Study of the Insulin Mimetic Sodium Orthovanadate in Glucosamine Induced Insulin Resistance in Primary Rat Hepatocytes

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STUDY OF THE INSULIN MIMETIC SODIUM ORTHOVANADATE IN GLUCOSAMINE INDUCED INSULIN RESISTNACE IN PRIMARY RAT HEPATOCYTES

by

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A thesis submitted to the Graduate College in partial fulfillment of the requirements for the degree of Master of Science
Department of Chemistry
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Insulin resistance and type II diabetes is a disease state characterized by a lack of sensitivity of insulin to provoke a proper response in insulin sensitive tissues. Currently, we utilize glucosamine to increase flux through the Hexosamine Biosynthetic Pathway (HBP) to induce an insulin resistance like state in primary rat hepatocytes. We tested the effectiveness of the insulin mimetic sodium orthovanadate to determine if it could elicit an insulin-like response under these conditions. Mixed results were obtained on the effectiveness of vanadate as an insulin mimetic in this insulin resistant model.
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Kalan McPherson
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INTRODUCTION

The word “diabetes” is Latin but ultimately derived from an ancient Greek word meaning “to pass through” or “siphon.” Mellitus is also derived from a Latin word mel or mellite meaning “honey” or “to sweeten with honey,” respectively. The term mellitus was first used by William Cullen in 1769 to distinguish between diabetes mellitus and diabetes insipidus [polyuria/excessive urination] (Sanders 2002).

Prevalence of Diabetes

Insulin resistance, a hallmark of diabetes, is a disease state that affects millions of people worldwide. The World Health Organization has declared that a “diabetes epidemic is underway.” Approximately 438 million people worldwide will be affected by insulin resistance by the year 2030 [WHO] (Smyth 2006). In this time, the U.S. is predicted to have the fourth largest rise in new cases of diabetes (Figure 1). Only China, India and the Middle East are predicted to have a greater
increase in new cases by population size. It is thought that this new trend is due to unhealthy lifestyles (lack of exercise), poor diets, aging and of course population growth (Smyth 2006). Right now in the United States, 25 million Americans have the metabolic disease state of diabetes and another 79 million have pre-diabetic symptoms. According to the Centers for Disease Control and Prevention (CDC), if this trend continues, 1 out of 3 Americans will have diabetes by 2050. The growing cost of health care associated with diabetes care in 2007 was estimated to be about 174 billion (total cost) in the United States and this figure represents a significant amount of the overall costs that are spent on health care every year. Much of these costs are associated with the direct care and treatment of the disease. However, many indirect costs (58 billion) such as disability, work loss, and premature death drive up the total cost of treatment according to the CDC. This figure represents a growing epidemic that will require new and novel methods of control or treatment to combat the disease state.
Figure 1- Global Projections for Diabetes Epidemic
(Permission to reuse figure obtained from IDF)
http://www.idf.org/diabetesatlas/5e/foreword

**Type I Diabetes Mellitus**

Diabetes can be attributed to many things which include hereditary or genetic, lifestyle, environmental or even viral causes. The first type, type I diabetes (IDDM) or Insulin Dependent Diabetes Mellitus is often linked to an inherited defect. Type I diabetes is an autoimmune response which destroys the insulin producing \( \beta \)-cells within the pancreas. There are two major hormones
produced in the pancreas and they regulate the metabolism and flux of glucose within the body. The hormones that regulate this highly organized process of glucose homeostasis are two small polypeptides, glucagon and insulin and they are produced by two different cell types within the pancreas, the α and β-cells, respectively. Both of these cell types are located in specific regions of the pancreas called the islets of Langerhans (Figure 2).
With the case of IDDM, the pancreatic β-cells are damaged by an immune response against oneself due to a response of the major histocompatibility complex (MHC). This MHC is a region of the genome that is present in all invertebrates and is responsible for production of cells associated with an immune response. Inherited genetic combinations within the MHC that are defective have been shown to produce phenotypes with autoimmune disorders. A specific type of MHC class II molecule is presented with a specific antigen that activates T cells through binding of this specific antigen (Ridgway 1998). It was shown that CD4- , CD8+ cytotoxic T cells have been found in the islets of patients with the onset of the disease (Foulis 1986). CD8+ cytotoxic T cells or killer T cells are leukocytes or white blood cells that express a specific glycoprotein called CD8. This affinity for CD8 and the MHC helps the cytotoxic T cell to recognize and target the cells for destruction. Also, autoantibodies or antibodies directed against the body’s own healthy cells which specifically target the islets of Langerhans are to blame and believed to be the cause of the β-cell
destruction. There are even a few laboratories emerging with evidence that Coxsackie B viruses (CVBs) can induce the type I form of diabetes although these theories are not very popular (Fairweather 2002).

Since insulin is no longer produced in the pancreas it must be supplemented and individuals are treated with multiple daily injections of insulin via needle or insulin pump. This treatment regiment can be difficult for patients to control blood glucose levels not to mention the physical burden associated with insulin injections. However advances have been made in the synthetic forms of insulin produced now and these have a longer lasting effect, which help patients control the blood glucose in a much narrower range with fewer injections.

**Type II Diabetes Mellitus and Insulin Resistance**

Non-Insulin Dependent Diabetes Mellitus (NIDDM) or Type II diabetes is a disease state whereby insulin is produced but nonetheless ineffective in stimulating glucose uptake and utilization. Often times this type of diabetes was referred to as adult onset diabetes. The
complications associated with Type II diabetes has been linked to obesity due to lack of physical exercise. In wealthier nations such as North America, Great Britain and the Middle East, the lifestyle has been heading toward more sedentary one. It is thought that inactivity along with a vast nutrient supply can cause overproduction of insulin, thereby leading to insensitivity. Cells that recognize and respond to insulin and its signal will become insensitive as well. When that lack of response or insulin resistance takes place the disease state can be characterized as NIDDM. It also seems that the β-cells responsible for the production of insulin eventually become overwhelmed and exhaust their production of functional insulin. When individuals are initially diagnosed with NIDDM, courses of treatment can involve exercise, change of lifestyle, learning how to control blood glucose with insulin injections as well as oral medications.

**Discovery of Insulin**

Diabetes is a disease that has been pondered by many cultures since the beginning of recorded history. Before
the discovery of insulin, a diagnosis of diabetes mellitus was a death sentence. So for nearly three millennia, patients with onset of the disease died early in life and often quickly and painfully. The research in this area became quite vigorous and has expanded exponentially in the last 100 years. Prior to that, scientists had difficulty describing diabetes let alone treating the disease. In 1893 two scientists, Oskar Minkowski and Josef von Mering, discovered that depancreatized dogs had lost the ability of digestion and ability to control blood glucose (Mering 1889). This was a significant finding that first proposed something within the pancreas controlled blood glucose. In the next twenty years numerous scientists such as Nicolae Paulescu (Paulescu 1921), George Zuelzer (Zuelzer 1908) and Isreal Kliener (Kliener 1919) came up with rudimentary extracts in an attempt to produce the mystery compound that could have a glucose lowering effect. The discovery of insulin is generally agreed upon to have happened in Toronto in 1922. Fredrick Banting and Charles Herbert Best, working under the expert tutelage of Dr. James Macleod, used an
experimental technique of duct ligation in the pancreas of dogs that was based on a paper from Moses Barron (Barron 1920). This series of experiments resulted in the isolation of extracts that were highly inconsistent between batches and as well as their ability to lower blood glucose. Frustrated with these problems the group sought the help of Dr. James Bertram Collip. It became a race between Banting/Best and Collip to purify the extract. Collip was a biochemist that eventually perfected the isolation and purification of insulin from the crude extract using the right concentration of ethanol to precipitate the peptide. Banting and Best wound up using beef pancreas from slaughter houses in the area to produce an extract. The crude extract isolated from the pancreas was named “isletin“(Banting 1922). On January 11, 1922 they injected Leonard Thompson with this extract and it failed miserably (Banting 1922). On January 23, 1922 the team resumed the injection on that same patient using the extract produced by Collip (Banting 1922). This time the patient flourished and was quite literally brought back from near death. This was
later produced and sold all over the world by the Indianapolis based pharmaceutical company, Eli Lily. Since then, the advances in the production and synthesis of insulin have made leaps and bounds. In 1936, Hans Christian Hagedorn discovered that the effects and duration of insulin action could be controlled with the use of protamine. These “slow-release” insulin compounds were able to control insulin action for a period of 24-36 hours. Later in the late 1950s, oral medications with anti-diabetic effects were developed that could treat NIDDM (Jerslid 1956) (Spencer 1956). From 1963-1966, insulin was chemically synthesized by several groups in Germany (Meienhofer 1963), China (Kung 1966), and the United States (Katsoyannis 1966).
Synthesis of Insulin

