University of Nevada, Reno

Molecular Mechanisms by which a Grape Seed Procyanidin Extract Modulates Glucose Homeostasis

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Abstract

Type 2 Diabetes is the most common form of diabetes and a prevalent health condition that resulted in 5.1 million deaths in 2013. Blood glucose levels are tightly regulated in order to maintain metabolic homeostasis. The hormone insulin, after binding to the insulin receptor, initiates a well-established controlled metabolic signaling cascade that consequently lowers blood glucose levels. There is considerable interest regarding effective natural therapies for combating glucose dysregulation. An example includes a grape seed procyanidin extract (GSPE), which has been demonstrated to decrease glucose levels in cell and animal models, however, the precise underlying molecular mechanisms are still under investigation.

One of the reported mechanisms by which GSPE regulates glucose homeostasis is via action as an "*insulinomimetic*", whereby, analogous to insulin, GSPE binds to the insulin receptor. Following binding to the insulin receptor, GSPE causes activation of the insulin signal transduction pathway, ultimately increasing glucose uptake. An additional mechanism by which GSPE is proposed to regulate blood glucose levels is via a direct effect on the primary glucose transporter in insulin-sensitive tissues, namely glucose transporter-4 (GLUT-4).

The physiological effects of unregulated blood glucose and the detrimental health consequences will be discussed, along with currently available treatment strategies and potential future remedies in the field of medicine. **The study of the literature presented in this thesis aims to provide evidence regarding the current knowledge base regarding the mechanisms identified thus far with respect to grape seed procyanidin extract and the regulation of glucose homeostasis.**

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First and foremost I would like to thank my Lord and Savior Jesus Christ for granting me the strength and abilities to pursue and complete an Honors Thesis.

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Abbreviations

α-cells:	alpha cells
Akt:	*Protein kinase B*
AMPK:	*Adenosine monophosphate-activated protein kinase*
ATP:	Adenosine triphosphate
β-cells:	beta cells
BMI:	Body Mass Index
CVD:	Cardiovascular disease
ERKs:	Extracellular signal regulated kinases
FXR:	Farnesoid x receptor
GLUT-1:	Glucose transporter-1
GLUT-4:	Glucose transporter-4 (Insulin-sensitive glucose transporter)
GSPE:	Grape Seed Procyanidin Extract
HDL:	High density lipoprotein
IR:	Insulin resistance
IRS-1:	Insulin receptor substrate-1
MAPK:	*Mitogen-activated protein kinase*
MFR:	Mean Fluorescence Ratio
mRNA:	messenger Ribonucleic Acid
PI3K:	*Phosphatidylinositol 3-kinase*
ROS:	Reactive Oxygen Species
SHP:	Small heterodimer partner
SREBP1c:	Sterol regulatory element-binding protein 1c
TBC1D1:	*Akt substrate GTPase activating protein*
TG:	Triglyceride
T1D:	Type 1 Diabetes
T2D:	Type 2 Diabetes Mellitus

Chapter 1:

Introduction

Chapter 1: Introduction

1.1 The Role of Insulin in Glucose Regulation

Blood glucose levels are tightly regulated to ensure that metabolic homeostasis is maintained. Humans consume food in order to provide energy to meet our daily requirements. After a meal is eaten, insulin, a hormone produced by the β-cells in the pancreas, is released into the bloodstream when blood glucose levels increase (Martini, Nath, & Bartholomew, 2011).

Insulin is an essential hormone that has many actions, most of which are directed towards the appropriate control of metabolism, including that of carbohydrates (sugars and starches), lipids (fats), and proteins. Insulin also regulates cell function, including growth, as well as being an absolute requirement for the use of glucose as energy (Martini et al., 2011).

The other essential hormone secreted by the pancreas, namely glucagon, regulates blood glucose concentration by counteracting the actions of insulin. Glucagon is released by α-cells in the pancreas in response to low blood glucose levels (Martini et al., 2011). Glucagon targets the liver and adipose tissue, primarily to mobilize different forms of energy storage; and mainly to facilitate the mobilization of lipids, in order to increase blood glucose levels. The pancreatic α- and β-cells are extremely sensitive to blood glucose levels. Hormones such as *gastrin* and *secretin*, which are released by the stomach and small intestine respectively, affect blood glucose levels indirectly by altering the amount of insulin and or glucagon produced and released by the pancreas (Martini et al., 2011).

1.2 Type 1 Diabetes

Type 1 diabetes (T1D) develops in individuals who no longer produce enough insulin to maintain glucose homeostasis (Martini et al., 2011). Patients diagnosed with T1D have long been thought to suffer from complete pancreatic β-cell destruction, leading to a dependence on insulin injections for the rest of their lives, in order to maintain glucose homeostasis (Davis et al., 2015). Several studies have previously reported the existence of a subset of individuals with T1D who continue to secrete a small amount of insulin after diagnosis, but they were thought to be exceptional cases (Keenan et al., 2010). However, a very recent study has shown that many patients with T1D still secrete residual levels of insulin (Davis et al., 2015). Insulin secretion has been used as a key diagnostic factor to differentiate between those with T1D and Type 2 diabetes (T2D). Although the American Diabetes Association in 2014 described T1D patients as having complete insulin deficiency, where they no longer secrete insulin, this recent study has again brought up the question of whether T1D patients still produce some insulin or not. This new study utilized a widely accepted method of measuring C-peptide in order to assess the amount of insulin secreted (Davis et al., 2015). C-peptide is a substance that is produced by pancreatic β-cells when proinsulin splits apart to produce one molecule of C-peptide and one molecule of insulin. Therefore, since C-peptide and insulin are produced at the same rate, C-peptide is widely recognized as a useful marker of insulin production (Jones & Hattersley, 2013). Another important finding in the study was that the detection of insulin secretion, via C-peptide measurement, correlates with age at diagnosis (Davis et al., 2015). For example, those diagnosed over 18 years of age, or as adults, were more likely to have detectable C-peptide levels than those diagnosed under 18 years of age (Davis et al., 2015).

There are many reasons for the development of T1D, including genetics, mutations in insulin receptors, mutations in insulin itself, or immunodeficiency (Martini et al., 2011). T1D is normally diagnosed during childhood and accounts for approximately 5-10 % of diabetic cases (Martini et al., 2011). Without appropriate insulin function, high blood glucose levels lead to serious clinical consequences (International Diabetes Federation, 2013). Since the body relies on glucose utilization for correct metabolic function, a disruption in insulin production leads to an alteration in whole body metabolism, including protein and triglyceride synthesis (Martini et al., 2011).

As mentioned above, individuals diagnosed with T1D require insulin injections for the remainder of their lives in order to appropriately control blood glucose levels (Martini et al., 2011), and they must also be diligent in their diet and lifestyle choices in order to remain healthy. Although the recent study mentioned above provides some promise that not all T1D patients may require lifetime insulin injections, further studies are clearly warranted to determine the length of time that the pancreas can still adequately function in order to produce sufficient quantities of insulin. Since T1D patients suffer from inadequate blood insulin levels (International Diabetes Federation, 2013) but are still sensitive to insulin's effects on the cells, the most likely outcome will be a treatment that helps to increase the efficacy of the insulin present or exogenous insulin, given via injections.

1.3 Type 2 Diabetes

Type 2 diabetes (T2D) has always been referred to as "*non-insulin*" dependent diabetes, and occurs when the body is either not able to produce insulin in sufficient amounts, or when the body is no longer able to respond to the effects of insulin, which leads to insulin resistance (IR),

and ultimately hyperglycemia (high blood glucose levels) (International Diabetes Federation, 2013). Hyperglycemia can lead to various health complications, such as neuropathy or peripheral vascular damage and can be a life-threatening condition if left untreated, and was responsible for a reported 5.1 million deaths in 2013 (International Diabetes Federation, 2013). Diabetic individuals with consistently high blood glucose levels are at tremendous risk for developing serious diseases such as cardiovascular disease, peripheral vascular disease, and retinopathy that affect important organs such as the heart, blood vessels, eyes, kidneys, and nerves (International Diabetes Federation, 2013).

