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MECHANISM BY WHICH INSULIN AND ITS MIMETICS, SELENATE AND VANADATE, REGULATE GLUCOSE-6-PHOSPHATE DEHYDROGENASE GENE EXPRESSION

by

Sanjay M. Jivraj

A Thesis
Submitted to the
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in partial fulfillment of the
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Department of Biological Sciences

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Sanjay M. Jivraj

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Sanjay M. Jivraj, M.S.

Western Michigan University, 1998

Glucose-6-phosphate dehydrogenase (G6PDH) is a key enzyme of the pentose phosphate pathway. It controls the carbon flow through this pathway, producing reducing equivalents in the form of NADPH to meet the cellular need for reductive biosynthesis and the maintenance of the cellular redox state. Hepatic expression of G6PDH has been shown to be regulated by hormones, nutrients and some growth factors, however the mechanism by which these factors regulate G6PDH gene expression has not been characterized. Here we investigate the mechanism by which insulin and its mimetics, selenate and vanadate, regulate G6PDH gene expression.

Insulin exerts its tissue specific affect by binding to the cell surface receptor initiating a phosphorylation cascade that reaches a variety of cytosolic and nuclear targets. Using well characterized inhibitors of the insulin signal transduction pathway we demonstrate that PI 3-K and S6K are essential for insulin to regulate G6PDH gene expression, where as the proteins of the Ras/Raf/MAPK pathway are not required. Furthermore, we show that the mimetics, selenate and vanadate, utilize different proteins of the insulin signaling cascade to regulate G6PDH gene expression.

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CHAPTER I

INTRODUCTION

Insulin

Insulin is the most potent physiological anabolic agent known that is released into the bloodstream by the β-cells of the pancreatic islets in response to high blood sugar levels. It regulates diverse physiological processes in a wide variety of cells and tissues (Figure 1), however in muscle, liver, and adipose tissues it primarily stimulates the synthesis and storage of carbohydrates, lipids, and proteins while inhibiting their degradation and release into circulation.

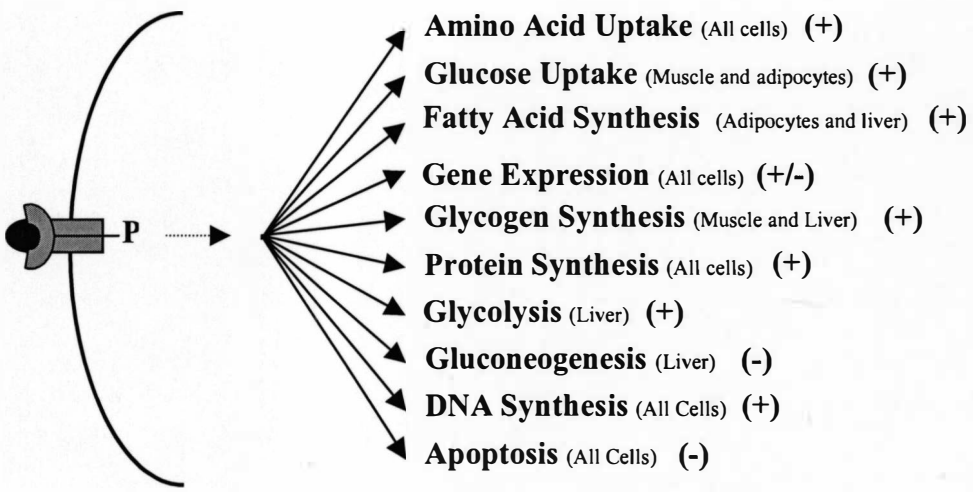


Figure 1. Physiological Processes Regulated by Insulin Upon Binding to Its Cell Surface Receptor. (+) = Processes Stimulated by Insulin, and (-) = Processes Inhibited by Insulin.

The physiological importance of insulin is revealed in diabetes mellitus, a disease that affects 5% of the population in the United States. Diabetes mellitus is classified into two basic types: Insulin-dependent diabetes mellitus (IDDM) and non insulin-dependent diabetes mellitus (NIDDM), both characterized by high levels of circulating glucose (Table 1).

Table 1

Comparison of Insulin Dependent Diabetes Mellitus (IDDM) and Non-Insulin Dependent Diabetes Mellitus (NIDDM)

Characteristic	**IDDM**	**NIDDM**
Level of insulin secretion	None or almost none	Normal or may exceed normal
Typical age of onset	Childhood	Adulthood
Percentage of diabetics	10-20%	80-90%
Basic defect	Destruction of pancreatic β cells	Reduced sensitivity of insulin's target cells
Associated with obesity	No	Usually
Genetic and environmental factors participating in disease?	Yes	Yes
Speed of development of symptoms	Fast	Slow

The mechanism by which insulin exerts its cell and tissue specific effect has only begun to be recently unraveled, as many investigators focus on the role of protein phosphorylation in insulin action. This has led to the identification of a complex network of interrelated and independent signaling pathways engaged in the cell by insulin.

Mechanism of Insulin Action

Following the release by the β-cells of the pancreas, insulin binds to its receptors on the surface of most cells. Classic insulin-responsive cells such as hepatocytes, adipocytes and muscle cells have relatively high levels of these receptors, however, most cells possess some insulin receptors and thus, insulin may affect physiological processes in these as well (Myers et al., 1993).

The insulin receptor is a heterotetrameric transmembrane glycoprotein consisting of two extracellular hormone-binding α subunits and two intracellular signaling β subunits containing tyrosine kinase activity (White et al., 1989). Binding of insulin to the receptor activates the tyrosine kinase, leading to the autophosphorylation of tyrosine residues on several regions of the intracellular β-subunit (Karlsson et al., 1979). This initiates a diverse metabolic and mitogenic signal that reaches its final destination via an elaborate network of intracellular signaling molecules (Figure 2). However, the precise intracellular routes of specific signals responsible for defined aspects of insulin action are not fully understood.

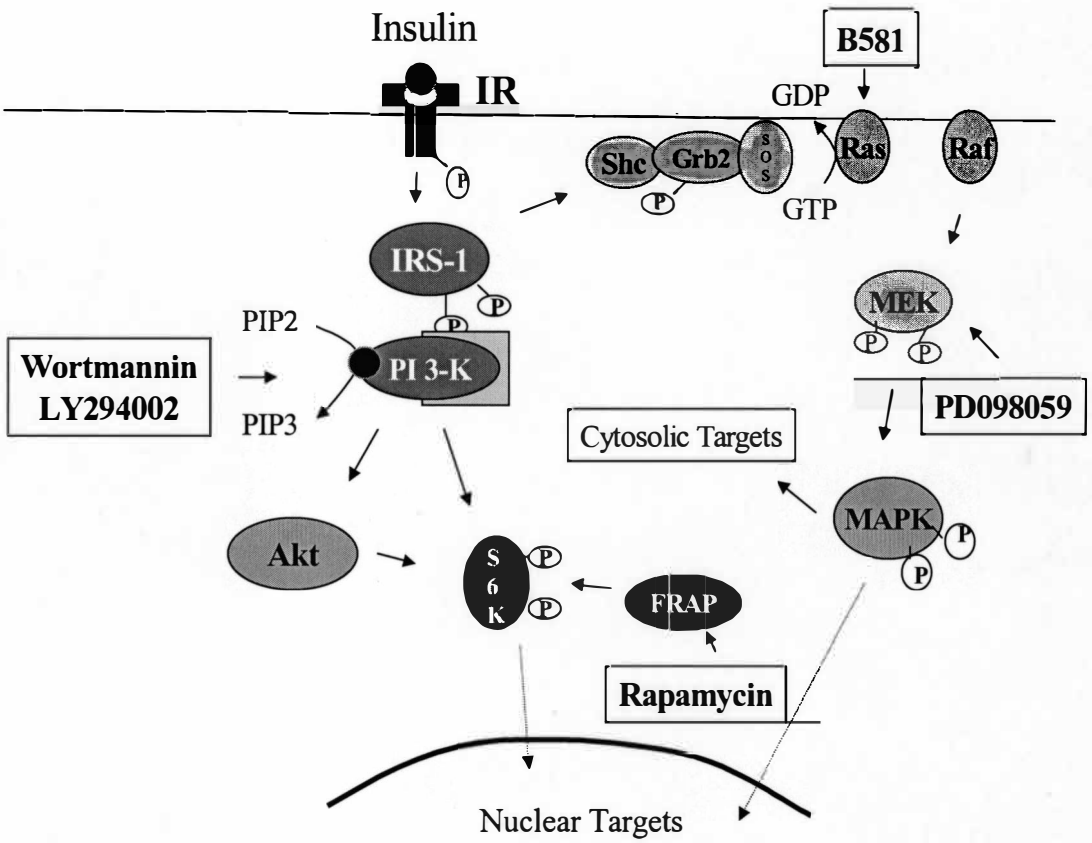


Figure 2. Insulin Signal Transduction Pathway.

Autophosphorylation of the receptor leads to the immediate tyrosine phosphorylation and activation of two families of intracellular proteins known as insulin receptor substrate (IRS) and Shc. Three isoforms of the IRS protein have been identified and designated IRS-1, IRS-2 and a less characterized IRS-3 (p60). IRS-1, a 135kda protein contains several potential tyrosine phosphorylation sites, at least eight

of which are phosphorylated immediately in response to insulin (Myers et al.,1993). Some of these sites reside in a presumed consensus sequence (YXXM) that binds to the *src* homology 2 (SH2) domain (a phosphotyrosine binding domain) of various signaling proteins such as the 85-kda regulatory domain of phosphatidylinositol 3 kinase (PI 3-K), SH-PTP-2, Grb 2 and Nck (Sun et al., 1992). IRS-2 and IRS-3 have been shown to behave in a similar manner in relaying the phosphorylation signal, however, differences in structural features and the levels found in cells suggest the possibility of important differences between IRS-1, IRS-2 and IRS-3 proteins (Lavan et al., 1997).

Both IRS-1 and IRS-2 have been shown to act as a docking protein that forms a signaling complex with PI 3-K, and thus regulates the activity of PI 3-K. Although the precise role of PI 3-K in insulin action remains unknown, activation of PI 3-K has been implicated in the regulation of a variety of insulin mediated metabolic and mitogenic cellular processes (Kappler et al., 1994). These include GLUT-4 mediated glucose uptake, regulation of glycogen synthesis, transport of amino acids, DNA synthesis, membrane ruffling, protein synthesis and transcription of genes, which are important in glucose metabolism (e.g. PEPCK).

The heterodimeric PI 3-K is composed of an 85kda regulatory subunit (p85) and a 110kda catalytic subunit (p110). The p85 regulatory subunit contains multiple domains that mediate interactions with regulatory proteins, such as two SH2 domains, one *src* homology 3 (SH3) domain, two proline rich domains and a Bcr homology domain (Figure 3). The region between the two SH2 domains of p85 is called the inter SH2 (iSH2) region and it binds to the N-terminus of p110 (Klippel et al., 1997).

