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Expression of Insulin Responsive Genes in Insulin Resistant Conditions, and the Effect of Selenium on Gene Expression

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EXPRESSION OF INSULIN RESPONSIVE GENES IN INSULIN RESISTANT CONDITIONS, AND THE EFFECT OF SELENIUM ON GENE EXPRESSION

by

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Chronically high blood glucose levels lead to many problems, such as insulin resistance, the hallmark of Type II diabetes. Increased flux through the hexosamine biosynthesis pathway is one mechanism by which high glucose as well as glucosamine has been shown to induce insulin resistance. This study tests the effects of glucosamine induced insulin resistance on insulin regulation of the metabolic genes glucose-6-phosphate dehydrogenase (G6PDH) and fatty acid synthase (FAS) as well as insulin responsive proteins tribbles homolog (TRIB3) and sterol regulatory element binding protein (SERBP-1c) 1c.

Selenium, a micronutrient has been shown to be an effective insulin mimetic in Type I diabetics, however its effectiveness has not been tested under Type II diabetic conditions. Thus we also assessed the ability of Se to act as an insulin mimetic agent under glucosamine induced insulin resistance.

In summary, our results show that glucosamine effects the insulin induction of the genes tested. The effect of Se, however was variable and is suggestive of different mechanisms by which it modulates gene expression.

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To God Be the glory! Great things He has done!

David L. Ruff II

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I. Introduction

Metabolism

Over the years, advancements in the study of metabolism have allowed us to increase life span and quality of life. Integral to the enhancement of our overall well being is the ability to confront and treat diseases, such as diabetes, that influence the utilization and production of nutrient resources. As a result of the study of metabolism, the understanding of how a properly working system provides energy for life's defining processes has increased. This knowledge has provided us with numerous benefits and is crucial to our well being.

When metabolism works as it should, each organ performs a specific role that is necessary for maintaining homeostasis. At the cellular level, carbohydrates, such as glucose, along with fatty acids provide energy to drive the body's processes or can be stored for later use. Prompted by newly available nutrients, the body's cells begin to produce proteins that can maximize the body's energy return. Proteins catalyze reactions and perform tasks that provide resources for the body.

Production of proteins utilizes significant amounts of resources and must be vigorously controlled (Neufeld & Arsham, 2010). One mechanism for control regulates mRNA transcription. Specifically, this mechanism allows for control over the types of proteins that are produced and for the timing of their production. In the presence of nutrients, production of mRNA for proteins such as phosphoenolpyruvate carboxykinase (PEPCK), which are active during starvation conditions, is decreased. Also, in nutrient rich conditions, proteins such as glucose-6-phosphate dehydrogenase (G6PDH) that are

needed for nutrient metabolism are produced in greater quantity. When metabolism does not function optimally, the mechanisms that control mRNA transcription receive mistimed or inappropriate signals. The result of these changes in cellular signals leads to the collapse of many cellular systems, which will eventually lead to the manifestation of many diseases such as diabetes.

Diabetes

There are two major types of diabetes: Type I, or insulin-dependent diabetes mellitus, and Type II, or insulin-independent diabetes mellitus. Type I diabetes occurs when insulin is not produced in sufficient quantities to perform its function. As a result, insulin is unable to exert control over proteins that mediate the uptake and metabolism of nutrients from the blood stream. Type II diabetes is signified by insulin resistance. Though insulin is still produced, it is no longer able to initiate the network of proteins involved in metabolism in insulin-sensitive tissues. As a result of insulin resistance, there is a marked increase in the concentration of carbohydrates and fatty acids in the blood stream. If left unchecked, this condition will develop into Type II diabetes.

The discovery of insulin in 1921 (Banting, Best, Collip, Campbell, & Fletcher, 1922) was a key step in treating both Type I and II diabetes and began a gradual uncovering of the extensive reach and complexity of proteins under insulin control. Proteins within insulin's signaling network take part in multiple aspects of metabolism and a myriad of cellular functions. Insulin exerts control over metabolic proteins through many different mechanisms including those involved in transcription. It has been suggested that insulin modulates the expression of over 800 proteins in the human skeletal muscle alone (Rome et al., 2003). Because of insulin's enormous influence, it is easy to see how resistance to insulin action could have a devastating effect and result in many deleterious complications.

Complications of Diabetes

The pains of diabetes are felt on the individual and societal levels. According to the Centers for Disease Control (Centers for Disease Control and Prevention [CDC],

2011), in 2010 there was an estimated 1.9 million new cases of diabetes here in the United States alone. According to a 2014 report by the CDC, that number increased by 1.7 million in 2012 alone (Centers for Disease Control and Prevention [CDC], 2014). The World Health Organization estimates that as of August 2012, approximately 29.1 million people in the United States have developed diabetes. In the coming years, the number of new cases is expected to grow at alarming rates.

Unless insulin resistance is properly managed, life-threatening complications of the disease such as kidney failure and heart disease will occur. Other complications associated with the disease such as lower limb amputations and blindness will add to the debilitating condition of an affected individual. Although other disease states can cause these complications, diabetes is by far the leading cause of all of these ailments in the United States (CDC, 2011). Ultimately, diabetes is a leading cause of death in the United States and, in this way, affects everyone (CDC, 2014).

The pains of diabetes are not only felt by disease victims or close relatives but are also felt in the economic strain diabetes places on society as a whole. In 2007 alone, $174 billion dollars were spent on diabetes and related issues (CDC, 2011). This cost has continued to grow since then and will continue to rise (Yang, Dall, Halder, Kowal, & Hogan, 2013). In 2012, the direct and indirect costs of diabetes in the United States reached 245 billion dollars (CDC, 2014). Climbing costs, sharp increases in the number of affected individuals, and the devastating effect of the complications associated with the disease are reasons why important new advances in the study of diabetes are imperative to our health and welfare.

History of Discovery

People over the ages have studied diabetes and its complications in an effort to understand its causes and how to deal with it. These studies led to a greater understanding of the disease. Discoveries from these efforts, which are reviewed by Awad Ahmed (Ahmed, 2002), eventually led to a greater understanding of insulin and the key role it plays in regulating the integration of nutrients into the body. The earliest available evidence of systematic studies of the disease came from documents found in archeological excavations of Egyptian burial sites revealing early Egyptian doctors' detailed descrip-

tions of many diseases (Ebbers, 1937). The discovery of these ancient documents marks the known beginning of what modern scientists have built upon to give us the knowledge of diabetes and its symptoms that we have today.

Before we knew what we know today, the onset of the disease was essentially a death sentence. Victims of the disease could expect to live short lives that were full of pain and agony. However, there were several discoveries over the years that changed our understanding and provided hope for those who were suffering.

One important discovery came when scientists realized that the problem originated from sugar that had not been properly incorporated from the bloodstream after a meal (Dobson, 1776). Important discoveries by Claude Bernard, reviewed by Young, helped to clarify the role of the liver and chronically high amounts of glucose in the bloodstream with his discovery of glycogen (Young, 1957). Building on the idea of poorly integrated nutrients, medical practitioners and researchers found some success in treating diabetes by restricting carbohydrate consumption (Rollo, 1798), thus reducing the amount of glucose that would be present in the blood stream as a result of digestion. This concept was the beginning of the employment of dietary modifications that are still useful today.

By the mid to late 1800s, scientists began to understand that a substance secreted from the pancreas, or "pancreatic juice" (Allen, 1913) had an unknown yet significant part to play in sugar metabolism. Minkowski and Von Mering (1889) would establish a firm connection between diabetes and pancreatic pathology in their experiments which showed that removal of the pancreas led to diabetes in dogs. This discovery helped the medical community usher in a new era in diabetes research.

A New Era

The next century saw the isolation of a substance within the "pancreatic juice," which came to be known as insulin. Insulin was isolated and purified by Fredrick Grant Banting and Charles Herbert Best with the help of James Collip in 1921 (Banting, Best, Collip, Campbell, & Fletcher, 1922) . The discovery that this insulin could be used to reduce blood glucose levels in humans and treat diabetes brought on the beginning of the insulin era. With this discovery, a new problem arose, and that was that not all people

suffering from diabetes responded to insulin in the same way. This observation led to the characterization of Type I and Type II diabetes by Dr. Harold P. Himsworth (Himsworth, 1936).

Himsworth noted that for a good number of those suffering from diabetes, it was not the concentration of insulin within the system or the lack thereof that was the primary contributing factor to the disease pathology but the ability to respond to the insulin that was present. Himsworth's work was verified by the work of Rosalyn Yalow and Solomon Berson, who in 1959–1960, discovered how to quantify the amount of insulin in a biological system (B. R. S. Yalow & Berson, 1960; R. S. Yalow, Glick, Roth, & Berson, 1965). In addition to verifying the observations of Himsworth, Yalow and Berson's discovery provided a method for doctors to scientifically differentiate between patients suffering from Type I and Type II diabetes, and to alert patients of abnormal insulin levels.

In 1984, Borgardus et al. compared insulin secretion and insulin action in nondiabetic and noninsulin-dependent diabetic subjects. Borgardus and associates observed that in the diabetic patients, the presence of insulin released by the pancreas was not able to efficiently change the plasma glucose concentration over time as it was able to do in the nondiabetic patients. He noted that as diabetes progressed, the hyperglycemia that was experienced resulted from both decreased insulin action as well as decreased peripheral insulin concentrations (Bogardus et al., 1984). This work helped to spark interest in the activity of insulin and its direct effect on insulin-responsive tissues.

Insulin Action

Discussion in the field arose concerning the mechanism that insulin used to cause the uptake of glucose from the blood stream. Some believed that the effect of insulin came from its acting directly on the metabolic enzymes that were responsible for the breakdown of glucose (Levine et al., 1950). This idea seemed to be supported by the finding that, in rats, insulin increased the activity of proteins called hexokinases (Price, et. al. 1945). These are enzymes which modify other molecules by the addition of phosphate groups, in this case, to glucose. Rachmiel Levine and associates designed an experiment to test whether insulin acted directly on the metabolic enzymes to increase the breakdown of glucose or if there was another explanation for insulin's effect (Levine, et. al., 1950).

Levine treated several dogs with galactose, a molecule that is structurally similar to glucose and is transported into cells in response to insulin. Two hours after these treatments, the dogs were treated with insulin, and the amount of galactose left in the bloodstream was measured and compared to the amount of glucose in the blood stream. Galactose is changed to glucose in the liver, kidneys and digestive tract, which can be converted to glucose-6-phosphate by hexokinases. Levine and associates removed these organs from their test subjects, to avoid any transformation of galactose by its interaction with metabolic enzymes, and injected galactose directly into the dogs' bloodstream.

They found that in the absence of insulin treatments, galactose concentration initially decreased slightly and then remained at a relatively constant level in the bloodstream. However, galactose was taken up by the tissues and its concentration in the bloodstream dropped abruptly after treating the animals with insulin. However, the structural molecule remained unchanged (Levine, et. al., 1950), which showed that insulin's primary mode of action was to stimulate the uptake of galactose and that this action was not limited by metabolism of galactose. Levine then deduced that the effect of insulin on glucose, which is so structurally similar to galactose, should not be much different and that there must be another explanation for insulin's blood glucose lowering ability other than the direct activity of insulin on metabolic proteins. Over time Levine began to turn his attention to the possibility of an external receptor as insulin's point of activation.

The Insulin Signaling Cascade

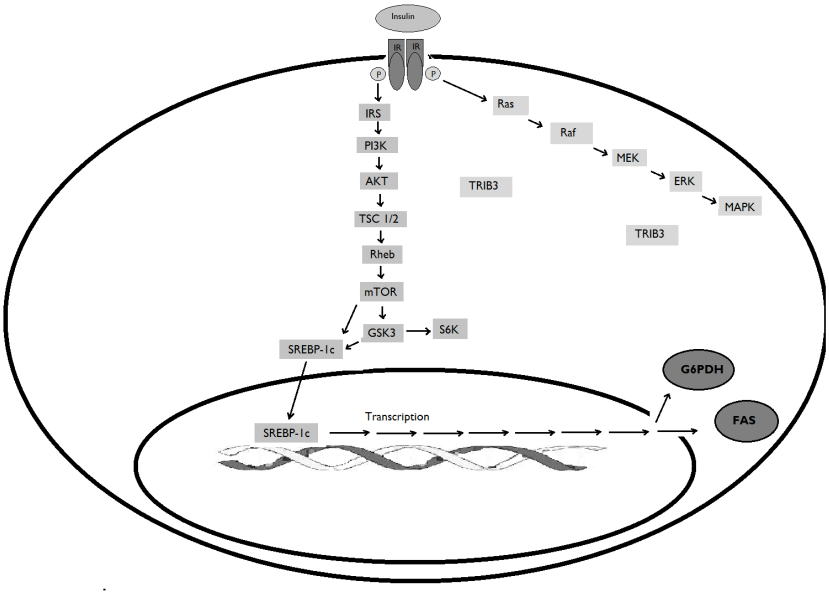

Figure 1. **Diagram of insulin signaling.** The insulin mediated signal is propagated along a protein cascade to regulate protein transcription.

Gradually, discoveries were made that substantiated Levine's idea of an insulin receptor, and the beginning of our knowledge of the insulin signaling pathway began to unfold (figure 1). Evidence began to show that there, in fact, was an insulin receptor which is a dimer comprised of two alpha and beta subunits. The insulin receptor was also found to be the start of a chain of proteins, now known as the insulin signal cascade, that undergoes phosphorylation (Petruzzelli et al., 1982). This chain of proteins leads to insulin's effect on metabolism and uptake of glucose.

Attention then turned to the activity of these signal proteins downstream of the receptor. In 1985, the insulin receptor substrate (IRS), a protein that interacts with the insulin receptor, was discovered by Morris White (White, 1985). Characterization of IRS1 followed shortly thereafter in 1991 (Keller, 1991). Research continued, and an intricate web of proteins downstream of IRS1 slowly began to emerge. Combined discoveries from several labs, including those of Lewis Cantley and his associates, and Yoshikazu Sugimoto et al., added the next piece to the puzzle. Through their work they were able to establish phosphotidal inositol-3,4,5-P3 (PIP3) as an important affecter of insulin action (Cantley, 2007) (Sugimoto, et. al., 1984).

Pulling together discoveries from several areas of interest, Malcolm Whitman et al. went on to describe how PIP3 was phosphorylated, identifying phosphotidal-inositol 3 kinase (PI3K) as its phosphorylating protein (Whitman et. al., 1988). This discovery was supported by the work of Neil Ruderman et al., who showed that insulin increased PIP3's abundance, and that PI3-K could be co-precipitated with antibodies specific for the insulin receptor (Ruderman, Kapeller, Whites, & Cantley, 1990). These findings demonstrated that insulin was indeed involved with the activation of PI3K and that this activation was a direct effect of PI3K's association with the insulin receptor.

In 1992, Jonathan Backer et al. shed more light on the activation of PI3K. His work showed that, when IRS1 was overexpressed, the activation of PI3K was increased in the presence of insulin (Backer et al., 1992). Additionally, Backer showed that tyrosyl phosphorylation of IRS1was crucial for PI3K activation, and identified the region of interaction between the two proteins.

With the discovery of the association of PI3K to insulin stimulation, members of a research team at Eli Lilly laboratories began working on research tools that would allow them to probe its function more closely. In 1994, they published a paper characterizing an inhibitor, 2-(4-Morpholinyl)-8-phenyl-4H-l-benzopyran-4-one (LY294002), that proved to be a successful and specific inhibitor of PI3K in primary cell lines (Vlahos, Matter, Hui, & Brown, 1994). The use of this and other inhibitors such as wortmannin, another inhibitor of PI3K, were invaluable tools that helped to provide a greater understanding of the insulin initiated cellular response.