In contrast to those who suffer from T1D, most patients who have T2D, can initially make lifestyle changes, e.g. with respect to diet and exercise, to help alleviate high blood glucose levels, because adequate insulin stores remain (International Diabetes Federation, 2013). Maintenance of normal blood glucose is extremely important for patients because it helps to prevent or delay the serious symptoms associated with a constant state of hyperglycemia, as mentioned above.

Insulin signaling lowers blood glucose levels via three main mechanisms: first by enhancing glucose uptake in peripheral tissues, e.g. adipose tissue, by inducing translocation of the main glucose transporter present in insulin-sensitive cells, namely glucose transporter-4 (GLUT-4), to the plasma membrane to facilitate cellular uptake of glucose; secondly by promoting normal utilization and storage of glucose in the liver, and thirdly, by inhibiting the breakdown of lipid and promoting lipogenesis in adipose tissue (Hanhineva et al., 2010). This thesis will focus on the first mechanism by which insulin signaling alleviates hyperglycemia via enhancement of glucose uptake in peripheral tissues via glucose transporters. Another glucose transporter namely, glucose transporter-1 (GLUT-1), will be discussed in detail towards the end of this chapter.

1.4 Insulin Resistance and the Associated Physiological Consequences

Insulin resistance (IR) is a key event in the development of T2D, which can damage the body systemically, particularly the cardiovascular system (Coughlan, Valentine, Ruderman, & Saha, 2013). When the body no longer responds to insulin and blood glucose levels rise, insulin resistance is the physiological consequence. As a result, higher levels of insulin are needed in order for insulin to function appropriately. Consequently, the pancreas compensates for the lack of glucose regulation by trying to produce more insulin. When insulin resistance is in the early stages, the pancreas produces more and more insulin, until the pancreas can no longer produce sufficient insulin for the body's demands, consequently leading to an inability to respond and therefore hyperglycemia ensues (International Diabetes Federation, 2013).

Therefore, IR is a risk factor for the development of diabetes and heart disease (Kendall & Bergenstal, 2001), and IR develops in multiple organs that are normally insulin sensitive, including skeletal muscle, liver, adipose tissue and the heart (Martini et al., 2011). IR, independent of body weight, as observed in individuals with a high fatty liver content, causes many symptoms that contribute to the loss of glucose homeostasis and cardiovascular disease (CVD), including hyperinsulinemia (high blood insulin levels), hypertriglyceridemia (high serum triglyceride levels), decreased high density lipoprotein (HDL) levels, and slightly increased ambulatory systolic blood pressure (Seppala-Lindroos et al., 2002). Consequently, when treating hyperglycemia, blood lipid levels are also routinely assessed and treated (Martinez, 2007). The relationship between lipids (fats) and glucose levels in the body involves gene expression and cellular signaling between insulin sensitive tissues (Cipriani, Mencarelli, Palladino, & Fiorucci, 2010), and will be discussed below.

1.5 Insulin Signaling at the Molecular Level

Insulin, secreted by pancreatic β-cells, regulates blood glucose levels by binding to the α-subunit of the insulin receptor located on the surface of the cell membrane (Khan & Pessin, 2002). The insulin receptor is present in insulin-sensitive tissues, such as the skeletal muscle, and the binding of insulin results in a conformational change in the receptor and activation of intrinsic *tyrosine kinase* activity (Pinent, Blay, Blade, et al., 2004). This *tyrosine kinase* activity leads to autophosphorylation of the β-subunit of the insulin receptor (Khan & Pessin, 2002), which is present on the inner cell membrane, as indicated in **Figure 1**. Autophosphorylation of the β-subunit of the insulin receptor is required in order to initiate the insulin-signaling pathway, leading to subsequent phosphorylation of downstream components, and in turn their activation in order to ultimately allow glucose entry into the cell from the bloodstream (Khan & Pessin, 2002).

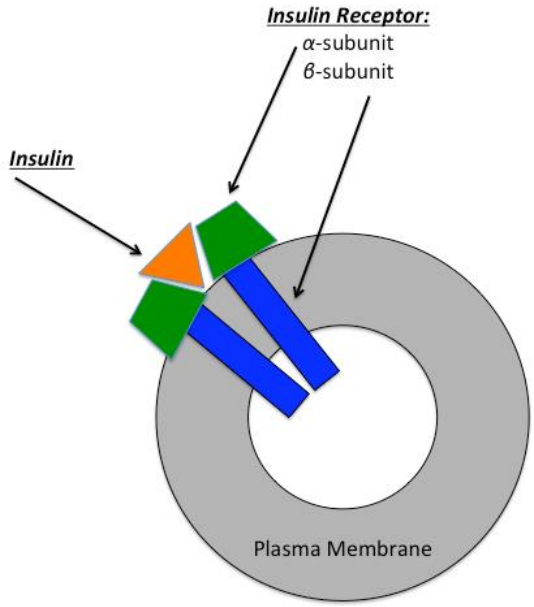

Figure 1: A schematic representation of the Insulin Receptor

Insulin receptors are proteins located in the plasma membrane of insulin-sensitive tissues. This image represents an individual cell. The α-subunit of the insulin receptor is located on the surface of the plasma membrane and the β-subunit transverses the plasma membrane. When insulin binds to the α-subunit, the conformation of the protein changes and autophosphorylation of tyrosine residues occurs on the β-subunit mediated via intrinsic tyrosine kinase activity, initiating the insulin-signaling cascade that ultimately leads to the entry of glucose into the cell.

Insulin receptor substrate-1 (IRS-1) is activated via phosphorylation, which is mediated by the action of the enzyme, *tyrosine kinase.* Then subsequently, IRS-1 phosphorylates and activates *phosphatidylinositol 3-kinase* (PI3-kinase), which is necessary for the movement of GLUT-4 from intracellular vesicles to the plasma membrane, subsequently facilitating entry of glucose into the cell (Khan & Pessin, 2002; Simpson, Whitehead, & James, 2001). The principal role of insulin is to target insulin-sensitive tissues to allow glucose to enter the cell, ultimately lowering blood glucose levels. GLUT-4, the insulin responsive glucose transporter, is expressed in insulin-sensitive tissues such as white adipose, cardiac tissue and skeletal muscle (James, Strube, & Mueckler, 1989). Insulin not only affects these intracellular substrates, but others that will be discussed below.

1.6 Insulin and Adenosine Monophosphate-Activated Protein Kinase (AMPK)

When insulin binds to the insulin receptor, the insulin-signaling cascade is initiated (**Figure 2**), leading to entry of glucose into the cell from the bloodstream, and one of the main signaling molecules activated is *adenosine monophosphate-activated protein kinase* (AMPK) (Coughlan et al., 2013). Although AMPK is known to cause many metabolic alterations, including that of cholesterol and lipid synthesis (Zhang, Zhou, & Li, 2009), this thesis will mainly focus on the role AMPK plays in glucose regulation. AMPK activation improves overall insulin sensitivity and glucose homeostasis (Coughlan, Valentine, Ruderman, & Saha, 2014). The ability of AMPK to improve insulin sensitivity in T2D patients is one of the mechanisms by which metformin, an antidiabetic medication, modulates glucose homeostasis (Ruderman, Carling, Prentki, & Cacicedo, 2013).