The insulin-induced activity of the PI 3-K is increased by binding of the two SH2 domaind to the phosphorylated YXXM motif on IRS-1. This dual association of the SH2 domains of the PI 3-K to IRS-1 leads to a conformational change in the p85 subunit resulting in the activation of the catalytic subunit (Figure 4). Upon activation,

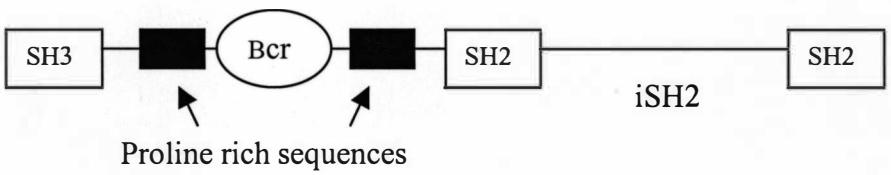


Figure 3. Structural Features of the PI 3-K p85 Regulatory Subunit.

the p110 catalytic subunit of PI 3-K is targeted to the membrane where it phosphorylates phosphatidylinositol-4-monophosphate (PI-4-P) and phosphatidyl-4,5-bisphosphate (PI-4,5-P_2) on the D-3 position forming phosphatidyl-3,4-bisphosphate (PI-3,4-P_2) and phosphatidyl-3,4,5-triphosphate (PI-3,4,5-P_3) , respectively (Whitman et al., 1998). An increase in their levels upon growth factor and insulin stimulation suggests that these D-3 phosphorylated phosphatidylinositides (PPI) are a novel class of secondary messengers (McIlroy et al., 1997).

Inhibition studies of PI 3-K have shown its importance in regulating the activation of several downstream kinases, such as Akt and S6 Kinase that have also been implicated in insulin mediated metabolic and mitogenic responses. Akt is a 60 kDa serine/threonine kinase that is composed of an N-terminal pleckstrin homology (PH) domain, followed by a catalytic domain and a short C-terminal tail. It is

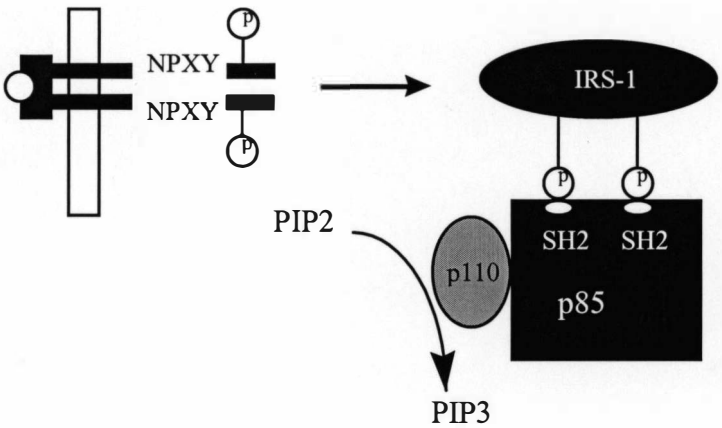


Figure 4. PI 3-K Activation by the IRS Protein.

activated by a variety of growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and insulin. This activation by the growth factors is prevented if the cells are preincubated with inhibitors of PI 3-K (wortmannin and LY 294002) or by overexpression of a dominant negative mutant of PI 3-K (Burgering et al., 1995), suggesting that Akt lies downstream of PI 3-K. The mechanism by which Akt is activated by PI 3-K is still unclear, however two models have been proposed. Franke et al. (1995) and Klippel et al. (1997) have suggested that the lipid products generated by PI 3-K partially activate Akt *in vitro* by binding to its NH_2 terminus PH domain. In direct contradiction to this proposed model, Kohn et al. (1996) have shown that the association of Akt with the iSH2 region of the PI 3-K was essential for the activation of Akt, while the PH domain was not important for its activation. Binding of the iSH2 region of the PI 3-K mediated the phosphorylation of serine/threonine residues on the C-terminus leading to the membrane targeting and the subsequent activation of Akt by insulin. To address this controversy, Andjelkovic

et al. (1996) have proposed that the lipid products of PI 3-K may be important in binding to the PH domain of Akt and recruiting it to the membrane, where serine/threonine kinases such as PI3-K phosphorylate and activate the Akt kinase.

Another downstream target of PI 3-K is S6 Kinase (S6K). It is a serine/threonine kinase that is responsible for phosphorylating the S6 protein of the 40S ribosome complex. Activation of S6K facilitates protein synthesis (Proud et al., 1994). It has also been shown that S6 phosphorylation correlates with a selective upregulation in the expression of a family of mRNAs containing a polypyrimidine tract at their 5' untranslated region. These mRNAs encode ribosomal protein and elongation factors (Terada et al., 1994). S6K has been shown to be directly phosphorylated by PI 3-K (Weng et al., 1995), Akt (Burgering et al., 1995) and FKBP12-rapamycin associated protein (FRAP) (Grammer et al., 1996). The mechanism by which the upstream kinases regulate S6K activation is unclear. However, a recent model suggests that the rapamycin sensitive protein FRAP is necessary for the mitogen stimulated phosphorylation of S6K at the C-terminus which "primes" the enzyme so it can be fully activated by the PI 3-K or Akt (Grammer et al., 1996). The precise role of FRAP and its mode of activation is not clear and needs further attention.

The proteins mentioned above IRS/PI 3-K/AKT/S6K/FRAP form one branch of the insulin induced signaling cascade commonly referred to as the PI 3-K pathway. On the other hand, autophosphorylation of the receptor can also lead to the activation of the Shc/Ras/Raf proteins and subsequent activation of the mitogen activated protein kinase (MAPK) cascade.

Upon phosphorylation by the insulin receptor, IRS-1 and Shc compete for the association with a 23 kda growth factor receptor binding protein (Grb2). Grb2 contains a single SH2 domain flanked by two SH3 domains. These SH3 domains direct the association of Grb2 with a guanyl-nucleotide exchange factor for Ras, termed son of sevenless (SOS), which facilitates the exchange of bound GDP for GTP on the protein Ras (Seger et al., 1995). This exchange causes the activation of Ras, which is further farnesylated and targeted to the membrane. Once membrane bound, Ras interacts directly with the N-terminus of Raf-1, a 75kda serine/threonine kinase and recruits it to the membrane where it is activated by an unknown mechanism (Wood et al., 1992). Subsequent to its activation, Raf-1 activates mitogen activated protein kinase kinase (MAPKK), commonly referred to as MEK. MEK is a serine/threonine kinase and is highly specific in the activation of ERK-1 and ERK-2, commonly known as MAP Kinase (MAPK).

The role of MAPK in insulin action is still controversial, however more recent work has shown MAPK activation to be important in cellular differentiation and mitogenesis. Activated MAPK has many nuclear and cytosolic targets. It has been shown to phosphorylate transcription factors such as $p62^{TCF}$, elk-1, c-jun, c-fos which mediate insulin-induced gene expression. Activated MAPK has also been implicated in the phosphorylation of upstream proteins in the pathway, such as SOS, causing the desensitization of Ras activity (Waters et al., 1995) ensuring that the MAPK pathway is not persistently activated.

Insulin Mimetics

In recent years, great interest has surfaced for using insulin mimicking agents or insulin mimetics as therapeutic agents for insulin-resistant or insulin-deficient forms of diabetes mellitus. Selenate (Se) and vanadate (V) are two such compounds that have been studied with regards to their insulin-mimetic properties.

Selenate

Selenium is a biologically essential trace element as it is an integral component of several enzymes. Several studies have recently shown selenate to effectively mimic the action of insulin. In isolated rat adipocytes, selenate has several insulin-like effects, including stimulating tyrosine phosphorylation of numerous endogenous cellular proteins, glucose transport, cAMP phospodiesterase activity, and ribosomal S6 phosphorylation (Ezaki et al., 1990). Subsequent experiments in streptozotocin (STZ) induced diabetic rats have confirmed these insulin-like effects of selenate in normalizing blood glucose levels (Becker et al., 1996) as well as the hepatic mRNA levels and activities of several enzymes that are important in carbohydrate and fatty acid metabolism. Some of these include glucokinase, L-pyruvate kinase, phosphoenolpyruvate carboxykinase (PEPCK) and fatty acid synthase, important enzymes of glycolysis, gluconeogensis and lipogenesis (Becker et al., 1996), respectively.

While selenate has been shown to be a potent insulin-mimetic in isolated adipocytes and *in vivo*, little work has been done on the mechanism by which selenate

mediates its insulin-like effects. Studies, in isolated adipocytes and hepatocytes have shown that several proteins of the insulin signal transduction pathway, such as the insulin receptor, IRS-1 and MAPK, are phosphorylated in response to treatment with selenate (Ezaki et al., 1990 and Stapleton et al., 1996). This suggests that selenate mediates its action on gene expression via a tyrosine phosphate signal transduction cascade in a manner similar to insulin.

Vanadate

Vanadate, in its various forms, has also been shown to be an effective insulin mimetic both *in vivo* and *in vitro*. Pugazhati et al. (1991) have shown that in diabetic rats, treatment with vanadate normalized the blood glucose levels by increasing glucose uptake and incorporation into glycogen. Furthermore, the decreased levels of key enzymes of carbohydrate and fatty acid metabolism such as glycogen synthase and malic enzyme were also normalized in these animals. *In vitro* studies with cultured rat adipocytes, hepatocytes and muscle cells have also reported several insulin-like properties of vanadate. These include an increase in glucose incorporation into lipid (Duckworth et al., 1988), increase in the levels of fructose-2, 6-bisphosphate, 6-phosphofructo 2-kinase (Miralpeix et al., 1990) and glucokinase (Gil et al., 1988), inhibition of gluconeogenesis (Gil et al., 1988) and lipolysis (Mooney et al., 1989). Non-insulin like effects of vanadate have also been demonstrated in rat hepatocytes where treatment with vanadate inactivated glycogen synthase and activated glycogen phosphorylase (Bosch et al., 1987). These effects are opposite to that observed with insulin.

Several studies aimed at investigating the mechanism of vanadate action have found vanadate to activate the insulin receptor (Pugazhenthi et al., 1991) and its downstream proteins IRS-1 (Fantus et al., 1995) and MAPK (Wagle et al., 1998). However, the mechanism by which vanadate regulates the expression of metabolic genes is still obscure and needs further investigation.

Use of Inhibitors to Elucidate Signaling Cascades

Chemical inhibitors have been instrumental in determining the role of certain signaling proteins in defined aspects of insulin action. Inhibitors of the protein PI 3-K showed that this protein was essential for insulin induced glucose uptake, amino acid uptake, protein synthesis, DNA synthesis and S6K activation in 3T3 L1 adipocytes (Cheatman et al., 1994). Wortmannin, a fungal metabolite, and a structurally distinct chemical 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (also known as LY 294002) (Vlahos et al., 1994), are two well-characterized inhibitors of PI 3-K. Wortmannin inactivates PI 3-K by binding and modifying the catalytic subunit (p110) of the enzyme (Wyamann et al., 1996), whereas LY294002 behaves as a competitive inhibitor for the ATP binding site of PI 3-K. Similarly, B581 (Cox et al., 1994) and 2-(2'-amino-3'-methoxyphenyl)-oxanapthalen-4-one (PD 098059) (Vlahos et al., 1994), inhibitors of proteins in the MAP Kinase pathway, have shown the importance of this cascade in cellular proliferation, differentiation and development (Seger et al., 1995). B581 mimics the CAAX binding site of farnesyltransferase and thus blocks the membrane localization of Ras and subsequent activation of downstream proteins. PD 098059 on the other hand is a specific inhibitor of MEK. Both B581 and PD 098059

are potent inhibitors of MAPK activation in a variety of cell types. An effective inhibitor of S6K, rapamycin has also been characterized. Rapamycin in association with FRAP causes the rapid dephosphorylation and thus inactivation of S6K.

