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Elucidation of the Pathway Required for the Stimulation of G6PDH Gene Expression by Insulin and Its Mimetics

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ELUCIDATION OF THE PATHWAY REQUIRED FOR THE STIMULATION OF G6PDH GENE EXPRESSION BY INSULIN AND ITS MIMETICS

by

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ELUCIDATION OF THE PATHWAY REQUIRED FOR THE STIMULATION OF G6PDH GENE EXPRESSION BY INSULIN AND ITS MIMETICS

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Insulin, an anabolic hormone, exquisitely controls cellular physiology and metabolism by modulating the activity of key proteins which regulate metabolic and mitogenic events. Insulin activates several cytosolic proteins via a phosphorylation cascade, but only some of these proteins are required for its specific action. We have elucidated the insulin signal transduction cascade in primary rat hepatocytes, and outlined some of the events that are required for the insulin induced stimulation of glucose-6-phosphate dehydrogenase (G6PDH) gene expression. Using well-characterized and structurally different inhibitors, we show that insulin stimulation of the gene requires the IRS-1/PI3K pathway and not the RAS/RAF/MAPK pathway. Gene expression is dependent on S^6Kinase, which is controlled by PI3K and as our results suggest, by Akt. Since previous studies have shown that the insulin mimetics, selenate and vanadate, control G6PDH gene expression, we studied the effect of these mimetics on the signaling proteins. Our results are consistent with the properties of vanadate as a phosphatase inhibitor. On the other hand, selenium signal transduction appears to be sequential and similar to insulin in the regulation of some of the proteins in the cascade.

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CHAPTER I

INTRODUCTION

Insulin

Insulin is a potent anabolic hormone, important in the regulation of a wide variety of metabolic events. It is secreted in the β cells of the pancreas in response to increased blood glucose concentration. In primary target tissues, liver, muscle and adipose, it is responsible for the influx of nutrients into the cells, synthesis and storage of carbohydrates, lipids and proteins. The hormone modulates the rates of DNA, RNA and protein synthesis and cellular growth and differentiation (White et al). It also regulates the expression of key metabolic enzymes like phosphoenolpyruvate carboxykinase (PEPCK), glycogen synthase and fatty acid synthase (FAS).

Lack of insulin or failure to respond to insulin causes the disease Diabetes mellitus which is normally classified into two types, Type-1 or IDDM (Lernmark A.) and Type-2 or NIDDM (Degroot L.J.), respectively. Both diseases are characterized by increased blood glucose concentrations which leads to further complications.

The broad spectrum of tissue specific responses generated by insulin can only be accounted for by a complex regulatory mechanism. Early on, investigators believed that the insulin signal was relayed via cyclic nucleotides and ion channels but as research continued, protein phosphorylation gained more significance and emerged as the molecular mechanism responsible for the varied actions of insulin (Avruch et al).

Insulin Signal Transduction

Insulin exerts its control on organismal and cellular physiology by activating several intracellular phosphorylation signals, generating a phosphorylation cascade (Figure 1).

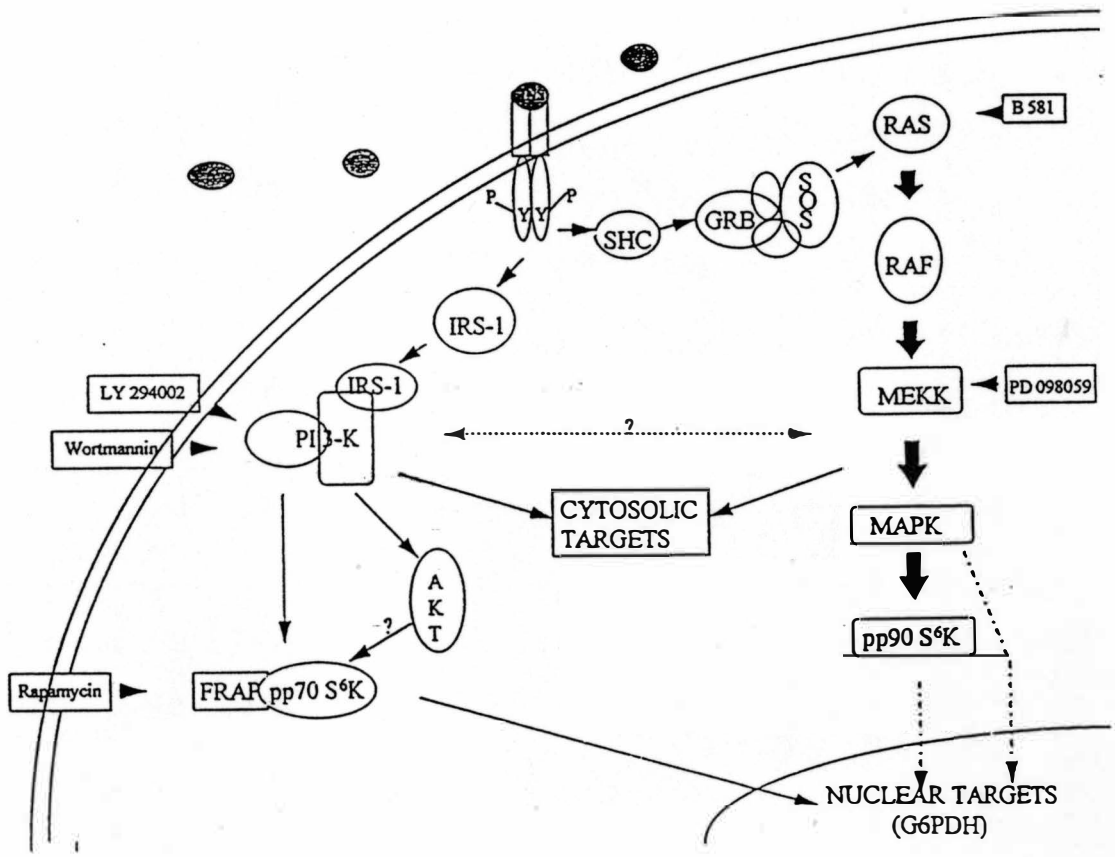


Figure 1. Insulin Signal Transduction Cascade.