There are two main ways that lead to the activation of AMPK. Firstly, when phosphorylated by *protein kinase B* (Akt), AMPK becomes activated and phosphorylates the Akt substrate GTPase activating protein (TBC1D1), which then increases GLUT-4 translocation to the plasma membrane, ultimately leading to lowered blood glucose levels (Sawada, Yamashita, Zhang, Nakagawa, & Ashida, 2014). The second way to activate AMPK is via the simultaneous increase in the adenosine monophosphate: adenosine triphosphate (AMP:ATP) ratio and phosphorylation of the amino acid Threonine 172 on the α-subunit of AMPK (Birnbaum, 2005). Increased levels of AMP cause AMP to replace ATP that is bound to AMPK, causing an allosteric change that leads to AMPK activation (Xiao et al., 2007), thus increasing glucose uptake (Coughlan et al., 2014). Since it is important to determine the exact molecular mechanism by which AMPK functions for therapeutic purposes, AMPK is currently under investigation by several researchers to determine the underlying mechanisms by which it increases GLUT-4 translocation

(Lien et al., 2014). Not only does AMPK have short-term effects on glucose regulation, but it has also been shown to affect transcription factors via phosphorylation (Lien et al., 2014).

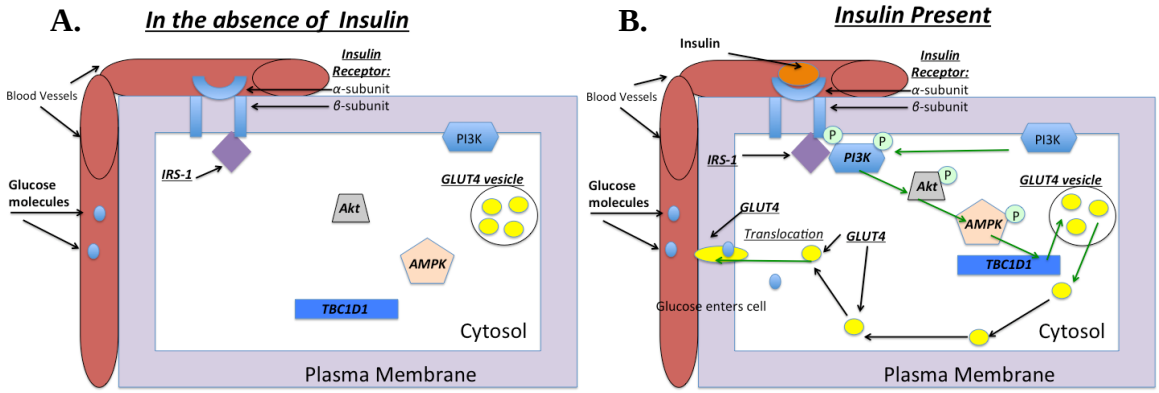

Figure 2: A schematic representation of the molecular regulatory effects that are induced by insulin to facilitate glucose uptake in an insulin-sensitive cell.

A. The insulin-signaling pathway components located in the cell in the absence of insulin and B. The insulin-signaling transduction pathway in the presence of insulin. When insulin binds to the α-subunit of the insulin receptor an insulin-signaling cascade ensues, ultimately causing glucose transporter-4 (GLUT-4) to translocate to the plasma membrane, allowing glucose to enter the cell from the bloodstream. GLUT-4 is found within vesicles that are within the cytoplasm of the cell. The green arrows signify steps in the pathway: insulin binds to the α-subunit of the insulin receptor, causing intrinsic *tyrosine kinase* to autophosphorylate the β-subunit of the insulin receptor. Insulin receptor substrate 1 (IRS-1) is then activated via phosphorylation by *tyrosine kinase*. This leads to the phosphorylation of *phosphotinositide 3-kinase* (PI3K) which causes its' activation and movement to the plasma membrane. PI3K then activates *protein kinase B* (Akt), which subsequently phosphorylates *AMP-activated protein kinase* (AMPK). AMPK then phosphorylates the Akt substrate *GTPase activating protein* (TBC1D1), which causes GLUT-4 translocation to the surface of the plasma membrane thereby allowing glucose to enter the cell. **Key for Figure 2:**

= GLUT-4 molecules

P = Phosphorylation (activation) of insulin-signaling pathway components

= Glucose molecules

1.7 Insulin and the Mitogen-Activated Protein Kinase (MAPK) pathway

When insulin binds to the insulin receptor, in addition to activation of the AMPK signaling pathway, an additional pathway is also activated; namely the *mitogen-activated protein kinase* (MAPK) pathway (Montagut, Onnockx, Vaque, et al., 2010), due to the fact that insulin acts as a mitogen (**Figure 3**). Mitogens act on cells to initiate cell growth (Martini et al., 2011). MAPKs are sometimes referred to as *extracellular signal regulated kinases* (ERKs), because they are activated via phosphorylation, which is mediated via the action of other protein *kinases* (Purves, 2012).

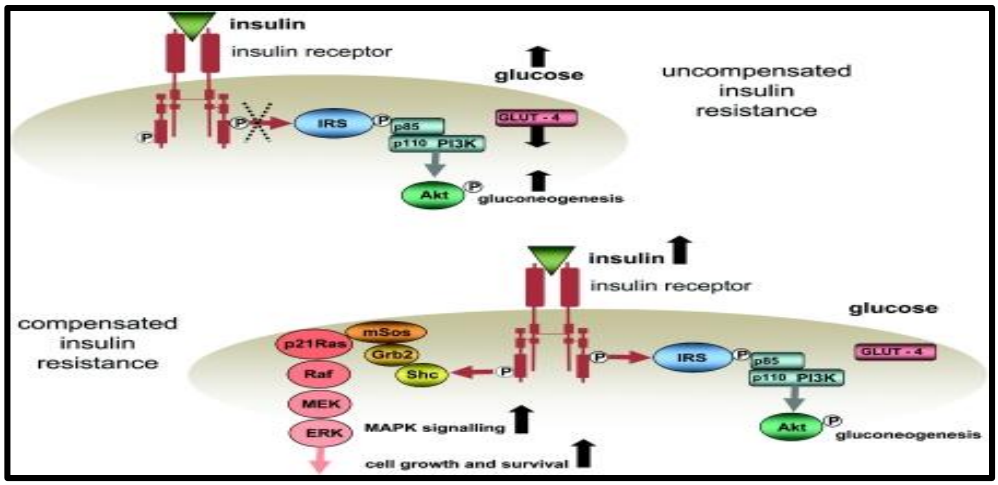

Figure 3: The MAPK signaling pathway

Insulin resistance occurs due to a reduction in the *phosphotinositide 3-kinase* (PI3K) signaling pathway. As a result, glucose levels rise since there are not as much glucose transporter-4 (GLUT-4) present at the plasma membrane, and gluconeogenesis is up-regulated. The pancreas secretes more insulin in response to the high blood glucose levels, which ameliorates the PI3K signaling pathway and brings gluconeogenesis back to homeostasis. The *mitogen-activated protein kinase* (MAPK) pathway is activated via increased insulin levels. [Reproduced from (Godsland, 2010)].

Like AMPK, once MAPK is activated, it can phosphorylate transcription factors and regulate gene expression (Purves, 2012). When first discovered, MAPKs were found to play a role in cell growth (Purves, 2012), but are now known for many other physiological roles. This thesis will principally focus on the role MAPK plays in metabolism.

Dexamethasone, a synthetic glucocorticoid, has been shown to induce insulin resistance in adipocytes, by reducing phosphorylation of p38 MAPK (Bazuine, Carlotti, Tafrechi, Hoeben, & Maassen, 2004), thereby reducing the amount of GLUT4 present in the plasma membrane (Bazuine et al., 2004). Consequently, Dexamethasone is commonly used as a negative control for glucose regulation in tissue culture studies (Bazuine et al., 2004).