Insulin is synthesized in the islets of Langerhans within the pancreas as a single polypeptide precursor known as preproinsulin (Figure 3). This precursor contains three distinct domains (A,B,C) and a signal sequence at the amino termini. Since the body does not store insulin in this form it must be converted to proinsulin by the action of a specific protease that removes the signal sequence. Proteolytic cleavage of the amino terminus is immediately followed by three distinct disulfide bond formations. This molecule, proinsulin, can now be stored in the pancreatic beta cells of the pancreas. Proinsulin is converted to mature insulin in response to elevated blood glucose levels. This happens when the C domain is removed and another specific protease cleaves two of the three disulfide bonds. Now insulin can be defined as two polypeptides (A and B chain) linked by a disulfide bond (Cox 2005).
The actions of insulin are very broad and specific (Figure 4). Insulin has the distinct ability to promote glucose uptake in muscle and fat, promote glycolysis and glycogen synthesis as well as protein synthesis. Insulin also has the ability to inhibit many other cell functions like de novo synthesis of glucose via gluconeogenesis, lipolysis and glycogenolysis.
Insulin is important for glucose metabolism and is secreted by the pancreas in response to an influx of glucose and elicits its response through binding to a cell surface receptor. The inherent nature of this hormone to stimulate insulin sensitive metabolic enzymes gives rise to numerous actions ranging from regulation of gene expression and eventual translation to glucose transport. An example of this would be insulin’s ability to promote the inhibition of gluconeogenesis or the
production of new sugars and the increased production of lipids via lipogenesis. Gluconeogenesis inhibition becomes problematic when the insulin signaling cascade fails to suppress the new production of sugar within the liver. When glucose concentrations in the blood are already high and new glucose production is not inhibited detrimental effects can be measured. This persistent state of elevated glucose levels is defined as diabetes.

**Insulin Signaling Phosphorylation Cascade**

Insulin binds to its cell surface receptor (Figure 5) initiating an insulin sensitive phosphorylation cascade that results in activation/deactivation of metabolic proteins or enzymes. The insulin receptor (IR) is comprised of two αβ monomers which have distinct regions and binding domains. The two alpha chains are on the outside of the plasma membrane. They are responsible for the binding of insulin. The two beta chains contain a region bound by the inner leaflet of the plasma membrane and a portion that protrudes out into the cytosol. This region contains a catalytic domain that transfers a phosphoryl group from ATP to multiple
different substrates containing a Tyr residue such as the insulin receptor substrate [IRS] (IRS-1, IRS-2, IRS-3, IRS-4, Cbl, Gab1, Gab2). Upon binding of insulin to the IR an auto-phosphorylation event occurs. This self phosphorylation occurs due to a conformational change in the alpha chains when insulin binds and corresponds with the activation of the tyrosine kinase activity of the beta chains. This conformational change exposes these Tyr residues to phosphorylation and thus activation of the IR. The IR is phosphorylated on three distinct tyrosine residues critical to its function. The insulin receptor then recruits the insulin receptor substrate one (IRS-1) to the protein complex. All IRS proteins contain two distinct regions at the amino terminus that allow binding to specific proteins and regions within the cell. The first binding specific region is the protein tyrosine binding (PTB) domain, which subsequently binds to the phospho-tyrosine residues in the IR. The second region is called a pleckstrin homology (PH) domain that is specific for the binding of certain lipids at the cytosolic side of the membrane (Parker 2001). Upon IRS-1
binding to the IR domain, Phosphotidylinositol 3 Kinase (PI3K) is recruited to the plasma membrane (Figure 5). This now becomes known as the point of nucleation for the protein complex. PI3K has two distinct domains, one for binding and one for catalytic activity. The p85 subunit contains a SH2 and SH3 domain which preferentially binds phospho-tyrosine residues. The p110 subunit is the domain responsible for the conversion of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$).

Figure 5- Insulin Signaling Cascade. Figure created in Microsoft PowerPoint.
Once PIP$_2$ is converted to PIP$_3$, a signal is recognized by Protein Kinase B (PKB) or Akt. Akt/PKB is a protein kinase that belongs to a subfamily of proteins called AGC protein kinases [CAMP-dependent protein kinase (PKA)/protein kinase G/protein kinase C (PKC) (Fayard 2005)]. Akt/PKB has three distinct regions that encompass its binding and catalytic and/or regulatory domain. This protein contains a binding domain similar to that of IRS-1. This N-terminal binding domain contains a PH domain that specifically binds phospholipids at the plasma membrane. The other domain at the C-terminus contains a hydrophobic motif (HM) (FxxF(S/T)Y), which happens to be the site of phosphorylation of a serine/threonine residue (Furtado 2002). Akt is then recruited to the plasma membrane by PI3K indirectly due to the conversion of PIP$_2$ to PIP$_3$. Upon arrival of Akt/PKB to the point of nucleation, it undergoes a phosphorylation by PDK-1 or phosphoinositide-dependent kinase 1. This phosphorylation occurs on a distinct residue Thr308. A second residue, Ser473, is thought to be phosphorylated by an unidentified kinase or possibly auto-phosphorylation by
Akt itself whereby causing a conformational change altering or increasing Akt activity 1000 fold within the cell. Akt is also involved in apoptosis or cell survival, cell motility and cell cycle progression.

Akt has been shown to activate pathways involving the mechanistic target of rapamycin (mTOR) and a large multi-protein complex. mTOR is activated by growth factors such as insulin or insulin like growth factor (IGF) as well as a nutrient sensing pathway involving the tuberous sclerosis complex (TSC1-TCS2) and the Rheb and Rag GTPases. mTOR is involved in two multi-protein complexes called mTORC1 and mTORC2. Akt also functions on numerous substrates that regulate glycogen synthesis and storage to glucose transport. Akt can phosphorylate a target protein in glycogen synthesis called glycogen synthase kinase 3 (GSK3). This phosphorylation of GSK3 inhibits it action. GSK3 is a protein which negatively regulates glycogen synthase (GS) and is directly involved in the new synthesis of glycogen from glucose. So upon phosphorylation and subsequent inactivation of GSK3 by Akt, GS can retain its activity
to produce long chain glucose polymers of glycogen. Akt can also influence the transport of glucose from outside the cell to areas within where it can be broken down and utilized as an energy substrate. There is a specific family of transporters that are responsible for glucose movement within the cell. They are referred to as glucose transporters (GLUTs). In muscle and adipose tissue, the predominant isoform of the protein responsible for the transport of glucose is Glut4. This protein, upon activation, translocates from an intracellular compartment to the cell surface where it is incorporated into the plasma membrane to facilitate glucose entering the cell (McCormick 1993).

Insulin can also stimulate a phosphorylation cascade that involves the insulin receptor substrate but is independent of the PI3K-Akt kinase pathway. This pathway is referred to as the extracellular-signal-regulated kinase (ERK) transduction pathway. The ERK pathway is involved in numerous processes such as cellular proliferation, differentiation, and survival. After insulin binds to the receptor tyrosine kinase it recruits
the insulin receptor substrate or IRS to the complex. GRB-2, a 23 kDa cytosolic accessory protein, recruits the son of sevenless (SOS) protein to the complex via its SH2 domain. The SOS protein is a nucleotide exchange factor that has an affinity for the SH2 domain of the Grb-2 protein due to its proline rich residues (Nelson 2005). Once this complex is formed it will recruit c-Ras to the plasma membrane where it can bind GTP. The SOS protein helps facilitate an increased rate of binding of bound GDP-Ras for GTP bound c-Ras. The Ras superfamily of small G proteins are highly conserved and exhibit GTPase binding activity. When Ras bound with GDP is replaced with GTP, a conformational change occurs. This binding activity usually functions similar to that of a molecular switch with a whole host of activities ranging from signal transduction, apoptotic prevention to secondary messaging. When it comes to insulin signaling, GTP bound Ras can stimulate the mitogen activated protein kinase pathway (MapK). The first protein activated in this cascade is Map kinase kinase kinase (Map3K) or Raf-1.
The binding of Raf-1 and its activation by Ras stimulates the phosphorylation of MEK on two Ser residues, thus activating it. MEK or Map kinase kinase (Map2K) continues on through the signal cascade to phosphorylate ERK or MapK at two distinct residues, Thr0202 and Tyr204. Once ERK is activated it can translocate to the nucleus where it can activate nuclear transcription factors like Elk1. Elk1 is responsible for regulating the transcription of over 100 insulin sensitive genes (Parker 2003; Parker 2004).