Insulin action is initiated at the cell surface receptor where binding to the receptor activates all downstream proteins. The insulin receptor is an

autophosphorylating tyrosine kinase (Kasuga et al). Its α-subunits, which are completely extracellular, bind to the hormone and lead to the phosphorylation of several tyrosine residues of the intracellular β-subunit (Ullrich et al). Tyrosine phosphorylation of the receptor is necessary for the full expression of the receptor kinase activity (White et al) which is responsible for phosphorylation and activation of downstream proteins

One of the immediate downstream proteins (Figure 1), first characterized and cloned from rat liver cells, was the 185 K-Da protein, Insulin Receptor Substrate-1 (IRS-1) (Rothenberg et al). This protein has several potential tyrosine phosphorylation sites in the C-terminus and at least eight are phosphorylated in response to insulin (Sun et al). Apart from this region, which contains multiple tyrosine residues, IRS-1 also contains characteristic motifs which provide binding sites for downstream proteins bearing src homology-2 (SH_2) domains (Sun et al).

Another known substrate of the insulin receptor is Shc (Figure 1), a product of the *shc* gene. The phosphotyrosine binding (PTB) domain of Shc is highly homologous to that of IRS-1 suggesting that the insulin receptor engages the two proteins in a similar fashion (Gustafson et al). Shc also contains several SH_2 domains which can serve as binding sites for downstream proteins.

The proteins with SH_2 domains thus serve as docking proteins and are termed molecular switches for tyrosine kinases. They make important contacts with the phosphate groups on phosphorylated tyrosine residues (Waksman et al) and are responsible for signal divergence in the cascade. They have been shown to have different affinities for receptors and substrates which is dependent on the primary sequence surrounding the phosphotyrosine binding (PTB) domain (Songyang et al).

A protein downstream of IRS-1 in the cascade, is phosphatidylinositol 3-kinase (PI3K), as shown in Figure 1. It is a dimeric enzyme containing two subunits, one of

which is catalytic (p110) and the other regulatory (p85) (Carpenter & Buckworth). The p85 subunit contains two SH_2 domains which bind to the tyrosine phosphorylated IRS-1 (Myer's et al). A region between the two SH_2 domains of p85, called the interSH_2 (iSH_2) region, binds to the N-terminus of p110, resulting in the activation of the catalytic subunit (Klippel et al). For p110 to be fully active, both SH_2 domains in p85 have to be fully occupied and hence insulin stimulation of PI3K requires the association with IRS-1 (Figure 2) (Backer et al).

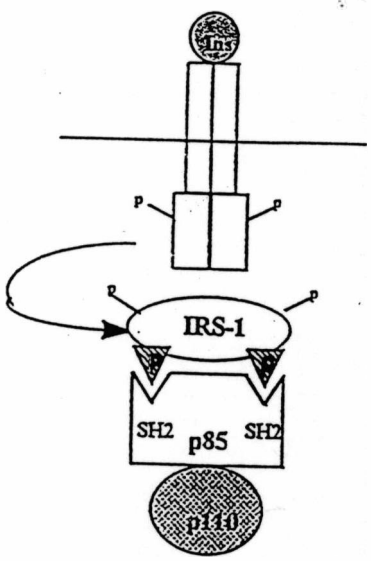
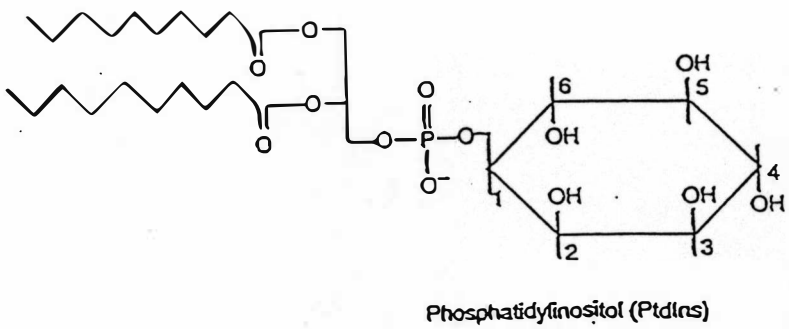


Figure 2. Association of p85 of PI3K With p110 and IRS-1.

PI3K, is a dual specificity kinase, which can phosphorylate proteins on tyrosine and serine/threonine residues and also use lipids as substrates (Dhand et al). Activation of PI3K is implicated in the regulation of a variety of metabolic and mitogenic cellular processes (reviewed by Kapellar & Cantley). It is cited as being required for DNA synthesis, cell growth and transformation, glucose transport, and regulation of glycogen synthase in various cell types including adipocytes, L6

myoblasts and CHO IR cells. Using constitutively active forms of PI3K, Klippel et al have shown that an active PI3K is sufficient to induce a number of different pathways. Activated PI3K is targeted to the membrane where it phosphorylates its lipid substrate, mainly inositol phosphate, on the 3 position of D-myoinositol ring (Whitman et al) to yield mono, di and tri phosphate derivatives (Figure 3), namely, phosphatidylinositol-3-phosphate, phosphatidylinositol-3,4-diphosphate and phosphatidylinositol-3,4,5-triphosphate. These products can themselves act as second messengers and assist in activating downstream events.



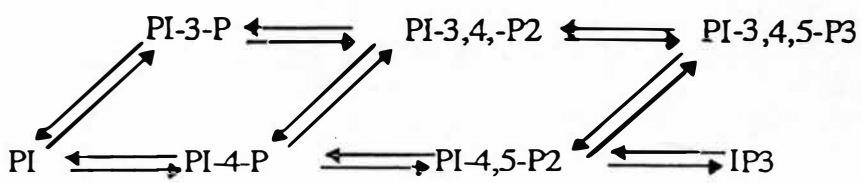


Figure 3. Phosphatidylinositol and Its Phosphorylated Derivatives.

