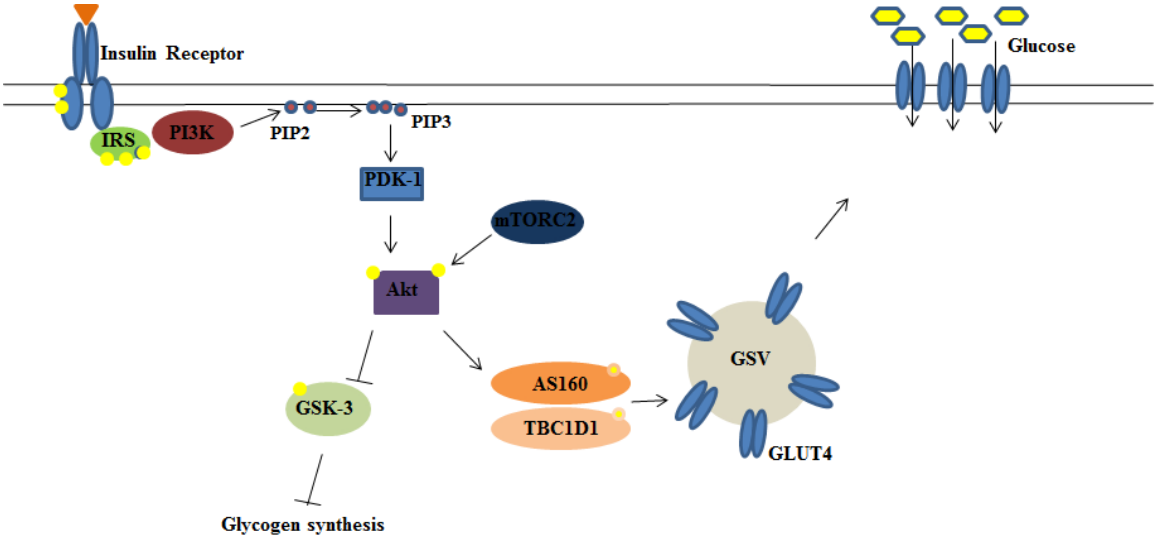


**Figure 1 GLUT4 translocation via the insulin-mediated PI3K pathway**

Schematic overview of GLUT4 trafficking through insulin signaling highlighting the key players in the process. Binding of insulin to its receptor at the cell surface causes a cascade of phosphorylation events via the PI3K pathway that ultimately lead to GLUT4 translocation [69]. Activation of the downstream protein Akt leads to the phosphorylation and subsequent dissociation of AS160 and TBC1D1 (two known negative regulators of GLUT4 translocation), causing GSVs to transport the GLUT4 protein to the cell surface [75, 80, 84-86].

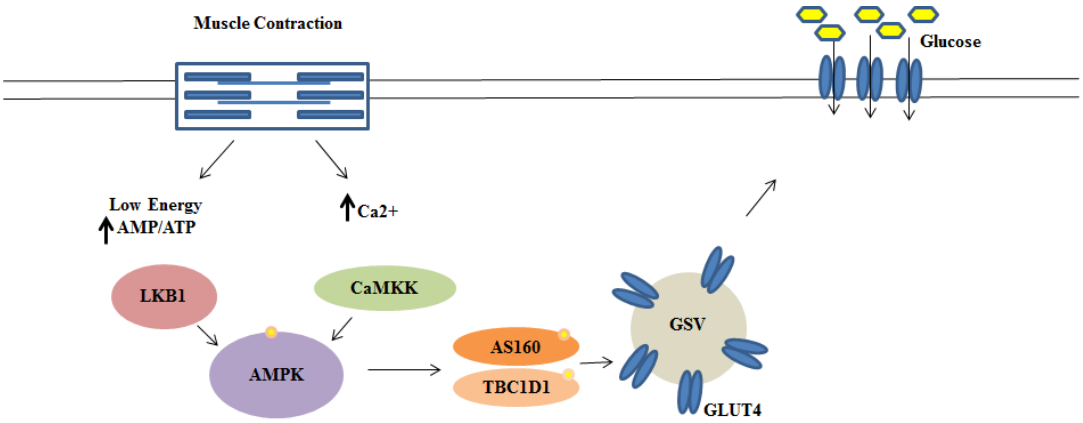


**Figure 2 GLUT4 translocation via the insulin-independent AMPK pathway**

Outline of GLUT4 trafficking via insulin-independent signaling highlighting the pivotal proteins involved in the process. Contractile activity of skeletal muscle (via exercise) causes an increase in AMP and cytosolic $Ca^{2+}$ concentrations which lead to activation of upstream kinases to AMPK, LKB1 and CaMKK, respectively [101-105]. Both kinases have the ability to stimulate AMPK activation at the α subunit (Thr172) which leads to the phosphorylation of AS10 and TBC1D1 and subsequent translocation of GLUT4 to the plasma membrane [121, 124, 125]. Although not depicted in this diagram, AMPK also increases GLUT4 transcription via phosphorylation of HDAC5, a negative regulator of MEF2, the transcription factor responsible for GLUT4 expression [120].