The p38-MAPK pathway is needed to ensure the maximal response to insulin, and therefore to facilitate efficient glucose uptake. Increased blood levels of free fatty acids has been shown to cause an up-regulation in the phosphorylation of the amino acid, Serine 307, in IRS-1, reducing the effects of tyrosine autophosphorylation, which lowers the affinity of IRS-1 to bind to the insulin receptor (Deng, Chang, Lee, & Lin, 2012). Increased Serine 307 phosphorylation has been shown to be an important marker for fatty-acid associated insulin resistance, which can also lead to a down regulation in the PI3K-Akt signaling pathway and decrease the response to insulin, thereby reducing glucose uptake (Deng et al., 2012).

1.8 Insulin and Glucose transporter-4 (GLUT-4)

Insulin is known to cause a decrease in blood glucose levels via increased expression of GLUT-4 at the plasma membrane in insulin-sensitive tissues, such as the skeletal muscle and adipose tissue, making insulin the obvious compound of interest in the modulation of blood glucose. GLUT-4 has also been shown to lower blood glucose levels in the absence of insulin, but interestingly, by still utilizing the insulin-signaling pathway (Montagut, Onnockx, Vaque, et al., 2010). Not only can insulin cause long-term changes in GLUT-4 to allow glucose to enter the cell when more GLUT-4 is made, but a recent study showed that insulin can affect the short-term speed

with which GLUT-4 moves to and from the surface of the cell (Brewer, Habtemichael, Romenskaia, Mastick, & Coster, 2014). Although rapid translocation of GLUT-4, via a post-transcriptional mechanism, ameliorates hyperglycemia via insulin as shown in **Figure 4**, insulin decreases GLUT-4 messenger RNA levels (Pinent, Blay, Bladé, et al., 2004).

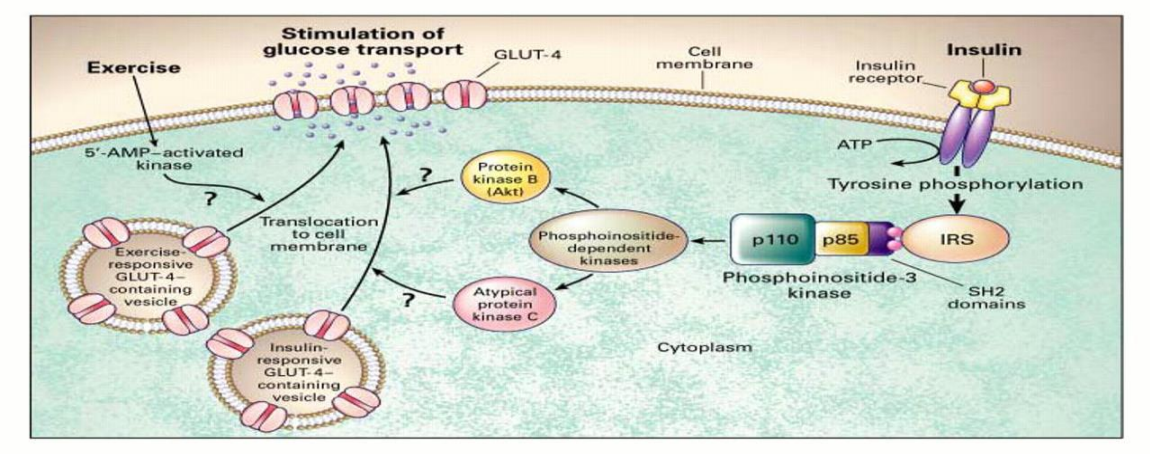

Figure 4. Schematic representation of the signaling effects leading to GLUT-4 movement to the plasma membrane in response to insulin. When insulin binds to the a-subunit of the insulin receptor, located on the surface of the plasma membrane, an insulin transduction pathway facilitates movement of glucose transporter-4 (GLUT4) to the plasma membrane, allowing glucose to enter the cell from the bloodstream. [Reproduced from (Shepherd & Kahn, 1999)].

This has led to the conclusion that there must be another mechanism by which the cell regulates glucose homeostasis in the absence of GLUT-4 at the plasma membrane. An insulin-independent mechanism, that involves another form of glucose transporter, will be discussed below.

1.9 Glucose transporter-1 (GLUT-1)

Glucose transporter-1 (GLUT-1), an "*insulin-independent*" transporter, is an additional glucose transporter expressed ubiquitously. GLUT-1 is expressed at the plasma membrane and allows glucose entry into the cell, and is regulated at both the transcriptional and post-

transcriptional levels (Carruthers, DeZutter, Ganguly, & Devaskar, 2009). Movement back and forth from intracellular compartments to the plasma membrane for GLUT-1 has been shown to be AMPK and PI3K-dependent (Luiken et al., 2004). Although AMPK and PI3K are components in the insulin-signaling pathway, GLUT-1 has been shown to move to the plasma membrane in a distinct manner compared to GLUT-4 (Martin, Lee, & McGraw, 2006). This led to the hypothesis that GLUT-1 expression is not completely dependent on insulin and that it may act in the absence or during states where GLUT-4 is down-regulated (Martin et al., 2006). GLUT-1 is expressed ubiquitously while GLUT-4 is only found in insulin-sensitive tissues and GLUT-1 was found to increase glucose uptake in an insulin-independent manner (Kim et al., 2007).

1.10 Grape Seed Procyanidin Extract (GSPE)

Grape seed procyanidin extract (GSPE), a dietary component, has been shown to act as an "*insulinomimetic*", i.e. by imitating insulin (Pinent, Blay, Blade, et al., 2004), and has been demonstrated to improve blood glucose uptake in cell culture studies (Montagut, Blade, et al., 2010). GSPE has been well studied with respect to the knowledge base regarding the underlying mechanisms by which it regulates lipid metabolism (Del Bas et al., 2008; Del Bas et al., 2009), and **this review of the literature aims to provide an overview of known mechanisms by which grape seed procyanidin extract regulates glucose homeostasis in cell and animal models.**

Chapter 2:

Type 2 Diabetes

Chapter 2: Type 2 Diabetes

2.1. Type 2 Diabetes and Public Health

As mentioned previously, diabetes was responsible for 5.1 million deaths in 2013 (International Diabetes Federation, 2013), while more than 382 million individuals were affected in one way or another by diabetes (Ghoshal & Bhattacharyya, 2015). Undiagnosed diabetics are estimated to constitute 46% of the individuals affected by diabetes (International Diabetes Federation, 2013). There are many factors that play a role in the development of diabetes, including genetics, obesity, and autoimmune pancreatic damage, however, this thesis will focus specifically on Type 2 diabetes mellitus (T2D). One of the earliest signs that a patient may be at risk for T2D is the development of insulin resistance (Ghoshal & Bhattacharyya, 2015). Another factor that plays a major role in the development of diabetes is abdominal weight gain (Consultation, 2004). According to the International Obesity Task Force, as many as 1.7 billion individuals are at an increased risk of developing T2D because of their high body mass index (BMI) (Ghoshal & Bhattacharyya, 2015). BMI, using the weight and height of the individual, is a ratio used to determine a patient's body fat content (Mahan, Escott-Stump, & Raymond, 2012). BMI is accurate for the majority of the population, however there are certain individuals, for example athletes, that will present with a higher BMI than classed as *"healthy"* due to their muscle content, which makes them heavier (Thompson & Manore, 2009). This presents physicians and patients with the need for consultation as to the best possible strategy for a positive healthy outcome for each individual patient. In order to better understand the treatment of T2D, an overview of the condition will be provided below.

2.2 Type 2 Diabetes and Comorbidities

The gradual development of T2D often co-presents with obesity (International Diabetes Federation, 2013), and cells become less and less responsive to the effects of insulin (Mahan et al., 2012). If the patient is overweight or obese, this condition is normally categorized as a comorbidity, as indicated in **Table 1** shown below. Comorbidities are when two or more chronic diseases, such as obesity and diabetes are present in the medical history of a patient.