It was observed that the activity of PI3K was accompanied by an increase in intracellular levels of $PI3,4P_2$ and $PI3,4,5P_3$ which correlated with the efficiency of PI3K to stimulate downstream responses such as cellular proliferation (Parker &

Waterfield), activation of Akt in COS-7 cells (Klippel et al), and other protein kinases like protein kinase C in CHO IR cells (Toker et al).

A direct target of PI3K is the serine/threonine kinase Akt (Franke et al). Stimulation of the kinase activity of Akt is dependent on an activated PI3K since inhibitors of PI3K also inhibit Akt kinase as shown by Burgering et al using NIH 3T3 cells stimulated by PDGF. Cells containing dominant negative mutants of PI3K exhibited no akt kinase activity (Jones P. F.). The phosphorylation sites at the C-terminus and a pleckstrin homology (PH) domain at the N-terminus are required for the activation of Akt while a central domain exhibits a high kinase activity (Franke et al). Akt exists as a homodimer in the nonactivated state (Datta et el). When stimulated by PDGF, the phospholipid products of PI3K have been shown to bind to the PH domain and activate Akt (Franke et al). However, Kohn et al showed that the association of the iSH_2 region of PI3K with Akt was essential for the phosphorylation on serine/threonine residues, membrane targeting and the subsequent activation of Akt by insulin while the PH domain was dispensable. One model which can explain the controversy regarding activation of Akt is that phosphatidylinositol-3-phosphate causes the dissociation of the inactive Akt homodimer and monomeric Akt is further activated via phosphorylation by PI3K (Bos, J.L.). Alternatively, phospholipid products of PI3K may be necesary for recruiting Akt to the membrane where membrane associated PI3K phosphorylates and activates Akt. It is also possible that insulin and PDGF have different mechanisms of action. The kinase activity of Akt is associated with regulation of glycogen synthase activity, increased glucose transport in 3T3 L1 adipocytes and activation of S^6Kinase (Burgering & Coffer).

Another downstream target of PI3K is S^6Kinase (Figure 1). It is a serine/threonine kinase responsible for phosphorylating and activating the 40S ribosomal protein S6, which leads to stimulation of protein synthesis. S^6Kinase can

either be directly stimulated by PI3K (Weng et al) or via Akt (Burgering & Coffer). The exact mechanism of activation of S^6Kinase by upstream kinases in not fully defined. S^6kinase activity can also be regulated by the kinase activity of rapamycin sensitive FKBP12-rapamycin associated protein (FRAP) (Brown et al).

The above mentioned proteins, IRS-1/PI3K/S^6K form one branch of the insulin signal transduction cascade. Alternatively, there exists a second branch originating downstream of Shc and constitutes the proteins Ras/Raf/MEK/MAPK (Figure 1).

On being phosphorylated by the insulin receptor, IRS-1 and Shc compete for association with the adaptor protein GRB-2 (growth factor receptor bound-2) (O'neill et al), but recent research suggests that Shc is the major protein in this branch (Pronk et al). The association of Shc with GRB-2 leads to the activation of son of sevenless (SOS) protein which is a guanine nucleotide exchange factor. SOS promotes the release of bound GDP from the protein RAS, the product of proto-oncogene *ras*, which is activated upon GTP binding (reviewed by Seger & Krabs). Farnesylation of RAS targets it to the membrane, a process essential for further activations. Once membrane bound, RAS interacts with the N-terminal region of Raf-1 (Matsuda et al), a ser/thr kinase and recruits it to the membrane, a process which activates Raf-1 (Stokoe et al). On activation Raf-1 activates MAP kinase kinase (MEK). MEK is a dual specificity kinase and is highly specific in the activation of map kinases ERK-1 and ERK-2 (Seger & Krebs), commonly called MAPKs. Phosphorylation of both tyrosine and threonine residues of MAPK is essential for the full expression of enzyme activity and for translocation into the nucleus. MAPKs can phosphorylate a wide variety of proteins, both in the cytoplasm as well as the nucleus. The main regulatory proteins that are targets of MAPKs are nuclear transcription factors like elk-1, c-jun, c-fos which mediate insulin induced gene expression (Davis, R). MAPKs are also known to control protein synthesis via phosphorylation of PHAS-1. Interestingly,

MAPKs can also phosphorylate upstream proteins of the cascade like SOS, Raf-1 and MEK to uncouple the SOS-GRB2 complex generating a feedback control on RAS activation (Waters et al).

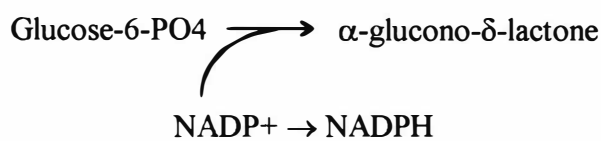
Elucidation of the Signal Pathway Necessary for Induction of G6PDH Gene Expression

Signal transduction cascades can be elucidated by using certain chemical compounds that are capable of inhibiting the enzyme activities of different proteins of the cascade. Inhibitors have been used to determine the necessity of a particular signal/protein in a signal transduction cascade. For example, the involvement of PI3K in cell trafficking (Kotani et al), glucose uptake and glycogen synthesis (Shepherd et al) in various cell types, including 3T3 L1 adipocytes and CHO IR cells, was determined using wortmannin, a fungal metabolite which is a well characterised inhibitor of PI3K. It inhibits PI3K by inhibiting the association between PI3K and IRS-1 (Norman et al). Another inhibitor of PI3K, LY 294002 has also been used to prove the requirement of PI3K for stimulation of S^6Kinase, DNA synthesis and glucose transporter translocation in 3T3 L1 adipocytes (Cheatham et al). LY 294002 is an analog of Quercetin and is structurally different from wortmannin (Vlahos et al). It is a more specific inhibitor of PI3K than wortmannin as it competes with p110 for ATP binding.