As a result of utilizing the PI3K inhibitors and a myriad of other useful tools that have been discovered over the years, we learned that there are three major branches within insulin's web of proteins that are used to mediate insulin's response. These branches, shown in fig.1, make up distinct but overlapping signaling pathways that can specialize in function. The branches are the MAPK, Cbl/Grb and PI3K/AKT pathways. Cell survival and response to stress is maintained through the MAPK pathway. Translocation of glucose transporters to the plasma membrane are mediated through the interaction of the insulin receptor and Casitas B-lineage (Cbl) protein and adapter protein with pleckstrin homology and Src homology 2 domains (APS) to affect the translocation of

glucose transporter 4 (GLUT4) in muscle (Chang, Chiang, & Saltiel, 2004) and GLUT2 in liver. Insulin-mediated metabolism mainly occurs through the PI3K pathway.

While each branch of the insulin signaling cascade specializes in a particular capacity, some of the functions of these pathways overlap to provide alternate routes for the flow of signals, creating a more robust system for cellular function. For example, the PI3K pathway also has an important role in cell survival, proliferation (Datta et al., 1997) and translocation of glucose transporter proteins to the cellular membrane (Calera et al., 1998).

Following the discovery of PI3K, protein kinase B (PKB), also known as AKT, was shown to be a part of the insulin signaling cascade by two labs in 1995 (Franke et al., 1995; Burgering & Coffer, 1995). AKT is a significant branch point for numerous pathways that are responsible for everything from metabolism to cell proliferation and survival. Anna Klippel and associates described the activation of AKT activation by PIP3 in response to PI3K in a cell free system in 1997 (Klippel, Kavanaugh, Pot, & Williams, 1997).

AKT is recruited by PI3K and is phosphorylated by PDK 1 and 2, on residues 309 and Ser 474 respectively (Meier & Hemmings, 1999; Hemmings & Restuccia, 2012). AKT then phosphorylates a myriad of other proteins responsible for a variety of functions, including insulin secretion, cell survival and the transcription of metabolic genes (Chang et al., 2004; Fayard, Tintignac, Baudry, & Hemmings, 2005).

Downstream of AKT are many other proteins such as glycogen synthase kinase 3 (GSK3). GSK3 is mainly known for its role in phosphorylating glycogen synthase but has also been shown to regulate at least 40 different proteins involved in numerous cellular functions (Jope & Johnson, 2004). The role of insulin in regulating GSK3 was characterized when scientists showed GSK3 to be inactivated in hamster ovary cells in response to the overexpression of the insulin receptors (Welsh & Proud, 1993). Other researchers established that insulin no longer had the ability to cause the inactivation of GSK3 in the presence of wortmannin (Cross et al., 1994).

Regulation of proteins such as GSK3 and AKT is key because it modulates the transcription of insulin-responsive proteins. For example, AKT regulates the transcription of peroxisome proliferator-activated receptor-gamma co-activator 1alpha (PGC-1a), both

directly by inhibitiing of PGC-1a's transcriptional activity (X. Li, Monks, Ge, & Birnbaum, 2007) and indirectly by AKT's phosphorylation of GSK3 (Bhalla et al., 2011). GSK3 also modulates the transcription of genes involved in gluconeogenesis such as PEPCK, and for genes involved in fatty acid metabolism like FAS (Bhalla et al., 2011). Loss of insulin sensitivity alters the propagation of the insulin signal to proteins such as AKT and effects transcription of metabolic genes (Michael et al., 2000).

Through the years, the development of insulin treatment for diabetics has saved the lives of many. Yet, treatment of diabetes with insulin is not without its pitfalls. Some individuals may experience debilitating complications as a result of problems with insulin treatment (Richardson & Kerr, 2003). At times, patients are required to switch between insulin types due to issues with sensitivity. Treatments that can aid in or potentially replace the use of insulin are crucial to explore and discover.

Selenium, an Insulin Mimetic

Selenium (Se) is a trace mineral and is a cofactor for several genes (Rayman, 2000), including those involved in glucose and lipid metabolism (Stapleton, 2000) and immune response. In 1990, Osama Ezaki was the first to show that Se could mimic the action of insulin in rat adipocytes (Ezaki, 1990). His experiments showed that 100μM Se was equivalent to 1nM insulin in the stimulation of glucose uptake, the increase of cAMP phosphodiesterase activity (Ezaki, 1990) and the reduction of the activity of cAMP responsive pathways. Other insulin-mimicking traits of Se include the ability to lower lipid levels and to restore transcriptional responses of metabolic proteins to, or close to, prediabetic levels in whole animals (Berg, Wu, Campbell, Kagey, & Stapleton, 1995; Becker et al., 1996).

Rats and mice with streptozotocin induced diabetes experienced several restored markers of insulin sensitivity including increased glucose uptake, weight gain and lowering of blood glucose following Se treatment (McNeill, Delgatty L.M., & Battell, 1991; Ghosh, Mukherjee, & Chatterjee, 1994). In addition to these markers, Se was also shown to behave like insulin in the metabolism of cholesterol and free fatty acids by reducing abdominal fat, cholesterol and adiponectin levels (J. E. Kim et al., 2012).

The insulin-like effect of Se has been attributed to increased phosphorylation of key signaling proteins within various branches of the insulin signaling network, including enhanced phosphorylation of the insulin receptor (Pillay & Makgoba, 1992) and IRS-1 (Ezaki, 1990; McNeill et al., 1991). Studies from our lab and others have shown several key proteins, such as AKT and MAPK, experience increased phosphorylation levels as a result of Se treatment in hepatocytes (Stapleton, Garlock, Foellmi-Adams, & Kletzien, 1997) and adipocytes (Hei, Farahbakhshian, Chen, Battell, & McNeill, 1998).

Se can be seen in nature in several forms: selenate, selenite, and selenide. Of the three, selenate has a faster and more straightforward rate of incorporation by enzymes and biological proteins (Mueller & Pallauf, 2006). In comparison, studies done in db/db mice between selenite and selenate, found that selenate resulted in increased glucose uptake and metabolism (Mueller & Pallauf, 2006).

Glucose Uptake and Metabolism in the Liver

The liver is essential to regulating the nutritional status of the body (Bémeur, Desjardins, & Butterworth, 2010). Its cells serve as nutritional reservoirs during fasting, or between meals, and are responsible for recycling carbohydrates and processing lipids. Nutrients that are stored in the liver are released into the blood stream and are available for other organs of the body during fasting and in the absence of insulin (Sherwin, 1980). The presence of insulin at its hepatic receptor site initiates the propagation of a signaling phosphorylation cascade that has influence over cellular activity, including the transcription of genes. Genes transcribed as a result of insulin's signal are incorporated into metabolic pathways that utilize nutrients such as glucose to provide building blocks for the cell (Wilcox, 2005).

Glucose Homeostasis

Glucose enters cells of the liver through a transporter protein that has a high capacity for glucose uptake from the bloodstream under physiological conditions. GLUT2, the dominant glucose transporter protein in the liver, does not depend on an ATP-driven system in order to allow glucose uptake (Gould & Holman, 1993); however, GLUT2 ex-

pression is modulated by diet (Miyamoto et al., 1993). Glucose molecules are retained within the cell by the addition of a phosphate molecule added by glucokinase.

Once glucose enters the cell, it is shuttled to several different metabolic pathways where it functions as a substrate for enzymatic reactions. Propagation of glucose as a substrate is controlled by several rate limiting factors. These factors include the availability of other substrates, the activity and availability of enzymes in the pathway and the needs of the cell at the time (Thomas & Fell, 1998). Changes in propagation brought on by decreases in insulin sensitivity disrupt the balance of byproducts produced by each pathway and result in deleterious effects.

Each pathway is orchestrated to provide for the timely production of byproducts that are vital for the life of the cell. For example, the glycogen and gluconeogenesis pathways provide a way that glucose can be available to produce energy during fasting. Glycolysis provides pyruvate, an important precursor involved in ATP production in the citric acid cycle. Glycolysis also provides substrates for several other metabolic pathways, such as glycogen and fatty acid synthesis, the making of several amino acids and the hexosamine biosynthesis pathway (HBP).

The HBP is a subsidiary pathway of glycolysis and metabolizes 3 to 5% of all cellular glucose molecules. The HBP is an active pathway in many different cell types but is especially active in the liver (Holts & Hart, 1986). Many proteins, including metabolic enzymes, are post-translationally modified as a result of the propagation of substrates through the HBP (Holts & Hart, 1986).

While the consequences of propagation through most metabolic pathways have been studied and are fairly defined, the effect of flux through the HBP is not as well understood. However, as a result of several recent studies, we have progressively begun to understand the impact that the HBP has on the body's insulin response mechanisms (W. Timothy Garvey, Olefsky, Matthaeig, & Marshall, 1987). By utilizing methods that have been shown to change the rate of flux through the HBP, we can gain a greater understanding of what this pathway does in the liver (Lombardi et al., 2012; Rossetti, Hawkins, Chen, Gindi, & Barzilai, 1995).

The Hexosamine Biosynthesis Pathway

Increased propagation of substrates through the HBP, outlined in figure 2, has been shown to have an effect on the insulin signaling cascade (Marshall, Bacote, & Traxinger, 1991). This increase occurs when glucose levels remain high for an extended period, creating a toxic condition in the blood stream. Chronically high glucose levels result in an inability of insulin to direct changes in carbohydrate metabolism and enzyme activity (Fujimoto, Torres, Donahue, & Shiota, 2006). As blood glucose increases to toxic levels, adjustments to regulate the amount of glucose being transported, retained and metabolized by insulin-responsive tissues have to be made. When the amount of glucose remains high in the blood stream, the adjustments of the cell can result in insulin resistance. Some of these adjustments come as a result of increased flux through the HBP (Marshall et al., 1991).

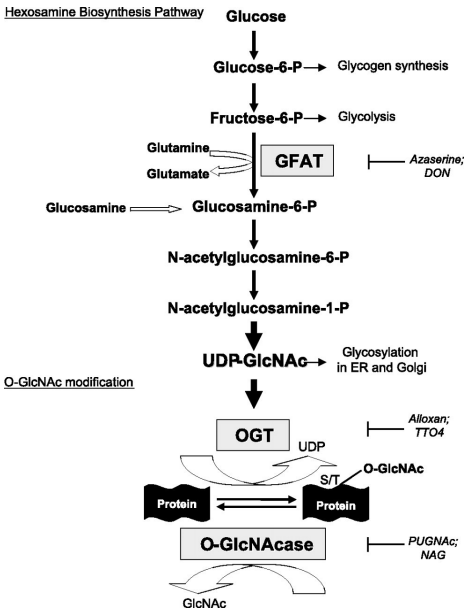

Figure 2. **Diagram of the hexosamine biosynthesis pathway**
(Used by permission of the author) (Laczy et al., 2009)

Discovery

Evidence that the HBP is part of the mechanism that regulates nutrient uptake and could lead to insulin resistance began with observations from experiments done in the 1980s. W. Timothy Garvey and colleagues noted that a reduction of glucose transporter translocation and activity in the plasma membrane could be used as a valid marker of insulin resistance in adipose tissue (W T Garvey, Huecksteadt, Matthaei, & Olefsky, 1988).

Physiologically high levels of glucose and insulin were initially investigated as agents that were involved in the onset of insulin resistance (W Timothy Garvey et al., 1987). In a paper published in 1989, Traxinger & Marshall showed that neither high concentrations of insulin or glucose alone could bring about the insulin resistant response observed in the glucose transport system (R Roger Traxinger & Marshall, 1989a). However, it is important to note that the desensitization came about because of concurrently and chronically high levels of glucose and insulin (W T Garvey et al., 1988). When either glucose or insulin was present by itself, an insulin resistant state was not fully achieved.

Upon examining the growth media used to culture their cells, a third and essential component was identified. Researchers observed that when growth media that did not contain amino acids was used, insulin resistance was not achieved. After experimenting with several different combinations, it was discovered that L-glutamine was the necessary amino acid that worked in combination with glucose and insulin to produce insulin resistance. This requirement was shown to be specific, as treatment with metabolites or derivatives of L-glutamine did not achieve insulin resistance (Roger R. Traxinger & Marshall, 1989b).

Marshall et al. noted that two of the components that are necessary for insulin resistance, glucose and glutamine, are both necessary for producing HBP byproducts (Marshall et al., 1991). Glutamine serves as a donor for the amine group that is transferred by glutamine:fructose-6-phosphate amidotransferase to fructose-6-phosphate to produce the glucosamine group on glucosamine-6-phosphate, the pathway's rate limiting step (Hassel P, Kimura, & Hascal, 1986).

Synthesis of hexosamine byproducts, as outlined in diagram 2, begins with the phosphorylation of glucose by glucokinases to produce glucose-6-phosphate, which is then converted to fructose-6-phosphate (F-6-P) by phosphoglucoseisomerase. F-6-P then enters the HBP and is converted to glucosamine-6-phosphate by glutamine:fructose amidotransferase (GFAT). N-acetylation of glucosamine-6-phosphate is then catalyzed by acetyltransferase, followed by the isomerization of N-acetyl-D-glucosamine-6-phosphate into N-acetyl-D-glucosamine-1-phosphate. N-acetyl-D-glucosamine-1-phosphate is then converted to uridine diphosphate-N-acetyl-D-glucosamine (UDP-GlcNAc) with the addition of uridine triphosphate by UDP-*N*-acetylglucosamine pyrophosphorylase. At this

point, UDP-GlcNAc can be relocated to the Golgi apparatus by UDP-GlcNAc transporters as a substrate to modify proteins and other molecules. In some cases it is changed into UDP-N-acetylgalactosamine by a reversible reaction catalyzed by UDP-galactose-4-epimerase (Shirato et al., 2011).

The HBP plays an essential nutrient sensing role that aids in the regulation of glucose uptake and metabolism (Daniels, Ciaraldi, Nikoulina, Henry, & Mcclain, 1996). Inhibition of GFAT, the rate-limiting enzyme of the HBP, by azoserine blocked diabetic symptoms in rats (Pang, Bounelis, Chatham, & Marchase, 2004). Conversely, overexpression of GFAT increases glycogen and fatty acid production in a manner that is independent of glucose levels (Daniels et al., 1996) and has a negative relationship on glucose tolerance in the liver and in pancreatic beta cells (Veerababu et al., 2000). Overexpression of GFAT also results in increased production of UDP-GlcNAc and correlates to several diabetic symptoms including obesity, insulin resistance and hyperlipidemia (McClain, 2002). Finally, when blood glucose levels remain high, an increase in protein modification can be measured in pancreatic beta cells (Andrali, Qian, & Ozcan, 2007).

Nutrient Sensing Role of the HBP

UDP-GlcNAc was first shown to modify proteins along the plasma membrane in 1984 (Torres & Hart, 1984). The modification of other proteins throughout the cell was shown by Holt and Hart in 1986 (Holts & Hart, 1986). Modification of certain proteins was later linked to insulin resistance by Marshall et al. in 1991 (Marshall et al., 1991). UDP-GlcNAc modification is a standard part of the cellular signaling that occurs on serine and threonine residues of proteins in many tissue types (Repeats, Kreppel, Blomberg, & Hart, 1997; Laczy et al., 2009; Hart, Slawson, Ramirez-Correa, & Lagerlof, 2011). Modification of a wide variety of proteins increases as a result of increased flux through the HBP due to high glucose levels in the bloodstream (Patti, Virkamäki, Landaker, Kahn, & Yki-Järvinen, 1999). In the liver, where the HBP is especially active, this can result in the modification of key insulin signaling proteins and can exacerbate the onset of insulin resistance (X. Yang et al., 2008)

Evidence shows that modification by UDP-GlcNAc can serve as a signal that can direct cellular activity (Laczy et al., 2009) and can have an adverse effect on insulin initi-

ated phosphorylation (Taylor et al., 2008; McClain, 2002; Vosseller, Wells, Lane, & Hart, 2002; Veetil, 2011). UDP-GlcNAc moieties can potentially compete for the same, or adjacent serine or threonine residues of signaling proteins as phospho moieties (Buse, Robinson, Marshall, Hresko, & Mueckler, 2002; Andrali et al., 2007; Laczy et al., 2009; Taylor et al., 2008; X. Yang et al., 2008). Thus, competing modification by UDP-GlcNAc can potentially serve a reciprocal purpose to that of phosphorylation (Kamemura & W, 2003; Issad, Masson, & Pagesy, 2010; Ruan, Singh, Li, Wu, & Yang, 2013). In the liver, the phosphorylation of several insulin signaling proteins have been shown to be affected by glycosylation (X. Yang et al., 2008).