**Fate of Glucose**

Glucose entering the cell can be bound for multiple fates. There are four different distinct pathways glucose can proceed down: glycolysis, pentose-phosphate pathway, glycogenesis, or the hexosamine biosynthesis pathway.

Typically, upon insulin induction, glucose is taken-up and immediately converted to glucose-6-phosphate (G6P) by hexokinase or glucokinase (liver). This step represents a rate limiting or controlling step in glycolysis. Glucose-6-phosphate can proceed through the glycolytic pathway which involves the catabolic breakdown
of glucose to pyruvate. Pyruvate can function as a substrate in numerous other metabolic processes.

Glucose-6-phosphate can also proceed along the pentose phosphate pathway. The pentose phosphate pathway (PPP) is a metabolic process that generates pentose sugars but more importantly generates NADPH. In this oxidative phase of PPP, G6P is converted to gluconolactone-6-phosphate and eventually to 6-phosphogluconate by G6PDH and gluconolactonase, respectively. 6-phosphogluconate is then converted to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase. This phase generates the two molecules of NADPH which can be used for reductive biosynthesis as in fatty acid synthesis. The non-oxidative phase of the PPP pathway involves the formation of ribose sugars used in nucleic acid synthesis from Ribulose-5-P. Other end products of this pathway are able to feed right into glycolysis.

Glycogenesis or glycogen biosynthesis is the production of glycogen from glucose entering the body. After glucose is converted to G6P by glucokinase it is then converted to glucose-1-phosphate (G1P) by
phosphoglucomutase. G1P is now converted to UDP-glucose by UDP-glucose pyrophosphorylase. This molecule of UDP-glucose can be directly added to the non-reducing end of a growing chain of glycogen branches by glycogen synthase.

**Hexosamine Biosynthesis Pathway (HBP)**

Glucose-6-phosphate can also proceed down the hexosamine biosynthesis pathway to generate UDP-GlcNac (Figure 6). G6P is converted to fructose-6-phosphate (F6P) by phosphofructokinase. F6P can now be shunted through the hexosamine biosynthesis pathway instead of glycolysis and be converted to glucosamine-6-phosphate by an enzyme called glutamine:fructose-6-phosphate amidotransferase or GFAT. GFAT is the rate limiting step in the HBP and therefore controls entry of glucose within the pathway. Glucosamine-6-phosphate can continue on through the pathway forming a uridine diphosphate N-acetyl glucosamine or UDP-GlcNAc. UDP-GlcNAc can be used in the production of various glycoproteins, glycolipids, proteoglycans and gangliosides. In the HBP there are two enzymes that regulate all post-translational
The first enzyme is O-GlcNAc transferase or OGT. OGT is responsible for the reversible O-linked GlcNacylation on numerous substrates such as transcription factors like Sp1 and glycogen synthase (Andreozzi 2004) and other enzymes involved in insulin signaling like IRS-1 (Buse 2002) and the glucose transporter Glut4 (Marshall 1991). It is interesting to note that GlcNacylation sites are typically on or near sites known to be phosphorylated. This can be problematic when these phosphorylation sites which are necessary for the insulin signaling cascade to proceed are occupied or sterically hindered by the presence of a GlcNac moiety. The other enzyme involved in the O-linked GlcNacylation events is O-GlcNacase. This enzyme is solely responsible for the removal of the post-translational modification of GlcNac of various substrates (Fantus 2006).

The hexosamine biosynthetic pathway is up-regulated when circulating levels of glucose within the body are high, which is the case for people with diabetes. In 1991 Marshall et al showed that there was indeed a correlation
between excess glucose flux being shunted through hexosamine biosynthesis pathway and eventual insulin resistance and or desensitization of the glucose transport system (Marshall 1991). In this study high levels of insulin and glucose in the presence of glutamine could cause impairment of glucose transport. Furthermore glucosamine can enter the hexosamine biosynthesis pathway distal to GFAT and cause that same impairment with glucose transport (Rossetti 1995). Since then numerous labs have shown that glucosamine can induce insulin resistance in a variety of model (Virkamaki 1997) (Fülöp 2007) by bypassing the rate limiting step GFAT which controls the flux through the HBP.

It could also be that the end products of this pathway are believed to be involved in altered gene expression and maybe a mechanism responsible for some of the disease states associated with Type II diabetes mellitus. High levels of UDP-GlcNAc generated through the HBP pathway is believed to be involved in the up-regulation of O-linked GlcNAcylation events which can
post-translationally modify proteins involved in gene transcription.

Figure 6- Hexosamine Biosynthesis Pathway. (McNeill 1992; Berg 1995). Copyright permission obtained from Oxford University Press provided by Copyright Clearance Center. (License # 2911400491159)

As indicated previously, the addition of glucosamine creates a pseudo-flux through the hexosamine biosynthesis pathway and ultimately provides an altered state similar to that of Type II diabetes. This is the insulin resistant state that we adapted for use throughout this thesis.
Insulin resistant states such as type II diabetes or NIDDM are typically treated with insulin injections that can be cumbersome and painful to the patient trying to control diabetes. The injection site for insulin delivery is in the abdomen and it can be challenging. Bruising, skin irritation, insulin leakage from injection site as well as forgetting to even take the insulin injection are all common problems. For this reason a compound that can be administered orally and mimic the effects of insulin are highly sought after in the medical world.

**Vanadium: An Insulin Mimetic**

Numerous types of insulin mimetic compounds have been introduced for study. These include various forms of vanadium (Poucheret 1998), selenium (Stapleton 1997) and even chromium (Wang 2010). Vanadium has a long history of discovery and rediscovery. Vanadium was originally discovered by a mineralogist, Del Rio in 1813 and so named it panchromium due to its formation of various colored complexes formed at different oxidation states (Arya 2011). Later in 1831, a chemist, Nils Gabriel
Sefstom rediscovered vanadium and so named it for a Germanic goddess of beauty, youth and luster “Vanadis” (Crans 2005). Vanadium exhibits numerous oxidations states ranging from -I, 0, +II, +III, +IV, and +V. The vanadyl(IV) and vanadate (V) forms are the predominant structure in solution at physiological pH (Rehder 2008). Under aerobic conditions the vanadate form exists. Under anaerobic conditions, like those similar to that of the interior of a cell i.e. the cytoplasm, the vanadyl ion exists. Inside the cell, vanadate (V) in the form of VO$_2^+$ is reduced to VO$_2^{2+}$ by reducing agents such as NADH and glutathione (Smith 1988).

Vanadium is a group V transition metal and is found in relatively low abundance in the earth crust (0.02%). There are over 60 different minerals that contain vanadium as well as many known vanadium containing compounds including “vanadoenzymes” or haloperoxidases from marine algae and a vanadium containing cofactor involved in nitrogenase activity in Azotobacter chroococcum (Smith 1988). In mammals vanadium is a trace element found in many dietary foods in relatively low
amounts. The total body pool of vanadium was determined to be around 100 μg and that accumulation of vanadium could be found in muscle, bone, fat and other tissues such as liver and kidneys (Byrne 1978). In some cases vanadium is considered an essential trace element and can be linked to cell growth and reproductive fitness (Ramasarma 1981).

Vanadium has been used in vitro and whole animal models with insulin mimetic like effects ranging from increased glucose uptake and glucose oxidation, inhibition of lipolysis, increased lipogenesis (Pugazhenthi 1991) and increased synthesis of glycogen (Rossetti 1989). Vanadium has also been shown to mimic insulin at the gene expression level by down regulating new glucose production in the liver by influencing pyruvate kinase in primary rat hepatocytes in culture (Miralpeix 1991) and phosphoenolpyruvate carboxykinase (PEPCK) in rat hepatoma cells (Bosch 1990). One of the major contributing insulin like effects of vanadate is its ability to normalize blood glucose (Meyerovitch 1987) and enhance glucose transport in
numerous tissue types such as adipocytes (Shechter 1980), skeletal muscle (Okumura 1992), brain (Meyerovitch 1989), and liver (Brichard 1993). Vanadate was also shown to normalize lipogenic enzymes such as G6PDH, Acetyl-CoA carboxylase, malic enzyme and ATP citrate lyase indicating that these actions are similar to insulin mediated pathways (Khandelwal 1995).