The MEK inhibitor PD 098059, was used to analyse the MAPK pathway. The inhibitor was used to show that MAPK is not involved in glucose utilization by insulin in 3T3 l fibroblasts and M6 myotubules (Lazar et al). B581, a peptidomimetic inhibitor is a tetrapeptide which mimics the CAAX tetrapeptide which is the farnesylation site of Ras (Garcia et al). By inhibiting Ras farnesylation, B581 inhibits membrane targeting which is responsible for downstream activations. Using B581,

Ras has been shown to be differentially involved in the activation of MAPK in adipocytes and fibroblasts (Whitehurst et al). An effective inhibitor of S^6Kinase is rapamycin, which on association with FRAP, inactivates S^6K. Phosphorylation of S^6K on threonine 229 and 389 is important for the catalytic activity of S^6K as it imparts a negative charge to S^6K, required for its functional activation. Rapamycin causes either dephosphorylation or impaired phosphorylation of threonine 229 thus inhibiting S^6K activity (Sugiyama et al). Using wortmannin and rapamycin, Sutherland et al showed that PI3K but not S^6K was required for the inhibition of PEPCK gene expression by insulin.

Use of inhibitors has allowed insulin action with respect to glycolysis, TCA cycle, lipogenesis and protein synthesis to be well characterized. Important enzymes have been shown to be regulated in these processes, for example, glycogen synthase kinase, PEPCK and fatty acid synthase. However, there has not been much effort to study the action of insulin on the pentose phosphate pathway (PPP), an important pathway that is responsible for the utilization of upto 60% of the glucose in the liver. The Pentose phosphate pathway is important for generating pentose sugars and other intermediates which are utilized for biosynthetic purposes. The reducing equivalence generated in the form of NADPH is used for reductive biosynthesis of fatty acids and for maintaining the redox state of the cell. The first enzyme of the pathway, Glucose-6-phosphate dehydrogenase (G6PDH), is the key enzyme of the pathway. It converts glucose-6-phosphate into its lactone, thereby generating a molecule of NADPH.



Although constitutively expressed in most tissues, expression of G6PDH is regulated in the liver, muscle and adipose tissue by insulin and other growth factors (Kletzien et al). The expression of modulated amounts of G6PDH is crucial. The hepatic enzyme activity changes as a result of insulin induced increase in gene transcription, mRNA stability and rate of protein synthesis (Katsurada et al and Manos et al). The mechanism by which insulin stimulates G6PDH gene expression has not yet been characterized. Our laboratory has shown previously that insulin mimetics can also induce G6PDH expression similarly to insulin (Berg et al) although the mechanism of action has not been determined.

Insulin Mimetics

Insulin mimetics are chemical compounds or naturally occuring elements that mimic the effects of insulin. Selenium and vanadium are two such essential trace elements which have been known to exhibit insulin like properties. Vanadate, in various oxidation states, has been proven to be a potent insulin mimetic and an anti-diabetic (Brichard et al) and is hence in a clinical trial for use as a therapy in NIDDM. Selenate also has been shown to exhibit similar anti-diabetic potentials (Ezaki O.).

Some of the well-known effects of these mimetic compounds are regulation of glucose homeostasis. Vanadate lowers blood glucose concentration in vivo mainly by channeling glucose into glycogen synthesis, which is accounted for by stimulation of glycogen synthase (Clark et al). Selenate also decreases blood glucose levels in streptozotocin (STZ) induced diabetic rats (Ezaki O.). Both vanadate and selenate act on muscle cells, adipocytes and hepatocytes. Vanadate increases the levels of fructose-2,6-bisphosphate, stimulates glucokinase (Gil et al), G6PDH and pyruvate kinase enzyme (Miralpeix et al) activity. Other in vitro effects of vanadate include inhibition

of lipolysis in adipose tissue (Dubyak et al) and inhibition of gluconeogenesis in the liver (Gil et al). Vanadate stimulates tyrosine phosphorylation of the kinase domains of the insulin receptor in primary rat hepatocytes and 3T3 L1 adipocytes (Stapleton et al). Selenate also stimulates the phosphorylation of the insulin receptor and IRS-1 in rat primary hepatocytes and activates MAPK (Stapleton et al).

In addition to these effects on cell regulation, both selenate and vanadate also regulate gene expression in a manner similar to insulin. In streptozotocin induced diabetic animals, vanadate increases pancreatic amylase mRNA (Johnson et al), phosphofructokinase and fructose-2,6-bisphosphatase mRNA (Inoue et al) and Glut 1 mRNA (Mountjoy & Flier) and inhibits phosphoenolcarboxykinase, pyruvate kinase (Brichard et al and Valera et al). Vanadate inhibits the expression of PEPCK gene (Bosch et al), while inducing the expression of pyruvate kinase and glucokinase genes (Miralpeix et al). Selenate also normalises mRNA levels of G6PDH and FAS in STZ-induced diabetic rats (Berg et al) and rat primary hepatocytes (Stapleton et al).

Objective of the Study

The objective of my research project was to determine which proteins of the insulin signal transduction pathway were necessary and in what sequence, in order activate of the G6PDH gene expression. Since the insulin response may be tissue specific, that is different proteins are involved in different sequences in different tissues, it was of interest to map out the pathway in primary rat hepatocytes, which are an excellent model for the primary target of insulin action, the liver. I have extensively studied the signal mechanism involved in the mimetic action to determine if the mimetics generated a cascade similar to insulin for the activation of G6PDH gene expression.