Summary of the underlying clinical factors for the development of hypoglycemia in patients with diabetes

1 Socioeconomic status (education, race)
2 Aging
3 State of diabetes (duration, HbA1c, body mass index)
4 Cognitive and mental function
5 Comorbidity
6 Failure of organ which influence on clearance of insulin and oral anti-diabetic drugs (Heart, liver, renal failure)
7 Hypoglycemia-associated autonomic failure

Table 1. Clinical factors involved in the development of hypoglycemia in patients living with diabetes. Many factors play a role in the development of low blood glucose levels in patients suffering from T2D. Hypoglycemia (low blood sugar) develops when blood glucose fluctuates out of control and can be life threatening. There are many factors that patients cannot control, such as socioeconomic status, aging, organ failure, and autonomic failure of glucose regulation, but lifestyle changes can be made to lessen the damage caused by other factors such as weight, dietary changes, and exercise. [Reproduced from (Yanai et al., 2015)].

Table 1 shows that there are many factors which are out of the patient's control with respect to glucose regulation, e.g. aging and genetic susceptibility to autonomic failure. However, many factors listed in the Table above, can be modified in the patient's favor. Patients who are in the early stages of developing T2D secrete far higher levels of insulin than normal, in order to lower the high circulating blood glucose levels, but ultimately, the pancreas wears out and stops secreting insulin (Mahan et al., 2012). As emphasized earlier, appropriate insulin signaling is required in

order to maintain blood glucose levels within the normal range (Coughlan et al., 2014). The gradual process in the development of T2D, due to insulin resistance, causes impairment of glucose entry into the cell. The patient with uncontrolled glucose levels and insulin resistance develops T2D and the end result is chronic elevated blood glucose (hyperglycemia). Chronic hyperglycemia can be extremely detrimental, causing many significant physiological and metabolic problems (International Diabetes Federation, 2013). Current treatments, including lifestyle changes, natural supplements, and pharmaceuticals are common approaches that are aimed at alleviating hyperglycemia, in order to protect patients from the consequential damage (Martinez, 2007).

2.3 Current Treatments for Type 2 Diabetes

The current treatments for T2D are straightforward from a clinical perspective. Initially, physicians advise patients to change their lifestyle habits, in order to improve blood glucose control (International Diabetes Federation, 2013). Lifestyle changes normally include modifications with respect to diet and exercise, as the predominant factors that aim to positively modulate blood glucose regulation (Kendall & Bergenstal, 2001). If the patient still presents with hyperglycemia, the physician will commonly then prescribe medication in order to effectively maintain glucose homeostasis (Yanai et al., 2015). Metformin is normally one of the first oral medications prescribed to try to control T2D (Rodbard et al., 2009). The main mechanism of action of metformin in the regulation of blood glucose levels is thought to be the inhibition of gluconeogenesis in the liver (Owen, Doran, & Halestrap, 2000). This means that metformin suppresses the production of glucose from sources other than carbohydrates, such as proteins or lipids, leading to a decrease in overall blood glucose (Owen et al., 2000). Another way in which metformin acts to control glucose homeostasis is by increasing the AMP:ATP ratio within cells,

thereby promoting AMPK activation (Owen et al., 2000). As a result, insulin sensitivity is increased and allows for increased glucose uptake within insulin-sensitive tissues. This is a good therapeutic outcome for those who suffer from T2D, but there are circumstances where too much medication can cause a rapid increase in insulin sensitivity, which can become detrimental (International Diabetes Federation, 2013). Medication also has to be taken at the correct time with a meal in order to exert the best effect on glucose regulation (Martinez, 2007).

Complications can arise if the medication is not used properly, for example, patients with T2D could take too much medication, causing low blood sugar levels (hypoglycemia), which can be fatal (Yanai et al., 2015). In order to protect patients with T2D from hypoglycemia, certain lifestyle changes need to be made and maintained in order to control overall blood glucose. Although even with medication and lifestyle changes, blood glucose levels must be constantly monitored (Martinez, 2007).

A misconception is that individuals with T2D are continually in a hyperglycemic state. However, it is the loss of control over homeostatic regulatory mechanisms associated with regulation, and not just high blood glucose levels, that affects the overall health of those who have T2D (Martini et al., 2011). Consequently, it is important to try and implement lifestyle changes first, because those who have to take medication are at a higher risk for developing hypoglycemia (Yanai et al., 2015), which is immediately life-threatening.

2.4 Physiological Consequences of Type 2 Diabetics

Physicians that treat patients with T2D have to diligently follow a treatment protocol that is customized for the patient's best possible prognosis (Martinez, 2007). The consequences for any

patient with uncontrolled blood glucose levels can be seen in **Figure 5**. Complications that could arise for those with long-term high blood glucose levels include: vision changes and possible retinopathy, neuropathy, kidney failure, foot ulcers, amputation of appendages, digestion issues, urinary problems, and severe cardiovascular effects (Martinez, 2007). Ultimately, since every cell in the body needs glucose, every cell in the body will be negatively impacted consequential to glucose dysregulation.

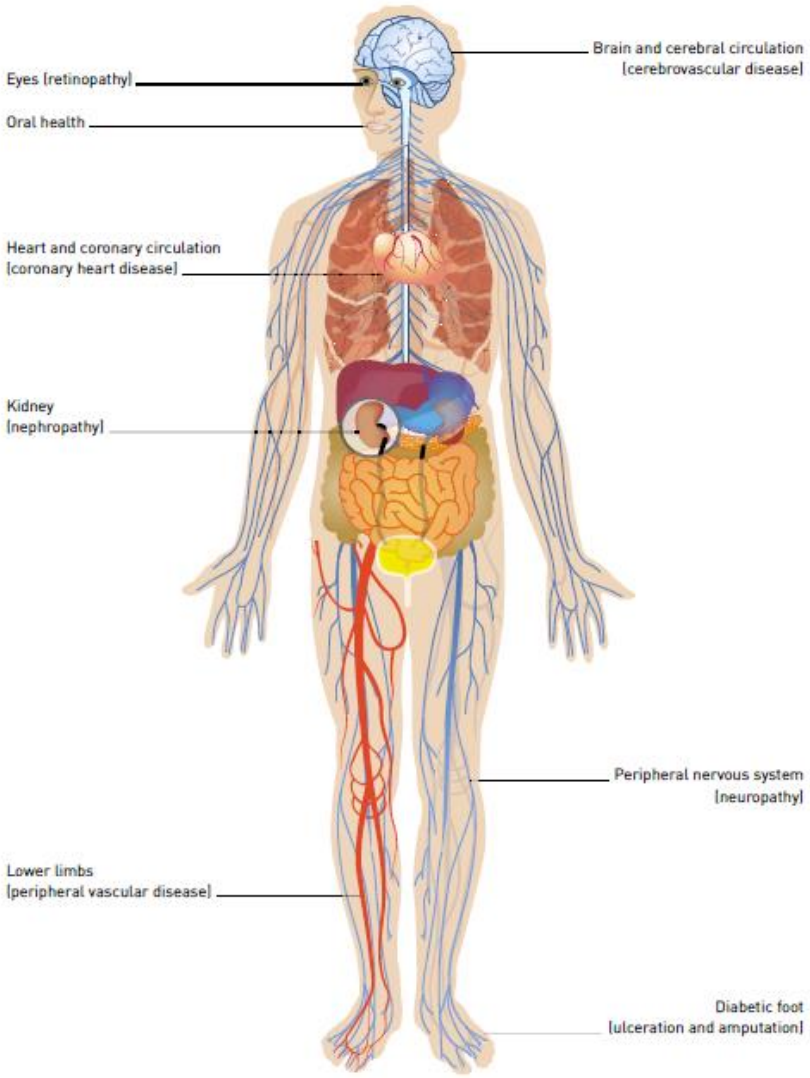

Figure 5. Physiological consequences of Type 2 Diabetes. Several detrimental consequences can occur in patients with uncontrolled Type 2 diabetes. They range from cardiovascular damage to amputation. All are serious consequences that may cause organ damage, loss, and eventually death. [Reproduced from (International Diabetes Federation, 2013)].