Vanadium and vanadium salts have been used to treat the symptoms of diabetes since as early as 1899. In this study, Lyonnet and Martin published an article in La Presse Medicale, roughly translated as the therapeutic uses of vanadium derivatives (Lyonnet 1899). However it was years before vanadium was again studied in this capacity. More recently vanadium and vanadium salts have been shown to stimulate glucose transport. Tolman et al. was the first to show that glucose oxidation and transport in rat adipocytes was altered upon treatment with vanadate (Tolman 1979). Many studies since then have shown that in cell types such as muscle and adipose tissue, vanadate has a positive effect on glucose transport. Interestingly enough, this positive up
regulation was shown to be independent of the insulin receptor substrate 1 or IRS-1 phosphorylation (Green 1986). In another study, it was also concluded that the orthovanadate mechanism of action lies distal or downstream of the insulin receptor. It was determined that after in vivo administration of vanadate in mice diaphragm, no increase in activation of the IR was detected (Strout 1989). Another group demonstrated that the IR activity was not affected in liver and muscle when STZ rats were treated with vanadyl sulfate for a period of 9-12 weeks (Venkatesan 1991). However one group had conflicting results showing that vanadate enhanced phosphorylation of the IR in rat adipocytes (Tamura 1983).

Somehow activation of the Glut 4 transporter and its activity is increased upon treatment with vanadate. It wasn’t until 1985 that the true effectiveness of vanadate was realized when Heyliger et al demonstrated that STZ (streptozotocin) diabetic induced female wistar rats treated over a period of 4 weeks with sodium orthovanadate showed normalized blood glucose levels
despite low plasma insulin levels compared to that of the control (Heyliger 1985). Additionally, vanadate restored male wistar STZ induced hyperglycemic rats to a state similar to that of normoglycemia (Sun 2000).

Some speculate that the action of vanadate is through inhibition of protein tyrosine phosphatases (PTPases) including one that is responsible for deactivation of IRS-1. If IRS-1 remains phosphorylated, then the activation of downstream targets ie PI3K and Akt retain signaling cascade induction. It has been shown that sodium orthovanadate in primary rat hepatocytes in culture can influence or inhibit the function of the Src homology 2 domain responsible for signal transduction (Pugazhenthhi 1995). Khandelwal et al also showed that PTPase activities in obese Zucker fatty rat livers were significantly decreased in rats treated with vanadate (Pugazhenthhi 1996). John McNeill’s group demonstrated that bis-maltolato oxovanadium (IV) inhibits the activity of the enzyme (PTP1B) responsible for de-phosphorylation of tyrosine residues on the insulin receptor (IR) in Zucker fatty rat skeletal muscle (Mohammad 2002). All of
these papers suggest that vanadium and its salts can be used for therapeutic benefits in the tissues associated with insulin resistance. As with any positive outcome negative aspects such as toxicity, needs to be evaluated.

**Vanadium and Toxicity**

Vanadium has been shown to have many beneficial and therapeutic effects with regards to insulin action. However, there is a great deal of evidence to suggest that vanadium is toxic with increasing concentration and increasing valance states (Llobet 1984). The most common effect of sodium vanadate in humans was mild gastrointestinal discomfort when administered to IDDM and NIDDM patients at 125 mg/day. It was also reported that in human trials, a tolerance of 75-300 mg doses of vanadyl sulfate for 6 weeks in NIDDM patients was observed (Goldfine 2000). Furthermore, insulin sensitivity in these NIDDM patients showed improvement for 2 weeks following the halting of treatment (Goldfine 2000). Sodium orthovanadate was shown to be toxic at a concentration of 2 mM in isolated perfused rat livers. Vanadate treatments showed a release of cytosolic
glutamate-pyruvate-transaminase (GPT) lactate dehydrogenase (LDH) and mitochondrial glutamate dehydrogenase (GLDH) enzymes as well as a large decrease of hepatic glutathione (Younes 1991). GPT and GLDH are used as a clinical diagnostic measurement of hepatocyte injury and overall health so these findings do indicate liver toxicity.

**LY294002 Inhibitor of PI3K**

Lilly Inhibitor 294002 or 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one is a reversible inhibitor of phosphatidylinositol-3-kinases (Vlahos 1994). LY294002 is a derivative of morpholine, a common additive in fossil fuels and nuclear power plants as well as an emulsifier in shellac based waxes for fruit preservation (McGuire 1999). It was shown that LY294002 had no effect on the tyrosine phosphorylation of the IR or IRS-1 in 3T3 mouse fibroblast over-expressing human insulin receptors (Sanchez-Margalet 1994). The LY294002 was used successfully to inhibit downstream actions of PI3K such as Akt (Jiang 2010). It has been shown that Akt can function in a number of pathways including apoptosis and...
survival mechanism. In many types of cancer, the Akt pathway is commandeered by the cancer metabolism to overcome the normal signal for cell death. It has been observed that many forms of cancer such as gastric, renal cell, ovarian (Shayesteh 1999) and lung cancers have constitutively activated pathways involving PI3K/Akt.

**History of Hepatocyte Isolation**

In 1951 the notion of isolating primary liver cells from adult Sprague-Dawley rats became evident (Anderson 1953). However the isolation to do so was crude and ineffective yielding only 5% of to total starting material. Later in 1953 the method was perfected by identifying substances such as citrate, pyrophosphate, versene or ethylenediaminetetraacetic acid (EDTA), and ATP additions to perfusion medium had equally beneficial effects (Anderson 1953). Upon blanching and perfusion of the liver, the liver was then processed by pressing the tissue in a Pyrex homogenizer tube via lucite pestal and then pressing it through “bolting silk.” The eventual isolation yielded only 40-60% of the actual tissue
started with. This mechanical force needed to separate the liver cells from the “cement” is believed to result in damage to the cells and loss in total cells harvested. In 1967, Howard et al discovered that the use of collagenase, a collagen digestive enzyme, to disrupt cellular cohesiveness of the liver slices ultimately yields intact liver cells at much higher viability (Howard 1967).

It wasn’t until much later in 1969 when Berry and Friend developed a re-circulating perfusion method that used collagenase and hyaluronidase buffer solutions. That method also employed the use of shaking and filtration of digested liver through nylon mesh gauze (Berry 1969). Several years later a breakthrough was made in the isolation technique. It was found that 5 mM concentrations of calcium 2+ ion is needed for effective dispersion of the liver tissue (Seglen 1973) and in fact it was a requirement of the collagenase. Since this time hepatocyte isolation and the frequency of success has increased dramatically. Numerous laboratories employ the
use of this technique, each with their own modification for improved isolation i.e. “trade secrets.”

**Objective of the Study**

The specific aim of this study was to continue to evaluate the ability of glucosamine to induce an insulin resistance state in primary rat hepatocytes. Furthermore, we hypothesized that the insulin mimetic sodium orthovanadate could overcome this glucosamine induced insulin resistant state. Our question became, can sodium orthovanadate stimulate key signaling proteins similar to the mechanism of action of insulin in a normal state when glucosamine is present.

**Specific Aims of the Study**

1. Determine effects of glucosamine on the activity or phosphorylation state of key metabolic enzymes in the insulin signaling cascade and establish insulin resistance.
2. Determine whether or not the insulin mimetic sodium orthovanadate can circumvent insulin resistance and
restore insulin signaling in key metabolic enzymes shown to be insulin resistant.