CHAPTER II

MATERIALS AND METHODS

Cell Culture, Maintenance and Processing

Hepatocyte Isolation and Maintenance

Hepatocytes were isolated from male Sprague-Dawley rats by the hyaluronidase (Sigma Chemical Co., St. Louis, MO), collagenase (Boehringer Mannheim, Indianapolis, IN) perfusion method as described by Stapleton et al. Rats were food-deprived 48 hours prior to isolation of hepatocytes. On the day of surgery, rats were anesthetised with an intraperitoneal injection of 30-40 units of pentobarbitol/Kg body weight. The liver was perfused in situ, excised and forced through 4 layers of sterile gauze to collect cells. The cells were washed 3 times with Waymouth's MB 752/l (Gibco, Grand Island, NY) medium containing bovine serum albumin (BSA). Cell viability was checked by brilliant blue die exclusion method. Cells with an average viability of greater than 80% were plated on sterile 60mm collagen coated plates falcon-3002 plates, to a confluency of 90%. After 3 hours of incubation in 4ml of Waymouth's MB 752/l medium containing 0.5% BSA, the cells were washed with BSA free Waymouth's medium and resuspended in the same. Hepatocytes were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell Treatment

Cell Stimulation

Cells were stimulated with either 80nM insulin (a gift from Eli Lilly) for 5 minutes, with 500μM sodium selenate for 3 hours, or with 500uM sodium vanadate (pH 6) for 3 hours.

Inhibitor Usage

Cells were pretreated with either 100nM wortmannin (Eli Lilly Inc.) for 3 hours, or with 50μM LY 294002 (Biomol) or 50μM B 581 (Biomol) for 30 minutes, or with 20μM PD 098059 (Parke-Davis) or 100nM rapamycin (Sigma Chemicals) for 1 hour prior to stimulation with either insulin or mimetics. (The concentrations and incubation times were obtained from literature.)

Cell Harvest

Cells were treated with either insulin, selenate or vanadate and pretreated with inhibitors as described. Two plates were used per condition for all treatments. Cell processing was carried out at 4°C or on ice. Cells were lysed in the respective lysis buffers as described in the individual assay procedures. All the cells were scraped into the buffer and centrifuged to collect the extranuclear extracts. Extracts from two plates of the same condition were pooled, flash frozen and stored at -20°C until further use.

Protein and Enzyme Assay Procedures

PI 3-Kinase Assay

PI 3-Kinase assay was carried out as described before by Serunian and Auger, with a few modifications. Each plate of hepatocytes was first washed twice in 5ml washing buffer containing 20mM Tris, pH 7.5, 137mM NaCl, 1mM $MgCl_2$, 1mM $CaCl_2$ and 100μM vanadate. Cells from each plate were then treated with 500μl of extraction solution containing 10% glycerol, 1% Tween-40, 1mM PMSF, 0.1mg/ml aprotinin and 1μg/ml leupeptin for 20 minutes with rocking at 4°C. Cells were scraped in the same solution, centrifuged and the extracts were collected as described previously. For the assay, PI 3-Kinase was immunoprecipitated from the cell extracts by incubating with 5μl of 1:2 diluted anti PI 3-Kinase, N-SH2 region antibody (anti-rat, monoclonal) (Upstate Biotrchnology, Inc.) per 200-400μg total protein, at 4°C overnight. Protein-A agarose beads (Gibco-BRL) were first incubated for 30 minutes in 10mM Tris buffer containing 1% BSA and subsequently washed 3 times with water. The beads were resuspended in phosphate buffered saline (PBS) containing 2% azide to give a 50%slurry. 50μl of the agarose slurry was added to the immunocomplex and the mixture was incubated at 4°C for two hours to precipitate the complex. The precipitate was washed 3 times with PBS containing 1% Tween-40 and 100μm vanadate, followed by 3 washings with 100mM Tris buffer, pH 7.5 containing 500mM LiCl and finally two washings with 10mM Tris, pH 7.5, containing 100mM NaCl, 1mM EDTA and 100μM vanadate. The precipitate was suspended in 50μl of the last wash solution. The substrate, phosphatidylinositol (Sigma Chemical Co.) was prepared by first dissolving the solid lipid in chloroform which was then evaporated under nitrogen. The lipid was first resuspended in a solution of 10mM HEPES, pH 7.5,

containing 1mM EGTA to give a final concentration of 2μg/ul and sonicated for 10 minutes by pulsing at a high speed. For assaying the enzyme activity, 10μl of sonicated substrate and 10μl of 100mM MgCl2 was added to the immunocomplex. The kinase reaction was initiated by adding 440μM ATP (Sigma Chemical CO.) containing 30uCi of γ-^{32}P ATP (Dupont-NEN, MA) and 10mM $MgCl_2$. The reaction mixture was incubated at 22°C for 10 minutes after which the reaction was stopped by adding 20μl of 8N HCl. The lipid products were extracted by adding 160μl of chloroform:methanol (1:1) mixture and then separated by thin layer chromatography. An aliqout of the organic phase was spotted on a 2mm silica gel plate coated with 2% potassium oxalate and subjected to thin layer chromatography. The plates were developed using chloroform, methanol, water and ammonium hydroxide in a proportion of 60:47:11.3:2. The plates were exposed to X-ray film for vizualising the spots which were further quantitated by scintillation counting.

Western Analysis

Association of PI 3-Kinase with IRS-1 was analysed by following the dual immunoprecipitation method. PI 3-Kinase was immunoprecipitated from the cell extracts as described in PI 3-Kinase assay protocol. The immunocomplex obtained by incubating the enzyme + antibody mixture with protein -A agarose beads was washed with PBS and suspended in 50μl of Laemmli sample buffer and subsequently subjected to SDS-PAGE in a 8% acrylamide gel. Electrophoresis was carried out at 100mV for 2 hours after which the proteins were transferred to a PVDF membrane using the directions provided by the manufacturer of the apparatus, NOVEX. The membrane was blocked overnight in 50ml. of blocking buffer containing 5% milk in Tris Buffered Saline containing 1% Tween-20 (TBST). It was then incubated with the primary

antibody (1:2000 dilution), anti IRS-1, carboxy terminal, anti rabbit, polyclonal (Upstate Biotachnology) for 1 hour after which the gel was washed 3 times with TBST. Secondary antibody (anti-rabbit) conjugated to horse raddish peroxidase (UBI) was added to the membrane (1:2500 dilution) after which the gel was washed again with TBST. The reactive bands were detected using enhanced chemiluminescence detecting system purchased from Amersham.