These inhibitors have been used to elucidate the mechanism by which insulin regulates key enzymes in a variety of metabolic processes. Some of these enzymes include hexokinase, glycogen synthase, PEPCK and fatty acid synthase which are important in glycolysis, glycogen synthesis, gluconeogenesis and fatty acid synthesis, repectively. However, key enzymes of the pentose phosphate pathway have not been characterized with regard to insulin action.

Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PDH) is a key regulatory enzyme of the pentose phosphate pathway. It controls the flow of carbon through this pathway catalyzing the net transfer of a hydride ion to $NADP^+$ from glucose-6-phosphate to produce NADPH and 6-phosphoglucono-δ-lactone.

G6PDH is present in most tissues and multicellular organisms where it is constitutively expressed, however in several tissues such as the liver, muscle, lung and adipose tissues, G6PDH gene expression and enzyme activity are regulated by diet, hormones and growth factors (Kletzein et al., 1995). In the liver, this enzyme plays an important role in both carbohdyrate and lipid metabolism. During periods of carbohydrate excess, G6PDH is the primary enzyme responsible for shuttling 50% of the glucose-6-phosphate down the pentose phosphate pathway (Kletzein et al., 1995).

The NADPH that is produced as a result is employed in reductive biosynthesis of fatty acids and cholesterol and in the maintenance of reduced glutathione. Therefore, understanding the mechanism by which G6PDH is regulated is of major importance, as it will provide us with a greater insight into the regulation of both carbohydrate and fatty acid metabolism.

Our lab has recently demonstrated that insulin and its mimetics, selenate and vanadate, regulate G6PDH gene expression and enzyme activity (Berg et al., 1995) both *in vivo* and in primary rat hepatocytes. The mechanism by which they regulate G6PDH gene expression, however, has not been characterized.

Objective of Study

The objective of this study is to elucidate the signal transduction pathway by which insulin and its mimetics, selenate and vanadate, regulate G6PDH gene expression. Of particular interest is the question whether the mimetics utilize similar signaling proteins/pathway as insulin to regulate G6PDH gene expression. The studies were carried out in primary rat hepatocytes which provided us with an excellent insulin-responsive model that would mimic *in vivo* conditions.

CHAPTER II

METHODS

Materials

Waymouth's MB 752/1 media was purchased from Gibco BRL, together with the TRIzol® and the Lipofectin® reagent. The hyaluronadase, BSA and the trypsin inhibitor were from Sigma. Collagenase D was purchased from Boehringer Mannheim. The Nucleobound AX kit was obtained from the Nest group, whereas the Luciferase Assay Kit and the Multiprime Labeling Kit were purchased from Promega and Amersham, respectively. The porcine insulin used in the study was a gift from Eli Lilly. Dr. Alan Saltiel of Parke-Davis Warmer Lambert generously provided PD 098059. B581 and LY 294002 were obtained from Biomol Research Laboratories. Wortmannin was purchased from Sigma and rapamycin was from Calbiochem. All chemicals used in the study were of the highest grade.

Hepatocyte Isolation and Maintenance

The hepatocytes were isolated from 8-10 week old male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) which were food-deprived for 2 days prior to sacrifice. Water was given ad libitum without restriction. The rats were anesthetized with pentobarbital, and the liver was then perfused via the portal vein with a digest solution containing 100 μg/ml colleganase D, 93 μg/ml hyaluronadase

and 160 µg/ml trypsin inhibitor in a Mg2+ free Hepes balanced salt solution. The partially digested liver was then forced through 4 layers of sterile gauze as previously described by Stapleton et al. The resulting cell suspension was washed three times with Waymouth's MB 752/1 media containing 0.5% bovine serum albumin (BSA). The viability of the cells was checked with a hemocytometer using the brilliant blue dye exclusion method. Cell preparations with an over 80% viability were then plated on sterile 60mm collagen-coated culture plates and maintained at 37°C in a humified environment of 5% CO_2 and 95% air. After 4 hours the media was changed to BSA free media and allowed to incubate overnight.

Plasmid DNA Preparation

The *E. Coli* bacteria containing the plasmid of interest was plated on Luria-Bertani (LB) agar plates containing 100 µg/ml of the antibiotic ampicillin. A single colony was used to inoculate 1 liter of LB Medium containing 100 µg/ml ampicillin and grown overnight at 37°C in a shaking incubator. The plasmid DNA was then isolated using the alkaline lysis method as described by Sambrook et al. (1989) and purified using the Nucleobond Ax silica based anion exchanger according to manufacturer's instructions. Visualization of the purified DNA using gel electrophoresis and the OD 260/280 absorption ratios confirmed the purity.

Transfection

The transfection of the hepatocytes was carried out using the Lipofectin® reagent. Lipofectin reagent is a 1:1 liposome formulation of the cationic lipids N-[-1-

(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phophotidylethanolamine (DOPE) in membrane filtered water. The lipofectin reagent (30 μg) was incubated for 10 minutes in 1 ml of BSA free waymouth's media. Then, 5 μg of the desired plasmid was added to the lipofectin media mixture and allowed to sit for 30 minutes. This lipid: DNA complex was diluted to 4 ml and added to the hepatocytes in culture. After 18 hrs, the media was changed and the cells were treated with various conditions. The expression of the plasmid was checked 24 hrs after dosing.

Cell Treatment

The cells were pretreated for 30 minutes with the concentrations of the various inhibitors listed on Table 1 prior to treatment with 300 ng/ml porcine insulin, sodium selenate (30 μM) or sodium vanadate (30 μM) for 22 hours. Two plates were used per condition for each animal sacrificed.

Table 2

List of Inhibitors Used in the Study, Their Area of Action and Concentration Used

Inhibitor	Area of Action	Concentration
B581	Inhibitor of Ras farnesylation	50 μM
PD 098059	Inhibitor of MEK	10 μM
LY 29004	Inhibitor of PI 3 Kinase	50 μM
Wortmaninn	Inhibitor of PI 3 Kinase	100 nM
Rapamycin	Inhibitor of FRAP	100 nM

Cell Processing

The primary rat hepatocytes in culture were first processed by removing the media and washing twice with cold phosphate-buffered-saline (PBS) solution. The cells were then lysed by adding 400 µl of 1x luciferase lysis buffer for 15 minutes and then scraped off the plates. The samples were centrifuged for 30 seconds at 14,000 x g to pellet the cell debris and the supernatant was transferred to a new tube and assayed for luciferase activity as described below. The protein content was measured using the Lowry method (Lowry et al., 1951).

Luciferase Assay

A 20 µl aliquot of the cell lysate was added to 100 µl of luciferase assay reagent. The reaction that occurs is characterized in Figure 5.

Luciferase + Luciferin + ATP ------→ Luciferase - Luciferyl-AMP +PPi

Luciferase - Luciferyl-AMP + O_2 ------→ Luciferase + oxyluciferin + AMP + CO2 + *hv*

Figure 5. Enzymatic Reaction Catalyzed by Luciferase.

The *hv* in the reaction represented in Figure 5 is then measured using the scintillation counter. The average of two one-minute measurements was then normalized to the amount of protein in the sample. The final values are in counts per minute per µg of sample (CPM/µg).

RNA Isolation

Total RNA was isolated using TRIzol® Reagent from Life Technologies (Grand Island). TRIzol® reagent is a monphasic solution of phenol and guanidine isothiocynate. To ensure absence of RNAse contamination, the work area was decontaminated with RNase ZAP (Invitrogen) and all glassware was baked. The primary hepatocytes were lysed in the culture dish by adding 2 ml of TRIzol® reagent and passing the cell lysate several times through a pipette. The lysate was then incubated at 15 to 30°C for 5 minutes to permit the complete disassociation of the nucleoprotein complexes. This was followed by a chloroform extraction and isopropanol precipitation. The precipitated RNA pellet was resuspended in diethylpyrocarbonate (DEPC) treated water, and the RNA concentration was quantified at optical density (OD) 260. The purity was assessed by the ratio of $(OD)_{260} / (OD)_{280}$ (<1.5).

Total RNA (15 µg) was subjected to electrophoresis on a 1% agarose/ 2.2 M formaldehyde gel. The separated RNA was then transferred to "GeneScreen" (NEN/DUPONT) and hybridized with [^{32}P] dCTP labeled G6PDH cDNA (Berg et al., 1995). The cDNA was labeled by the random primer method using the mutiprime labeling kit as described in the manufacturer's instructions. The membranes were then washed and subjected to autoradiography at –70°C with Kodak XAR-5 film and intensifying screens. The exposed films were quantified using densitrometric scanning. The amount of G6PDH mRNA was normalized to the control ß-actin probe.

Statistical Analysis

The results are expressed as the means $\pm$ S.E.M. of N number of animals per group with each determination being performed in duplicates. Statistical significance was evaluated by the 1-tail student's t test which is significant at $p < 0.05$.

CHAPTER III

RESULTS

To elucidate the signaling proteins and /or pathways involved in the insulin induced regulation of G6PDH gene, we used a series of well defined inhibitors (Table 1) to determine their effects on the signaling proteins of the insulin signal transduction pathway as well the G6PDH gene expression. The expression of the G6PDH gene was measured by (a) a G6PDH promoter driven luciferase gene construct (Figure 6) tranfected into primary rat hepatocytes, and (b) the endogenous G6PDH mRNA levels.