Examples of insulin sensitive proteins that are known to be affected as a result of increased UDP-GlcNAc modification include AKT and IRS1 (Park, Ryu, & Wan, 2005; Patti et al., 1999), metabolic enzymes such as hexokinase (Virkamäki & Yki-Järvinen, 1999) and transcription factors such as specificity protein 1 (Sp1) (Laczy et al., 2009) (Hart et al., 2011). Blocking the removal of UDP-GlcNAc from protein residues has been shown to reduced insulin responsiveness in insulin-responsive tissues and to decrease the translocation of glucose transport proteins to the plasma membrane (Vosseller et al., 2002; Park et al., 2005).

Glucosamine

In 1991, Stephen Marshall and his associates proposed and successfully showed that flux through the HBP could be increased by treating cells with glucosamine (Marshall et al., 1991). Glucosamine circumvents the pathway's rate limiting step and thereby leads to insulin resistance. Early studies showed that glucosamine had an adverse effect on glucose uptake, metabolism and glycogen accumulation (Bekesi, Bekesi, & Winzler, 1969). Even with these findings, scientists had not made a solid connection between glucosamine, glucose toxicity and insulin resistance.

Glucosamine is commonly used as a supplemental treatment for patients with joint injuries, many of whom have diabetes. The entry of glucosamine into hepatocytes is very similar to glucose in that it enters through the same receptor (GLUT2) and is phosphorylated by glucokinase (Uldry, Ibberson, Hosokawa, & Thorens, 2002). While

GLUT1, 2, and 4 all can transport glucosamine, GLUT2 has a higher affinity for glucosamine than for glucose (Uldry et al., 2002).

Following the work by Marshall et al., evidence that glucosamine promoted O-GlcNac modification of proteins as a result of increased flux through the HBP and could lead to insulin resistance was shown in several models including adipocytes in culture (Vosseller et al., 2002) and rat skeletal muscle (Patti et al., 1999; Robinson, Sens, & Buse, 1993). In the liver, O-GlcNac modification has been shown to occur on several insulin signaling proteins (Virkamaki, 1997), and, in cases where modification is increased, the phosphorylation of several signaling proteins such as IRS and PI3K (Patti et al., 1999) and transcription proteins such as SP1 is negatively affected (Hart et al., 2011)

Several labs also demonstrated that glucosamine impaired the insulin initiated translocation of the GLUT4 transporter protein to the plasma membrane (Baron et al., 1995) (Rossetti et al., 1995; Giaccar et al., 1995). Antti Virkamaki showed that glucosamine affected propagation of phosphate signals for proteins in the insulin signaling cascade in multiple insulin sensitive tissues (Virkamaki, 1997). This modification has been shown to occur at multiple sites along the insulin signaling cascade including PI3K (Patti et al., 1999), which plays a role in regulating insulin-responsive GLUT4 translocation (Baron et al., 1995). In the liver, where glucose is retained as a result of phosphorylation by glucokinase, glucosamine was shown to reduce the activity of glucokinase in a concentration dependent manner (Barzilai, Hawkins, Angelov, Hu, & Rossetti, 1996). Glucosamine treatment has also been shown to modulate the expression of genes involved in both fat and glucose metabolism in adipocytes (Rumberger, Wu, Hering, & Marshall, 2003) and hepatocytes (Hirahatake, Meissen, Fiehn, & Adams, 2011).

Insulin Responsive Proteins

Understanding the effect that glucosamine has on the insulin-mediated expression of proteins can provide valuable information about the role that high glucose concentration has on insulin-induced expression. Expression of proteins is crucial to the cellular response to its nutrient environment. This study looks at the expression of several of those proteins to characterize their response under glucosamine induced insulin resistant conditions, namely TRIB3, SREBP-1c, G6PDH, and FAS.

Tribbles3

Tribbles3 (TRIB3) (also known as neuronal cell death-inducible protein kinase) plays a role in regulating the insulin signaling pathway. Since its discovery in humans in 1999 (Mayumi-Matsuda, Kojima, Suzuki, & Sakata, 1999), TRIB3 and its family members, TRIB1 and TRIB2, have been implicated in numerous functions including cellular proliferation and regulation of chemotaxis (Ostertag et al., 2010; Ding, Kato, & Du, 2008).

TRIB3 is the most characterized member of the Tribbles family and possesses a 30% identity to other proteins in the serine/threonine kinase family (Wilkin et al., 1997). However, unlike other members of this family, it possesses no ATP binding site which is why it is called a pseudokinase. TRIB3 is expressed throughout the body with significantly high levels of expression in the liver (Liu et al., 2010). It is regulated at both the transcriptional and posttranscriptional levels (Du & Ding, 2009; Hegedus, Czibula, & Kiss-Toth, 2006; Wilkin et al., 1997).

Several pieces of evidence have firmly connected TRIB3 to insulin signaling and the diabetic disease state. Numerous models of diabetes, including streptozotocin (STZ) induced diabetic rats, *db/db* mice, Zucker fatty rats, liver specific receptor knockout mice and patients with Type II diabetes mellitus, show increased TRIB3 expression (Liu et al., 2010) (Koo et al., 2004). Additionally several labs have shown that TRIB3 protein and mRNA are increased during insulin resistance (He, Simmen, Mehendale, Ronis, & Badger, 2006; Du & Ding, 2009; Wasef, Robinson, Berkaw, & Buse, 2006). Recent studies have identified TRIB3 as an inhibitor of several members of the insulin signaling pathway, including AKT, ERK1/2, IRS1 and MAPK (Du, Herzig, Kulkarni, & Montminy, 2003; Liu et al., 2010; Tang et al., 2008; Wilkin et al., 1997; Liu et al., 2010). In the cytosol of insulin resistant, animals studies have shown increased co-localization of TRIB3 and AKT (He et al., 2006; Yao & Grégoire Nyomba, 2007).

TRIB3's influence over AKT is of particular interest since AKT occupies a gatekeeping role in propagating insulin signaling to many arms of the insulin signaling cascade. TRIB3 inhibits the phosphorylation of AKT at Ser473 and Thr308 in AKT's activation loop (Zhang et al., 2013) during an insulin resistant state (Hegedus, Czibula, & Kiss-Toth, 2006; Liu et al., 2010). Studies that overexpressed TRIB3 show a clear decrease in

AKT phosphorylation, which promotes hyperglycemia and reduces glucose tolerance in *db/db* mice (Du et al., 2003). Conversely, knockdown of hepatic TRIB3 has been shown to improve glucose tolerance and increase insulin sensitivity in liver and muscle (Koo et al., 2004; Liu et al., 2010) Knockdown of TRIB3 also enhances AKT phosphorylation in HepG2 cells (Du et al., 2003)

We are still learning about the many roles of TRIB3. From the evidence that we presently have, it is clear that TRIB3 plays a part in many aspects of metabolism. In addition to activation and increased expression caused by insulin resistance, TRIB3 is also increased as a result of conditions that are associated with insulin resistance, such as changes in blood sugar concentration (Liu et al., 2010; Schwarzer, Dames, Tondera, Klippel, & Kaufmann, 2006), increased reactive oxygen species in kidneys of diabetic mice (Morse et al., 2010), ER stress (Nicoletti-Carvalho et al., 2010; Liu et al., 2010) and sustained high levels of free fatty acids due to a high fat diet (Y.-G. Wang, 2009; Ostertag et al., 2010). And, as with many elements that affect insulin resistance, there is evidence to show that TRIB3 expression and activity also responds to changes in insulin levels (Ding et al., 2008) in a tissue specific manner. Still some controversy remains over the specific role that TRIB3 plays. At least two research groups have challenged findings that TRIB3 has any role to play in the regulation of the insulin signal pathway at all (Iynedjian, 2005; Okamoto et al., 2007) contradicting the findings of many other research groups.

Sterol Regulatory Element Binding Protein-1c

In 1993 Tontonoz et, al. isolated a novel protein called adipocyte determination and differentiation protein 1 or ADD1 (Tontonoz, Kim, Graves, & Spiegelman, 1993). Today ADD1 is known as sterol regulatory element binding protein-1c (SREBP-1c) and is one of three isoforms that are part of the basic helix-loop-helix leucine zipper (bHLH-Zip) family of proteins. SREBPs belong to the basic-helix-loop-helix leucine zipper family of DNA-binding proteins and play a fundamental role in cholesterol and fatty acid metabolism. Three isoforms that vary in structure, regulation and function have been identified in mammalian tissues; they are SREBP-1c, SREBP-1a and SREBP2. SREBP2 is primarily involved in cholesterol metabolism (H Shimano et al., 1999) and is transcribed

from an alternate gene (*srebp2)* (Giandomenico, Simonsson, Gro, & Ericsson, 2003). SREBP-1a and SREBP-1c are products of alternative promoter usage of the *srebf1* gene (Tontonoz et al., 1993).

SREBP-1c is the principal isoform in liver (Rome et al., 2008; Yellaturu, Deng, Park, Raghow, & Elam, 2009) and regulates transcription of genes involved in fatty acid metabolism, such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (**ACC**) (Hitoshi Shimano et. al., 1999). Therefore, it is foreseeable that any potential changes in SREBP-1c regulation would also affect fatty acid metabolism and gene expression. SREBP-1c cooperates with other metabolic transcription factors like SP1 and CAAT binding factors (Mueller et al., 2008) to regulate proteins involved in carbohydrate metabolism such as G6PDH (Sirek et al., 2009; Azzout-marniche et al., 2000; Dentin et al., 2004; H Shimano et al., 1999; Korczynska et al., 2004).

Regulatory control of SREBP-1c is achieved by several mechanisms. While SREBP-1c is not regulated exclusively by insulin, numerous studies support the regulation of SREBP-1c's activity and expression by the insulin responsive PI3K/AKT pathway (Azzout-marniche et al., 2000; Foretz et al., 1999; Yellaturu et al., 2009; Worgall, Sturley, Seo, Osborne, & Deckelbaum, 1998; Krycer, Sharpe, Luu, & Brown, 2010; Fleischmann & Iynedjian, 2000; Horton, Bashmakov, Shimomura, & Shimano, 1998; Foretz et al., 1999). Inhibition of both AKT and PI3K results in a significant reduction in SREBP-1c gene expression (Fleischmann & Iynedjian, 2000; K. H. Kim et al., 2004; Rome et al., 2008; Hasty et al., 2000). Phosphorylation of SREBP-1c by GSK3 beta, a point downstream of AKT, plays a role in regulating SREBP-1c stability and transcription. (Punga, Bengoechea-Alonso, & Ericsson, 2006; Krycer et al., 2010; He et al., 2006; Hirano, Yoshida, Shimizu, & Sato, 2001).

In addition to its regulation by insulin, SREBP-1c is also regulated by glucose levels in the liver (Yamamoto et al., 2007). Liver X receptor (LXR), an essential component of SREBP-1c transcriptional regulation, is a target for glucose-regulated *O*-linked GlcNAc modification (Anthonisen et al., 2010). Increases in O-GlcNAcylated LXR as a result of increased glucose flux through the HBP has been shown to modulate SREBP-1c expression in H2-35, a mouse liver cell line, and Huh7, a human liver cell line (Anthonisen et al., 2010). These factors point strongly to the possibility that expression

levels of SREBP-1c are directly influenced by glucosamine induced insulin resistance as a result of increased flux through the HBP.

Glucose-6-Phosphate Dehydrogenase

The pentose phosphate pathway (PPP) is the dominant carbohydrate metabolizing pathway in the liver, metabolizing nearly 50% of all the glucose that enters this organ (Shonka, 1960). It is crucial to many essential processes in the cell including maintaining the balance of reducing compounds such as NADPH needed for lipid metabolism and producing building blocks for nucleic acids. Glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting enzyme for the PPP and has been shown to respond to insulin's signal, both at the level of activity and expression (Berg et al., 1995).

Rate limiting proteins, like G6PDH, are acted upon directly by elements of the insulin signaling cascade to control activity, or are indirectly regulated through transcriptional regulation. The regulation of proteins in this manner helps to control fat and glucose and assure that they are metabolized in a way that meets the biological needs at the time.

Regulation of G6PDH transcription and activity plays an important part in directing hepatic glucose metabolism and the nutrient state of the cell. Because of its importance, it is understandable that hormones, such as insulin, play a significant role in the regulation of this rate limiting enzyme. Effort has been made to uncover the mechanism for regulation of G6PDH by insulin and validate its effect on G6PDH expression. Some evidence suggests that G6PDH is predominately regulated at the posttranscriptional level (Salati et al., 2004; Kurtz & Wells, 1981). However, there is evidence to show that expression of G6PDH is also regulated by the presence of insulin (Kurtz & Wells, 1981; Stumpo & Kletzien, 1984; Stapleton et al., 1993; Wagle, Jivraj, Garlock, & Stapleton, 1998).

Evaluation of the G6PDH promoter region has revealed binding sites for several important nutrient and hormone directed transcription factors, such as SREBP-1c (Arkwright-Keeler, 2005; Thekkat, 2007; Laliotis, Bizelis, Argyrokastritis, & Rogdakis, 2007), implicating the PI3K/AKT pathway in G6PDH regulation. Dampening the insulin

induced response by inhibiting members of the PI3K/AKT pathway has been shown to have an effect on G6PDH mRNA expression (Wagle et al., 1998).

Fatty Acid Synthase

Several factors relate hepatic fatty acid synthesis and glucose metabolism. Glucose metabolized through the PPP produces reducing molecules for the oxidation of fatty acids (FAs), making it possible for the body to store excess energy as FAs (Postic & Girard, 2008). The activity and expression of enzymes involved in both pathways also share a commonality in that they respond similarly to insulin and increased glucose availability. For example, expression of fatty acid synthase (FAS), a multi-enzyme complex that utilizes malonyl-CoA as a building block to synthesize 16 carbon FAs, is known to increase in response to insulin and increased glucose availability (Foufelle, Girard, & Ferré, 1996; Rufo et al., 2001). Insulin mediated increase of FAS expression has been shown to occur via the PI3K/AKT and MAPK pathways (Memon, Grunfeld, Moser, & Feingold, 1994; Wang & Sul, 1998b; Radenne et al., 2008) and to decrease in response to fasting and cAMP (Goodridge et al., 1989; Paulauskis & Sul, 1989). FAS expression was also decreased in whole animals treated with streptozotocin and was reversible with the addition of Se (Berg et al., 1995).