The association of PI 3-Kinase with Akt was checked using the same procedure except Akt was immunoprecipitated from the cell lysate using anti-Akt, carboxy terminal, anti-sheep, monoclonal antibody (Upstate Biotechnology) and the primary antibody used was anti-PI 3-Kinase, N-SH2 region.

MAPK Assay

Map Kinase assay was performed either as described by Stapleton et al or as indicated in the kit purchased from New England Biolabs. Detailed description of the use of the kit will be outlined here. Cells were treated as described in the figure captions and harvested after 48 hours of incubation. They were then lysed with 600µl of the cell lysis buffer provided in the kit. The extracts were sonicated 5 times for 5 seconds each, then centrifuged and stored as described above. MAPK was immunoprecipitated fom 200µl of the cell lysate containing 500-600µg total protein by incubating with the phospho-MAPK antibody (1:100 dilution) at 4°C overnight. The immunocomplex was precipitated by incubating with 20µl of a 50% protein-A agarose slurry in PBS, for 2 hours at 4°C. The pellet was washed twice with 500µl of cell lysis buffer and then twice with 500µl of kinase buffer as mentioned in the kit. The pellet was suspended in 50µl of kinase buffer supplemented with 100µM ATP and 1µg Elk 1 fusion protein, suppplied in the kit. After incubation for 30 minutes at room

temparature for 30minutes, the reaction was terminated by adding 25µl of 3X SDS sample buffer. The sample was then boiled for 5 minutes, vortexed and centrifuged for 2 minutes. 20µl of the sample was subjected to SDS-PAGE as described by Laemmli, at 100mV for 2 hours. Western analysis was carried out as described above using phospho-specific elk-1 antibody provided in the kit. The antibody was diluted 1:1000 in antibody dilution buffer (TBS containing 0.05% tween 20 and 5% BSA). Proteins were detected using phototope-HRP western detection kit provided by the manufacturer. Kinase activity was quantitated using scanning densitometry.

S^{6}Kinase Assay

S^{6}Kinase assay was performed using the S^{6}K assay kit and following the directions supplied by the manufacturer (Upstate Biotechnologies, Inc). 5µg of S^{6}Kinase antibody was first incubated with 100µl of protein-A agarose slurry previously washed in PBS, for 30 minutes at 4°C. The beads were then washed with Buffer A (50mM Tris, pH7.5, 1mM EDTA, 0.5mM sodium orthovanadate, 0.1% B-mercaptoethanol, 1% triton X-100, 50mM NaF, 5mM sodium pyrophosphate, 10mM sodium glycerophosphste, 0.1mM PMSF, 1µg/ml each of leupeptin and aprotinin and resuspended in 100µl of buffer A. 75 µl of cell lysate containing 200-300 µg of total protein was added to the beads and the reaction mixture was incubated for 2 hours at 4°C. The immunoprecipitate was washed with 500µl of buffer A containing 0.5M NaCl, and then with buffer A alone, followed by a wash with the assay buffer (20mM MOPS, pH 7.2, 25mM B-glycerophosphate, 5mM EGTA, 1mM sodium orthovanadate and 1mM DTT). Kinase reaction was carried out on the beads by adding 10µl of assay buffer, 10µl of substrate cocktail (substrate peptide in assay buffer), 10µl of inhibitor cocktail and 10µl of ATP containing 30 µCi of γ-^{32}PATP.

All reagents were provided in the kit. The reaction was allowed to continue for 10 minutes at room temparature. The beads were spun down and an aliqout of the supernatant was spotted on phosphocellulose paper. Any non-bound radioactivity was washed away by washing the assay squares with phosphoric acid and consequently with acetone. The radioactivity incorporated into the product was qauntitated by scintillation counting. Appropriate controls as described in the kit were used to correct for background counts.

Protein Estimation

Total protein concentration of cell extracts was determined by the method of Lowry et al using BSA as the standard protein. Protein values were expressed as μg/μl.

Statistical Analysis

Statistical significance was tested by the 1-tail t test and all results, expressed as +/- standard deviations of the mean, are significant at $p < 0.05$.

CHAPTER III

RESULTS

Insulin response is a result of a series of phosphorylation events. To define the pathway necessary for the activation of a specific response, activation of G6PDH gene expression, we used inhibitors of certain signal proteins and analysed their effect on insulin signal transduction in primary rat hepatocytes. The inhibition of the signal proteins was then correlated to the effect of inhibitors on gene expression to determine the necessity of a signal/protein in the cascade.