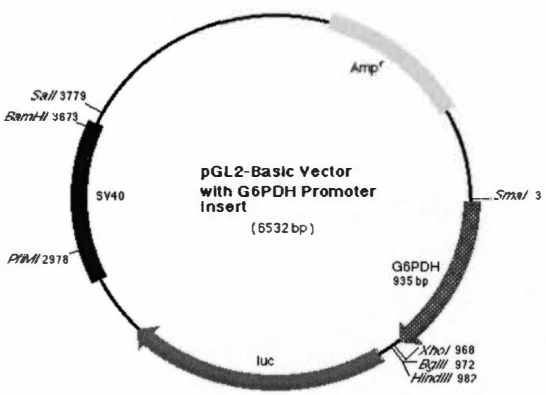


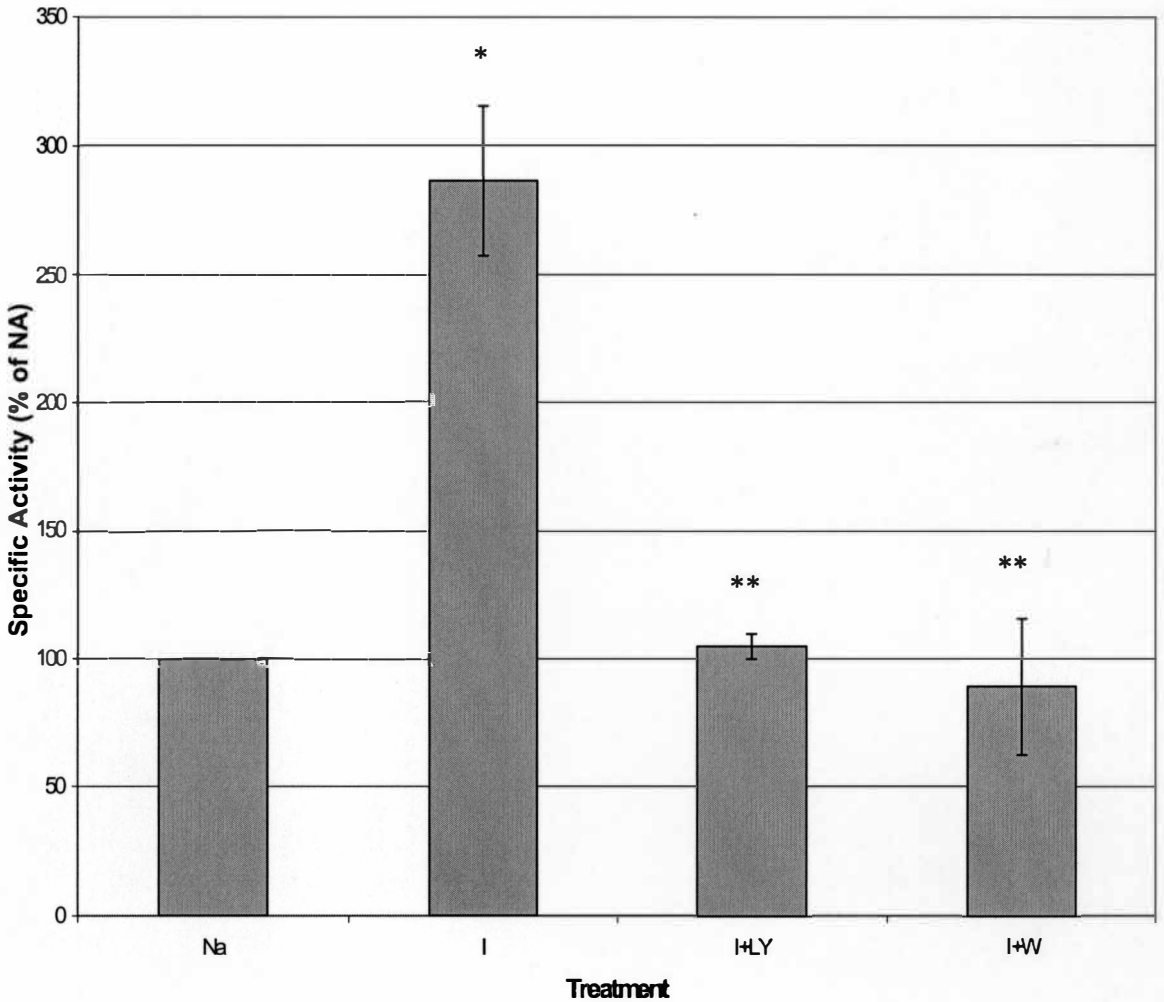
Figure 6. PGL-2 Basic Vector With a G6PDH Promoter Gene Insert.

Insulin Induced Stimulation of G6PDH Gene Expression Is Sensitive to LY 294002 and Wortmannin

LY 294002 and wortmannin are two structurally distinct inhibitors of PI 3-K. PI 3-K has been shown to be important in a variety of insulin and growth factor induced metabolic and mitogenic responses (Kapellar et al., 1994). As shown in Figure 7 and 8 respectively, incubation of primary rat hepatocytes with insulin led to a 2-3 fold increase in the G6PDH promoter driven luciferase activity as well as the endogenous G6PDH mRNA levels. In the presence of either LY 294002 or wortmannin, however, this insulin-induced stimulation is completely abolished.

Insulin Induced Stimulation of G6PDH Gene Is Sensitive to Rapamycin

S6K, a direct downstream target of PI 3-K (Weng et al.,1995), has been shown to be essential for insulin and/or growth factor mediated regulation of some metabolic genes important in glucose metabolism. With the aid of rapamycin, a potent inhibitor of FRAP which is important in the activation of S6K, we analyzed the importance of S6K activity on insulin induced G6PDH gene expression. Our results demonstrate that the 2-3 fold increase in the insulin induced G6PDH promoter driven luciferase activity (Figure 9), as well as the endogenous G6PDH mRNA levels (Figure 10), are sensitive to rapamycin. This suggests a role for S6K in insulin induced G6PDH gene expression.

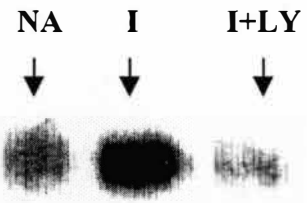


Legend. NA = No Addition, I = Insulin, LY = LY 294002, W = Wortmannin

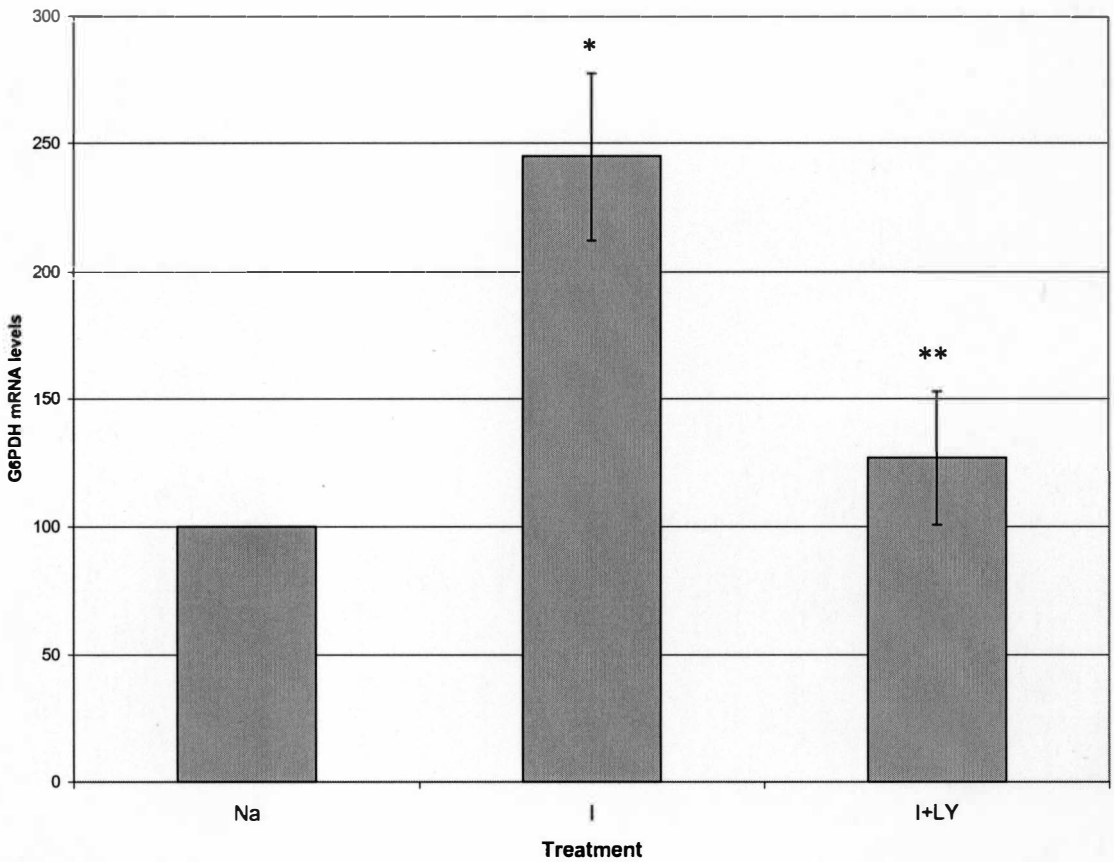
*= Significant Stimulation (P<0.05) **=Significant Inhibition. (P< 0.05)

Figure 7. Role of PI-3K on Insulin Stimulated G6PDH Promoter Driven Luciferase Activity. Primary Hepatocytes were Pretreated with Either LY 294002 (50μM) or Wortmannin (100nM) for 30 Minutes Prior to Stimulation with Insulin (80nM) for 22 Hours. N =4

A.



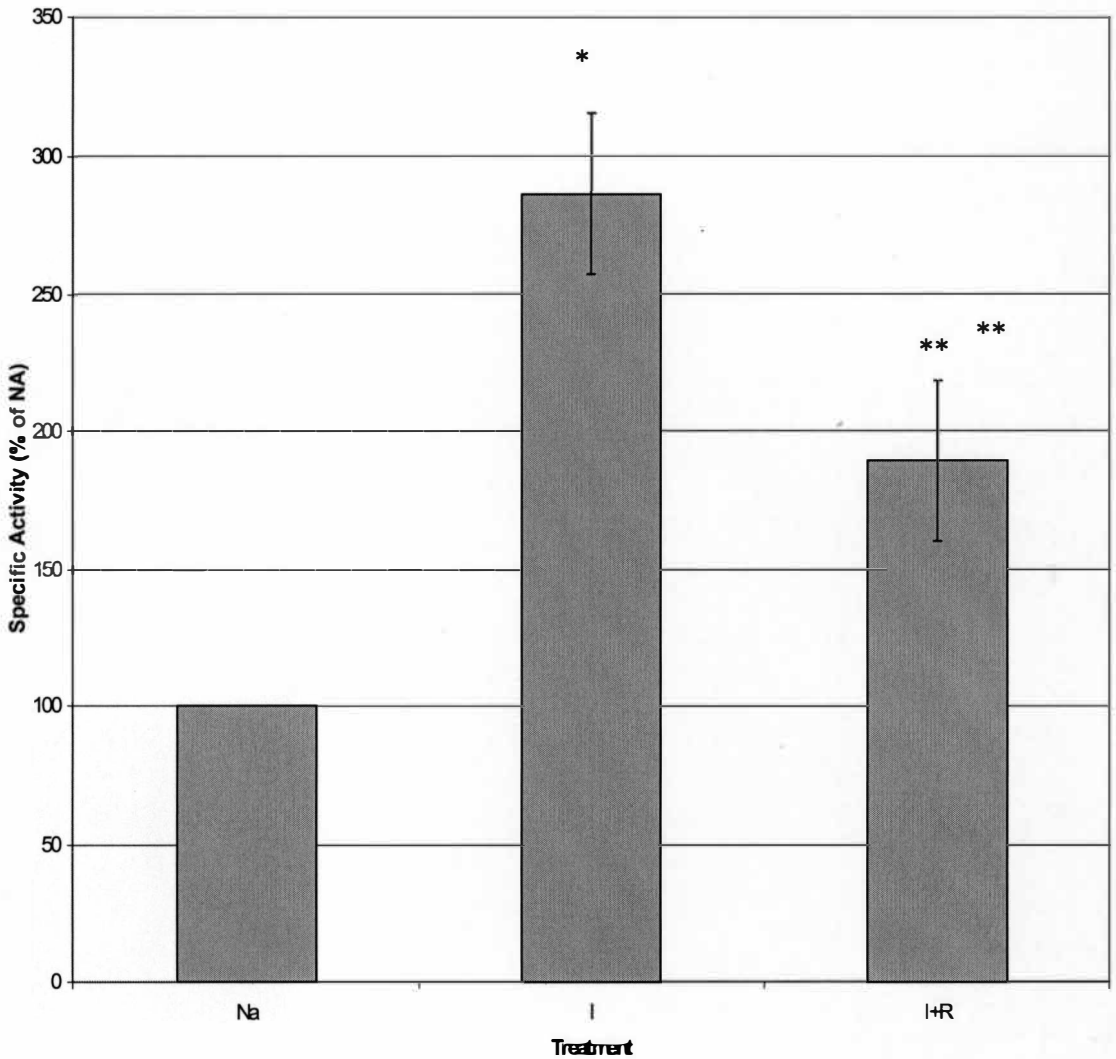
B.