Promising evidence shows that glucosamine is linked to the glucose response of lipogenic genes. A study in rat adipocytes revealed that combined glucosamine and insulin treatment significantly increased FAS, and acetyl-CoA carboxylase (ACC) and glycerol-3-P dehydrogenase in primary adipocytes, producing a somewhat additive effect when compared to glucosamine or insulin alone (Rumberger et al., 2003). Evidence also shows that FAS expression is significantly increased in high carbohydrate diets of diabetic patients who are insulinimic (Schwarz, Linfoot, Dare, & Aghajanian, 2003; R. A. Memon, Grunfeld, Moser, & Feingold, 1994). Further, this same study showed that removal of glutamine from cell culture media reduced the increase of FAS and acetyl-CoA carboxylase expression in the presence of glucose and insulin. The reduction in gene expression, along with other evidence indicate that in adipose tissue, the HBP is a mechanism for nutritional control of expression of FAS in primary rat adipocytes (Rumberger et al., 2003). Another study showed that activation of the HBP is associated with increased

FAS expression and ACC-1 expression in HepG2 cells (Hirahatake et al., 2011). Even though the potential for differential expression in different model types is well known, it is reasonable to expect a similar expression profile in primary rat hepatocytes.

Objectives of Study

The effects of glucosamine induced insulin resistance on metabolism are manifested in a myriad of ways including the transcription of genes. This study evaluated the effect of glucosamine on the insulin initiated transcription of TRIB3, SREBP-1c, G6PDH and FAS, which are involved in signal transduction, transcription and metabolism respectively.

Se's ability to behave as an insulin mimetic agent has been established in vivo models of Type I diabetes supporting its use as a therapeutic agent in Type I diabetes. However, Se's effectiveness as a therapeutic agent and insulin mimetic in Type II diabetes or insulin resistance is less understood. This study investigated whether Se could restore the transcription of insulin responsive genes that has been lost or dampened due to glucosamine induced insulin resistance.

We hypothesize that increased flux through the hexosamine biosynthetic pathway can inhibit or dampen the transcription of select genes involved in signal transduction, transcription and metabolism, and that selenium, an insulin mimetic, can overcome the insulin resistant state.

Objectives of Study

1. To assess the effect of glucosamine on the insulin induced gene expression of key insulin induced signaling protein, transcription factor and metabolic enzymes; namely TRIB3, SREBP-1c, G6PDH and FAS.

2) To test the ability of selenium to mimic insulin's effect on gene expression and circumvent insulin signaling pathway in glucosamine induced insulin resistance, once the glucosamine effect was characterized for our genes of choice.

II. Materials and Methods

Male Sprague-Dawley SASCO rats weighing 150-170 grams were obtained from Charles River, Portege, MI. Rats were maintained on lab diet 5001 standard rodent chow. Animal protocols were approved by the WMU Institutional Animal Care and Use Committee (IACUC). Prior to hepatocyte isolation, rats weighing >200 grams were fasted (provided drinking water ad libitum) for approximately 48 hours. Animals were anesthetized with an injection of pentobarbital (approximately 50mg/Kg) (Sigma-Aldrich). Hepatocytes were isolated using a collagenase-hyalurodinase perfusion and digestion method (Stapleton et al., 1993). The perfusion solution was introduced to the liver via a catheter through the portal vein and contained 0.148M NaCl, 0.01M HEPES, 0.017M fructose, 0.049mM EGTA, 0.5% phenol red, and 6unit/ml heparin with pH-7.4. Then a digestion solution that contained l00ug/ml collagenase D, 93unit/ml hyaluronidase, 160unit/ml trypsin inhibitor, and 0.2% BSA was passed through the portal vein.

The liver was removed and cut into smaller pieces and placed on gauze covering a beaker. Digestion solution was poured over the resulting segments while they were digested and forced through the gauze. Following this, the solution containing the digested tissue was decanted into 50 ml tubes and centrifuged at $4^{o}C$ for 3 minutes at 50xg. The supernatant was aspirated, and the pellet was resuspended, washed and centrifuged twice with cold Waymouth's MB 752/1 medium (Gibco, Grand Island, NY) containing 0.5% bovine serum albumin (BSA) from Sigma. After the final washing, the cell pellet was gently resuspended in fresh BSA/Waymouth's medium. Cell viability was determined using trypan blue exclusion. Cells with greater than 75% viability were plated on sterilized 60 mm collagen (Sigma) coated plates at approximately 2.0×10^{6} cells per plate. The cells were incubated in 4 ml BSA/Waymouth's medium at $37^{o}C$ in an incubator (5% CO_2 and 95% air) for a 4 hour attachment period. After the attachment period, the media was aspirated, and cells were washed with 1 ml BSA free Waymouth's MB 752/1 media. Finally, cells were incubated in 4ml of BSA free Waymouth's MB 752/1 media (Invitrogen, Carlsbad, CA) at $37^{o}C$ in an incubator (5% CO_2 and 95% air) for 18 hours until treatment.

Treatment

To assess the effect of glucosamine on insulin or selenium induced expression of insulin responsive genes, the media was aspirated, and cells were washed with DMEM low glucose media (5mM) (Invitrogen, Carlsbad, CA). Cells were then separated into two treatment groups, with or without 1mM glucosamine (Sigma) and incubated for 18hrs in DMEM low glucose media (Invitrogen, Carlsbad, CA) at 37^{o}C in an incubator (5% CO_2 and 95% air).

Marshall et al., demonstrated that glucosamine treatment could be utilized to successfully induce insulin resistance and result in minimal damage, in (Marshall et al., 1991) thus, we mirrored these conditions in our study. To test for glucosamine's effect on insulin induction, 44nM insulin (Sigma) was added to designated insulin treatment groups in both glucosamine and non-glucosamine groups at the time of media change and incubated for 18hrs. 500uM sodium selenate was added for 6hrs to media of cells undergoing mimetic treatment in both glucosamine and non-glucosamine treatment groups.

To test the role of the insulin pathway on glucosamine induced insulin resistance and mimetic activation, the PI3K inhibitor LY294002 (Sigma) was added to sample plates where indicated for 1 hour prior to an18hr incubation period with insulin.

Harvesting and Isolation of Cellular mRNA

Prior to harvesting of cellular mRNA, media was removed and cells were then washed twice with 2ml of cold phosphate buffer saline (PBS). Cells were then scraped from the plate, lysed and the cellular mRNA was isolated using 1ml of TRIzol/60mm dish (TRIzol reagent, Invitrogen). The lysate was then placed in Eppendorf tubes. Chloroform (Sigma) (.2-.3ml/1ml TRIzol) was added to the cell suspension and pulled up and down through the pipette tip for several seconds, followed by a 10min incubation at room temperature. The cell lysate was then centrifugation for 15 min (=14000 Xg at 4ºC). Following centrifugation, the upper aqueous phase that contains RNA was transferred to a fresh Eppendorf tube and 1ml isopropyl alcohol was added to each sample tube followed by a 10min incubation period at room temperature. Samples were then centrifuged for ten more minutes (10000 Xg @4ºC). After removing the supernatant, the RNA precipitate

was then washed with 1 ml of 75% ethanol. The RNA pellet was again centrifuged (5000 Xg @4ºC) for 5 minutes and resuspended in water.

Amplification

mRNA concentration was measured using a NANO Drop (Thermo Scientific, ND 100). For the quantification of the RNA isolate, 1ul of RNA was placed on the NANO drop and 230 nm, 260nm and 280 nm readings were taken.

For quantification of the RNA isolated, a spectrophotometer was used, and readings at wavelengths of 230nm, 260 nm and 280 nm were taken. The reading at 260 nm allows for quantification of nucleic acid concentration in the purified sample. The 230nm and 280nm reading allows for the determination of solvent and protein contaminants levels. The RNA concentration read was then calculated by using the following formula:

1 O.D. (optical density) at 260 nm for RNA molecules = 40 ng/ul of RNA

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRTPCR)

Real-time quantitative PCR (qRTPCR) was done using a two-step method. All reagents for both steps of the qRTPCR were purchased from Life Technologies, Inc. First, DNase enzyme was utilized (Deoxyribonuclease I, Amplification Grade, Cat. No. 18068-015, Life Technologies) to eliminate interfering signals from genomic DNA in preparation of the RNA sample prior to qRTPCR. Then mRNA was reverse transcribed into cDNA on a thermocycler (Eppendorf Master Cycler gradient) using the Taqman reverse transcription kit. Reverse transcription reactions were performed in a total volume of 100 µl that contained 10ul 10x Taqman buffer, 22 ul of 25mM MgCl2, 5ul random hexamers, 200ng of mRNA, 10 mMdNTP's, 2ul RNase inhibitor, 18.5 ul water and 2.5 10X reverse transcriptase enzymes (TaqMan Reverse transcription reagents, part no. N808-0234). Amplification of cDNA was intermittently confirmed by running cDNA out on an electrophoresis gel.

The second step was performed on the Step One^{TM} System (Life Technologies). qRTPCR reaction was carried out in a 96-well plate containing the following reaction; 12.5ul of 2X Taqman Universal PCR Master Mix, 1.25 ul of 20X Taqman Gene expres-

sion assays-containing specific PCR primers and FAM Tm dye labeled probes, cDNA obtained from the first strand synthesis reaction and 6.25ul of water to a final reaction volume of 25ul. After an initial incubation step for 2 minutes at 50ºC and denaturation for 10 minutes at 95 ºC, qRTPCR was carried out using 40 cycles of PCR (95ºC for 15s. 60ºC for 60s). βactin was used as a reference gene. Primer/probe sets for the genes studies were commercially obtained from Life Technologies and are included in table 1.

Gene	Assay ID
G6PDH	Rn00566576_m1
FAS	Rn00569117_m1
SREBP-1c	Rn01495769_m1
TRIB3	Rn00595314_m1
bACT	Rn00667869_m1

Table 1. List of oligonucleotide primers

Statistical Analysis

The results were expressed as the mean ± SEM of treatment groups or animals used for each experiment. The comparisons within groups were performed non-parametric paired t-test (Wilcoxon signed rank test) and non-parametric ANOVAs (Kruskal Wallis). Statistical significance was at $p<0.05$.

III. Results

An essential part of insulin's role is the mediation of protein production in response to newly ingested nutrients. The process of protein production is energy intensive and must be vigorously controlled by multiple mechanisms (Neufeld & Arsham, 2010). One avenue for control is the regulation of transcription. Transcriptional control allows the cell to manage the types of proteins that are produced and the timing of their production.

Chronically high glucose concentration can lead to breakdowns in the mechanisms that control transcription as well as result in miss-timed or inappropriate signals. Many of these miscues can lead to diabetes. In the presence of chronically high glucose concentrations, cells employ systems that control the rate of glucose influx and regulate sensitivity to insulin activation.

In 1991 Marshall and colleagues implicated the HBP as a mechanism that is utilized by the cell to control insulin sensitivity and glucose uptake in insulin responsive tissues in the presence of chronically high circulating glucose concentration (Marshall et al., 1991). Studies showed that by treating adipocytes in culture with glucosamine, the pathways' rate limiting step was bypassed resulting in increased flux through the HBP (Marshall et al., 1991; Hassel, Kimura, & Hascal, 1986). This increased pathway flux resulted in an increase in the byproducts, uridine diphosphate-N-acetyl-D-glucosamine and UDP-N-acetylgalactosamine concentrations and also increased modification of multiple cellular proteins (Buse et al., 2002; Andrali et al., 2007; Laczy et al., 2009). Several studies showed that the increased protein modification by HBP byproducts, that occurred in glucosamine treated animals, interfered with phosphorylation along the insulin signaling cascade (Ruan et al., 2013; Marshall et al., 1991) by competing for phosphorylation sites and blocking phosphorylation signals (Patti et al., 1999). Glucosamine has been successfully used to mimic the effect of high glucose concentration in many models and to induce insulin resistance.

To date, most studies utilizing glucosamine as a model of high glucose concentration have not concentrated on liver. Therefore, substantially less is known about the effect of glucosamine on insulin mediated expression in liver. Our laboratory has established that in primary hepatocytes, incubation with glucosamine can indeed yield insulin resistance. The results of this study both confirm and expand our previous findings.

Effect of Glucosamine Induced Insulin Resistance on Insulin Mediated G6PDH Gene Expression

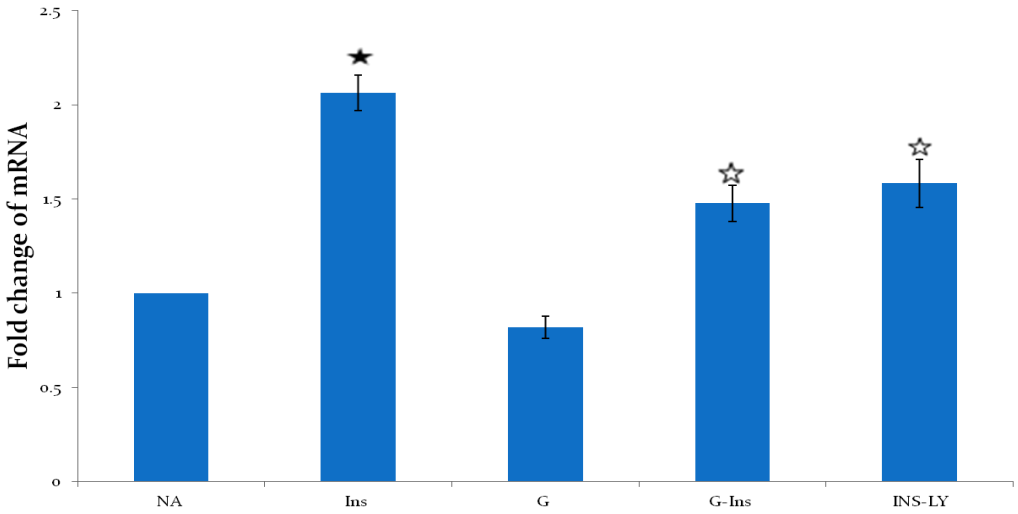

Figure 3. **Insulin mediated expression of G6PDH in the presence of glucosamine.** Cells were incubated for 18h in low glucose media (5mM) with or without 1mM glucosamine. 44nm of insulin was added where indicated and incubated with cells for 18h. Values indicate the fold increase of mRNA as compared to the non-treated control (NA, n=16; INS, n=15; GLU, n=12; G-INS, n=14; INS-LY, n=5) n= number of subjects. ★ =significant difference from NA; ☆ =significant difference from INS. The comparisons within groups were performed non-parametric paired t-test (Wilcoxon signed rank test) and non-parametric ANOVAs (Kruskal Wallis). Statistical significance was at $p<0.05$.

Insulin mediates expression for metabolic genes such as G6PDH. This rate limiting enzyme of the pentose phosphate pathway is vital for both carbohydrate and fatty acid metabolism in the liver. Expression of G6PDH has been shown to respond to insulin though activation of the insulin signaling cascade (Berg et al., 1995).

Glucosamine Suppresses Insulin Mediated Expression of G6PDH

To verify the effect of glucosamine in establishing insulin resistance in hepatocytes and in diminishing the insulin induction of G6PDH expression, liver cells were co-treated with 1mM glucosamine and 44nM insulin. Expression levels of G6PDH (fold increase/decrease in mRNA, quantified using RT-PCR) from all treatments were compared

to the expression level of G6PDH in non-treated control (NA). Expression of G6PDH increased approximately two fold over the control in the presence of 44nM insulin, confirming the insulin induction of G6PDH expression that we have previously observed (Veetil, 2011; Thekkat, 2007; Berg et al., 1995).

The insulin induction of G6PDH was significantly suppressed in the presence of 1mM glucosamine (figure 3). A 0.53 reduction in expression in the presence of insulin with glucosamine was observed when compared to incubation with insulin alone. Glucosamine alone had no significant effect on the expression of G6PDH, thus the action of glucosamine is truly in interfering with the process by which insulin induces expression. While there was not a complete suppression of the insulin action, these results are comparable to previous data from our lab that show a reduction in insulin mediated G6PDH expression in the presence of glucosamine in primary hepatocytes in culture (Veetil, 2011).