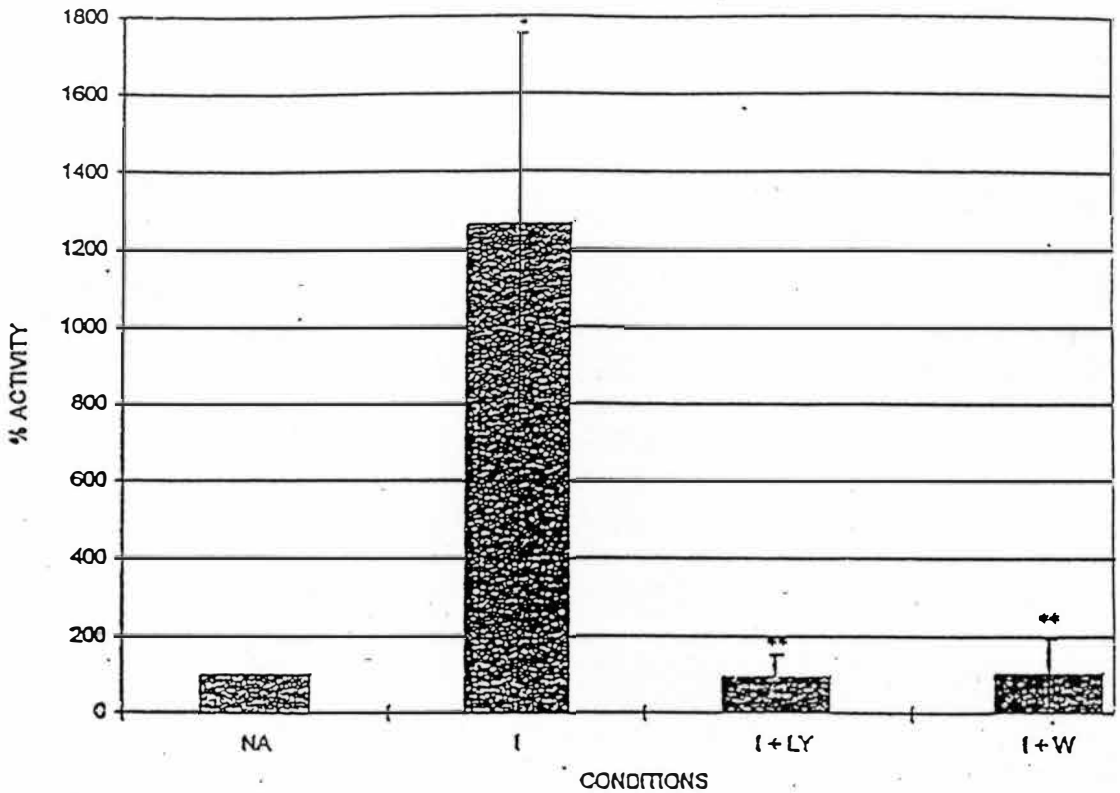
Insulin Induced Stimulation of PI3K is Sensitive to Wortmannin and LY 294002

PI3K, an enzyme involved in the regulation of metabolic and mitogenic regulation in respnse to insulin and other growth factors (Kapellar et al), is known to be inhibited by wortmannin and LY 294002. As shown in Figure 4, incubation of primary rat hepatocytes with insulin showed a 7-10 fold induction in PI3K activity which was sensitive to both LY 294002 and wortmannin. In the presence of either of the two inhibitors, insulin induction of PI3K was completely blocked.

S^6Kinase Activity

S^6K is a downstream target of PI3K (Weng et al). Rapamycin, an immunosuppresant blocks the PDGF and insulin induced stimulation of S^6K in a variety of cell types including adipocytes and fibroblasts. Since the effect of rapamycin in primary rat hepatocytes has not been studied, we analysed the activity of S^6K in response to insulin in the presence and absence of rapamycin. Our results show

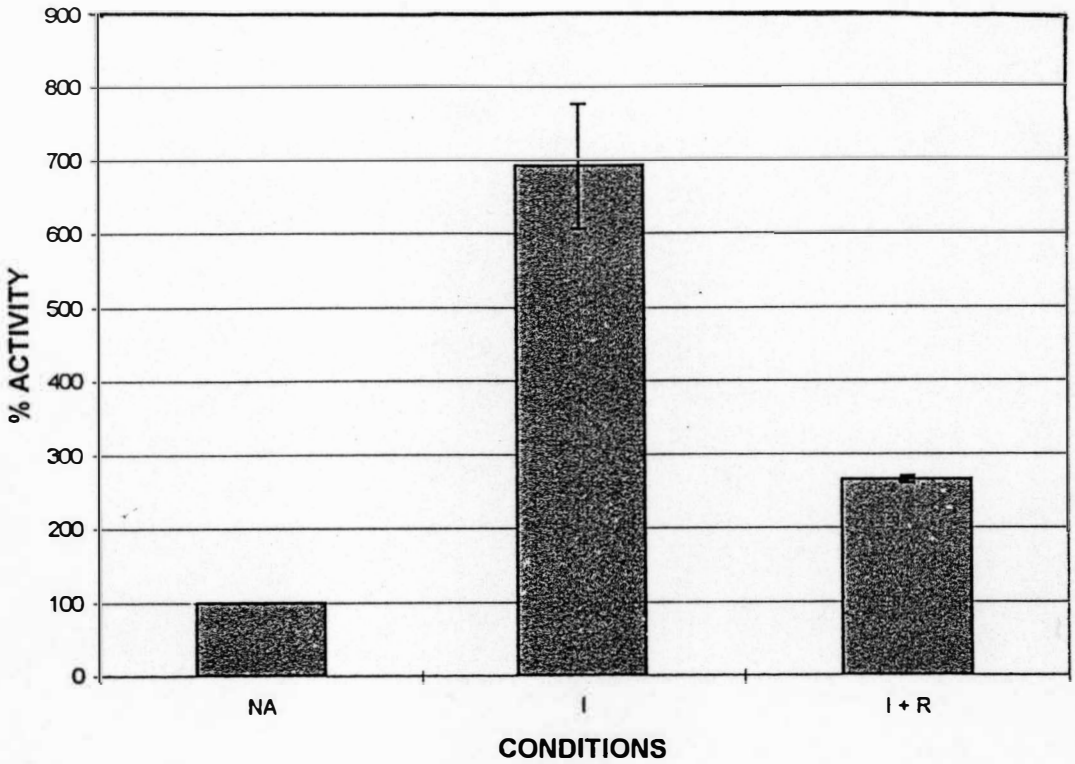
that insulin induced stimulation of S^6K activity is only partially inhibited by rapamycin (Figure 5). This suggests the involvement of a rapamycin insensitive pathway mediated by another signal protein that transmits the signal from PI3K to S^6K.



Legend. NA = No addition, I = insulin, W = wortmannin, LY = LY 294002.

* = significant stimulation. ** = significant inhibition. $p < 0.05$.

Figure 4. PI3Kinase Activity. Primary rat hepatocytes were pretreated with either (100nM) wortmannin for 3 hours or 50μM LY 294002 for 30 minutes prior to stimulation by 80nM insulin for 5 minutes. n = 5.