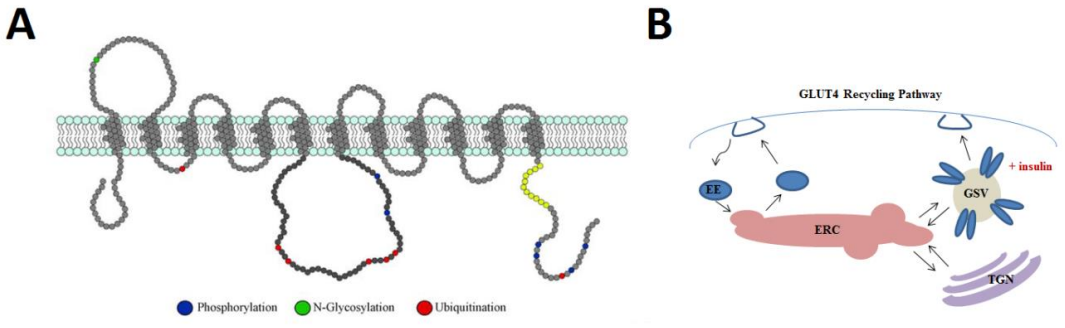


**Figure 3 The GLUT4 transporter protein**

(A) Structure of the GLUT4 transporter protein [188]; GLUT4 is a 12 transmembrane protein that forms aqueous pores within the plasma membrane to allow glucose entry [129, 130].

(B) The GLUT4 Recycling Pathway; Diagram of the GLUT4 transportation routes through different subcellular compartments. Initially, translated GLUt4 protein is transported from the ER to the trans Golgi Network (TGN) where post-translational modifications are made [132, 145]. From there, the GLUT4 protein is embedded into GLUT4-storage vesicles (GSVs) which remain in the cytosol at basal conditions. Upon insulin stimulation (or AMPK), GSVs get transported to the plasma membrane where they fuse and subsequently allow glucose to enter the cell [132, 145]. The GLUT4 protein can also be recycled to and from the cell membrane through being recycled via the endosome system (both at basal and stimulated conditions) [132, 144]. The protein first gets endocytosed and subsequently fused to early endosomes (EE). It then gets sorted into the endosomal recycling compartment ERC where it will either (1) be recycled back to the surface, (2) be retained in the TGN, or (3) get targeted for lysosomal degradation [132].