Legend. NA = No Addition, I = Insulin, LY = LY 294002

*= Significant Stimulation (P<0.05) **=Significant Inhibition (P< 0.05)

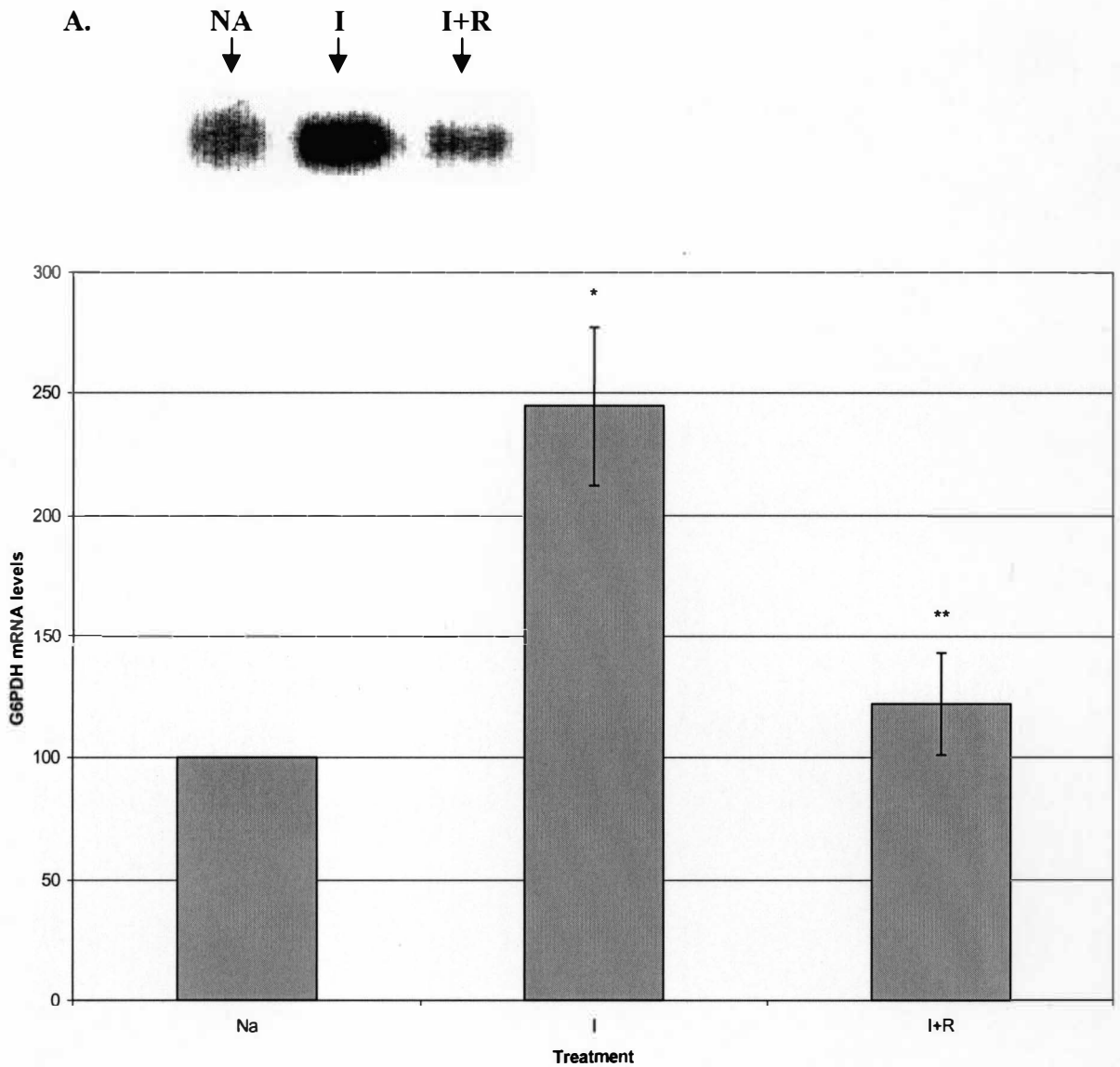
Figure 8. Role of PI 3-K on Insulin Stimulated G6PDH mRNA Levels. **A.** Northern Blot Showing G6PDH Gene Expression. **B.** G6PDH mRNA Levels. Primary Hepatocytes were Pretreated with LY 294002 (50μM) for 30 Minutes Prior to Stimulation with Insulin (80nM) for 22 hours. N =4



Legend. NA = No Addition, I = Insulin, R=Rapamycin

*= Significant Stimulation (P<0.05) **=Significant Inhibition (P< 0.05)

Figure 9. Role of S6K on Insulin Stimulated G6PDH Promoter Driven Luciferase Activity. Primary Hepatocytes were Pretreated with Rapamycin 100nM) for 30 Minutes Prior to Stimulation with Insulin (80nM) for 22 Hours. N =5.



Legend. NA = No Addition, I = Insulin, R=Rapamycin

*= Significant Stimulation (P<0.05) **=Significant Inhibition (P< 0.05)

Figure 10. Role of S6K on Insulin Stimulated G6PDH mRNA Levels. **A**. Northern Blot showing G6PDH gene expression. **B.** G6PDH mRNA Levels. Primary Hepatocytes were Pretreated with Rapamycin 100nM for 30 Minutes Prior to Stimulation with Insulin (80nM) for 22 Hour. N =5.

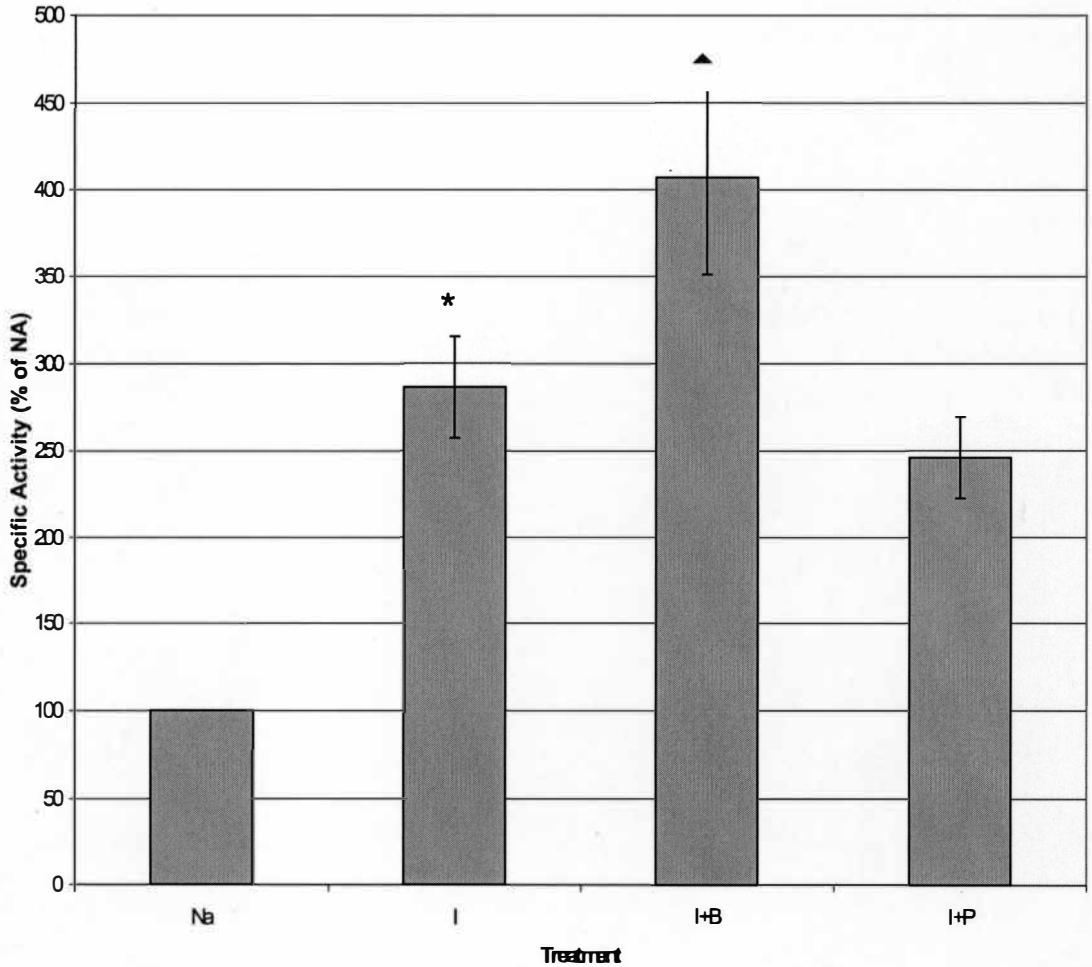
Insulin Induced Expression of the G6PDH Gene Is Not Sensitive to Inhibitors of Ras Farnesylation or MEK Activation

After establishing the importance of PI 3-K and S6K in the insulin induced regulation of G6PDH gene expression, the role of proteins of the MAPK pathway in the regulation of the G6PDH gene was determined. B581, an inhibitor of Ras farnesylation and the MEK inhibitor PD 098059 have been proven to be effective inhibitors of insulin induced MAPK activation in a variety of cell types. As seen in Figures 11 and 12, neither inhibitor was effective in inhibiting the insulin induced increase in the G6PDH promoter driven luciferase activity or the endogenous G6PDH mRNA levels. In fact, pretreatment with the ras farnesylation inhibitor, B581, before stimulation with insulin, led to a significant increase (over insulin) in the G6PDH promoter driven luciferase activity. This can be attributed to the fact that treatment of primary rat hepatocytes with B581 by itself, led to a significant increase in the G6PDH promoter driven luciferase activity over the basal (data not shown).

Together these data suggest that Ras/Raf/MAPK pathway is not involved in the insulin mediated regulation of the G6PDH gene.