It has been shown that insulin regulation of G6PDH is achieved largely through the insulin activation of the PI3K/AKT pathway (Wagle et al., 1998). Indeed, in these studies, activation of PI3K was determined to be a key protein for the insulin induction of G6PDH. LY294002 is a known inhibitor of PI3K and thus we compared its effect to the effects of glucosamine. Interestingly in these results LY294002 diminished the effect of insulin on the induction of G6PDH in a manner comparable to results obtained from co-incubation of glucosamine and insulin. (figure 3) Indeed it might be attractive to speculate that glucosamine has some effect on activation the PI3K pathway by insulin.

Effect of Glucosamine Induced Insulin Resistance on Insulin Mediated FAS Gene Expression

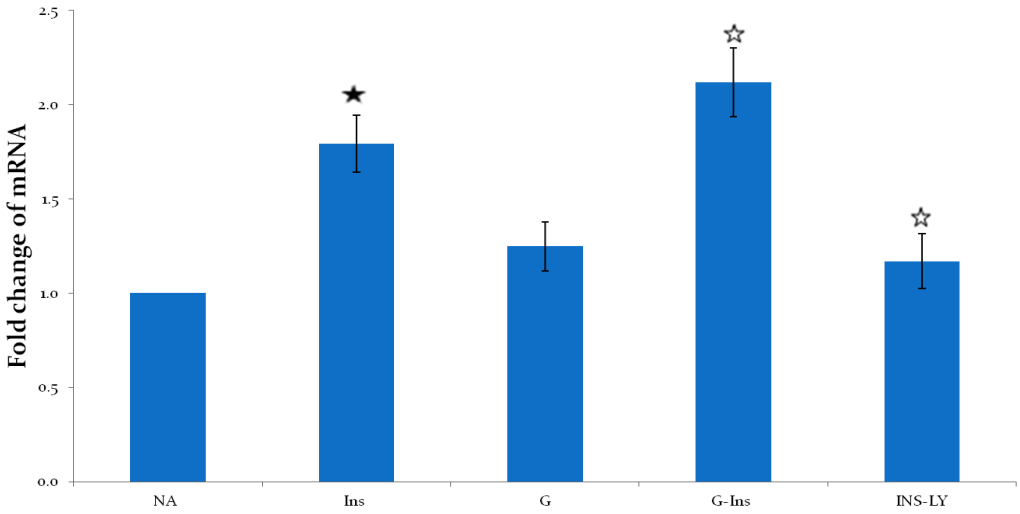

Figure 4. **Insulin mediated expression of FAS in the presence of glucosamine.** Cells were incubated for 18h in low glucose media (5mM) with or without 1mM glucosamine. 44nm of insulin was added where indicated incubated with cells for 18h. Values indicate the fold increase of mRNA as compared to the non-treated control (NA, n=11; INS, n=10; GLU, n=9; G-INS, n=8; INS-LY, n=6). ★ =significant difference from NA; ☆ =significant difference from INS. The comparisons within groups were performed non-parametric paired t-test (Wilcoxon signed rank test) and non-parametric ANOVAs (Kruskal Wallis). Statistical significance was at $p<0.05$.

FAS expression is known to increase in response to insulin and increased glucose availability (Rufo et al., 2001). FAS converts acetyl CoA and malonyl CoA into a 16 carbon fatty acid chains for energy storage in response to insulin activation. Treatment with insulin resulted in a 1.88 fold increase of FAS gene expression over the non-treated control, while glucosamine treatment alone showed a small, but significant difference from the non-treated control (1.25 fold increase). Treatment with insulin in the presence of glucosamine resulted in an increase of FAS expression (2.34 fold increase over the non-treated control) over that of insulin alone (1.88 fold) (figure 4). The results of this study differ from a previous study in our lab showed that glucosamine plus insulin resulted in a decrease in FAS expression (Veetil, 2011). However, in a study done by John Rumberger et al., in adiposities it was found that the presence of insulin, glucose and glutamine enhanced the expression of FAS in adiposities, implicating the HBP as important for the

positive transcriptional response of FAS to both insulin and glucose responses (Rumberger et al., 2003). Evidence also points to similar increases in FAS expression in the mouse liver and HepG2 cells in normal media with fetal bovine serum (Hirahatake et al., 2011).

Effect of Glucosamine Induced Insulin Resistance on Insulin Mediated SREBP-1c Gene Expression

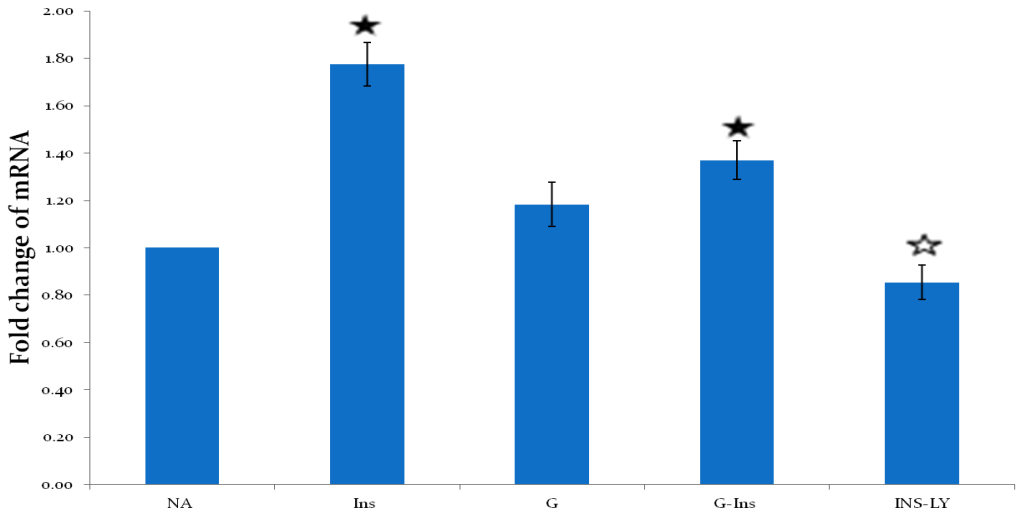

Figure 5 **Insulin mediated expression of SREBP-1c in the presence of glucosamine.** Cells were incubated for 18h in low glucose media (5mM) with or without glucosamine. 44nm of insulin was added where indicated incubated to cells for 18h. Values indicate the fold increase of mRNA as compared to the non-treated control. (NA, n=13; INS, n=11; GLU, n=12; G-INS, n=12; INS-LY, n=5). ★ =significant difference from NA; ☆ =significant difference from INS. The comparisons within groups were performed non-parametric paired t-test (Wilcoxon signed rank test) and non-parametric ANOVAs (Kruskal Wallis). Statistical significance was at $p<0.05$.

SREBP-1c is critical in the response to newly acquired nutrients and is one of several transcription factors that respond to insulin action. It has been shown to regulate expression of genes involved in fatty acid metabolism (Hitoshi Shimano et. al., 1999) and to some extent carbohydrate metabolism. While SREBP-1c has been shown to respond to insulin, it has also been shown to be active in insulin resistant conditions. In HepG2 cells where GFAT was over expressed causing symptoms of insulin resistance, one study showed a 7 fold increase of SREBP-1c and a 19 fold increase of FAS (Sage et al., 2010).

Glucosamine has also been shown to cause increased glycosylation of several proteins involved in the regulation of SREBP-1c (Kim et al., 2004; Laplante & Sabatini, 2010; McPherson, 2013) and in some cases increase their activity (Anthonisen et al., 2010). One example of a glycosylated transcriptional regulator of SREBP-1c is liver X receptor (LXR). LXR is glycosylated in the presence of glucosamine and positively regulates SREBP-1c expression (Yamamoto et al., 2007; Anthonisen et al., 2010).

Insulin stimulated SREBP-1c expression to approximately two times the level of the non-treated control (1.92 fold) and insulin induced SREPB-1c expression was diminished (1.37 fold) in the presence of glucosamine plus insulin (figure 5). These results show the ability of glucosamine through the HBP to modulate the expression of transcription factors such as SREBP-1c that are pivotal in the regulation of metabolic genes such as G6PDH and FAS.

Some models have shown increased transcription and activity of key regulatory proteins by activation of the HBP in the absence of insulin. A study done by Anthonisen et al., noted increased levels of SREBP-1c mRNA as well as higher glycosylated and activated forms of LXR, a key regulator of SREBP-1c, in fasted refed streptizotazinizin mice. The same increase was observed in mice treated with glucosamine and in mice treated with PUGNAc, which inhibits the removal of glycosylation by O-GlcNAcase (Anthonisen et al., 2010). In this model, transcription of SREBP-1c was not increased in the presence of glucosamine alone indicating that there are other factors at play in the transcriptional response of SREBP-1c. Further study will have to be done in order to discover the reason for these differences.

Inhibition of both AKT and PI3K results in a significant reduction in SREBP-1c gene expression in numerous models (Kim et al., 2004; Rome et al., 2008; Hasty et al., 2000) including transfected primary hepatocytes (Fleischmann & Iynedjian, 2000). Yet, it is also true that several Type II diabetic models, including those that utilize glucosamine treatment and that do not employ direct AKT and PI3K inhibition, show an increase in SREBP-1c expression (Anthonisen et al., 2010; Kakuma et al., 2000; Sage et al., 2010). It is reasonable to expect that the suppression of the insulin signal to its signaling cascade will affect the expression of SREBP-1c in this model, but it is also possible that treatment with glucosamine may produce and effect on the expression of SREBP-1c through flux

through the HBP. In this study, we compared the effect of glucosamine on insulin mediated expression of SREBP-1c with that of LY294002, an inhibitor of PI3K to see if similar results could be obtained on SREBP-1c expression when inhibiting PI3K and glucosamine was used to induce insulin resistance.

Insulin increased expression of SREBP-1c expression was completely blocked by treatment with LY294002 ($P<0.05$) (figure 5). These results indicate that the regulation of SREBP-1c in this model is strongly dependant on the regulatory influence of insulin through PI3K. These results also show that the effect that treatment with glucosamine has on the insulin signaling pathway results in decreasing the insulin mediated expression of SREBP-1c potentially through PI3K. The lack of correlation between FAS and SREBP-1c presents the likely conclusion for compensating by other fatty acid regulatory transcription factors in our conditions.

It is interesting to note that the expression patterns of SREBP-1c and G6PDH were somewhat similar in their response to insulin and to glucosamine treatments. SREBP-1c has been shown to be a transcriptional regulator of G6PDH (Arkwright-Keeler, 2005; Thekkat, 2007) and so a similarity in their expression pattern with these agents might be expected.

Effect of Glucosamine Induced Insulin Resistance on Insulin Mediated TRIB3

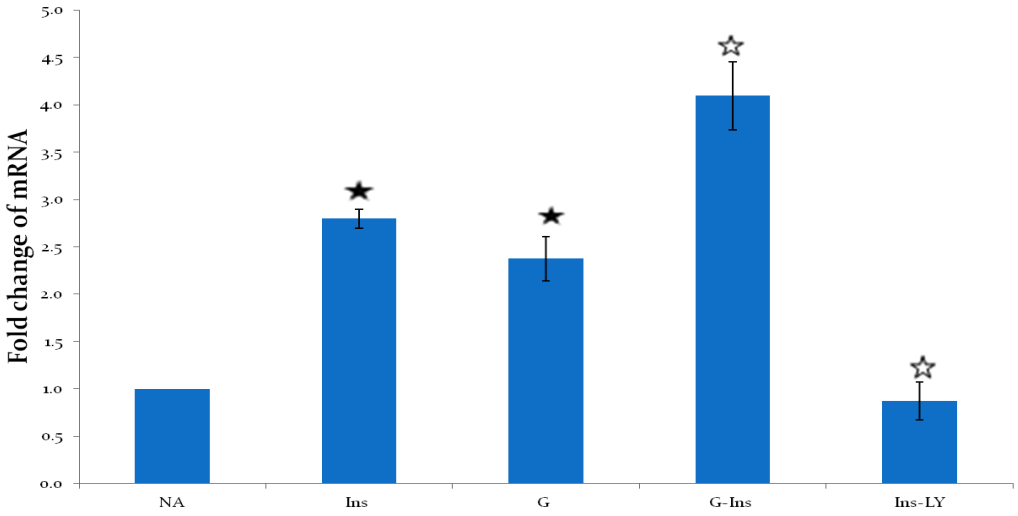

Figure 6. **Insulin mediated expression of TRIB3 in the presence of glucosamine.** Cells were incubated for 18h in low glucose media (5mM) with or without glucosamine. 44nm of insulin was added where indicated and incubated with cells for 18h. Values indicate the fold increase of mRNA as compared to the non treated control (NA, n=13; INS, n=11; GLU, n=11; G-INS, n=11; INS-LY, n=4). ★ =significant difference from NA; ☆ =significant difference from INS. The comparisons within groups were performed non-parametric paired t-test (Wilcoxon signed rank test) and non-parametric ANOVAs (Kruskal Wallis). Statistical significance was at $p<0.05$.

In several model systems, TRIB3 plays a role in regulating the phosphorylation of proteins in the insulin signaling pathway including, for example, AKT, ERK1/2, IRS1 and MAPK (Du et al., 2003; Hegedus, Czibula, & Kiss-Toth, 2006; Liu et al., 2010; Tang et al., 2008; Wilkin et al., 1997; Liu et al., 2010). The regulation of phosphorylation and subsequent inhibition of AKT by TRIB3 is of particular interest and is well studied. Expression and activation of TRIB3 has been shown to occur in both Type1 and Type2 diabetic models and to result in inhibition of AKT by TRIB3 (Liu et al., 2010). However, the initiating factors that result in TRIB3 expression are not completely understood.

TRIB3 expression was stimulated in the presence of insulin alone (2.78 fold) and also in the presence of glucosamine alone (2.38 fold). Co-treatment with insulin and glucosamine produced an additive effect on TRIB3 expression, resulting in a 4.09 fold increase in expression (figure 6). These results indicate that TRIB3 plays a role in the re-

sponse to high glucose, which would coincide with findings from other studies done in other labs (Zhang et al., 2013).

The PI3K inhibitor, LY294002 completely inhibited insulin mediated expression of TRIB3 and reduced expression to a level similar to the non-treated control (1.15 fold compared to the non-treated control) (figure 6), verifying that insulin induced TRIB3 expression is regulated by the PI3K pathway. More research is needed to discover the mechanism of PI3K activation of TRIB3 with glucosamine treatment. The response of TRIB3 to both insulin and insulin resistant conditions suggests that TRIB3 may also play a complex role in regulating insulin signaling and glucose metabolism. Future research could also look to see if the glucosamine induced expression is blocked by LY264002, which will help to shed more light on the mechanism of glucose regulation of TRIB3.

In summary, insulin stimulated the expression of all genes examined in this study. There was no induction of expression in the presence of glucosamine alone for G6PDH and SREBP-1c. However, glucosamine alone contributed to gene expression for FAS and TRIB3 and resulted in an additive effect on gene expression when combined with insulin instead of suppressing gene expression as was hypothesized. Expression levels for G6PDH and SREBP-1c were suppressed when insulin and glucosamine treatments were combined. Results are summarized in table 2.