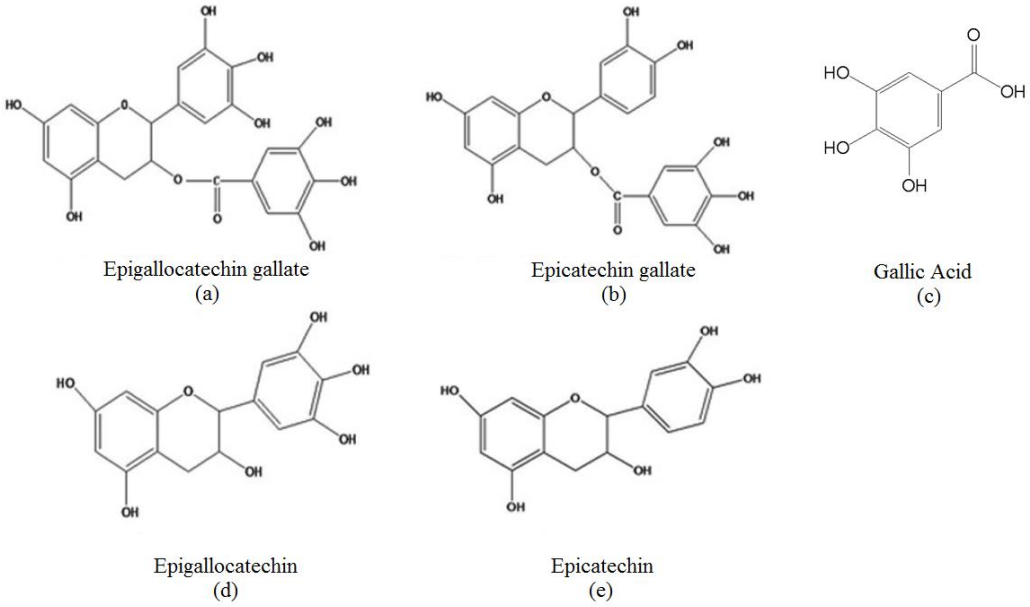


**Figure 4 Structures of the major tea catechins and gallic acid [189].**

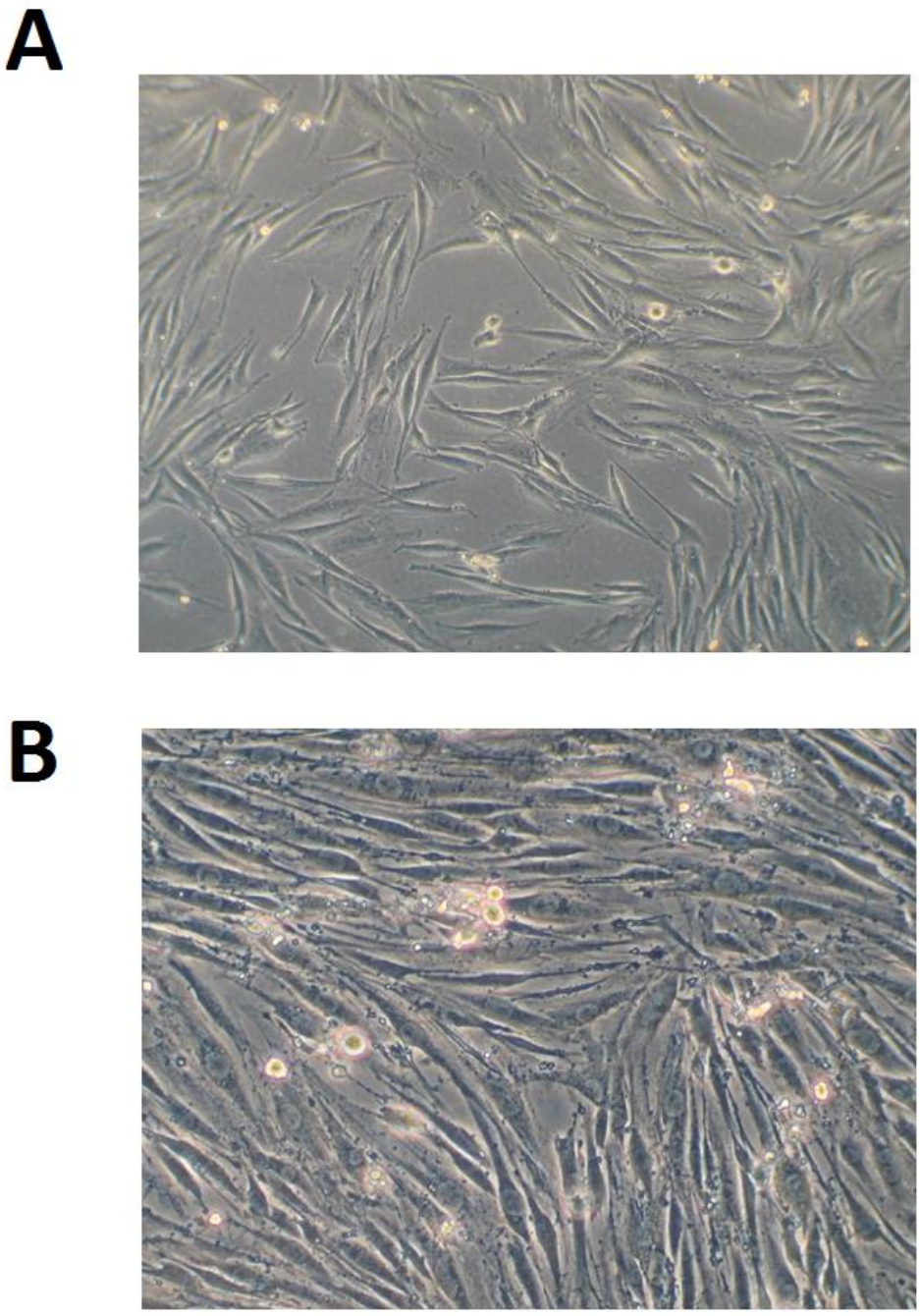


**Figure 5 The L6 rat skeletal muscle cell line**

(A) L6 myoblasts at high confluence in DMEM + 10% FBS + 1% Streptomycin (4 days, 10X); (B) Differentiated L6 myotubes in DMEM + 2% FBS + 1% Streptomycin (8 days; 40X)