3. Use the inhibitor LY294002 to potentially elicit mechanistic action of sodium orthovanadate.
CHAPTER II

MATERIALS AND METHODS

Materials

Sodium orthovanadate, glucosamine-HCl, heparin sodium salt from porcine intestinal mucosa (158 U/mg), Waymouth MB 752/1 medium, hyaluronidase (801 U/mg) from bovine tests, acrylamide, N,N′-(1,2 Dihydroxyethylene) bisacrylamide, sodium chloride, calcium chloride, potassium chloride, sodium dodecyl sulfate, tetramethylethylenediamine (TEMED), ammonium persulfate (APS), phenol red, sucrose, and fructose were purchased from Sigma Aldrich (St. Louis, MO). Collagenase type IV (272 U/mg) was obtained from Worthington Biochemicals (Lakewood, NJ). Porcine insulin was obtained from Eli Lilly (Indianapolis, IN). The Phototope-HRP Western Blot Detection System which includes Anti-rabbit IgG, HRP-linked antibody, biotinylated protein ladder along with Lumiglo detection reagents A and B were obtained from Cell Signaling Technology, Inc (Danvers, MA). Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) antibody, phospho-Akt
(Ser473) rabbit monoclonal antibody and Phospho-GSK-3α/β (Ser21/9) (D17D2) Rabbit mAb were obtained from Cell Signaling Technology, Inc (Danvers, MA). Phospho-IRS-1 Antibody (Tyr 989) (sc-17200-R) rabbit polyclonal antibody were obtained from Santa Cruz Biotechnology. Supplies for cell culture include Dulbecco's Modified Eagle Medium (DMEM) which contains 1,000 mg/L D-glucose and 110 mg/L sodium pyruvate, but no L-glutamine or phenol red (Gibco). Materials and supplies for Western blot analysis include SeeBlue Plus2 protein standard, NuPAGE LDS sample buffer, Novex Tris-Glycine Transfer Buffer (25X), Novex 10% Tris-Glycine and 4-12% Bis-Tris Mini Gels 1.0-1.5 mm, 10 well gels. iBlot system with iBlot transfer stacks regular nitrocellulose and polyvinylidene fluoride (PVDF) 0.2 μM pore size membrane (Invitrogen). Kodak Biomax XAR film (8 in x 10 in) (Sigma) was used to image chemiluminescence of bound antibodies reacting to substrate in immunoblot assay. Falcon tissue culture dishes (60mm x 15mm) round polystyrene vacuum gas plasma treated culture plates
(Becton Dickinson) were used to culture primary rat hepatocytes.

**Cell Culture**

Male Sprague Dawley (SD) rats (Charles Rivers) were housed in an approved (IACUC) animal facility at Western Michigan University (WMU). SD rats were feed Lab Diet 5001 standard rat chow and drinking water ad libitum. The rats were housed one per cage on a 12 h light/dark schedule. SD rats weighing approximately 200-400 grams were food deprived two days prior to surgery. The rats were anesthetized through an injection into the peritoneal cavity with approximately 35-50 cc of pentobarbital sodium salt depending on overall weight (45-50 mg/kg).

Perfusion and digestion of the liver was previously described (Wagle 1998). The liver is cut into many pieces and placed onto sterile gauze covering a 600 mL beaker. The digestion solution is poured over the tissue which is then pushed through gauze to isolate primary liver cells within the digestion solution. Approximately 300 mL of cell suspension/digestion solution are poured
into six 50 mL conical tubes and spun for 3 mins at 100xg. The media is then aspirated leaving the mass of cells behind. Cells are then resuspended in Waymouth serum free media + BSA, concentrated into two smaller volumes and transferred to 50 mL concical tubes. Once again the cells are spun for 3 mins at 100xg and media aspirated. Cells are then resuspended in Waymouth media and washed for a third time using the same process stated above. The primary rat hepatocytes are combined into one conical tube and suspended in 5-10 ml of media depending on the size of the pellets. Cells can now be counted and plated.

**Trypan Blue Exclusion**

Viable cells are counted using a hemocytometer and the trypan blue exclusion method (Tennant 1964). The trypan blue is able to penetrate the compromised cells due to membrane disruption therefore viability can be determined. Once the viability and number of cells is determined, cells are plated to yield a confluence of 90% or greater if possible. Cells below 70% viability are not used. Typically, between 2.0-3.0 x 10^6 cells per
plate are used. The primary cells are plated on collagen-coated plates which allows for cell adherence in Waymouth media with the addition of BSA. After 4 hrs the media is changed to a BSA free Waymouth serum and left for 18 hrs prior to treatment in a 37°C humidified incubator containing 5% CO₂.

**Preparation of (10x) Perfusion and Digestion Solutions**

The perfusion 10x solution is made by combining 43.5 g NaCl, 12.0 g HEPES, 15.0 g Fructose, 0.95 g EGTA or ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid. The volume is brought to 450 mL along with the subsequent addition of 6 mL of a 0.5% Phenol red solution. Solution is then brought to a pH of 7.4 with NaOH, diluted to 500 mL and passed through a 0.22 μm sterile filter.

The digestion 10x solution is made by combining 2.5 g KCl, 41.5 g NaCl, 12.0 g HEPES, 3.7 g CaCl dihydrate, 15.0 g Fructose. The volume is then brought to 450 mL along with an addition of 6 mL of a 0.5% Phenol red solution. The solution is then brought to a pH of 7.4
with NaOH, diluted to 500 mL and passed through a 0.22 μm sterile filter.

**Preparation of (1x) Perfusion and Digestion Solutions**

The perfusion 1x solution is made by diluting the 10x stock. 30 mL of the 10x stock solution is taken and diluted to 250 mL. To this solution an anticoagulant heparin sulfate is added at 10 Units/mL. This solution is then brought to a pH of 7.4, diluted to 300 mL and passed through a 0.22 μm sterile filter. Solutions are incubated at 37° C in a water bath.

The digestion 1x solution is made by diluting 50 mL of the 10x stock to 450 mL along with the addition of 3% BSA, 93 Units/mL of hyaluronidase from bovine testes, 160 Units/mL of trypsin inhibitor. The 1x digestion solution is brought to a pH of 7.4, diluted to 500 mL after the addition of the tissue digestive enzyme Type IV collagenase at a final concentration of 100 Units/mL. The solution is then passed through a 0.22 μm sterile filter and incubated at 37° C in a water bath.
Preparation of Collagen Coated Tissue Culture Plates

Rat tail collagen is diluted from a stock concentration ranging from 4.0-5.0 mg/mL down to 0.2 mg/mL. 1 mL of the diluted collagen solution is then added to 60 mm x 15 mm round tissue culture dishes that have been vacuum gas plasma treated by the manufacturer. The solution is swirled and allowed to sit for approximately 2-3 mins. This step is repeated following removal of the collagen solution. Plates are allowed to dry overnight in the Baker biological safety cabinet under UV light.

Treatment Schedule

Primary rat hepatocytes are either cultured in DMEM low glucose media or with DMEM low glucose media + 1 mM glucosamine. After an 18 hr incubation in glucosamine, the cells are used to study insulin resistance. Cells that were not incubated in DMEM + glucosamine were used as the normal control model for purposes of comparison. (N) = no addition, (G) = (1 mM) glucosamine, Ins = (80 nM) Insulin
Table 1- Treatment Schedule of Primary Rat Hepatocytes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.Na</td>
<td>DMEM low glucose media for 24 hrs.</td>
</tr>
<tr>
<td>N.Ins</td>
<td>DMEM low glucose media for 24 hrs. Insulin (80nM) at hr 23.</td>
</tr>
<tr>
<td>G.Na</td>
<td>DMEM low glucose media + 1 mM glucosamine for 24hrs.</td>
</tr>
<tr>
<td>G.Ins</td>
<td>DMEM low glucose media + 1 mM glucosamine for 24 hrs. Insulin (80nM) at 23 hrs.</td>
</tr>
<tr>
<td>N.500.1</td>
<td>DMEM low glucose media for 24 hrs. Vanadate (500 uM) at 18 hrs.</td>
</tr>
<tr>
<td>G.500.1</td>
<td>DMEM low glucose media + 1 mM glucosamine for 24 hrs. Vanadate (500uM) at 18 hrs.</td>
</tr>
<tr>
<td>N.LY.Ins.1</td>
<td>DMEM low glucose media for 24 hrs. LY294002 (50 uM) at 22 hrs. Insulin (80nM) at 23 hrs.</td>
</tr>
<tr>
<td>G.LY.500.1.1</td>
<td>DMEM low glucose media + 1 mM glucosamine for 24 hrs. LY294002 (50 uM) at 17 hrs. Vandate (500 uM) at 18hrs.</td>
</tr>
</tbody>
</table>
**BCA Protein Assay**

Protein concentrations were determined using the Pierce BCA protein assay kit. The BCA protein assay employs the reagent bicinchoninic acid. The reagent forms a copper complex which exhibits strong absorbance. The protein/copper complex absorbance is measured spectrophotometrically at 562 nm wavelength and is proportional to the amount of protein present. A series of BSA standard solutions ranging from 25-2000 µg were made with the provided 2 mg/mL stock solution of BSA. These standards and their measured absorbance at 562 nm were used to create a standard curve to determine unknown protein concentration in whole cell lysates from the treated primary rat hepatocytes. Concentrations were determined by employing the protocol above. This protein assay and its procedure were originally described in a paper by Smith et al (Smith 1985).