	G6PDH	SREBP-1c	TRIB3	FAS
Ins	↑	↑	↑	↑
G	–	–	↑	↑
G + Ins	↑	↑	↑	↑
LY + Ins	↑	–	–	–

Table 2. Summary of results of insulin gene expression

Effect of Selenium on G6PDH Expression

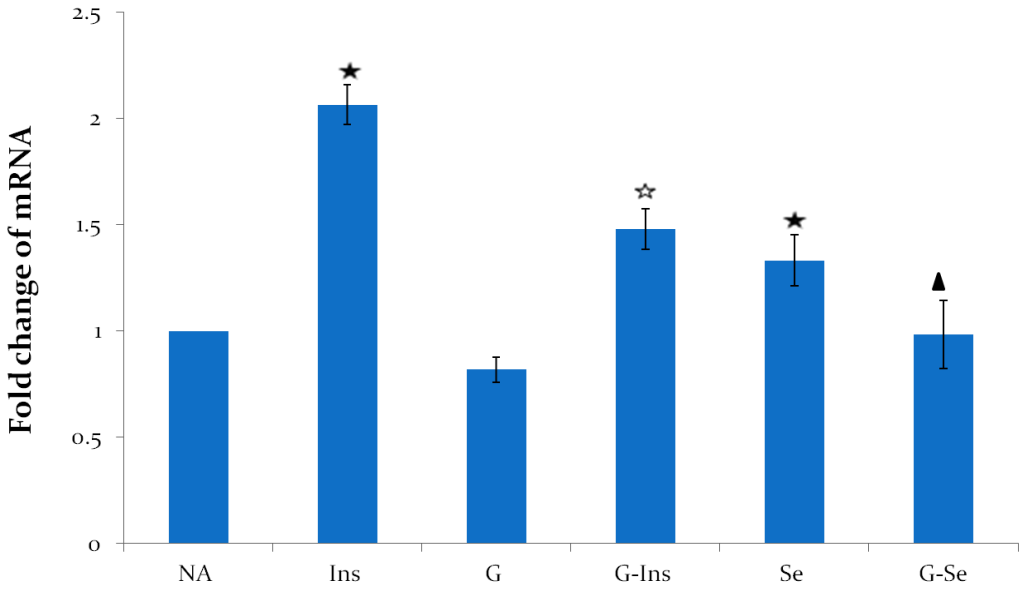

Figure 7. **Insulin and Se mediated expression of G6PDH.** Cells were incubated for 18h in low glucose (5mM) media with or without 1mM glucosamine. 500um of Se was added where indicated and incubated with cells for 6h. Values indicate the fold increase of mRNA cells (NA, n=16; Se, n=9; G-Se, n=14; Se-LY, n=3). ★ = Significant difference from NA, ☆ = Significant difference from INS, ▲ = Significant difference from Se. The comparisons within groups were performed non-parametric paired t-test (Wilcoxon signed rank test) and non-parametric ANOVAs (Kruskal Wallis). Statistical significance was at $p<0.05$.

Insulin resistance compromises the ability of insulin to effectively regulate the expression of critical genes such as G6PDH. Se has long been known to act as an insulin mimetic and to effect the expression of insulin responsive genes (Berg et al., 1995) (Becker et al., 1996) thus providing a method for restoring the effects of insulin action under diabetic conditions. The ability of selenium to mimic insulin's effect on G6PDH expression has been established by our lab in a Type1 diabetic model (Berg et al., 1995).

To test Se's effect on G6PDH transcription in our Type 2 model, cultured hepatocytes were treated for 6 hours in low glucose media, with or without glucosamine. Expression levels of G6PDH increased 1.33 fold above the non-treated control (figure 7) in the presence of 500um Se for 6 hours, indeed, verifying Se's ability to stimulate G6PDH

expression. Se was not, however able to stimulate G6PDH expression in the presence of glucosamine. Expression of G6PDH was equal to the non-treated control in the presence of glucosamine and Se. This finding is different from previous results from a similar study in our lab, which found that Se induces expression of G6PDH in the presence of glucosamine paralleled the expression of G6PDH with Se treatment in the absence of glucosamine (Veetil, 2011). Reasons are not obvious presently as to why these results are contradictory.

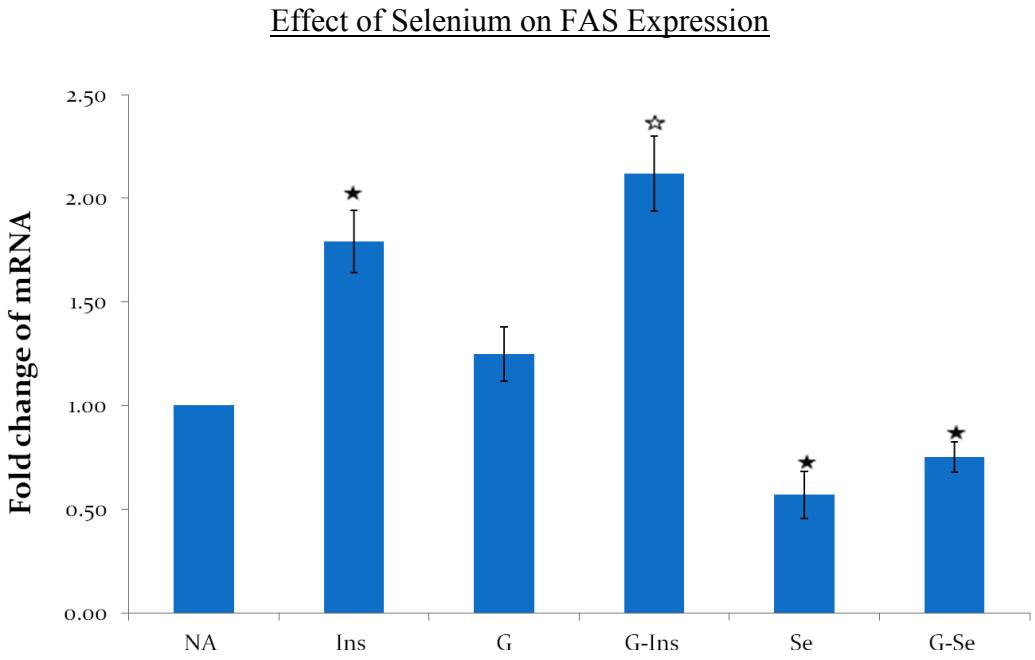

Figure 8. **Insulin and Se mediated expression of FAS.** Cells were incubated for 18h in low glucose media (5mM) with or without glucosamine. 44nm of insulin was added where indicated incubated with cells for 18h. Values indicate the fold increase of mRNA as compared to the non-treated control (NA, n=11; Se, n=4; G-Se, n=3; Se-LY, n=2; G- Se-LY, n=2). ★ =significant difference from NA; ☆ =significant difference from Se. The comparisons within groups were performed non-parametric paired t-test (Wilcoxon signed rank test) and non-parametric ANOVAs (Kruskal Wallis). Statistical significance was at $p<0.05$.

Expression of FAS has been shown to positively respond to Se in a Type I diabetic model (Berg et al., 1995). We hypothesized that expression of FAS would respond similarly to Se in a Type II model. Cells treated with Se alone for 6h showed a decrease (0.57 fold) in FAS expression levels when compared to the non-treated control. Six hour

treatments of Se in cells incubated with glucosamine for 18h also showed no increase in FAS expression compared to the non-treated control (0.75 fold). The inability of Se to stimulate expression diverges from what has been shown in the literature and was not expected in these results (figure 8).

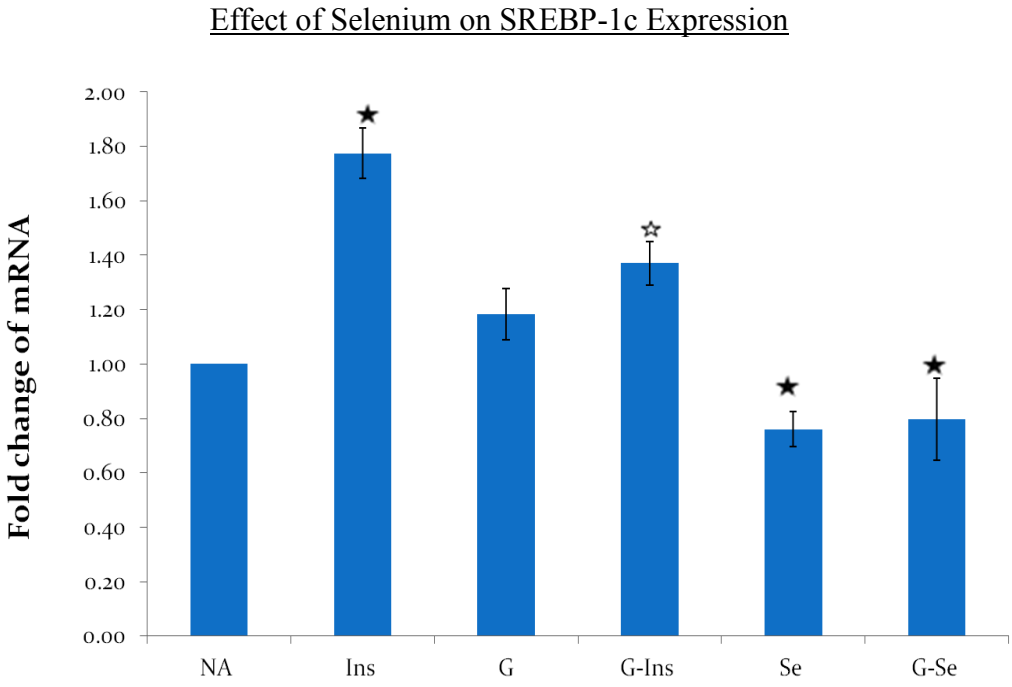

Figure 9. **Insulin and Se mediated expression of SREBP-1c.** Cells were incubated for 18h in low glucose media (5mM) with or without glucosamine. 500um Se was added where indicated incubated with cells for 18h. Values indicate the fold increase of mRNA as compared to the non-treated control (NA, n=13; INS, n=11; Se, n=9; G-Se, n=5). ★ =significant difference from NA; ☆ =significant difference from INS. The comparisons within groups were performed non-parametric paired t-test (Wilcoxon signed rank test) and non-parametric ANOVAs (Kruskal Wallis). Statistical significance was at $p<0.05$.

Treatment with 500um Se for 6h did not result in induction of SREBP-1c but resulted in a slight but significant reduction in its expression (0.5 fold when compared to the non-treated control). Given these results, it was not surprising to find that Se showed no ability to induce SREBP-1c expression in the presence of glucosamine (figure 9). This result was also unexpected, since SREBP-1c is a mediator of G6PDH in its expression and G6PDH is induced by Se.

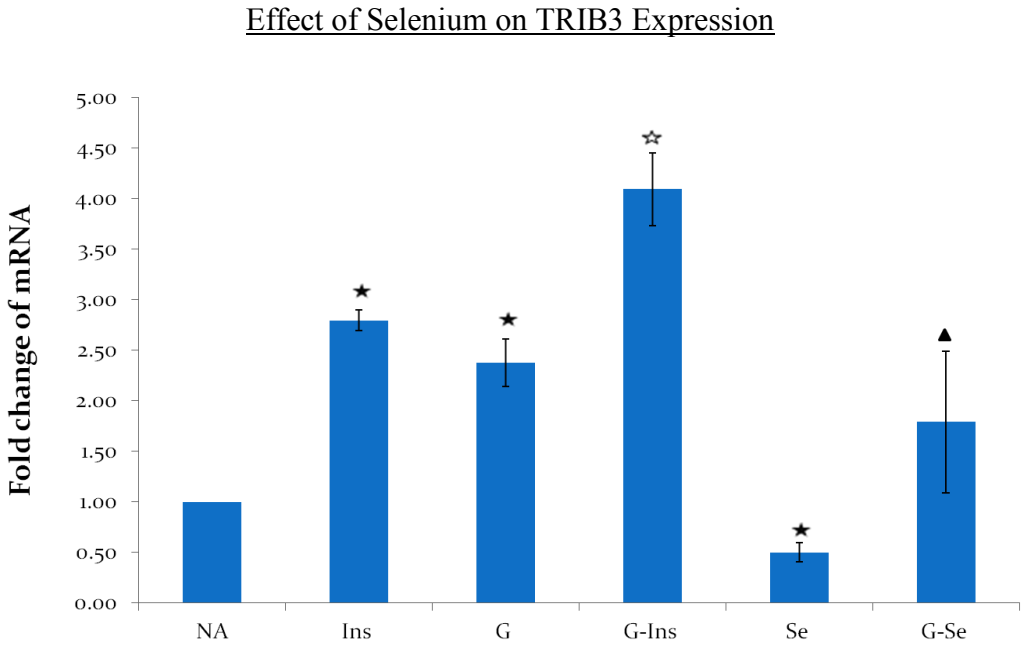

Figure 10. **Insulin and Se mediated expression of TRIB3.** Cells were incubated for 18h in low glucose (5mM) media with or without 1mM glucosamine. 500um of Se was added where indicated and incubated with cells for 6h. Values indicate the fold increase of mRNA cells (NA, n=16; Se, n=9; G-Se, n=14; Se-LY, n=3). ★ = Significant difference from NA, ☆ = Significant difference from INS, ▲ = Significant difference from Se. The comparisons within groups were performed non-parametric paired t-test (Wilcoxon signed rank test) and non-parametric ANOVAs (Kruskal Wallis). Statistical significance was at $p<0.05$.

Se alone was not able to stimulate TRIB3 expression. We hypothesized that Se would behave like insulin and stimulate the expression of TRIB3, but found that its expression was instead reduced 0.48 fold when compared to the non-treated control. The decrease that resulted from Se was not expected. Combined Se and glucosamine treatment did, however, show a slight increase in TRIB3 expression above the non-treated control at 1.79 fold (figure 10) which was less than glucosamine alone which increased over two fold when compared to the control. These results suggest Se may have a dampening effect on the glucosamine induction of TRIB3 by glucosamine. It seems reasonable to speculate that reduction of TRIB3 expression by Se treatment could result in reduced inhibition of AKT and MAPK by TRIB3. In other studies, when hepatic TRIB3 expression was reduced, improved glucose tolerance and increase insulin sensitivity in liver and muscle was observed (Koo et al., 2004) (Liu et al., 2010).

In summary, Se showed an insulin mimetic effect on expression of G6PDH, but did not show an insulin mimicking effect on any other gene of interest in the present study. Se also did not mediate the expression of any of our genes of interest in the presence of glucosamine but in most cases had a suppressing effect even on proteins where glucosamine alone showed a stimulatory effect on gene expression. Results are summarized on table 3.

	G6PDH	SREBP-1c	TRIB3	FAS
Ins	↑	↑	↑	↑
G	–	–	↑	↑
G + Ins	↑	↑	↑	↑
LY + Ins	↑	–	–	–
Se	↑	↓	↓	↓
Se + G	–	↓	↑	↓

Table 3. Summary of results of Se on gene expression

IV. Discussion

Much of the health-related trauma experienced in diabetes can be traced back to chronic hyperglycemia or chronically elevated blood glucose levels. Hyperglycemia results in increased flux through metabolic pathways, the accumulation of metabolic byproducts and the buildup of reactive glucose molecules in the bloodstream, resulting in reduction of insulin-mediated events such as protein signaling and transcription (Chandarlapaty et al., 2011) (Buse, 2006). Increased flux through the HBP has been shown to play a role in mediating the effects of hyperglycemic in the liver. Increased flux through the HBP is a contributing factor wich leads to hepatic insulin resistance and eventually, diabetes. During hyperglycemia, flux through the HBP is increased resulting in increased protein glycosylation by HBP byproducts (Marshall et al., 1991). Glycosylation of proteins often affects phosphorylation as common amino acids are utilized for both types of protein modification.