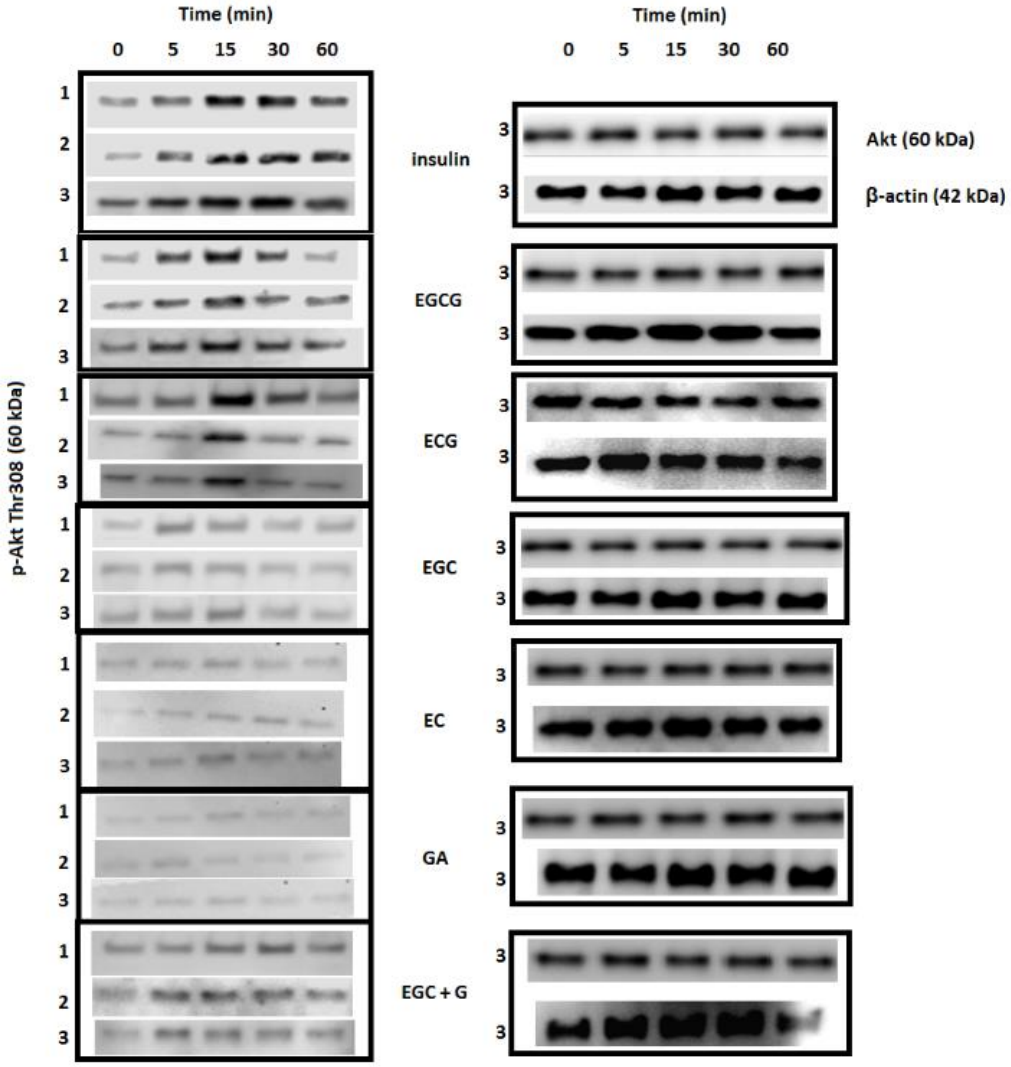


**Figure 6 Akt (Thr308) activation via stimulation from insulin and tea polyphenols**

Western blotting results for L6 myotubes treated with 10 nM insulin, 30 µM EGCG, 30 µM ECG, 30 µM EGC, 30 µM EC, 30 µM GA, and 30 µM EGC + 30 µM GA at times 0 (untreated), 5, 15, 30, and 60 minutes. Blots were probed for p-Akt (Thr308), Akt, and β-actin. Numbers (1, 2, 3) on the left indicate the trial of each experiment run (n = 3).

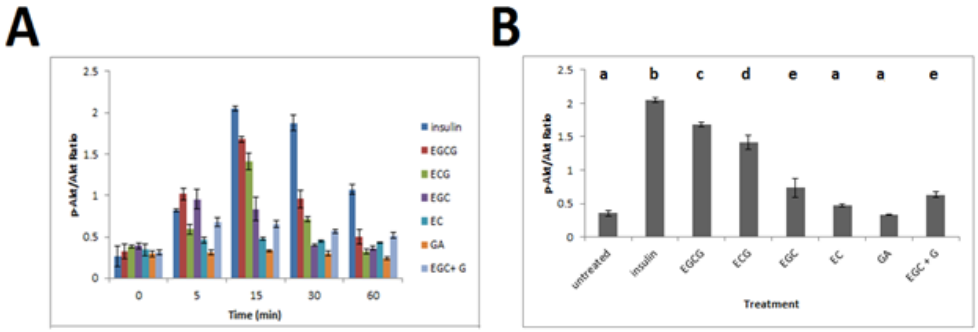


**Figure 7 Densitometric representations of the p-Akt/Akt ratio**

(A) Bar graphs obtained by averaged densitometric analysis of Western blotting data using ImageJ show the comparison of Akt activation between treatments at each specified time point. Results (mean±S.E.M.; n=3) represent the ratio between p-Akt and Akt after normalization to β–actin. Standard error bars for each group are included. Data were analyzed by mixed-design ANOVA using SPSS21.

(B) Post-hoc Tukey results are represented by the bar graph on the right using the 15 minute time point. Means not sharing the same letter are significantly different ($p = < 0.05$).

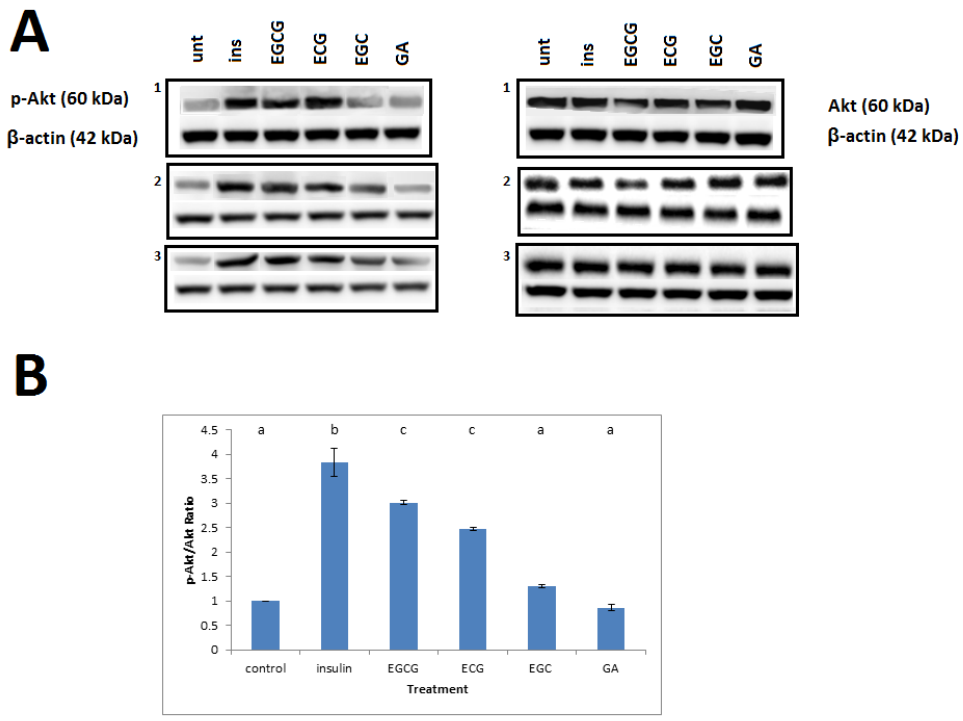


**Figure 8 Akt (Thr308) activation via stimulation from insulin and tea polyphenols**

(A) Western blotting results for L6 myotubes treated with 10 nM insulin, 30 μM EGCG, 30 μM ECG, 30 μM EGC, and 30 μM GA, at 15 minutes. Samples were loaded next to an untreated control. Blots were probed for p-Akt (Thr308), Akt, and β-actin. Numbers (1, 2, 3) on the left indicate the trial of each experiment run (n = 3).