**SDS-PAGE**

Sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) employs the use of a strong
detergent and a separating matrix composed of acrylamide and bisacrylamide chemically crosslinked. This polymerization is catalyzed by the addition of TEMED or tetramethylethylenediamine and ammonium persulfate (APS). APS is a strong oxidizing agent that creates radical species which facilitate the polymerization. SDS is a strong detergent that denatures the protein from its tertiary and quaternary structures down to the primary protein structure of single amino acids bound by polypeptide bonds. This detergent also coats the protein in an overall negative charge. This allows for the separation of all the proteins in the whole cell lysate to be solely based on the molecular weight. Since the proteins are negatively charged they will migrate toward the positive cathode in a polyacrylamide gel matrix. The SDS-PAGE is composed of two layers: a stacking gel and a separation gel. The stacking gel typically contains only 4% polyacrylamide while the separating gel was composed of 10-12% based on protein of interest. Latter studies employed the use of pre-poured gels from Novex and consisted of 10% Tris-Glycine or 4-12% Bis-Tris.
**Western Blot Analysis**

Upon separation of whole cell lysate/protein slurry via SDS-PAGE, the proteins are transferred to a nitrocellulose membrane. This technique is performed in an electroblotting apparatus with Tris glycine transfer buffer or an iBlot western blotting transfer system. Once protein has been transferred to this membrane, immunoblot analysis can begin. The membrane is first incubated in 0.1% non-fat dry milk in Tris buffered saline + Tween 20 (200 mM Tris Base, 150 mM NaCl, 0.05% Tween-20, pH 7.6) (TBS-Tween) overnight at 4°C with gentle agitation to saturate non-specific binding sites. The membrane is then incubated in primary antibody overnight at 4°C with gentle agitation. Once the primary antibody is bound, the membrane is washed 3x in TBS-Tween for 5-45 mins. The membrane is then incubated in a secondary (2°) antibody which contains horseradish peroxidase (HRP) linked anti-rabbit IgG and HRP-linked anti-biotin antibodies. After incubation in 2° antibody, the membrane is again washed 3x in TBS-Tween for 5-45 mins. A luminol substrate containing hydrogen peroxide is added to the membrane.
causing a chemiluminescent reaction which emits light. The luminol is converted to 3-aminophthalate in the presence of hydrogen peroxide to produce photons of light. This emitted light is due to 3-aminophthalate decaying from an excited triplet state to a lower energy level. This radiation or light can be captured on XAR film.
CHAPTER III

RESULTS AND DISCUSSION

Effect of Glucosamine on Insulin Induced IRS-1 Phosphorylation

In an insulin resistant state, the addition of insulin is no longer able to stimulate the phosphorylation cascade of key proteins that are part of the insulin signal cascade or at least not to the same extent that is observed under normal conditions. In our study, we utilized glucosamine, a substrate that enters the hexosamine biosynthesis pathway downstream of the rate limiting enzyme GFAT to mimic excess glucose in the system and flow through this pathway to generate an insulin resistant model in primary rat hepatocytes.

If insulin resistance is generated then insulin should no longer be able to stimulate to the same extent as insulin under normal condition the increase in phosphorylation of a key signaling protein in the insulin signal cascade, IRS-1. As shown in Figure 7, the
addition of 80 nM insulin to primary rat hepatocytes for 1 hr shows an increased phosphorylation of IRS-1 1.4 fold over control as measured by western blot analysis. Incubation of these cells with 1 mM glucosamine for 24 hrs has no significant effect compared to the cells with no addition. In the presence of glucosamine, the addition of 80 nM insulin for one hour was not able to increase the phosphorylation of IRS-1 compared to the cells treated with insulin alone. Thus, insulin resistance was confirmed. Glucosamine alone had no toxic effects on the cells as no morphological changes were observed. This shows the effectiveness of the compound, glucosamine, to induce a model for insulin resistance for further study.
Figure 7 - Effect of glucosamine on insulin induced IRS-1 phosphorylation. Primary rat hepatocytes were incubated in DMEM low glucose media without (N) or with (G) 1 mM glucosamine for 24 hrs. Cells were either treated with 80 nM Insulin (Ins) after 23 hrs or left untreated (NA). Cells were processed, with whole cell lysate collected. The results are expressed as the mean ±SEM of N number of animals used for each experiment. The difference between nontreated and treated cells were evaluated by one-tailed student’s t test. Statistical significance * was tested at p<0.05.

Effect of Sodium Orthovanadate on Glucosamine Induced Insulin Resistance and the Phosphorylation of IRS-1

Insulin resistance continues to be a growing medical issue world-wide. It is important to not only understand possible mechanisms that cause the disease but also
explore new avenues of treatment. Insulin mimetics are naturally occurring compounds that have been used for over a century. With these insulin mimetics and model of insulin resistance, through the use of glucosamine, the signaling pathway and the mechanism of action for the insulin mimetic vanadate can be studied. As was demonstrated, insulin increased the phosphorylation nearly 1.5 fold of the key signal protein IRS-1 when compared to the control (Figure 7). However, when glucosamine is added to establish the insulin resistant state the effects of insulin to stimulate phosphorylation of IRS-1 is markedly decreased. As seen in Figure 8, the insulin mimetic sodium orthovanadate was able to mimic the effects of insulin in the cells without glucosamine treatment by increasing the phosphorylation of IRS-1. Furthermore, it was also able to increase the phosphorylation of IRS-1 in the cells treated with glucosamine. In the non-diabetic model, cells treated with LY294002 an inhibitor of PI3K a downstream protein of IRS-1, insulin showed little to no effect on IRS-1 phosphorylation. This result was unexpected. Since this
inhibitor works downstream of IRS-1, it was expected that
treatment with LY294002 and insulin would show an
increase in phosphorylation similar to that of insulin
alone. Also, in the glucosamine induced diabetic model,
cells treated with LY294002 and vanadate showed a marked
decrease compared to that of the no addition non-diabetic
control. Thus, while the effect of LY294002 was
unexpected, vanadate and insulin in the presence of this
compound mirrored each other.
Figure 8- Effect of sodium orthovanadate on glucosamine induced insulin resistance and the phosphorylation of IRS-1 TYR989. Primary rat hepatocytes were incubated in DMEM low glucose media without (N) or with (G) 1 mM glucosamine for 24 hrs and treated with 500μM sodium orthovanadate (500.V) added at 18 hrs. Cells were either treated with 80 nM Insulin (Ins) after 23 hrs, LY294002 (LY) or left untreated (NA). LY294002 was added 1 hr prior to Ins or Van. Cells were lysed and processed with whole cell lysate collected. The results are expressed as the mean ±SEM of N number of animals used for each experiment. The difference between nontreated and treated cells were evaluated by one -tailed student’s t test. Statistical significance * was tested at p<0.05.
Effect of Sodium Orthovanadate on Glucosamine Induced Insulin Resistance and the Phosphorylation of GSK3β

It has been shown that phosphorylation on Ser 9 of GSK3β by the insulin signaling cascade is the underlying mechanism of its inhibition (Sutherland 1993) (Summers 1999). In the case of insulin resistance, the ability of insulin to stimulate the synthesis of new glycogen in the liver and muscle is diminished. Insulin fails to elicit a normal function and promote glycogen synthesis. Any compound that could restore this function could represent a possible therapeutic target. Many labs have implicated an insulin mimetic effect of vanadate on restoring glycogen synthesis in a variety of tissues (Tamura 1984) (Rossetti 1989) (Pugazhenthi 1991) (Khandelwal 1995) (Semiz 2002). We set out to determine if sodium orthovanadate could restore the phosphorylation and deactivation of glycogen synthase kinase. Our results indicate that insulin was able to stimulate over a 2 fold increase in GSK3β phosphorylation when compared to the control. Sodium orthovanadate was also able to increase phosphorylation of GSK3β almost 2 fold.
as well. Interestingly, the mimetic did not restore the insulin resistant state. The results for GSK3α are very similar to what we observed with GSK3β, a lack of phosphorylation induction in the glucosamine induced insulin resistant state. The LY294002 inhibitor was able to decrease the effects of the insulin induction compared to the insulin stimulated induction in the non-diabetic model.
Figure 9- Effect of sodium orthovanadate on glucosamine induced insulin resistance and the phosphorylation of GSK3β Ser9. Primary rat hepatocytes were incubated in DMEM low glucose media without (N) or with (G) 1 mM glucosamine for 24 hrs and treated with 500μM sodium orthovanadate (500.V) added at 18 hrs. Cells were either treated with 80 nM Insulin (Ins) after 23 hrs, LY294002 (LY) or left untreated (NA). LY294002 was added 1 hr prior to Ins or Van. Cells were lysed and processed with whole cell lysate collected. The results are expressed as the mean ±SEM of N number of animals used for each experiment. The difference between nontreated and treated cells were evaluated by one-tailed student’s t test. Statistical significance * was tested at p<0.05.
Figure 10- Effect of sodium orthovanadate on glucosamine induced insulin resistance and the phosphorylation of GSK3α Ser21. Primary rat hepatocytes were incubated in DMEM low glucose media without (N) or with (G) 1 mM glucosamine for 24 hrs and treated with 500μM sodium orthovanadate (500.V) added at 18 hrs. Cells were either treated with 80 nM Insulin (Ins) after 23 hrs, LY294002 (LY) or left untreated (NA). LY294002 was added 1 hr prior to Ins or Van. Cells were lysed and processed with whole cell lysate collected. The results are expressed as the mean ±SEM of N number of animals used for each experiment. The difference between nontreated and treated cells were evaluated by one -tailed student’s t test. Statistical significance * was tested at p<0.05.
Effect of Sodium Orthovanadate on Glucosamine Induced Insulin Resistance and the Phosphorylation of Akt