In liver cells or hepatocytes, protein glycosylation by O-linked N-acetylglucosaminyltransferase (OGT) (Cheatham, 2004) (Shirato et al., 2011) occurs at higher rates because of the predominance of the HBP in the liver (Holts & Hart, 1986). Byproducts of the HBP have not only been associated with yielding a reduction of insulin mediated events but have also been implicated as a cause of insulin resistance (Marshall et al., 1991). The role of glucosamine in increasing HBP flux by bypassing the rate controlling step was first shown in adipocytes and has been implicated in the induction of insulin resistance (Marshall et al., 1991). Similarly, we and others have utilized glucosamine to induce insulin resistance in liver cells in culture to study not only the role of HBP in insulin resistance but also the mechanism(s) by which insulin resistance is caused. The first study by our lab to look at the glucosamine induced insulin resistance found that there is a general increase in protein glycosylation as a result of glucosamine treatment, as well as on specific proteins (Veetil, 2011). Additionally proteins associated with insulin signaling such as AKT, GSK3 and IRS1 were found to have their phosphorylation states are altered by glucosamine treatment (Veetil, 2011) (McPherson, 2013). Other labs have also shown sigificant decreases on insulin mediated phosphorylation of insulin signaling proteins such as PI3K. For example, Hawkins et al., showed a significant effect on PI3K and an increase in UDP-GlucNAc in human skeletal muscles cells

after only 30 minutes of incubation with glucosamine, which resulted in a loss of glucose uptake shortly after decreased phosphorylation of PI3K was noted (Hawkins et al., 1999). Not only is there an effect on insulin signaling proteins, but there has also been shown to be an effect on the insulin mediated expression of metabolic genes. It is reasonable to believe that the changes in insulin mediated expression seen in this study are likely the result of increased flux through the HBP caused by glucosamine treatment.

The Insulin Mediated Gene Expression

Early studies in metabolism showed a link between insulin levels and regulation of the expression of both G6PDH and FAS. For example, several groundbreaking studies found that increases in G6PDH activity and mRNA levels were observed in livers from fasting and refed rats (Spolarics, 1999) and in primary rat hepatocytes in response to insulin (Fritz, Stumpo, & Kletzien, 1986). Similar increases of FAS mRNA expression due to insulin were observed in 3T3-L1 adipocytes (Paulauskis & Sul, 1989), chick hepatocytes (Goodridge et al., 1989), mouse liver and streptozoticin induced diabetic mice (Moustaïd, Beyer, & Sul, 1994). In 1995, our laboratory studied the expression of G6PDH and FAS in streptozoticin treated rats in response to insulin as well as compounds such as Se that mimic the effect of insulin. The results of this study showed that the expression patterns of G6PDH and FAS were similar in response to insulin (Berg et al., 1995). In this present study the transcriptional response of G6PDH and FAS concurs with observations of previous research by our lab and others for both FAS and G6PDH (Paulauskis & Sul, 1989) (Goodridge et al., 1989) (Moustaïd et al., 1994) (Sul, Latasa, Moon, & Kim, 2000) (Berg et al., 1995).

With the use of inhibitors such LY294002 that block the insulin induced activation of PI3K, studies have confirmed that the effect of insulin on both G6PDH and FAS expression occurs through the PI3K signaling cascade (Wagle et al., 1998) (Wang & Sul, 1998a).

To confirm the role of the PI3K signal pathway in the insulin induction of FAS and G6PDH, we utilized LY294002 in the present study and observed, when the inhibitor was present, a lack of insulin induction of each gene (figure 3) and (figure 4), al-

though the magnitude of the inhibition for each varied as the presence of LY 294002 completely inhibited the insulin induction of FAS but only partially blocked insulin induction of G6PDH.

Insulin has been shown to mediate the expression of FAS and G6PDH through not only the PI3K pathway but also through the regulation of insulin responsive transcription factors. SREBP-1c is a transcription factor that is responsive to insulin and regulates the expression of both glycolytic and lipogenic genes in the liver. For example, research with rats with chronic renal failure has shown that an increase in SREBP-1c protein concentration resulted in an increase in both FAS and G6PDH mRNA in rat white adipose tissue (Korczynska et al., 2004). Others have observed that SREBP-1c cooperates with other transcription factors such as SP1, CCAAT-enhancer-binding proteins (C/EBP) and peroxisome proliferator-activated receptor gamma (PPARGAMA) in response to insulin and carbohydrates to regulate lipogenic genes (Payne et al., 2012). Indeed previous research from our lab identified a potential binding site for SREBP-1c along with SP1 within the G6PDH promoter (Thekkat, 2007) (Arkwright-Keeler, 2005).

While evidence supports the role of SREBP-1c in insulin regulation of lipogenic genes like G6PDH and FAS conflicting data exists. Bertile and Raclot showed that expression of lipogenic genes did not neccesarily correspond with an increase in SREBP-1c expression (Bertile & Raclot, 2004).

The regulation of SREBP-1c by insulin and the necessity for the PI3K pathway is confirmed in this present study (figure 3). Blocking the insulin induction of PI3K with LY294002 (figure 3) resulted in suppression of SREBP-1c expression. This finding is in agreement with evidence from numerous reports from several model systems including rat hepatocytes, (Azzout-marniche et al., 2000) H2-35 cells (Hasty et al., 2000) knockout mice for the insulin receptor (H Shimano et al., 1999).

While PI3K has been shown to be important in the regulation of SREBP 1C, downstream signal proteins of PI3K have also been identified and include mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3 (GSK3). Both proteins have been shown to also require PI3K and AKT activation by insulin (Ono et al., 2003) (S. Li, Brown, & Goldstein, 2010). The phosphorylation by AKT in the presence of insulin inhibits GSK3's ability to phosphorylate its targets: glycogen synthase (GS) and

SREBP-1c. In the absence of insulin initiated phosphorylation of AKT, GSK3 inhibits the activity of GS and marks SREBP-1c for ubiquitination. The absence of insulin also inhibits the GSK3 mediated phosphorylation CCAAT-enhancer-binding protein (C/EBP), another insulin-regulated transcription factor that mediates the expression of both SREBP-1c and TRIB3 (Payne et al., 2012). C/EBP also regulates the expression of TRIB3, a signaling protein discussed later (Du & Ding, 2009). Previous research by our lab has shown that glucosamine blocks phosphorylation of GSK3 by AKT in primary hepatocytes (McPherson, 2013). Although some research suggests the possibility that GSK3 mediated control of SREBP-1c and fat metabolism my be activated apart from mTOR, (Bengoechea-Alonso & Ericsson, 2009) (D. Wang & Sul, 1998a) it is reasonable to believe that the GSK3 mediated regulation of SREBP-1c may be affected by treatment with glucosamine.

SREBP-1c is an important transcription factor for a number of genes beyond those for classic metabolism proteins. TRIB3, also known as neuronal cell death-inducible putative kinase, has been shown to modulate the phosphorylation of several insulin sensitive proteins and is regulated by insulin through SREBP-1c in cooperation with several other cotranscription factors such as peroxisome proliferator-activated receptor gamma (PPARgamma) (Oberkofler et al., 2010).

A study looking at the response of TRIB3 to insulin in Fao cells, a hepatocyte cell line, showed that the insulin mediated expression of TRIB3 was completely abolished as a result of PI3k inhibition with LY294002 (Du & Ding, 2009) demonstrating that PI3K is essential for TRIB3 expression. Similarly to other studies that confirmed a role for the PI3K pathway for TRIB3 induction by insulin (Du & Ding, 2009) (Ding et al., 2008), we did not observe TRIB3 induction with insulin in the presence of LY294002 (figure 4). In this present study we have also shown that TRIB3 is induced by insulin in both insulin sensitive and insulin insensitive conditions caused by glucosamine treatment. Future research should examine the role of PI3K in expression of TRIB3 by insulin in insulin resistant conditions.

While **t**he expression of TRIB3 is modulated through the PI3K pathway, data that has examined the role of TRIB3 in modulating insulin mediated phosphorylation of insulin signaling proteins has shown conflicting evidence.

Although most research to date shows that increased expression of TRIB3 leads to suppression of insulin initiated phosphorylation of AKT and its downstream proteins, (Matsushima, Harada, Webster, Tsutsumi, & Nakaya, 2006) two independent reports show conflicting evidence. Patrick Iynedjian et al. showed that overexpression of TRIB3 in primary rat hepatocytes had no effect on AKT phosphorylation (Iynedjian, 2005), and a second report by Haruka Okamoto et al. showed that double negative mutant mice for TRIB3 demonstrated no noticeable difference from their wildtype littermates (Okamoto et al., 2007). Additional reports show that even in the face of reduced phosphorylation of AKT, S6K was still phosphorylated in an insulin dependent manner (Sharma, Guthrie, Chan, Haq, & Taegtmeyer, 2007). This phosphorylation of S6K in insulin resistant conditions presents the likelihood of a permissive function of AKT in diabetic conditions (Sharma, Guthrie, Chan, Haq, & Taegtmeyer, 2007). This possibility is also evidenced by the findings of one report which showed the inability of TRIB3 overexpression to block SREBP-1c expression in 3T3-L1 cells (Takahashi, Ohoka, Hayashi, & Sato, 2008). Future research will help us more fully understand TRIB3's role in regulating AKT and its downstream proteins.

Effect of Glucosamine Induced Insulin Resistance on Gene Expression

Based on the recent findings of our lab and others, it was our expectation that glucosamine would block the insulin-mediated expression of the insulin responsive genes examined in this study, and would have the same or similar effects as inhibition of PI3K with LY294002. In the present study, inhibition of PI3K by LY249002 resulted in suppression of mRNA expression for all the genes studied: G6PDH, FAS, SREBP1c and TRIB3. However, the glucosamine-induced insulin resistant state did not uniformly inhibit insulin-mediated expression. While the insulin induced expression of two genes of interest, G6PDH and SREBP-1c, saw a decrease in insulin induced expression in the presence of glucosamine, the other two genes in this study, FAS and TRIB3, actually saw increased expression as a result of glucosamine, and this increase was additive in the presence of insulin.

Changes in insulin initiated expression are due in part to a rise in protein glycosylation caused by increased flux through the HBP (Li et al., 2010). As a result of the work of Marshall et al., we now know that increased glycosylation as a result of increased HBP flux reduces the phosphorylation of insulin responsive signaling proteins (Marshall et al., 1991). Subsequent studies have shown that many end results of the insulin signal are blocked as a consequence of glycosylation in a manner comparable to the use of chemical inhibitors. Two previous studies from our lab have supported the findings of Marshall et al. and demonstrated that there is reduced phosphorylation of AKT and other proteins within the insulin signaling cascade as a result of the increased HBP flux (Veetil, 2011) (McPherson, 2013), possibly due to an effect on the phosphorylation of PI3K (Hawkins et al., 1999).

The onset of insulin resistance results in drastic changes in glucose and lipid metabolism. Insulin, though it is still present in the bloodstream in high amounts, loses its ability to suppress hepatic glucose synthesis and break down glucose (S. Li et al., 2010) but maintains its ability to regulate and increase the production of lipids (R A Memon et al., 1994). These factors are a signficant cause of hyperglycemia and hyperlipidemia in diabetic individuals.While the accumulation of fat in the body and the liver can come from several sources (Fong, Nehra, Lindor, & Buchman, 2000) (Fong et al., 2000), increased lipid production in the liver during insulin resistance is a significant contributor to the overall accumulation of fat in the body (Postic & Girard, 2008). This increase is contributed to significantly by hyperglycemia and increased HBP flux (Rumberger et al., 2003). Memon et al. looked at hepatic synthesis of lipids in two mice models, one genetically predisposed for diabetes and the other genetically predisposed for obesity and hyperinsulinemia. Their study found that, despite the onset of diabetes in the one model, hepatic lipid production was increased 9 fold in the presence of insulin and 2.4 fold in mice genetically predisposed for obesity (Memon et al., 1994).

The contrasted responses of FAS and G6PDH to glucosamine induced insulin rsistance could be due to variations observed within the lipogenic family where genes associated with lipid metabolsim differ from those associated with carbohydrate metabolism. In insulin resistant conditions, proteins that metabolize carbohydrates no longer respond to insulin as they would in insulin sensitive cells; however, genes involved in

producing lipids are still able to respond to the presence of insulin (Li et al., 2010). This phenomenon is mirrored in the results of this study. For example, in the presence of insulin, the expression of G6PDH, a carbohydrate metabolizing protein, was suppressed under insulin resistant conditions, while the expression of FAS, a protein involved in the production of fat, continued to be increased in response to insulin. The similarity in G6PDH expression levels in the presence of LY294009 and glucosamine plus insulin were echoed in the results of a previous study which also showed a reduced insulin-mediated expression of G6PDH as a result of glucosamine treatment that was similar to the result of treatment with LY294002 (Veetil, 2011).

The continued positive regulation by insulin of fat production and its release from the liver in the face of confirmed insulin resistance, in combination with the loss of regulatory control over carbohydrate metabolizing genes, is one of the mysteries of diabetes. This phenomenon has led some to propose that hyperglycimia induced insulin resistance is not complete (Brown & Goldstein, 2008) (Owen et al., 2012) (Vatner et al., 2015) and that hyperglycemia induced insulin resistance is not a complete oblation of insulin sensitivity (Li, Brown, & Goldstein, 2010). Speculation arises as to how these mechanisms differ.

Some evidence also indicates that flux through the HBP as a result of hyperglycemia may be a factor that contributes to continued regulation of FAS expression and the increase in fatty acid production. Studies in adipocytes and HEPG2 cells have shown that treatment with glucosamine is sufficient to increase FAS expression even in the absence of insulin (Rumberger et al., 2003) (Hirahatake et al., 2011) (Sage et al., 2010). Studies have also shown that increasing HBP pathway flux by overexpression of GFAT, the rate limiting step in the HBP, results in increased fat production and expression of FAS as well as several other proteins associated with fat synthesis (Veerababu et al., 2000) (McClain, Hazel, Parker, & Cooksey, 2005) (Sage et al., 2010) such as acetyl-CoA carboxylase (ACC) (Rumberger et al., 2003) and leptin (Jiali Wang, Hawkins, Barzilai, & Rossetti, 1998) (Foufelle et al., 1996). Additionally, when glutamine, the amino source utilized by GFAT to make glucosamine, was removed, glucose lost its ability to regulate FAS expression in primary adipocytes (Rumberger et al., 2003) (Veerababu et al., 2000).

In the present study, we observed a minimal yet significant increase in FAS expression with the treatment of glucosamine alone. The expression of FAS induced by the combined glucosamine and insulin treatment was greater than expression of FAS with insulin alone. This finding coincides with findings in other studies that show that HBP flux is a critical part of the mechanism of glucose regulation of FAS expression (Hirahatake et al., 2011) (Foufelle et al., 1996) (Sage et al., 2010).

Increased glycosylation due to glucosamine treatment may have a role to play in the induction of FAS expression. In the present study, treatment with LY294002 led to suppression of insulin-induced FAS expression, while treatment with glucosamine alone resulted in a slight increase in FAS expression.

The difference in expression between FAS and G6PDH in the presence of glucosamine alone points to the possibility that glucosamine influences the expression of FAS at a point distal to PI3K, while PI3K or upstream or immediate downstream factors may have more of a role for insulin induced G6PDH expression. Evidence points to the interaction between mTOR and GSK3 as the potential point at which the difference between regulation of G6PDH and FAS expression occurs (S. Li et al., 2010).

The invovlement of mTOR in the metabolism of proteins is well established; however, gaining a full understanding of what mTOR does has shown to be a challenge. What is known is that mTOR is activated as a result of nutrient abundance and is regulated by insulin through the PI3K/AKT (Yea & Fruman, 2011) pathway as LY294002 was shown to suppress the insulin activation of mTOR (Penuel & Martin, 1999) (Schwarzer et al., 2006). The increased activity of mTOR leads to increased expression of SREBP-1c and lipid synthesis (Iii & Birnbaum, 2012). Research has shown that rapamycin of mTOR was able to block the insulin-mediated control of SREBP-1c transcription in mouse embrionic fibroblasts (Düvel et al., 2010). The role of mTOR in regulating lipogenic gene expression was shown by Li et al., who showed that there was a concentration-dependent reduction in mTOR's control of lipogenic genes such as LXR, SREBP-1c and FAS in fasted and refed rat hepatocytes that were treated with rapamycin (S. Li et al., 2010). This same study showed that the regulation of mTOR was specific to lipogenic genes as no effect was noted on insulin's control of phosphoenol carboxy kinase (S. Li et al., 2010).