(B) Bar graph obtained by averaged densitometric analysis of Western blotting data using ImageJ show the comparison of Akt activation between treatments at 15 minutes. Results (mean±S.E.M.; n=3) represent the ratio between p-Akt and Akt after normalization to β–actin. Standard error bars for each group are included. Data were analyzed by one-way ANOVA using SPSS21. Post-hoc Tukey results are indicated by letters at the top.

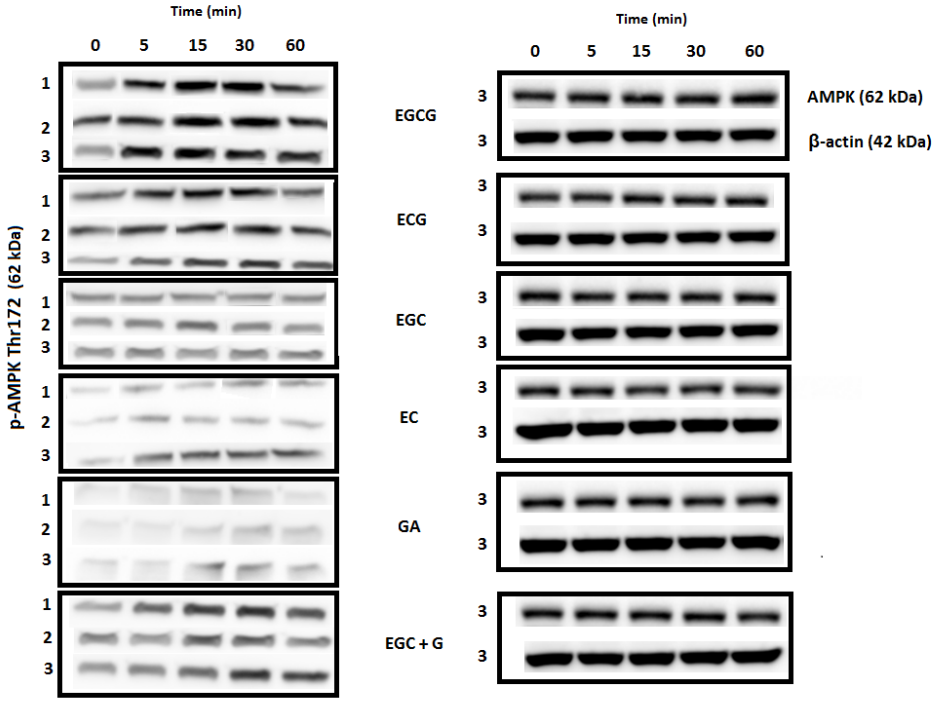


**Figure 9 AMPK activation in myotubes stimulated with polyphenols**

Western blotting results for L6 myotubes treated with 30 µM EGCG, 30 µM ECG, 30 µM EGC, 30 µM EC, 30 µM GA, and 30 µM EGC + 30 µM GA at times 0 (untreated), 5, 15, 30, and 60 minutes. Blots were probed for p-AMPK (Thr172), AMPK, and β-actin. Numbers (1, 2, 3) on the left indicate the trial of each experiment run (n = 3).

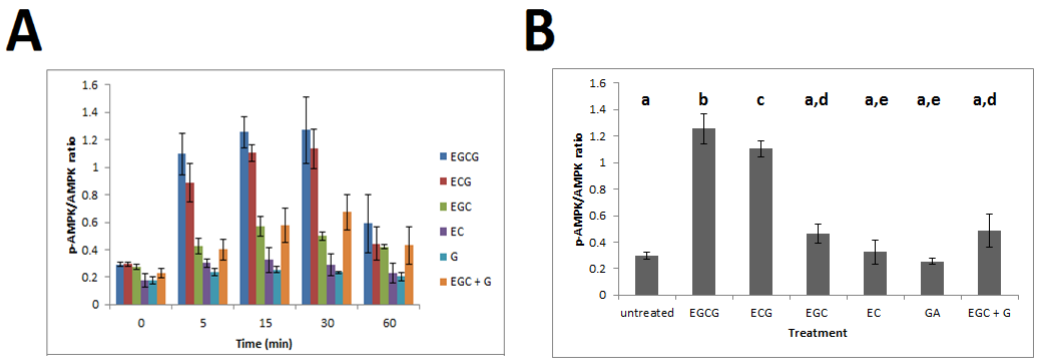


**Figure 10 Densitometric representations of the p-AMPK/AMPK ratio**

(A) Bar graphs obtained by averaged densitometric analysis of Western blotting data using ImageJ show the comparison of AMPK activation between treatments at each specified time point. Results (mean±S.E.M.; n=3) represent the ratio between p-AMPK and AMPK after normalization to β–actin. Standard error bars for each group are included. Data were analyzed by mixed-design ANOVA using SPSS21.