Interestingly, the insulin mimetic sodium orthovanadate has little to no effect on the phosphorylation of Akt in the non-diabetic control state or the glucosamine induced insulin resistant state. Insulin was able to increase the induction of phosphorylated Akt 1.8 fold over the no-addition control at a concentration of 80 nM. However, when the cells were treated with vanadate no significant increase in phosphorylated Akt was observed using western blot analysis. The same results were obtained for a non-diabetic model and treatment with sodium orthovanadate by Mehdi et al. (Mehdi 2005). In this study the group used CHO-HIR cells (Chinese hamster ovary cells over-expressing human insulin receptor) incubated with 100 nM insulin for 5 min or 1 mM of vanadium compounds for 10 min. They demonstrated vanadium compounds such as vanadyl sulfate, BMOV induced phosphor-Akt Ser473 phosphorylation. However, sodium metavanadate (NaMV) and sodium orthovanadate did not illicit any response. It
appears that there is a difference in the action of inorganic vanadium compounds like sodium orthovanadate (used in this study) and vanadium metal-organic such as BMOV. This phenomenon of higher potency of organic vanadium compounds over vanadium salts has been observed before (Reul 1999). The data suggest that differences in potency between compounds are due to differences in their insulin-like properties.
Figure 11- Effect of sodium orthovanadate on glucosamine induced insulin resistance and the phosphorylation of Akt Ser473. Primary rat hepatocytes were incubated in DMEM low glucose media without (N) or with (G) 1 mM glucosamine for 24 hrs and treated with 500μM sodium orthovanadate (500.V) added at 18 hrs. Cells were either treated with 80 nM Insulin (Ins) after 23 hrs or left untreated (NA). Cells were lysed and processed with whole cell lysate collected. The results are expressed as the mean ±SEM of N number of animals used for each experiment. The difference between nontreated and treated cells were evaluated by one -tailed student’s t test. Statistical significance * was tested at p<0.05.

**Discussion**

With 285 million cases worldwide a growing epidemic of insulin resistance is in our “midst.” Alarmingly, the number of new cases of diabetes is projected to increase
to 435 million by the year 2030. According to the International Diabetes Federation, a person dies every seven seconds from diabetes. New and novel treatments are needed to combat this disease state. Insulin and other anti-diabetic agents are listed as the 4th most in therapeutical class spending (Kleinrock 2011). Human insulins and synthetic analogues have contributed to 64% of growth in this area with a 1.3 billion dollar increase in total spending, according to the IMS Institute for Healthcare Informatics. Individuals diagnosed with insulin resistance are faced with a myriad of treatment regimes. Some are used alone or in combination with daily injections of insulin, which can be troublesome and painful. One of the more commonly prescribed anti-diabetic drugs is metformin. According to a report by the IMS Institute (Kleinrock 2011), metformin was the 7th most prescribed drug in the United States. In 2010 there was a 16.9 billion dollar increase in oral anti-diabetic medicines. Other anti-diabetic medication such as Thiazolidinediones, a class of glitazones drugs along with sulfonylureas are two other commonly prescribed
drugs to treat insulin resistance that helped to contribute to the escalation in diabetic drugs being used.

Other compounds, such as naturally occurring or synthesized molecules that contain transition metals, have been observed to have insulin mimetic action. While these compounds have offered potential in alleviating diabetic symptoms the basic science behind their mechanism of action still remains elusive. For over a century, scientists have implicated the use of vanadium and vanadium salts to treat insulin resistance in numerous animal models. In many cases glucose homeostasis was corrected and symptoms were managed using vanadate and its derivatives (Heyliger 1985; Meyerovitch 1987; Sekar 1996; Crans 2005). However the mechanism for these treatments and the mode of action that allows vanadate to mimic the actions of insulin remain unclear. Insulin signaling is disrupted in patients with NIDDM. In our case we utilized the increased flux through the HBP pathway to establish an insulin resistant state in primary rat hepatocytes. Our question was to determine
the mode of action or influence vanadate has on the insulin signaling cascade at key metabolic enzymes (IRS-1, Akt and GSK3) and determine whether or not vanadate could still elicit insulin like action even in this insulin resistant. Previously in our laboratory, it was established that glucosamine, in very low concentrations, can induce an insulin resistant state in rat hepatocytes (Sandhya 2011). Our laboratory has a long standing interest in not only understanding how insulin affects key metabolic processes but also how insulin mimetics might stimulate these same processes. In addition to Vanadate (Berg 1995), our laboratory has studied the insulin mimetic selenium (Stapleton 2000; Stapleton 2000). Selenium has insulin mimetic properties that increase PI 3-kinase activity in rat hepatocytes in culture (Stapleton 1998). Other evidence supports the involvement of MAPK and S6 kinases in the insulin-mimetic actions of vanadium and selenium (Hei 1998). Stapleton et al. demonstrated similar results with MAPK. In this study they demonstrated that both the p42 and p44 MAP kinases
are activated when either hepatocytes or adipocytes are incubated in the presence of selenate (Stapleton 1997).

Other recent work from our laboratory demonstrated, in primary rat hepatocytes, that cells incubated with selenium showed an increase in the phosphorylation of Akt Ser473 under glucosamine induced insulin resistance similar to that observed with insulin alone conditions (Sandhya 2011). Since we have observed parallel results with selenium and vanadate in previous studies, it was hypothesized that the insulin mimetic properties of vanadium and its salts could also function in an insulin resistant state similar to that of a normal state. To test this question, primary rat hepatocytes were isolated using the heparin perfusion-collagenase digestion method and vanadate and insulin were tested on key signal proteins under control and glucosamine induced insulin resistance conditions.

The first use of vanadium as an insulin mimetic was demonstrated in the laboratory of Ruth Partridge in 1979 (Tolman 1979). Her group demonstrated that vanadate could stimulate glucose oxidation and transport in rat
adipocytes. Furthermore they measured an increase in the radio-labeled glucose-$^{14}$C conversion to glycogen-$^{14}$C in isolated hepatocytes and diaphragm sections. It was also determined that the uptake of tritiated 2-deoxyglucose in rat adipocytes was increased 6-12 fold, similar to that of a maximally activating concentration of insulin (Tolman 1979).

In 1983 Tamura et al demonstrated that vanadate was able to stimulate glycogen synthase in a manner similar to that of insulin but not identical to that of insulin in rat adipocytes. The group determined that vanadate stimulated the phosphorylation of the insulin receptor on tyrosine residues both in intact adipocytes and in solubilized insulin receptor fractions.

This showed that insulin and vanadate could have similar initial actions on receptor phosphorylation and also act similarly on an intracellular event, such as the activation of glycogen synthase (Tamura 1983; Tamura 1984). Our results support these findings of vanadate increasing glycogen synthase activation indirectly. While we did not directly measure the phosphorylation of
the insulin receptor in the presence of vanadate in our cells, the increase in phosphorylation of the IRS-1 could be indicative of the phosphorylation of the receptor as IRS-1 is downstream in the signal cascade. We showed that vanadate could stimulate IRS-1 phosphorylation (Figure 8) and downstream enzymes responsible for inhibiting glycogen synthase was phosphorylated and thus inhibited (Figure 9, 10).