Typically changes in FAS mRNA expression are accompanied by similar changes in its transcriptional regulator, SREBP-1c. One finding of the present study that is challenging to interpret is that we observed an induction of FAS in spite of a SREBP-1c decrease when glucosamine is present. Research has shown that regulation of SREBP-1c is the result of an intricate balance between carbohydrate levels and insulin signaling (Horton et al., 1998) (Kim et al., 1998) (Shimano et al., 1999) (Yamamoto et al., 2007) (Hasty et al., 2000). Yet the role of the relationship between insulin and increased flux of glucose through the HBP during hyperglycemia and in relation to the regulation of the genes studied is not known.

What is known is that insulin can modulate SREBP-1c expression through the transcription factor LXR (Xiao & Song, 2013), whose regulation of expression is influenced through post-translational processing, and the signal proteins mTOR, GSK3 and S6K. Several reports have shown that in the presence of high glucose concentrations, and with glucosamine treatment, increased glycosylation activates LXR and mTOR activity (Foretz et al., 1999) (Matsuzaka et al., 2004) (Anthonisen et al., 2010), and at least two reports showed that azaserine, an inhibitor of GFAT, reduces the production of SREBP-1c, implicating the HBP in SREBP-1c regulation (Hasty et al., 2000) (Hirahatake et al., 2011).

Conflicting data, however, from another study shows that increased glycosylation may not lead to increased SREBP-1c expression. Yang et al. found that overexpression of O-GlcNAc transferase (OGT), which is responsible for removal of glycosyl groups from proteins, caused suppressed insulin-mediated SREBP-1c mRNA expression in mice hepatic cells (X. Yang et al., 2008). This same study also showed that, as a result of overexpression of GFAT in whole mouse, hepatic expression of SREBP-1c and its target genes was decreased (X. Yang et al., 2008). These data appear to indicate that increased glycosylation due to increased HBP flux suppresses insulin-mediated expression of SREBP-1c. It is difficult to interpret the reason for the conflicting results between previously mentioned studies. Future research will be needed to tease apart the specific mechanisms involved in regulation of SREBP-1c expression as a result of glycosylation.

In the present study, we show that co-treatment with glucosamine and insulin resulted in a significant decrease in insulin initiated SREBP-1c mRNA expression. Expres-

sion of SREBP-1c, as seen in figure 3, followed the same trend as G6PDH expression seen in figure 3. Glucosamine alone and glucosamine with insulin treatments showed no significant difference, indicating that glucosamine was able to block the insulin initiated induction of SREBP-1c.

Regulation of SREBP-1c targets is also nuanced as we see in the case of FAS and G6PDH. It is known that SREBP-1c is needed for G6PDH expression (H Shimano et al., 1999) and so it makes sense that their expression patterns are similar. However, FAS is also regulated by SREBP-1c but did not correlate with SREBP-1c expression patterns in response to glucosamine treatment. The reason for this divergence in expression patterns could lie within the effect of glucosamine on the activity of mTOR, which is responsible for the insulin-mediated phosphorylation of GSK3 and S6K (Xiao & Song, 2013) (Li et al., 2010). Phosphorylation of SREBP-1c by GSK3 has been shown to result in ubiquitination of SREBP-1c, and inhibition of this phosphorylation by GSK3 was shown to increase SREBP-1c stability (Xiao & Song, 2013). S6K, a protein that is downstream of mTOR, aids in directing the processing of the immature proteins and activation of the nuclear version of SREBP-1c (Xiao & Song, 2013). In the presence of insulin, GSK3 is inhibited by AKT phosphorylation, promoting SREBP-1c stability. Synthetic inhibitors of GSK3 activity also enhanced SREBP-1c protein stability and resulted in the increased transcription activity of its targets, FAS and ACC (K. H. Kim et al., 2004). Evidence from our lab and by others showed that, in insulin-resistant conditions caused by glucosamine, phosphorylation of GSK3 by AKT at Ser9 is inhibited (McPherson, 2013) (Hawkins et al., 1999). The evidence from the previous studies by McPherson and Hawkins et al. suggests that the decreased phosphorylation of GSK3 would lead to increased SREBP-1c degradation and an inability of SREBP-1c to regulate targets. However, the decrease in SREBP-1c did not appear to have an effect on one of SREBP-1c's targets: FAS expression.

A likely explanation for the lack of effect on the expression of FAS in the absence of SREBP-1c expression is that there is compensation by other transcription factors including other isoforms of SREBP-1c. Evidence shows that in SREBP-1c (-/-) mice liver, SREBP2 and SREBP-1a were able to compensate for the loss of SREBP-1c (Liang et al., 2002). Additionally, there are other transcription factors such as PPARgama and

CCAAT-enhancer-binding proteins (C/EBP) that respond to insulin's initiated phosphorylation of mTOR and positively promote the expression of lipogenic genes (Payne et al., 2012). It is also likely that these transcription factors were able to compensate for the lack of SREBP-1c expression and promote the expression of FAS. Future research will have to be done to clarify the transcriptional mechanism for FAS expression in the absence of SREBP-1c. In addition to mediating the increase in FAS and SREBP-1c expression, PAPARgama and C/EBP have also been shown to contribute to the increase in TRIB3 expression (Selim, Frkanec, & Cunard, 2007) (Cunard, 2013) (Du & Ding, 2009) in hepatoma cells (Du & Ding, 2009) and liver (Koo et al., 2004).

The relationship between insulin sensitivity and TRIB3 regulation by members of the insulin signaling cascade is a complicated one, exasperated by a further misunderstanding of the effect that hyperglycemia has on these insulin mediated mechanisms. While high glucose concentration has been shown to induce the expression of TRIB3 in hepatic models and to result in the inhibition of AKT (Du et al., 2003), no previous study has examined the effect of glucosamine and increased HBP flux on TRIB3 in hepatocytes.

In the present study there was an additive effect of insulin and glucosamine on the expression of TRIB3. Literature shows that both insulin excess and hyperglycemia (Liu et al., 2010) (Zhang et al., 2013) increase the expression of TRIB3. Interestingly, we found glucosamine to induce TRIB3, which would suggest this may be a mechanism for AKT inhibition and loss of insulin signalling. Additional studies would need to confirm an interaction between TRIB3 with AKT under glucosamine conditions.

The next logical direction would be to test for association of TRIB3 with not only AKT but also other insulin signaling proteins. What can be concluded from the observations of this study is that TRIB3 expression increases as a result of glucosamine treatment which is commonly used as a model for high blood glucose levels or hyperglycemia and that insulin also has a direct effect.

The Effect of Se on the Expression of Insulin Responsive Genes

Se is a micronutrient that is involved in many life processes and is a cofactor for numerous genes (Rayman, 2000) (Stapleton, 2000) (Puchau, Zulet, Gonzalez de Echavarri, Navarro-Blasco, & Martinez, 2009). Deficiency of Se causes multiple and serious health problems, such as neurodegenerative and cardiovascular disorders, and has also been shown to cause loss of insulin sensitivity (Wang et al., 2014).

Se is one of several compounds, researched over the years, that has been shown to mimic effects of insulin, such as the lowering of plasma glucose, the transporting of proteins to the plasma membrane for glucose uptake and restoring of expression of insulin responsive genes, in both in vitro and in vivo models (Becker et al., 1996) (McNeill et al., 1991) (Ghosh et al., 1994; Kim et al., 2012; Hei et al., 1998; Stapleton, 2000; Aboul-Soud et al., 2011). Research by Osama Ezaki and associates in 1990, first showed the insulin mimicking ability of Se to mediate the translocation of glucose transporters to the cell surface membrane of rat adipocytes (Ezaki, 1990). As a result of their findings, selenium has been widely studied as a method to combat symptoms associated with diabetes and insulin resistance. Part of the effectiveness of Se is attributed to its ability to mediate increased phosphorylation to signaling proteins in the insulin signaling cascade.

In several cellular models, research has shown that Se induces increased phosphorylation of several signaling proteins associated with the insulin signaling cascade, including IR (Pillay & Makgoba, 1992) PI3K, (Stapleton et al., 1997) (Heart & Sung, 2003) and IRS1(Stapleton et al., 1997). This increase in phosphorylation by Se has been shown to lead to increases in expression of FAS and G6PDH in rat liver, in a Type I diabetic model (Berg et al., 1995) (Ghosh et al., 1994) (Hei et al., 1998). While it has been proposed that the increase in phosphorylation in the presence of Se is a result of inhibition of phosphatases the actual mechanism remains unknown (Pillay & Makgoba, 1992) (Ezaki, 1990) (McNeill et al., 1991).

Recently our lab has begun to explore the ability of Se to increase metabolic gene expression in the glucosamine model of chronic hyperglycemia. In the present study, we hypothesized that Se may have insulin mimetic properties in an insulin resistant state. Thus we tested whether or not Se could restore the expression of our genes of interest in glucosamine induced insulin resistant conditions.

The results of this present study show significant increase in G6PDH mRNA with Se treatment alone and are in concert with what was shown in our early whole animal studies by Berg et al. (Berg et al., 1995) and in hepatocytes by Veetil (Veetil, 2011). Glucosamine treatment reduced Se induced G6PDH expression equal to the non-treated control, showing that Se was not able to overcome changes in expression brought on by glucosamine. The inability of Se to overcome the effect of glucosamine was a result that was not expected, as another study in our lab suggested Se continued to elicit an increase in G6PDH (Veetil, 2011). More work will have to be done in order to delineate the reasons for the differences in these results.

Evidence demonstrates that in mice Se decreases the plasma lipid, triglyceride and cholesterol concentrations, while increasing the liver lipid concentration (Mueller & Pallauf, 2006) (Steinbrenner, 2013). There is evidence to show that Se also modulates the expression of lipogenic genes such as FAS (Berg et al., 1995) (Veetil, 2011). The results from this study conflicted with our expectations that Se alone would increase the expression of FAS as previous studies have shown. In fact, there was a slight decrease in expression with Se alone and an even slighter decrease with Se and glucosamine, although the difference between Se alone and Se plus glucosamine did not achieve significant levels. The differences between this study and previous work with respect to Se's ability to stimulate the expression of metabolic genes are difficult to interpret. More study will have to be done to determine the role of Se in regulating the expression of metabolic genes in primary hepatocytes. However, as we have discussed previously in this report, the increase in FAS and many other lipogenic proteins is a symptom of diabetic conditions and leads to increased lipogenesis and obesity in diabetic individuals. The suppression of FAS by Se could be advantageous in diabetic conditions and may help to fight complications of diabetes.

Several studies, including one by Mueller, have shown that rats fed a selenate supplemented diet saw an increase in hepatic SREBP-1c expression (Mueller et al., 2009). The present study showed a slight suppression of SREBP-1c expression when cells were treated with Se and demonstrated no ability to overcome the glucosamine effect. These results differ from those found in a study done by Mueller et al. and by other research groups (Zhoua, Huanga, & Lei, 2013). This difference may have been because

our study was done in cells in culture instead of whole animals and because Se is directly available to the cells, without interference from other organs and systems. However, if Se is behaving as a mimetic on G6PDH, it is difficult to explain why Se did not behave as an insulin mimetic and induce the expression of SREBP-1c, FAS and TRIB3.

Although, from previous literature, we have a partial understanding of the mechanism that insulin uses to mediate the expression of TRIB3, to my knowledge at this time there is no literature that looks at the expression of TRIB3 in response to Se, so an interpretation of these results in light of other relevant research is difficult. However, the effect of Se on the expression and regulation of insulin signaling proteins has been well studied. Se has been shown to increase phosphorylation and expression of several signaling proteins. One protein to note that experienced increased phosphorylation in another study, as a result of Se treatment, is AKT (Veetil, 2011). Under insulin resistant conditions AKT is thought to be inhibited by TRIB3 (Liu et al., 2012). In this study, treatment with Se did not stimulate TRIB3 expression but instead reduced its expression to below the non-treated control. A dampening of glucosamine mediated stimulation of TRIB3 expression was observed with combined Se and glucosamine treatment. This effect by Se, though unexpected, in a very practical sense, could lead to reduced inhibition of AKT and MAPK by TRIB3. In studies, where hepatic TRIB3 expression was reduced, improved glucose tolerance and increased insulin sensitivity in liver and muscle was observed (Koo et al., 2004)(Liu et al., 2010). It would be an interesting direction for future studies to take in looking at the mechanism by which Se controls TRIB3 expression.

In the present study, we hypothesized that if Se is truly a mimetic, then it should increase expression of genes in a manner that is similar to insulin. While the effects of Se alone on G6PDH were in concert with what was shown in earlier studies (Berg et al., 1995) (Veetil, 2011), we were not able to replicate other studies that showed Se increased basel expression of FAS, and, in no case, was Se able to reverse suppression of gene expression that resulted from glucosamine treatment. However, the increase of certain genes as a result of insulin resistance serves to compound the diabetic condition. The suppression of certain genes by Se could be advantageous in diabetic conditions and may help to fight complications of diabetes.

To date, we have only a partial understanding of the mechanism that Se uses to mediate the expression of genes. We have broken new ground looking at the effect of Se on TRIB3 and the expression transcription factors. It is my hope that the results of this research will spark new questions about the mechanisms utilized by Se to combat insulin resistance and to suppress genes that compound the insulin resistant condition.

Summary

In conclusion, we have utilized glucosamine as a tool to simulate chronic hyperglycemia induced insulin resistance in primary rat hepatocytes and have measured the effect on insulin mediated gene expression. After determining the effects of insulin resistance on our proteins of interest, we tested the ability of insulin and Se to act on gene expression under these conditions. While our results add some clarity to this ongoing discussion, other questions are also emerging. Further research must be done in order to shed more light on insulin regulation of genes under hyperglycemic conditions.

APPENDIX

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

PROJECT OR COURSE TITLE: Regulation Of Gene Expression In Hepatocytes
IACUC Protocol Number: 13-04-06
Date of Review Request: 04/01/15 Date of Last Approval: 5/10/2014
Purpose of project (select one): ☐Teaching ☒Research ☐Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: Susan Stapleton Title: Professor
Department: Chemistry Electronic Mail Address: susan.stapleton@wmich.edu
CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Jaafar Hachem Title: Grad Student
Department: Chemistry Electronic Mail Address: jaafar.hachem@wmich.edu

1. The research, as approved by the IACUC, is completed:
   ☐Yes (Continue with items 4-5 below.) ☒No (Continue with items 2-5 below.)

If the answer to any of the following questions (items 2-4) is "Yes," please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☐Yes ☒No
3. Have there been any new findings or publications relative to this research that require you to alter your study? ☐Yes ☒No
   Describe the sources used to determine the availability of new findings or publications:
   - ☐No search conducted (Please provide a justification on an attached sheet.)
   - ☐Animal Welfare Information Center (AWIC)
   - ☒Search of literature databases (select all applicable)
     - ☐AGRICOLA ☒Current Research Information Service (CRIS)
     - ☒Biological Abstracts ☒Medline
     - ☐Other (please specify):
     - Date of search: Years covered by the search: 2005-2015
     - Key words:
   - ☐Additional search strategy narrative:
4. Are there any adverse events, in terms of animal well-being, or mortalities to report as a result of this research? ☐Yes ☒No
   Cumulative number of mortalities:
5. Animal usage: Number of animals used during this quarter (3 months): 4 Cumulative number of animals used to date: 20

✏ Susan J. Stapleton 3/11/15
Principal Investigator/Faculty Advisor Signature Date

✏ 3/18/15
Co-Principal or Student Investigator Signature Date

IACUC REVIEW AND APPROVAL
Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature.

✏ 4-20-15

Revised 01/2012 WMU IACUC All other copies obsolete.

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