(B) Post-hoc Tukey results are represented by the bar graph on the right using the 30 minute time point. Means not sharing the same letter are significantly different ($p = < 0.05$).

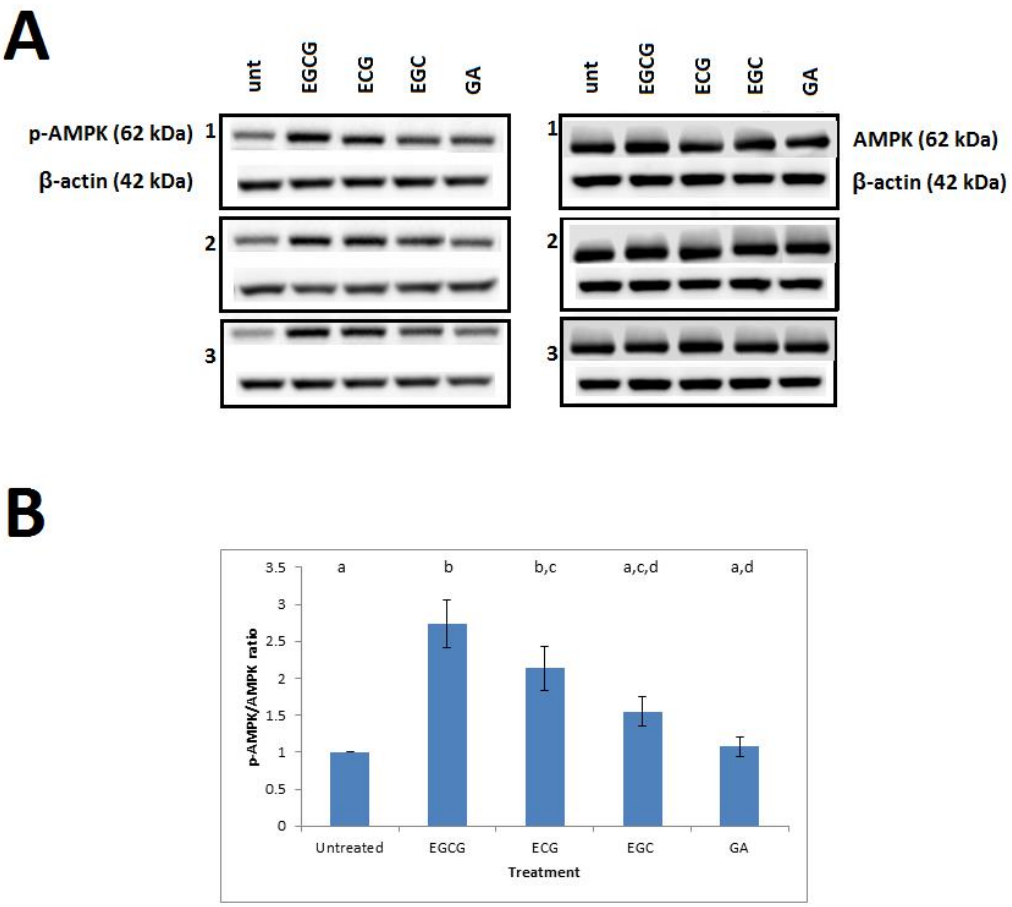


**Figure 11 AMPK (Thr172) activation via stimulation from various tea polyphenols**

(A) Western blotting results for L6 myotubes treated with 30 µM EGCG, 30 µM ECG, 30 µM EGC, and 30 µM GA, at 15 minutes. Samples were loaded next to an untreated control. Blots were probed for p-AMPK (Thr172), AMPK, and β-actin. Numbers (1, 2, 3) on the left indicate the trial of each experiment run (n = 3).

(B) Bar graph obtained by averaged densitometric analysis of Western blotting data using ImageJ show the comparison of AMPK activation between treatments at 15 minutes. Results (mean±S.E.M.; n=3) represent the ratio between p-AMPK and AMPK after normalization to β–actin. Standard error bars for each group are

included. Data were analyzed by one-way ANOVA using SPSS21. Post-hoc Tukey results are indicated by letters at the top.