Vanadate was able to stimulate the phosphorylation and thus deactivation of glycogen synthase kinase 3 which is responsible for inhibiting glycogen synthase. Western blot analysis of IRS-1 phosphorylation on Tyrosine 989 showed an increase upon treatment with 500 μM sodium orthovanadate in the control model without the presence of glucosamine. IRS-1 phosphorylation also increased as well in cells treated with 80nM insulin compared to that of the no addition. This demonstrates that insulin and vanadate have the ability to increase phosphorylation of IRS-1 (Figure 9). In contrast to these findings, Shechter et al has implicated a membranous non-receptor tyrosine kinase that is involved with the insulin like response
produced with vanadate (Elberg 1997). Furthermore, they also determined that the insulin mimetic response of vanadate is independent of the actions of insulin via the IR and IRS-1 (Shechter 1995). The group used quercetin, a cell-permeable inhibitor of the IR, to show that insulin activation of the receptor was blocked and that vanadate induced activation was increased even though the inhibitor was present. This activation was observed in rat adipocytes and may not apply to rat hepatocytes and the action of Vanadate within these cells.

During the 1980’s all these effects of vanadium and its ability to mimic the action of insulin were all demonstrated in “in vitro” studies. With the development and the use of these compounds such as alloxan and streptozotocin (STZ) to induced diabetic models in rodents, it became possible to look at “in vivo” models for study. STZ induced diabetes simulates a model of type 1 diabetes by destroying pancreatic beta cells responsible for secreting insulin. One of the most monumental and pioneering studies was introduced by the McNeil group. They decided to administer sodium
orthovanadate to the drinking water female wistar rats (non-diabetic control) and STZ induced diabetic rats for 4 weeks. They showed that blood glucose levels after treatment were not significantly different when compared to non-diabetic rats despite low insulin levels in STZ induced rats. However, diabetic rats that did not receive the treatment had a blood glucose level that was three times higher than the non-diabetic control group (Heyliger 1985).

The McNeill group also produced two separate papers that concluded BMOV or bis(maltolato)oxovanadium (IV) had insulin mimetic activities that are independent of Akt/PKB. In the first paper the group looked at changes in PI3K expression and activity in rat skeletal muscle from STZ-diabetic and fa/fa obese Zucker rats treated with BMOV for 3 weeks. They determined that glucose utilization was restored in the diabetic model with vanadate but basal levels of PI3K as well as insulin stimulated PI3K were unaffected by treatment with BMOV as compared to the control. These results suggest that BMOV does not influence or reduce hyperglycemia in a PI3K
dependent manner (Mohammad 2001). These results are monumental to the field of study because all studies thus far implicated PI3K in glucose uptake. The second paper used STZ-diabetic rats and determined that chronic treatments of BMOV normalized plasma glucose levels without having an effect on PKB activity. It was concluded that the glucoregulatory effects of BMOV were independent of PKB activity (Marzban 2001). Thus there is prior evidence to suggest that vanadate and its insulin mimetic action and involvement in stimulation of glycogen synthesis via inhibition of GSK3 could be independent of Akt/PKB.

In contrast to these finding a group from the Montreal Diabetes Research Center had observed a different phenomenon. This group observed that organovanadium compounds (OVC) such as BMOV or VAC (vanadium oxo-bis acetyl acetionate) were able to activate IRS-1, Akt/PKB as well as GSK3β in Chinese hamster ovary cells overexpressing human insulin receptor (CHO-HIR) (Mehdi 2005). However, consistent with our findings (Figure 11) inorganic compounds such as sodium orthovanadate and
sodium metavanadate failed to exert any effect on Akt/PKB phosphorylation (Mehdi 2005), suggesting a different mechanism for organic and inorganic variations of vanadium.

If sodium orthovanadate is proposed to be an insulin mimetic then it should perpetuate the same signaling events similar to that of insulin. Our results seemed to indicate that the insulin mimetic agent sodium orthovanadate is able to stimulate the phosphorylation and inactivation of GSK3 independently of Akt (when measured at Serine 473) and thus activating glycogen synthesis (Figure 8 and 9).

It has been demonstrated that Akt activation is dependent on two phosphorylation sites, Thr308 and Ser473 with the latter phosphorylation site being a requirement for full kinase activity (Fayard 2005). Akt has many proteins and substrates that are subject to regulation by its kinase activity including GSK3, forkhead box protein O1 (FOXO1) and tuberous sclerosis complex 1/2 (TSC2). As mentioned earlier in the introduction, PDK1 controls the phosphorylation of the Thr308 residue on Akt/PKB and a
previously unidentified protein deemed PDK2 was thought to be responsible for the Ser473 phosphorylation (Chan 2001; Dong 2005). New evidence has emerged that shows a link to a nutrient sensing pathway involving the multi-protein complex mTORC2 to Akt phosphorylation at Ser473 (Sarbassov 2005). Although relatively little is known about this complex, many have demonstrated a clear link between the mTORC2 complex and many biological processes that include cell cycle progression, cell survival and metabolism. The mTORC2 complex involves the master regulator (mTOR) mechanistic (formerly mammalian) target of rapamycin, rictor, mLST8, mammalian stress-activated protein kinase interacting protein (mSIN1), Protor-1 and Deptor.

It was shown in 2005 that mTOR and the accessory protein rictor were directly responsible for phosphorylation of Akt at Ser473 in Drosophila and numerous human cancer cell lines (Sarbassov 2005). More recently another group demonstrated that mSIN1 was a critical factor responsible for the stabilization of the mSIN1-mTOR-rictor complex ie mTORC2 and that complex is
likely the sole “PDK2” or phosphoinositide-dependent protein kinase 2 responsible for the Akt phosphorylation at Ser473 (Jacinto 2006). It was also shown that the mTORC2 complex is involved in insulin signaling to FOXO but not to TSC2 or GSK3B and it requires mLST8 and rictor. They demonstrated that mTORC2 complex is involved in insulin signaling and subsequent activation of Akt at Ser473 and that signal can be abolished by disruption of the complex (Guertin 2006). Furthermore, that ablation of the complex did not disrupt insulin signaling for GSK3 (Guertin 2006). This becomes very significant due to the fact that many groups have implicated the phosphoserine 473 on Akt as a marker for insulin signaling and activation of GSK3. Due to a lack of an increase in Akt phosphorylation by treatments with sodium orthovanadate and a response by signaling proteins upstream and downstream of the major metabolic regulator we began to question the validity of these findings (Figure 11). However, experiments with inhibitor LY294002 which were intended to shed light on the mechanistic action of vanadate were inconclusive. In conclusion
vanadate was able to stimulate key signaling proteins in the insulin signaling cascade similar to that of insulin. However, the mechanism of action for vanadate is different with respect to insulin inducing phosphorylation at Akt-Ser473 and vanadate did not. Vanadate and Insulin has similar effects on the insulin receptor substrate IRS-1 Tyr989 and GSK3α/β Ser21/9. The future direction of this study should investigate the activation of mTOR along with all the components of the mTORC2 complex in a diabetic model of glucosamine induced insulin resistance and vanadate. The mTORC2 complex has been shown to phosphorylate and activate Akt on Ser473. Vanadate may interact in some way with this multi-protein complex and cause downstream effects circumventing the insulin resistant state.
Date: March 30, 2010

To: Susan Stapleton, Principal Investigator

From: Robert Eversole, Chair

Re: IACUC Protocol No. 10-02-01

Your protocol titled “Regulation of Gene Expression in Hepatocytes” has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals.

Approval Termination: March 30, 2011
REGULATION OF GENE EXPRESSION IN HEPATOCYTES

IACUC Protocol Number: 1002-01
Date of Review Request: 02/01/12
Date of Last Approval: 02/11

1. The research, as approved by the IACUC, is completed:
   [ ] Yes (Continue with items 4-5 below.)  [x] 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? [ ] Yes  [ ] No

Describe the sources used to determine the availability of new findings or publications:

[x] No search conducted (Please provide a justification on an attached sheet.)
[ ] Animal Welfare Information Center (AWIC)
[ ] Search of literature databases (select all applicable)
  [ ] AGRICOLA
  [ ] Biological Abstracts
  [ ] Current Research Information Service (CRIS)
  [ ] Medline
  [ ] Other (please specify):

Date of search: 02/01/12  Years covered by the search: 5
Key words: insulin, insulin-mimetics, G6PDH, PEPCK, AKT, SREBP, insulin resistance, glucosamine

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): 10
Cumulative number of animals used to date: 35

Principal Investigator/Faculty Advisor Signature  Date

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.

IACUC Chair Signature  Date
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