2.5 Future Treatments for Type 2 Diabetes

The ideal therapeutic treatment for hyperglycemia and T2D would be to create or modify a molecule that could cure diabetes mellitus without any adverse side effects. Natural dietary compounds are potential candidates for multiple reasons, including, but not limited to, cost,

potentially fewer side effects compared to pharmaceutical compounds, and their abundance around the globe (Babu, Liu, & Gilbert, 2013). An example of such a dietary compound is grape seed procyanidin extract (GSPE), which has been shown to lower blood glucose levels in a way that mimics insulin, thereby exerting effects on the insulin signaling pathway (Pinent, Blay, Blade, et al., 2004).

Since T2D develops gradually in patients, diet and exercise modifications will likely continue to be the first recommendation from a physician, unless the blood glucose levels are found to be extremely high (Martinez, 2007). Although natural anti-diabetic compounds such as GSPE are being tested in cell culture and animal models, for T2D patients, future treatments could rely on a combination of treatments in order to effectively maintain glucose homeostasis.

The treatment plan for each individual patient will depend on how significant the insulin resistance has become. In the later stages of T2D when patients have overworked their pancreas in response insulin resistance, or there is a lack of response to insulin in insulin sensitive tissues, a combination of medications that includes metformin will likely be warranted. Metformin increases the sensitivity of tissues to insulin by modifying the energy levels within each cell (Owen et al., 2000). Since GSPE works well in the presence of low blood insulin levels (Pinent, Blay, Bladé, et al., 2004), it would be interesting to test whether or not GSPE and metformin are a good combination treatment to ameliorate hyperglycemia.

Chapter 3:

Procyanidins and their Physiological Effects

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3.1 Flavonoid Chemistry

Flavonoids, as shown in **Figure 6**, are a large group of >6,000 phenolic compounds found in various foods and beverages such as fruits, vegetables, and red wine (Williams, Spencer, & Rice-Evans, 2004). This thesis will focus on a subgroup of flavonoids called procyanidins. Procyanidins are phenolic compounds that have certain attributes due to their structure, and are well known for their antioxidant properties (Castrillejo et al., 2011).

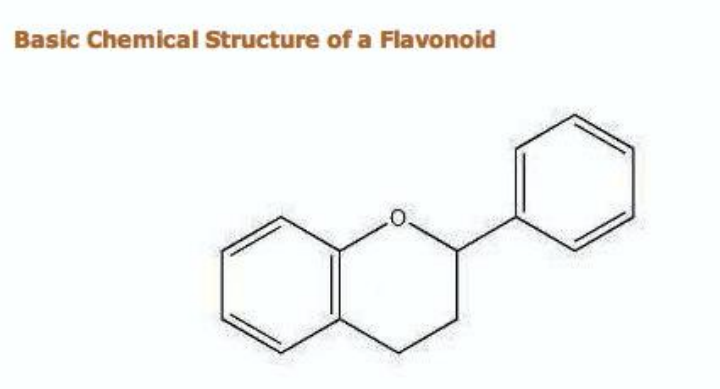

Figure 6. The Chemical Structure of a Flavonoid Compound.

[Reproduced from http://lpi.oregonstate.edu/infocenter/phytochemicals/flavonoids/basicflav.html].

3.2 Procyanidins and their Antioxidant Properties

Grape seed procyanidin extract (GSPE), by acting as a signaling molecule, has been shown to reduce the levels of reactive oxygen species (ROS) and regulate antioxidant enzyme activity (Castrillejo et al., 2011). GSPE alters oxidative chemistry, mainly due to the polyphenolic structure, which has many hydrogens and hydroxyl (OH) groups present on the outside of the

phenolic ring structures. As shown in **Figure 7**, procyanidins are comprised of the monomers, *catechin* and *epicatechin*, which polymerize to from procyanidin dimers, trimers, tetramers, pentamers, etc, all the way up to polymeric tannins.

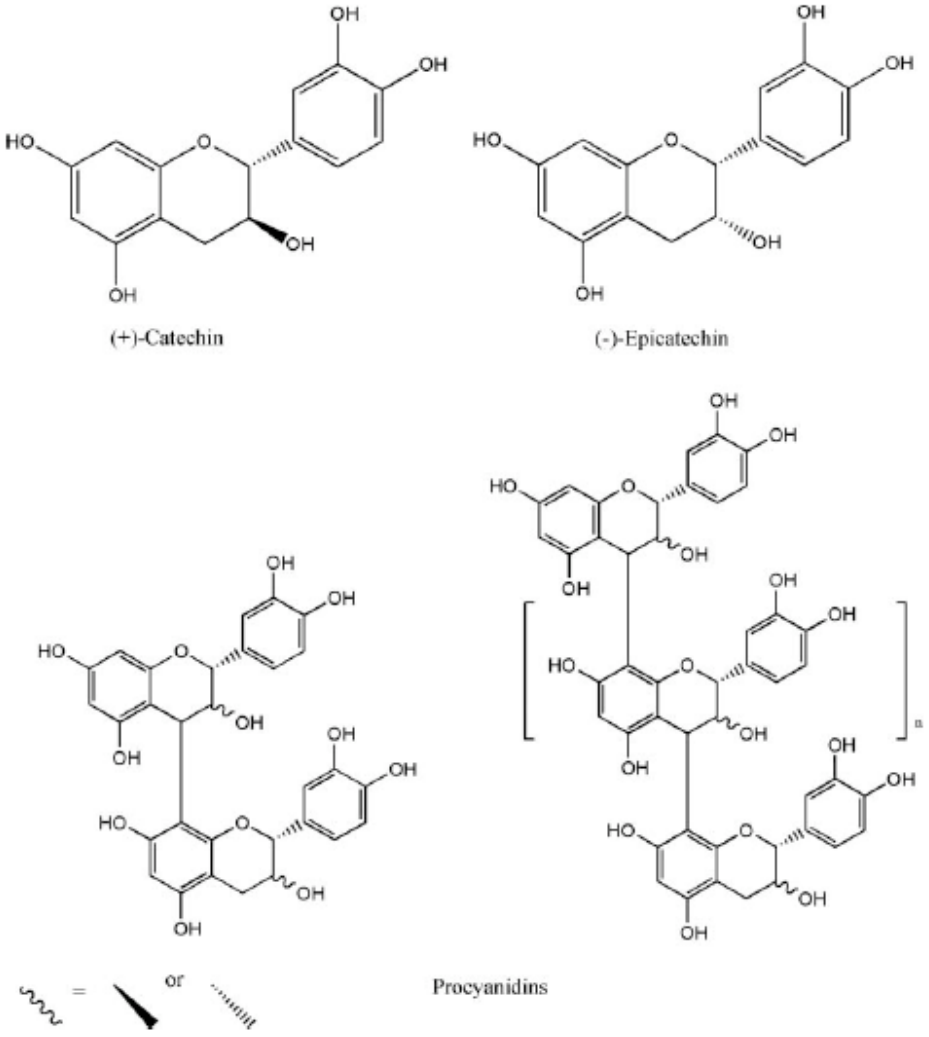

Figure 7: The Structures of Monomeric, Dimeric and Trimeric Procyanidins. The monomers of procyanidins are catechins and epicatechins and are shown above the procyanidins.

[Reproduced from (Pinent, Cedo, Montagut, Blay, & Ardevol, 2012)].

Note: OH = (hydroxyl group).