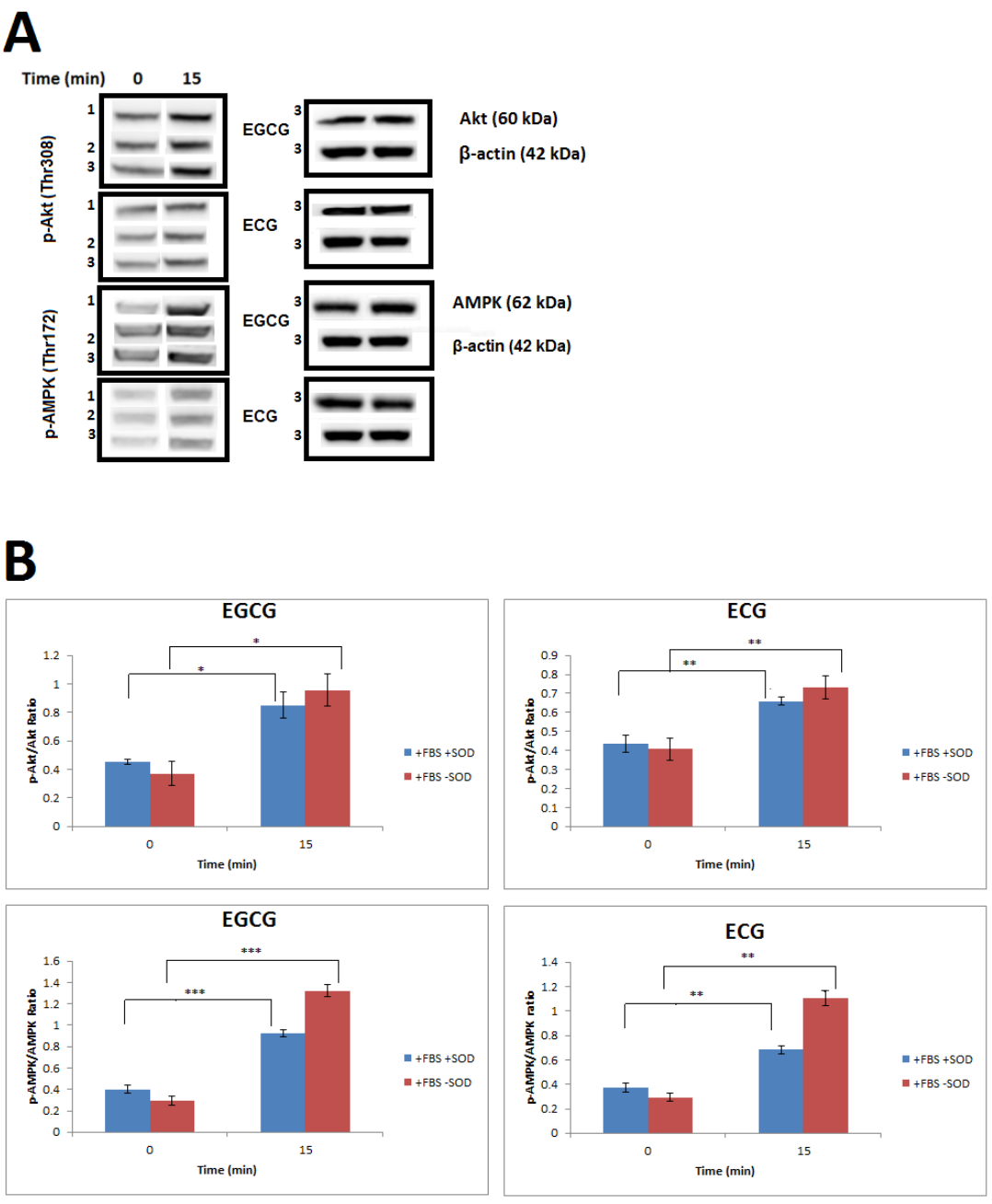


**Figure 12 Effects of SOD and catalase on Akt and AMPK activation in L6 myotubes**

(A) p-Akt and p-AMPK Western blotting analysis; L6 cells were treated with either EGCG or ECG in 10% FBS + SOD/catalase for 15 minutes. Untreated samples (0 minutes) were run as controls.

(B) Bar graph obtained by densitometric analysis of Western blotting data using ImageJ show the comparison of Akt and AMPK activation at 0 (untreated) and 15 minutes between cells treated with EGCG and ECG (in 10% FBS, (-) SOD/catalase [red]; 10% FBS, (+) SOD/catalase [blue]). Results (mean±S.E.M; n=3) represent the ratio between p-Akt and Akt (and p-AMPK to AMPK) after normalization to β–actin. Standard error bars for each group are included. Data were analyzed by a paired t-test using Excel 2013.