Selenate and Vanadate Do Not Utilize the Same Pathway as Insulin to Regulate G6PDH Gene Expression

Previous studies by our laboratory have shown that the insulin mimetics selenate and vanadate induce G6PDH enzyme activity and the mRNA levels (Berg et al., 1995), both *in vivo* and in cells in culture. Both of these mimetics increase the overall phosphorylation state of the cell, including phosphorylation of proteins

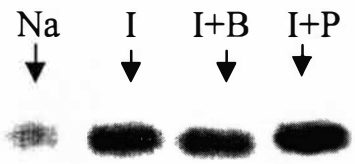


Legend. Na =No Addition, I=Insulin, B=B581, P=PD098058

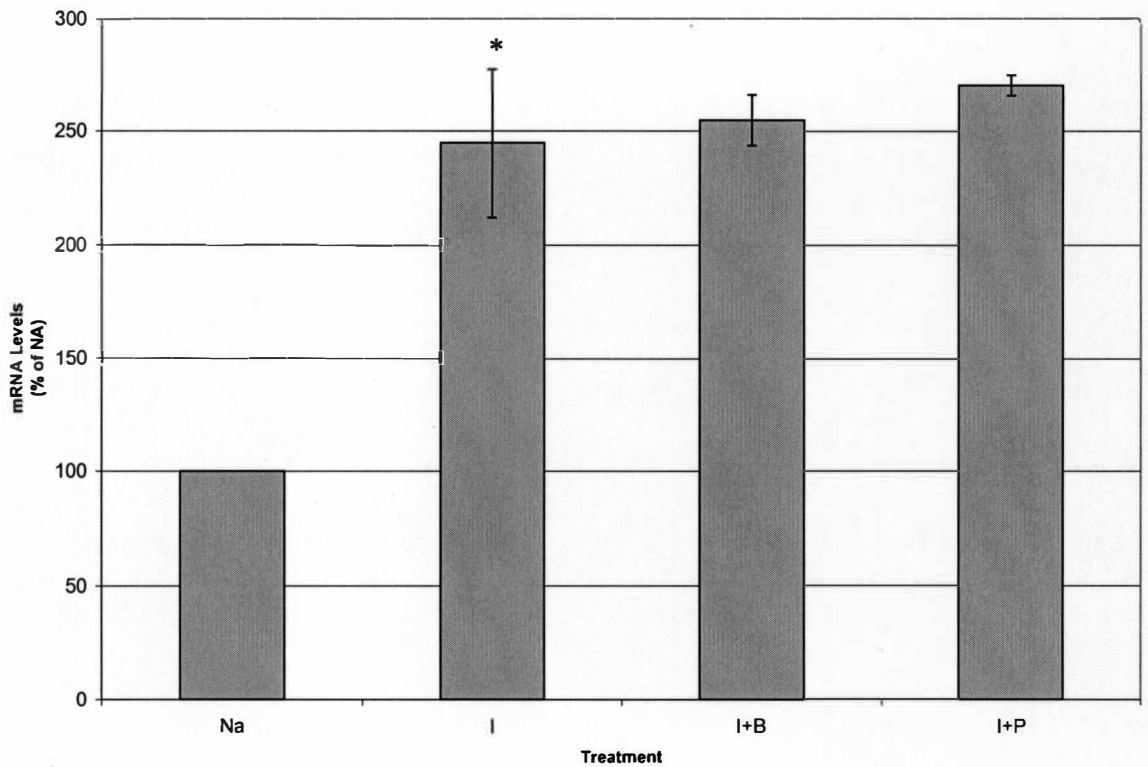
*= Significant Stimulation (P<0.05) **=Significant Inhibition (P <0.05)
▲=Significant Stimulation over Insulin (P< 0.05)

Figure 11. Role of MAPK Activation on Insulin Stimulated G6PDH Promoter Driven Luciferase Activity. Primary Hepatocytes were Pretreated with Either B581 (50μM) or PD098059 (10μM) for 30 Minutes Prior to Stimulation with Insulin (80nM) for 22 hours. N=5

A.



B.



Legend. NA = No Addition, I = Insulin, B=B581, P=PD098059

*= Significant Stimulation (P<0.05) **=Significant Inhibition (P< 0.05)

Figure 12. Role of MAPK Activation on Insulin Stimulated G6PDH mRNA Levels. **A**. Northern Blot Showing G6PDH Gene Expression. **B.** G6PDH mRNA Levels. Primary Hepatocytes were Pretreated with PD 098059 (10μM) or B581 (50μM) for 30 Minutes Prior to Stimulation with Insulin (80nM) for 22 Hours. N =3.

identified in the insulin signal cascade. To determine whether the mimetics utilize similar signaling proteins/pathway in the regulation of the G6PDH gene, we carried out inhibition studies similar to that previously described for insulin.

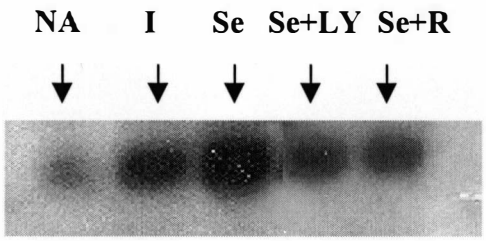
Selenate

Treatment of primary rat hepatocytes with selenate led to 2-3 fold increase in G6PDH gene expression as demonstrated in Figures 13 and 14. This 2-3 fold increase in G6PDH gene expression, similar to insulin, requires the activation of PI 3-K as well as S6K (Figure 13), but not MAPK (Figure 14). More interestingly however, using an inhibitor of Ras activation, it appears that selenate requires the membrane targeting and subsequent activation of the protein Ras (Figure 14) to mediate its effect on G6PDH gene expression. Together, these results demonstrate a unique mechanism by which selenate regulates G6PDH gene expression by utilizing the protein Ras in addition to PI 3-K and S6K.

Vanadate

The mechanism by which vanadate regulates G6PDH gene expression is also unique and interesting. The 2-3 fold increase in G6PDH gene expression in response to vanadate is PI 3-K dependent (LY 294002 sensitive) but S6K independent (rapamycin insensitive) (Figure 15). Vanadate, in a mechanism similar to that of insulin, however does not require the activation of the proteins Ras and / or MEK for regulating G6PDH gene expression, since both B581 and PD 098059 have no effect on vanadate induced G6PDH gene expression (Figure 16).

A.



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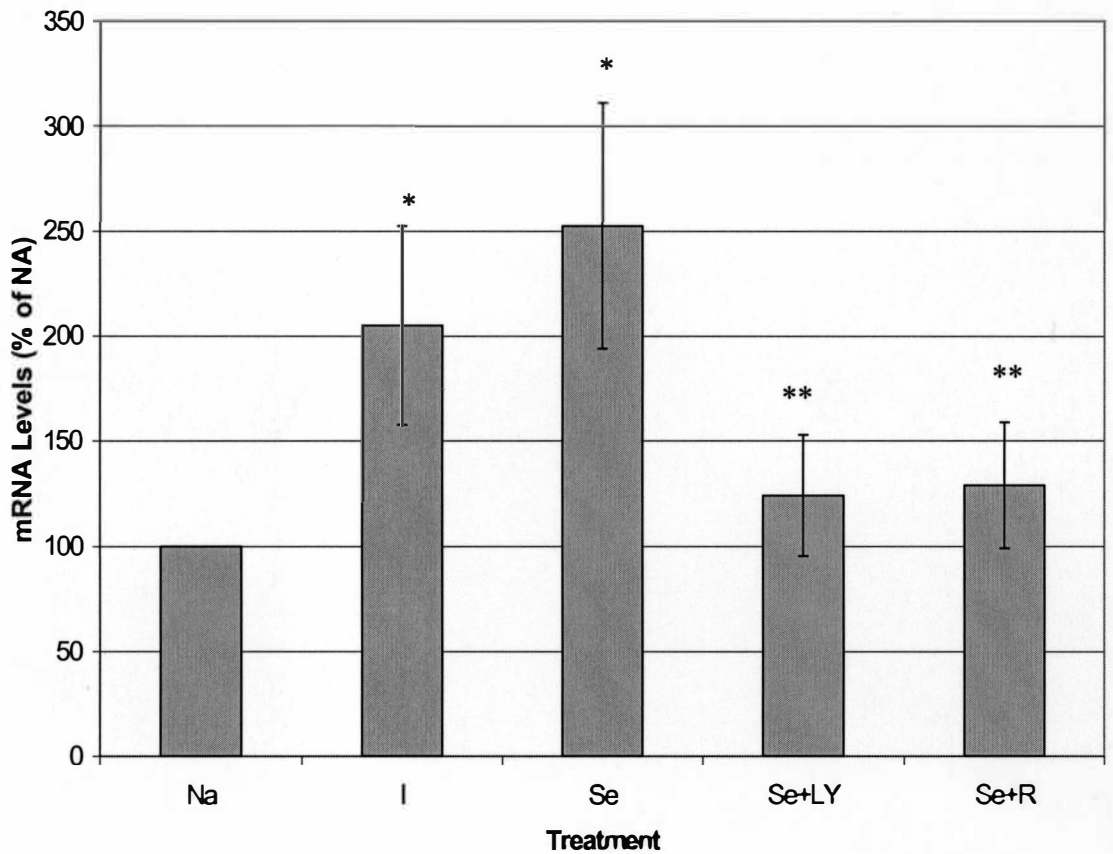
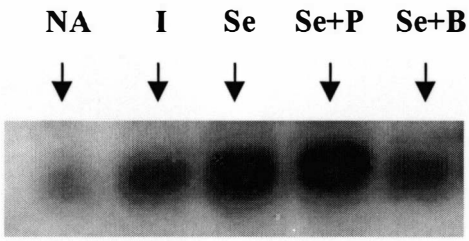


Legend. NA = No Addition, I = Insulin, Se = Selenate, LY = LY 294002, R=Rapamycin

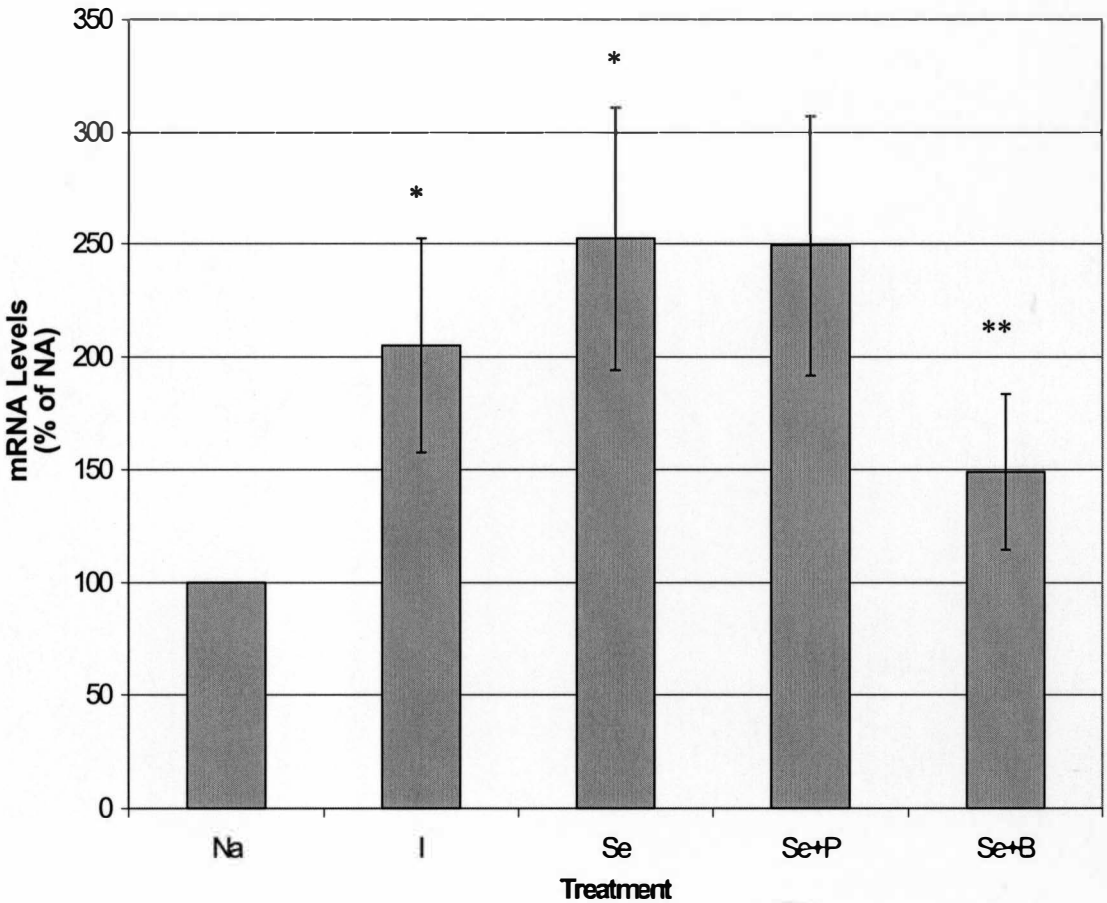
*= Significant Stimulation (P<0.05) **=Significant Inhibition (P< 0.05)

Figure 13. Role of PI 3-K and S6K on Selenate Stimulated G6PDH mRNA Levels. **A**. Northern Blot showing G6PDH Gene Expression. **B.** G6PDH mRNA Levels. Primary Hepatocytes were Pretreated with LY204002 (50μM) or Rapamycin (100nM) for 30 Minutes Prior to Stimulation with Selenate (30μM) for 22 Hours. N =3.