GSPE has received the most attention due to the well-known flavonoid antioxidant properties and because of this, potential beneficial therapeutic applications in treating cancer (Williams et al., 2004). Flavonoids have been shown to be potent antioxidants acting as reducing agents within the body by donating electrons (Williams et al., 2004). This antioxidant property reduces the possibility of ROS, commonly found in association with cancer, ultimately destroying the plasma membrane and leading to apoptosis, or cell death. Although the focus of this thesis is primarily on glucose regulation, the applications of GSPE for many conditions that involve disruption in metabolic homeostasis, including cancer, are promising (Babu et al., 2013). Since the molecular mechanisms of many biochemical pathways have been elucidated, flavonoids were proposed to modulate protein and lipid kinase cascades (Williams et al., 2004) such as MAPK (Schroeter et al., 2002) and PI3K signaling, respectively (Matter, Brown, & Vlahos, 1992).

3.3 Procyanidins and Lipid Metabolism

GSPE has been shown to lower serum triglyceride (TG) levels via modulation of a transcription factor, resulting in alterations in gene expression (Del Bas et al., 2009). GSPE was identified as a co-agonist ligand for the transcription factor, farnesoid x receptor (FXR) (Del Bas et al., 2009), a member of the nuclear hormone receptor superfamily (Aranda & Pascual, 2001). FXR is critical in the regulation of cholesterol (Makishima et al., 1999; Parks et al., 1999), triglyceride (Watanabe et al., 2004), and glucose homeostasis (Cariou et al., 2006; Ma, Saha, Chan, & Moore, 2006). In combination with bile acids, the endogenous ligands for FXR, GSPE has previously been reported to increase FXR transactivation, resulting in reduced serum triglyceride levels. This is achieved via increased expression of small heterodimer partner (SHP), which ultimately inhibits the expression of sterol regulatory element-binding protein 1c (SREBP1c), the

master regulator of lipogenesis (Del Bas et al., 2008; Del Bas et al., 2009). Since GSPE has been shown to regulate lipid metabolism (Del Bas et al., 2008; Del Bas et al., 2009) there is a distinct possibility that GSPE could be involved in whole body energy metabolism, since many biochemical pathways are interconnected, although the intricate details are yet to be fully elucidated.

3.4 Future Applications for Procyanidins involved in Glucose Modulation

Several studies have been conducted using procyanidins in order to better understand their effect on glucose homeostasis, however, the overall results have sometimes been conflicting. For example, when there is low blood insulin levels, procyanidins act as "*insulinomimetics*" (Pinent, Blay, Blade, et al., 2004), however in the presence of normal blood insulin levels, procyanidins were found to have no significant effect on glucose homeostasis (Pinent et al., 2012). This observation does not affect the decision to investigate procyanidins as a therapeutic treatment for those who suffer from T2D, because these patients will, by definition, have inadequate blood insulin levels.

Within the scope of treating patients with diabetes, those who have T1D and T2D, are going to differ in the etiology with respect to glucose dysregulation, because disrupted glucose homeostasis has many different molecular origins (Pinent et al., 2012). This could ***lead to the hypothesis that procyanidins may have different physiological effects, depending on the type and severity of diabetes affecting the patient.***

Procyanidins have been shown to act in many ways to affect glucose metabolism, but it is still unclear as to which area procyanidins exert the most effect. Procyanidins were previously thought to have the largest effect on glucose homeostasis by regulating glucose uptake in the small

intestine (Pinent et al., 2012) where the majority of nutrient absorption occurs (Martini et al., 2011). Although procyanidins do have effects on intestinal cells, this doesn't appear to be the major regulatory point for glucose homeostatic modulation by procyanidins because studies conducted on rats (Al-Awwadi et al., 2004; Pinent, Blay, Bladé, et al., 2004) showed an increased difference in tissue sensitivity in response to insulin, compared to that of a reduced glucose absorption in intestinal cells.

Procyanidins were also hypothesized to modulate glucose homeostasis by repairing damage to pancreatic β-cells that are responsible for secreting insulin (Pinent et al., 2012), but this has been shown not to be the case, based on current evidence in the literature (Pinent et al., 2012).

The final hypothesis proposed regarding the involvement of procyanidins in glucose homeostasis is that they may have direct effects on insulin-sensitive tissues (Pinent et al., 2012). This seems to be the most reasonable way in which procyanidins modulate glucose homeostasis because GSPE has been shown to alleviate hyperglycemia and increase the amount of GLUT-4 after treatment (Pinent, Blay, Blade, et al., 2004).

A new and exciting proposition is that, based on its known effects via FXR with respect to lipid metabolism, GSPE may also modulate glucose homeostasis via FXR. Research is currently on-going to elucidate whether there is a molecular regulatory mechanism by which GSPE and FXR function together to modulate glucose homeostasis. Since GSPE and FXR have previously been shown to function together in order to regulate lipid metabolism (Del Bas et al., 2008; Del Bas et al., 2009), and that FXR regulates glucose homeostasis (Cariou et al., 2006; Ma et al., 2006) it could be *hypothesized* that they may also work together to regulate glucose metabolism.

3.5 GSPE as an "*insulinomimetic*"

GSPE, analogous to insulin, has been shown to require many similar intracellular components of the insulin signaling pathway (Montagut, Onnockx, Vaqué, et al., 2010; Pinent, Blay, Bladé, et al., 2004). The insulin receptor is activated in the presence of GSPE and low insulin levels (Montagut, Onnockx, Vaque, et al., 2010). IRS-1 is also activated by GSPE, although not to the same extent as that seen with insulin (Montagut, Onnockx, Vaque, et al., 2010). The substrate Akt was also confirmed to be required for GSPE-induced glucose transport (Montagut, Onnockx, Vaque, et al., 2010). The statement that GSPE acts as an *"insulinomimetic"* is extremely relevant because GSPE also requires PI3K to reduce blood glucose levels (Pinent, Blay, Blade, et al., 2004). Taking into account all of the overlapping effects on intracellular components, by insulin, and the requirements of GSPE for glucose regulation, it is highly possible that GSPE takes over the function of insulin in insulin-sensitive tissues in the states of insulin deficit.

Chapter 4:

Grape Seed Procyanidin Extract modulates Glucose Homeostasis

Chapter 4: Grape Seed Procyanidin Extract modulates Glucose Homeostasis

4.1 The Potential of Grape Seed Procyanidin Extract as a Treatment for Type 2 Diabetes

Grape seed procyanidin extract (GSPE) is a dietary compound that can affect many different processes throughout the body, including triglyceride synthesis and glucose regulation (Del Bas et al., 2008; Del Bas et al., 2009; Montagut, Bladé, et al., 2010; Pinent, Blay, Bladé, et al., 2004). The role of GSPE in glucose regulation will be the main focus of this final chapter. There is a high demand for a natural therapeutic treatment for those with T2D, and GSPE has been shown to modulate glucose homeostasis (Montagut, Blade, et al., 2010). Although T2D has detrimental physiological consequences, T2D develops slowly (Mahan et al., 2012) and can be reversed in the early stages by lifestyle changes to diet and exercise (International Diabetes Federation, 2013). Future treatments are needed to help those who have already developed T2D and are in the midst of battling the detrimental physiological consequences.

As mentioned previously, GSPE acts as an "*insulinomimetic*" (Pinent, Blay, Blade, et al., 2004) and it could also potentially share qualities with the potent diabetic drug metformin, which indirectly increases AMPK activation (Lien et al., 2014). **Although GSPE has not been shown to act via AMPK to regulate glucose homeostasis, this is another hypothesis by which GSPE may alleviate hyperglycemia**. Consistent with this hypothesis, a different grape seed extract has been shown to increase AMPK in human umbilical vessel cells in a study assessing the effects of grape seed extract on hypertension (Cui, Liu, Feng, Zhao, & Gao, 2012). Therefore, a dietary compound such as GSPE is viewed as a favorable candidate to combat hyperglycemia in T2D patients (Caiozzi, Wong, & Ricketts, 2012). Although in studies using both cell culture and animal models, GSPE has shown considerable promise, human clinical trials are needed in order for GSPE

to be considered a clinically relevant dietary compound in the treatment of T2D (Hanhineva et al., 2010).