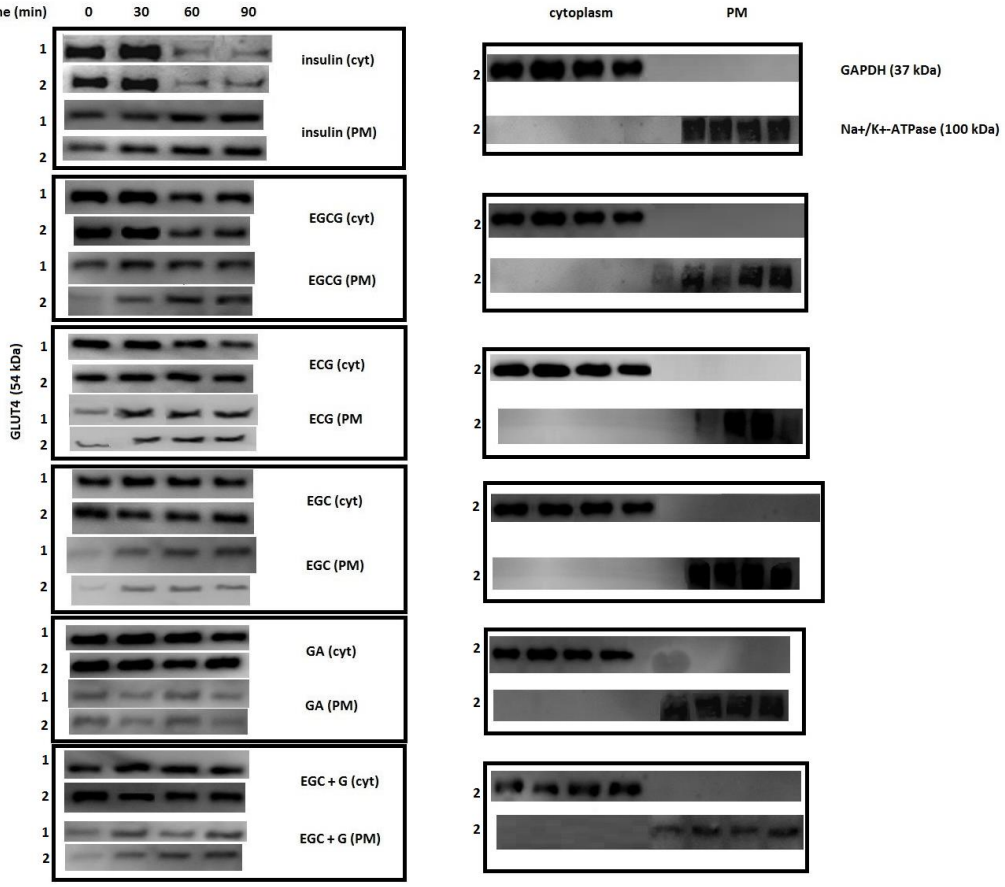


**Figure 13 GLUT4 translocation upon stimulation with insulin and tea polyphenols**

Western blotting results for L6 myotubes treated with 10 nM insulin, 30 μM EGCG, 30 μM ECG, 30 μM EGC, 30 μM GA, and 30 μM EGC + 30 μM GA at times 0 (untreated), 30, 60, and 90 minutes. Blots were probed for GLUT4, $Na^+/K^+$-ATPase, and GAPDH. Numbers (1 and 2) on the left indicate the trial of each experiment run (n = 3).

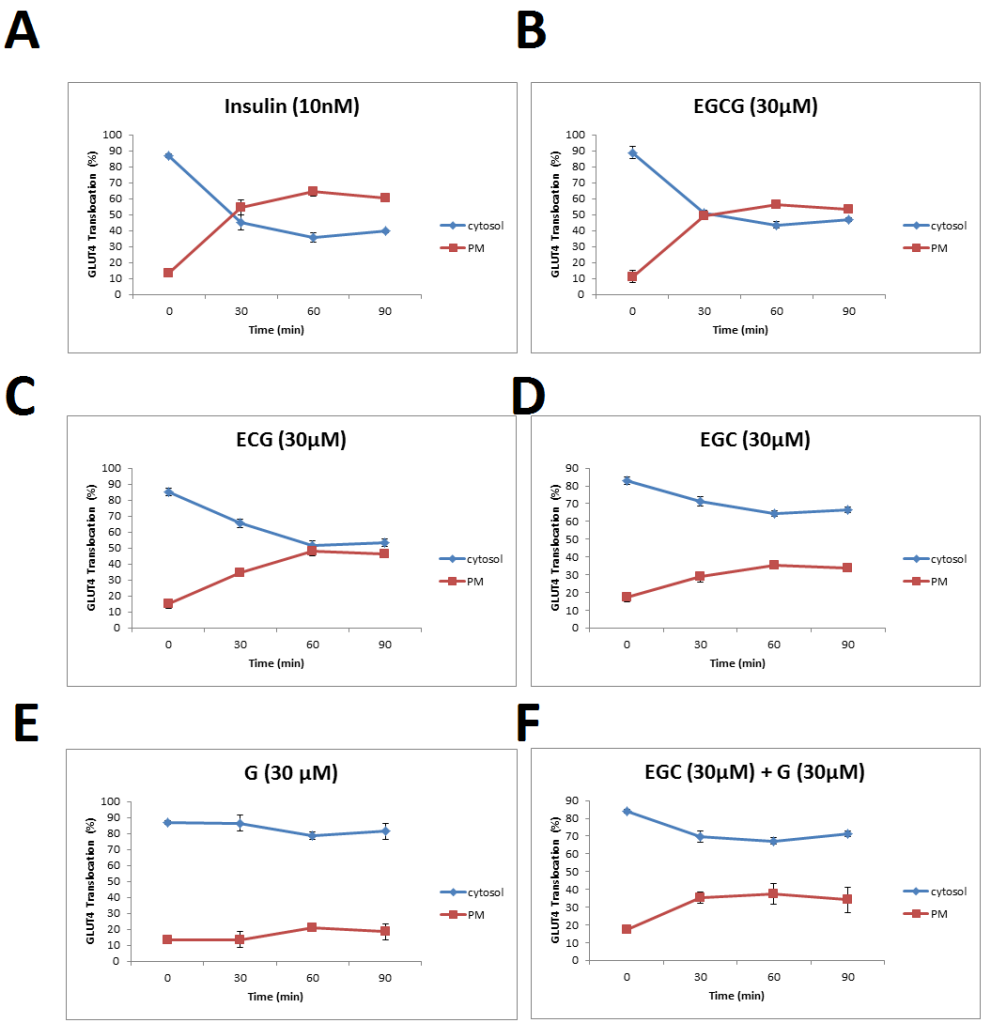


**Figure 14 GLUT4 translocation over time**

Trends of GLUT4 expression in both the cytosol (blue) and plasma membrane (red) over time in cells treated with (A) 10 nM insulin; (B) 30 µM EGCG; (C) 30 µM ECG; (D) 30 µM EGC; (E) 30 µM G; (F) 30 µM EGC + 30 µM G. Results for each time point are expressed as averages (n=3). Standard error bars for each time point are included. Data was plotted from averaged densitometric analysis of Western blotting data using ImageJ.

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