A.



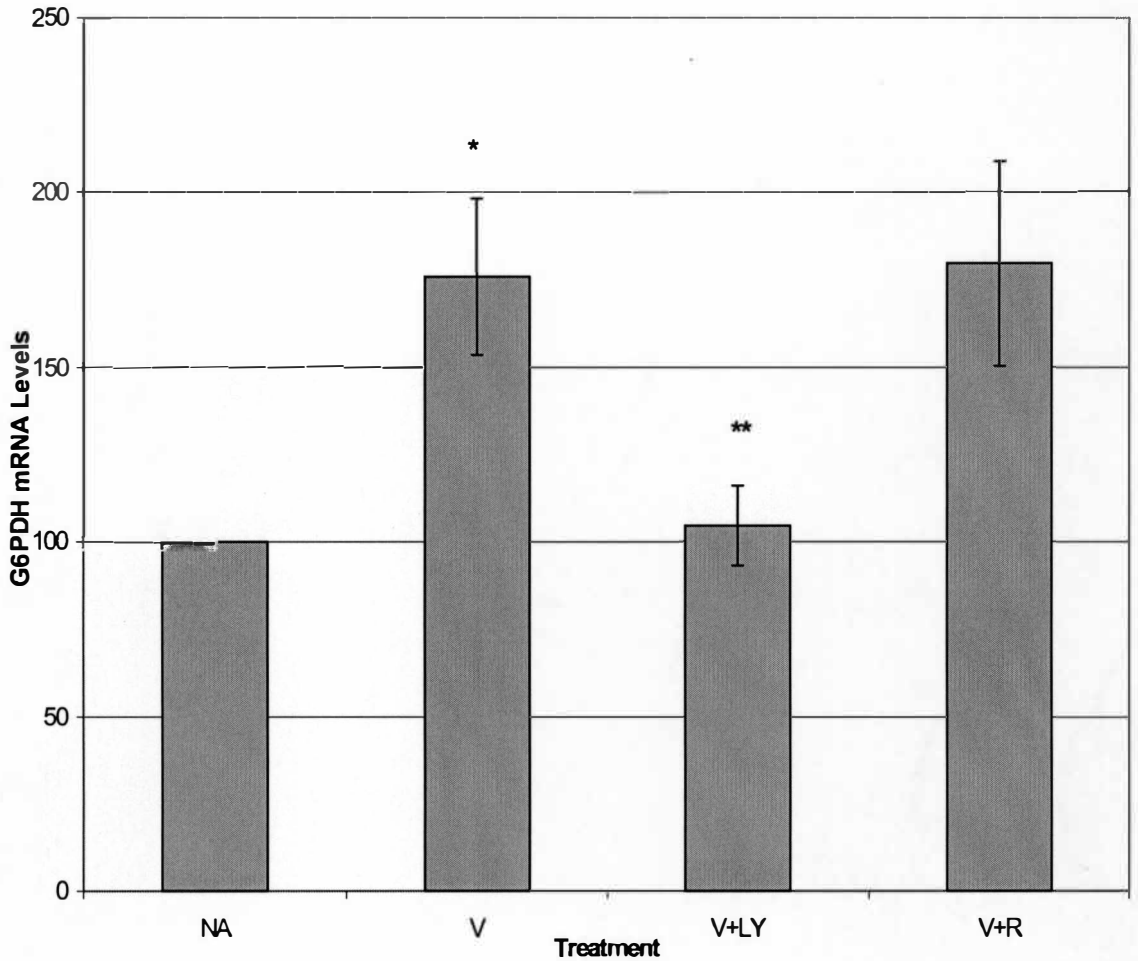
B.



Legend. NA = No Addition, I = Insulin, Se = Selenate, B=B581, P=PD098059

*= Significant Stimulation (P<0.05) **=Significant Inhibition (P< 0.05)

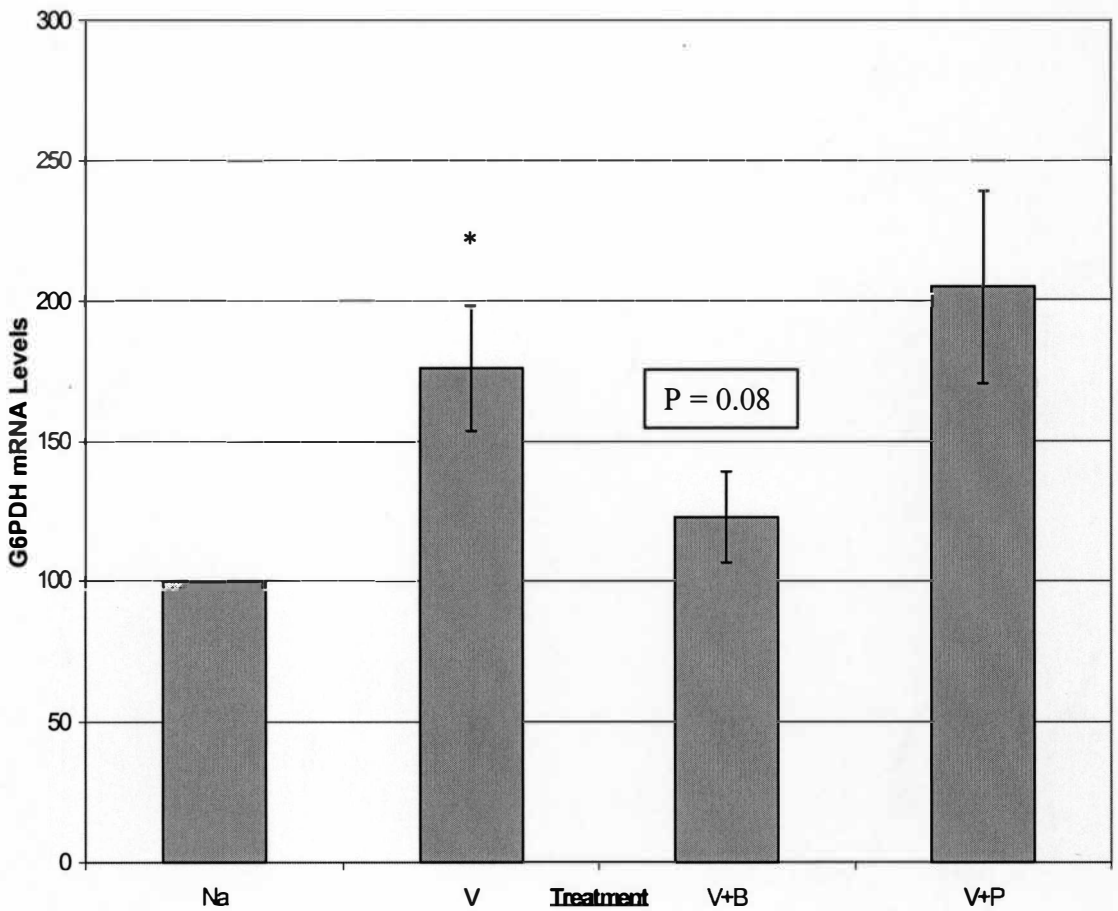
Figure 14. Role of the MAPK Pathway on G6PDH mRNA Levels. **A**. Northern Blot Showing G6PDH Gene Expression. **B.** G6PDH mRNA Levels. Primary Hepatocytes were Pretreated with PD 098059 (10μM) or B581 (50μM) for 30 minutes Prior to Stimulation with Selenate (30μM) for 22 Hours. N =3.



Legend. NA = No Addition, V = Vanadate, LY = LY 294002, R = Rapamycin

*= Significant Stimulation (P<0.05) **=Significant Inhibition (P< 0.05)

Figure 15. Role of PI 3-K and S6K on Vanadate Stimulated G6PDH mRNA Levels. Primary Hepatocytes were Pretreated with Either LY 294002 (50μM) or Rapamycin (100nM) for 30 Minutes Prior to Stimulation with Vanadate (30 μM) for 22 Hours. N =4



Legend. NA = No Addition, V= Vanadate, B = B581, P = PD 098959

*= Significant Stimulation (P<0.05) **=Significant Inhibition (P< 0.05)

Figure 16. Role of the MAPK pathway on vanadate Stimulated G6PDH mRNA Levels. Primary Hepatocytes were Pretreated with Either B581 (50μM) or PD 098059 (10μM) for 30 Minutes Prior to Stimulation with Vanadate (30μM) for 22 Hours. N =4

CHAPTER IV

DISCUSSION

Insulin affects the expression of a variety of metabolic genes by an elaborate network of signaling molecules. The precise molecular mechanism by which it exerts its tissue specific effect is an area of intense interest and study. Metabolic genes such as PEPCK (Gabbay et al., 1996) and FAS (Moustaid et al., 1994), important in carbohydrate and fatty acid metabolism respectively, have been extensively characterized in this respect and have revealed diverse regulatory mechanisms. G6PDH, a key rate-limiting enzyme of the pentose phosphate pathway plays a pivotal role in both carbohydrate and fatty acid metabolism. Apart from controlling the carbon flow through the pentose phosphate pathway, it produces reducing equivalents in the form of NADPH, which are utilized in synthesis of fatty acids and cholesterol. Understanding the molecular mechanism by which insulin regulates G6PDH gene expression is therefore important, as it provides us with an ideal model to study the regulation of both carbohydrate and fatty acid metabolism by insulin. The physiological roles of proteins of the insulin induced signaling cascade in glucose metabolism have been extensively characterized using chemical inhibitors. The inhibition of the insulin-induced PI 3-K activity by wortmannin and a structurally distinct chemical LY 294002 have revealed the obligatory role of this enzyme in insulin induced glucose uptake, amino acid uptake, DNA synthesis, protein synthesis,

glycogen synthesis as well S6K activation (Sanchez-Margalet et al., 1994). Furthermore, the PI 3-K inhibitors have also been shown to effectively block the action of insulin on gene 33 and PEPCK gene expression in hepatoma cells (Yang et al., 1995). Consistent with these studies, which demonstrate the obligatory role of PI 3-K in a variety of insulin mediated metabolic processes, our results show that PI 3-K activity is essential for insulin to regulate G6PDH gene expression.