4.2 Grape Seed Procyanidin Extract and Glucose transporter-4

GSPE has been shown to increase the amount of GLUT-4 in the plasma membrane of adipocytes (Pinent, Blay, Blade, et al., 2004). Since GLUT-4 is an insulin sensitive glucose transporter, this raises the possibility that GSPE may affect the translocation of GLUT-4, therefore bypassing a requirement for transcriptional regulation, since GSPE has been shown to decrease the amount of GLUT-4 messenger RNA (mRNA) following treatment (Montagut, Blade, et al., 2010). Although GSPE lowered GLUT-4 mRNA levels, glucose uptake into the cell was increased, which was proposed to be mediated via increased levels of GLUT-4 protein in the cell membrane fraction (Montagut, Blade, et al., 2010).

Despite the observation of increased GLUT-4 protein in the plasma membrane fraction of cells (Montagut, Bladé, et al., 2010), to date, no studies regarding the actual translocation of GLUT-4 to the plasma membrane in the presence of GSPE has been determined. Consequently, in preliminary studies conducted during the course of this thesis we have shown that indeed GSPE does increase translocation of GLUT-4 in 3T3-L1 mouse adipocytes (**Figure 8**) (Brewer et al., 2014), unpublished observations).

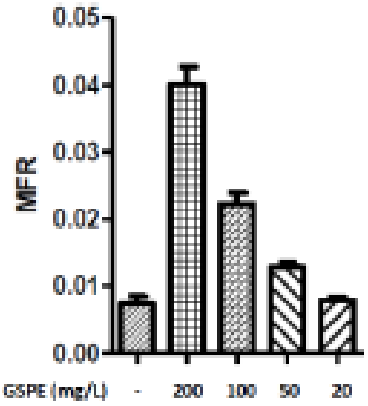

Figure 8. Determination of the ability of a Grape Seed Procyanidin Extract (GSPE) to induce GLUT-4 translocation in differentiated 3T3-L1 adipocytes. (MFR: Mean Fluorescence Ratio). A dose-dependent response to GSPE was seen in GLUT-4 translocation as measured by Mean Fluorescence Ratio (MFR).

(Brewer, Yancey, Mastick, & Ricketts, 2014).

4.3 Grape Seed Procyanidin Extract and Future Applications

With the current research underway to understand the full extent of GSPE's involvement in glucose regulation, there is still much to be learned before GSPE can be applied in a therapeutic setting. Patients may reap the benefits someday of a dietary compound, such as GSPE, once the active components are identified and the underlying molecular mechanisms are fully understood. Research is currently on-going to understand which component in GSPE is the active constituent that contributes to the beneficial physiological effects. Once this is known, there will still need to be clinical trials conducted in human cohorts (Hanhineva et al., 2010) in order to be used as a therapeutic strategy. GSPE has been shown to have significant short-term effects on the insulin-signaling pathway and glucose regulation (Pinent, Blay, Blade, et al., 2004), however, it has not been studied in depth as a long-term treatment for glucose homeostasis (Pinent et al., 2012).

T2D patients normally suffer from inefficient insulin signaling, due to the lack of insulin produced and elucidation of beneficial therapeutic compounds that can be used for the amelioration of hyperglycemia, that may be valuable in treating T2D (Krishnapuram et al., 2013). Although GSPE shares many similar actions to insulin, with respect to the regulation of glucose homeostasis, GSPE treatment does result in different levels of phosphorylation for several proteins involved in

the insulin-signaling pathway, as compared to insulin, e.g. IRS-1 (Montagut, Onnockx, Vaque, et al., 2010).

In conclusion, GSPE has been shown to lower blood glucose levels in insulin-sensitive tissues, but the exact molecular mechanisms underlying this observation are still under investigation. GSPE increases GLUT-4 translocation, although GSPE has been shown to have conflicting results at the transcriptional level for GLUT-4 mRNA levels, leading to the hypothesis, from the literature review conducted herein, that the major effects exerted by GSPE on glucose homeostasis are not at the transcriptional level, but that the most significant effect mediated by GSPE occurs via a direct, acute and rapid regulatory effect, namely that of increased GLUT-4 translocation to the plasma membrane. This is particularly exciting since the time-frame during which blood glucose homeostasis needs to be regulated is rapid. Directly after a meal it is critical that the body responds to the rise in blood glucose (Pinent et al., 2012) and GSPE, based on preliminary data presented in this thesis, appears an exciting prospect for the rapid regulation of glucose homeostasis.

4.4 Future Directions for Grape Seed Procyanidin Extract and Glucose Homeostasis

GSPE has been shown to decrease GLUT-4 mRNA levels after administration (Pinent, Blay, Bladé, et al., 2004), and consistent with this observation, was also shown to initially lower GLUT-4 mRNA levels (Flores-Riveros, McLenithan, Ezaki, & Lane, 1993). Since both GSPE and insulin have been shown to rapidly increase GLUT-4 translocation in 3T3-L1 adipocytes (Brewer et al., 2014;Brewer et al., 2014), the ability of GSPE to modulate glucose homeostasis seems to be immediate, although further studies are needed in order to establish the critical time for GSPE-induced effects on GLUT-4 mRNA expression. Although insulin initially decreased

GLUT-4 mRNA levels, at about 24 hours post-treatment, GLUT-4 mRNA expression leveled off seen below in **Figure 9** (Flores-Riveros et al., 1993).

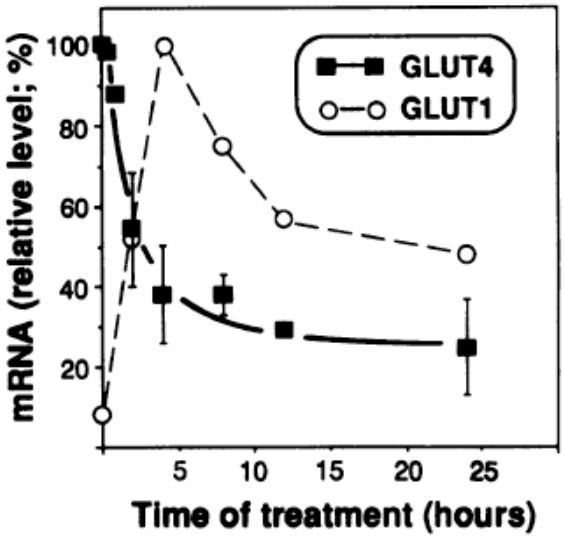

Figure 9. Glucose transporter mRNA levels in response to insulin treatment. GLUT-1 shows a maximal response around 4 hours in direct correlation with GLUT-4 mRNA levels decreasing. GLUT-4 mRNA levels become steady around 24 hours post-treatment. Reproduced from (Flores-Riveros et al., 1993).

In contrast, expression of GLUT-1, the insulin independent glucose transporter, increased in the first 5 hours following insulin treatment in 3T3-L1 adipocytes (Flores-Riveros et al., 1993). GLUT-1 transcription increased while GLUT-4 transcription decreased in the presence of insulin, despite the increased amount of GLUT-4 found at the plasma membrane (Flores-Riveros et al., 1993). Further studies are needed in order to evaluate the effects on GLUT-1 in response to GSPE treatment. GSPE have similar effects on GLUT-1 as insulin and future studies could lead to the discovery of an alternative mechanism for glucose modulation at the transcriptional level. GSPE has previously been described as an "*insulinomimetic*"(Pinent, Blay, Bladé, et al., 2004), however,

the full picture beyond this statement requires additional studies in order to fully elucidate the molecular mechanisms by which GSPE modulates glucose homeostasis.

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