The signaling events that lie downstream of PI 3-K are still unclear. One protein that has been demonstrated to lie downstream of PI 3-K is S6K. S6K is a serine/threonine kinase responsible for insulin induced phosphorylation of the 40S ribosomal protein S6 (Chung et al., 1994). We used the inhibitor rapamycin to examine the physiological role of S6K in the propagation of the signal to the nucleus. Rapamycin is a potent inhibitor of insulin stimulated S6K activity and has no affect on the PI 3-K activity. In 3T3 L1 adipocytes, insulin stimulated glycogen synthase activity, as well as glycogen synthesis, is sensitive to rapamycin (Sheperd et al., 1995). The insulin stimulated hexokinase II mRNA levels in skeletal muscle cells, was also shown to be inhibited by rapamycin (Osawa et al., 1996). More recently however, several reports have also suggested that the metabolic affects of insulin are mediated by rapamycin insensitive pathways that diverge from PI 3-K and do not require S6K as the downstream effector. In 3T3 L1 adipocytes, insulin induced DNA synthesis, regulation of glucose transport and activition of glycogen synthase kinase have all been shown to be regulated in this rapamycin insentive manner (Lazar et al., 1995 and Cohen et al., 1997). Furthermore, expression of insulin regulated metabolic genes such as PEPCK (Sutherland et al., 1996), gene 33 and glyceraldyhde-3-

phosphate dehydrogenase (O'Brien et al., 1991) are all rapamycin insensitive. In contrast to these findings, our results demonstrate that insulin induced G6PDH gene expression is sensitive to rapamycin.

Akt, a serine/ threonine kinase, is also a downstream target of PI 3-K. It has been shown to mediate some of the rapamycin insensitive responses of insulin. The association of Akt with PI 3-K is required for insulin stimulated activation of Akt (Kohn et al., 1996), and this activation is sensitive to PI 3-K inhibitors without being affected by rapamycin. Wagle et al. (1998) have shown that in primary rat hepatocytes insulin activated PI 3-K associates with Akt, which is abolished by inhibitors of PI 3-K. Since Akt can stimulate S6K activity (Burgering et al., 1995) it provides us with an attractive model of dual control of S6K by PI 3-K in primary rat hepatocytes, directly or indirectly via Akt.

Activation of the Ras → Raf → MEK → MAP kinase pathway is another major component of insulin signal transduction. Previous studies have suggested that the Ras/MAPK pathway plays a pivotal role in insulin induced mitogenesis and *c-fos* gene expression (Davis et al., 1993). This pathway however, may not be required for many of the metabolic effects of insulin. To elucidate the importance of this pathway in the regulation of G6PDH gene expression we used two well characterized inhibitors, B581 and PD098059, that have been shown to block the activation of MAPK in various cell types (Dudley et al., 1995) (Cox et al., 1994). B581, a recently identified inhibitor prevents the post-translational farnesylation and the subsequent membrane localization and activation of Ras. Although B581 blocks insulin induced activation of MAPK, it fails to suppress G6PDH gene expression in primary rat

hepatocytes. In addition to B581, we used an inhibitor of a downstream kinase MEK, PD098059, to further assess the role of MAPK activation in the insulin-mediated regulation of the G6PDH gene. Our results show that the insulin stimulated MAPK activity is partially but significantly inhibited in the presence PD098059 (Wagle et al., 1998) and this inhibition does not affect the insulin induced G6PDH gene expression in primary rat hepatocytes. This partial inhibition of MAPK by PD098059 is not affected by increasing concentrations of the MEK inhibitor, suggesting that a MEK independent pathway in primary rat hepatocytes can activate MAPK. This MEK independent pathway does not involve PI 3-K since PI 3-K inhibitors have no effect on insulin stimulated MAPK activity (Wagle et al., 1998).

In light of the evidence, we conclude that insulin regulation of the G6PDH gene does not require the activation of the Ras/MAPK pathway but is dependent on PI 3-K and S6K activation.

In recent literature, selenate and vanadate have both been reported to exhibit insulin-mimetic properties. Several studies, both *in vivo* and in isolated cells in culture, have shown selenate and vanadate to have many insulin like effects, including stimulating tyrosine phosphorylation of endogenous cellular proteins, glucose transport and expression of various genes important in glucose metabolism. However, very little work has been done concerning the mechanism by which these mimetics mediate their action. Our study focused on the mechanism by which selenate and vanadate regulated G6PDH gene expression in primary rat hepatocytes.

Previous studies have demonstrated that selenate induces rapid phosphorylation of intracellular proteins involved in the insulin signal transduction

cascade. In NIH 3T3 HIR3.5 cells selenate induced the phosphorylation of the insulin receptor β-subunit and IRS-1 (Pillay et al., 1992). Stapleton et al. (1996) have further shown that in addition to the insulin receptor β-subunit and IRS-1, selenate also stimulates the phosphorylation and activation of MAPK in primary rat hepatocytes and 3T3 L1 adipocytes. More recently, Wagle (1998) demonstrated that selenate stimulates PI 3-K activity by a mechanism unlike insulin in that it does not require IRS-1 association for activation. This suggests that PI 3-K is activated by an IRS-1 independent mechanism. Our results suggest the possibility that Ras is responsible for the selenate induced PI 3-K activity. Ras has been shown to regulate PI 3-K activity both *in vivo* and *in vitro* by several investigators (Rodriguez-Viciana et al., 1994). Although the relationship between selenate induced Ras activity and PI 3-K activity is unclear in primary rat hepatocytes, it is interesting to observe that the inhibition of Ras farnesylation with B581 abolishes the selenate induced G6PDH gene expression. In comparison to insulin, further studies with inhibitors have revealed that activation of PI 3-K as well as S6K is also necessary for selenate to stimulate G6PDH gene expression, whereas activation of MAPK is not. Together these results reveal a unique mechanism to that of insulin, by which selenate regulates G6PDH gene expression through the protein Ras in addition to PI 3-K and S6K.

The mechanism by which vanadate regulates G6PDH gene expression in primary rat hepatocytes is also very unique and interesting. Vanadate, in a manner similar to insulin and selenate, required the activation of PI 3-K for regulating G6PDH gene expression. However, the activation of the protein S6K, which is

essential for insulin and selenate to regulate G6PDH gene expression, is not required by vanadate to mediate its action. This reveals a rapamycin insensitive mechanism by which vanadate regulates G6PDH gene expression. It is possible that Akt, the protein downstream of PI 3-K, may be responsible for mediating these rapamycin insensitive affects since Akt activity has been shown to increase in response to treatment of rat adipocytes with vanadate and peroxvanadate (Wijkander et al., 1997). However, further studies need to be done to show the effects of vanadate on Akt activity in primary rat hepatocytes.

Vanadate, in a manner similar to that of insulin and selenate, did stimulate MAPK activity, however, this activation occurred in a Ras and MEK independent fashion, since inhibition with B581 and PD 098059 did not affect the vanadate induced MAPK activity (Wagle, 1998). Therefore, the importance of MAPK activation on vanadate induced G6PDH gene expression is not known. This MEK independent activation of MAPK, was also seen previously by Band et al. using bisperoxovanadium 1,10-phenentrolin (bpV(phen)). It has been suggested that vanadate, a potent phosphatase inhibitor, inhibits a specific tyrosine/ threonine phosphatase involved in the negative regulation of MAPK, such that activation of MAPK (by a MEK independent pathway and/ or autophosphorylation) (Misra-Press et al., 1994) is unopposed. One such group of phosphatases that mediate the dephosphorylation of MAPK are the MAPK phosphatases (MKP). Several isoforms of the MKP have been identified (MKP-1, MKP-2, MKP-3, MKP-4) and they differ in levels found in cells and tissue distribution (Misra-Press et al., 1994). Furthermore, the importance of Ras activation as measured by inhibiting Ras with B581 on

vanadate induced G6PDH gene expresssion is not clear ($p= 0.08$) and also needs further investigation.

From the results presented here, we conclude that the mechanism by which insulin regulates G6PDH gene expression requires the proteins IRS-1/PI 3-K/S6K and not the proteins of the Ras/Raf/MAPK pathway. Furthermore, it is interesting to observe that the mechanism(s) by which the insulin mimetics, selenate and vanadate, regulate G6PDH gene expression are different from that of insulin.

Appendix A

Investigator IACUC Certificate

IACUC Number 97-07-03

Date of Receipt

Date of Approval 9/5/97

RECEIVED
JUL 25 1997
I.A.C.U.C.

WESTERN MICHIGAN UNIVERSITY
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

Application to use Vertebrate Animals for Research or Teaching

The use of any vertebrate animals in research and/or teaching without prior approval of the Institutional Animal Care and Use Committee (IACUC) is a violation of Western Michigan University policies and procedures. This Committee is charged with the institutional responsibility for assuring the appropriate care and treatment of vertebrate animals.

Mail the signed original and five (5) copies of the typed application and any supplements to Research and Sponsored Programs, 301 Walwood Hall, (616) 387-8270.

Any application that includes use of hazardous materials, chemicals, radioisotopoes or biohazards must be accompanied with SUPPLEMENT A.

Any application that includes survival surgery must be accompanied with SUPPLEMENT B.

Susan R. Stapleton	Chemistry	387-2853
Principal Investigator/Instructor	Department	Campus Phone
Susan R Stapleton	7/24/97	
Signature	Date	
Responsible Faculty Member (if PI not faculty member)	Department	Campus Phone
Signature	Date	

Title of Project/Course Regulation of Gene Expression by Insulin, Insulin-mimetics and other metals

Check One: Teaching ____ Research X Other ____

I. ANIMAL USE CATEGORIES (check ONLY one category)

A. X Projects that involve little or no discomfort (including injections).

B. ____ Projects that may result in some discomfort or pain, but of short duration. Anesthetics, analgesics or tranquilizers will be used.

C. ____ Projects that may result in significant discomfort or pain. Anesthetics, analgesics, or tranquilizers will not be used.

WESTERN MICHIGAN UNIVERSITY
INVESTIGATOR IACUC CERTIFICATE

Title of Project: Regulation of gene expression by insulin, insulin-mimetics and other metals 44

The information included in this IACUC application is accurate to the best of my knowledge. All personnel listed recognize their responsibility in complying with university policies governing the care and use of animals.

I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. Technicians or students involved have been trained in proper procedures in animal handling, administration of anesthetics, analgesics, and euthanasia to be used in this project.

If this project is funded by an extramural source, I certify that this application accurately reflects all procedures involving laboratory animal subjects described in the proposal to the funding agency noted above.

Any proposed revisions to or variations from the animal care and use data will be promptly forwarded to the IACUC for approval.

_____ Disapproved _____ Approved ✓ Approved with the provisions listed below

Provisions or Explanations:

① specific name of database searched (e.g. Medline) to determine non-duplication

② specific item number where animals will be housed

IACUC Chairperson — 6/26/97 Date

Acceptance of Provisions

Signature: Principal Investigator/Instructor — 9/1/97 Date

IACUC Chairperson Final Approval — 9/5/97 Date

Approved IACUC Number 97-07-03

Rev. 3/92

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