Legend. NA = No addition, I = insulin, R = rapamycin

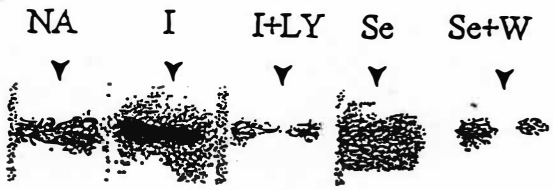
* = significant stimulation, ** = significant inhibition at $p < 0.05$

Figure 5. Insulin Induced S^6Kinase Activity is Partially Inhibited by Rapamycin. Hepatocytes were incubated with 100nM Rapamycin for 1 hour prior to stimulation with 80nM insulin for 5 minutes.

Akt is a Direct Target of PI3K

Akt has been shown to be a candidate for mediating the effects of PI3K on downstream processes since it is shown to be a direct target of PI3K and can stimulate S^6K activity (Burgering et al). Insulin stimulation of Akt requires its association with the i-SH2 region of PI3K (Kohn et al). To determine whether activated PI3K associates with Akt in primary rat hepatocyes, we carried out a double immunoblot and western analysis on cells stimulated by insulin in the presence or absence of PI3K

inhibitors. As shown in the picture of the electrograph, the dark band seen in insulin stimulated cells denotes PI3K associaion with Akt. Our results show that insulin induced PI3K does bind to Akt and interestingly, the inhibitors of PI3K activation, inhibit this insulin induced association (Figure 6). Little or no association is observed in the control cells that have not been stimulated by insulin.



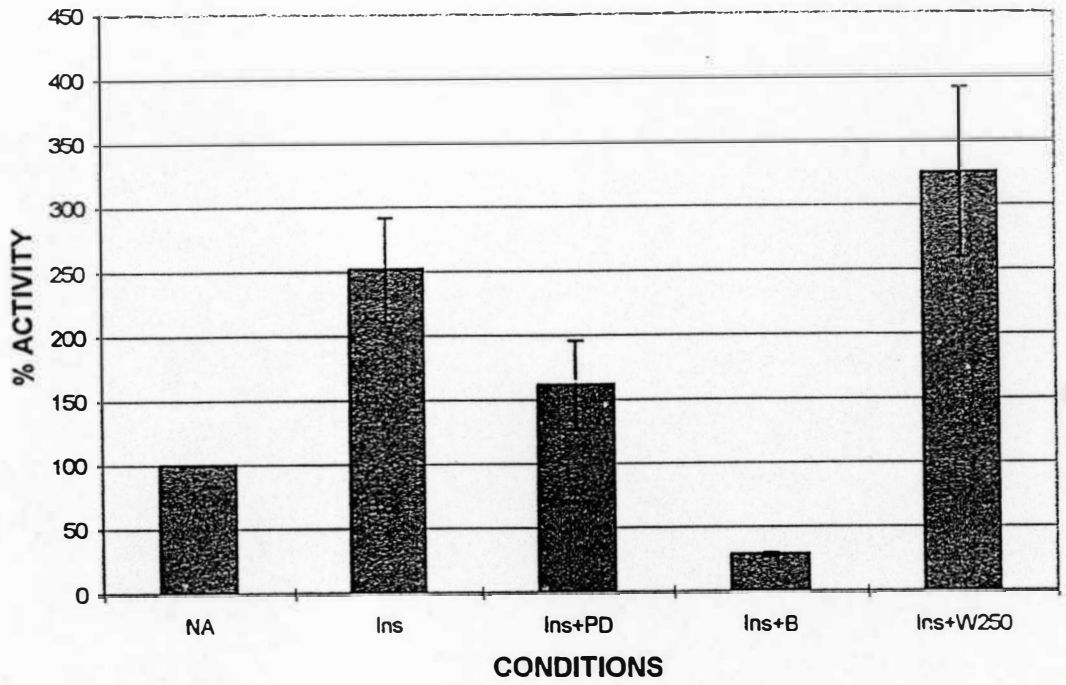
Legend. NA = No addition, I = insulin, Se = selenate, W = wortmannin, LY = LY 294002.

Figure 6. Insulin and Selenate Stimulate Association of PI 3-K With Akt. Cells were treated as described for PI 3-K assay. The dark band seen with insulin treatment represents the association of PI 3-K with Akt.

MAPK Activity

MAPK, being a serine/threonine phosphorylating agent could also potentially stimulate S^6K activity. The effect of the inhibitors of MAPK pathway, PD 098059, which inhibits MEK and B581 which inhibits RAS farnesylation, has not been determined in primary rat hepatocytes. As shown in Figure 7, in primary rat hepatocytes, MAPK activation is definitely RAS dependent since B581 completely inhibited the insulin induced 2-3 fold stimulation of MAPK. Interestingly, PD 098059 only partially blocked the insulin induction of MAPK. PI3K has been known to stimulate MAPK activity in response to insulin in adipocytes (Suga et al). We

observed that the insulin induced MAPK activity was not inhibited by wortmannin (Figure 7). Therefore we conclude that in this system, PI3K is not involved in MAPK stimulation.



Legend. NA = No Addition, I = Insulin, PD = PD 098059, B = B 581.

* = significant stimulation. ** = significant inhibition at $p < 0.05$.

Figure 7. Insulin Induced MAPK Activity is Dependent on RAS and MEK, But Not on PI 3-K. Primary rat hepatocytes were incubated with 80nM insulin either in the presence or absence of 20μM PD 098059 for 1 hour or 50μM B 581 for 30 minutes or 250nM wortmannin for 3 hours. n = 3.

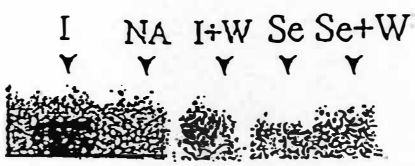
Correlating the above studies with the effect of inhibitors on insulin induced stimulation of G6PDH gene expression as studied by my collegue Sanjay Jivraj, we

show that the pathway utilized by insulin is the IRS-1/PI3K/S6K pathway and not the RAS/RAF/MAPK pathway.

Insulin Mimetics Utilize a Different Pathway for Signal Transduction

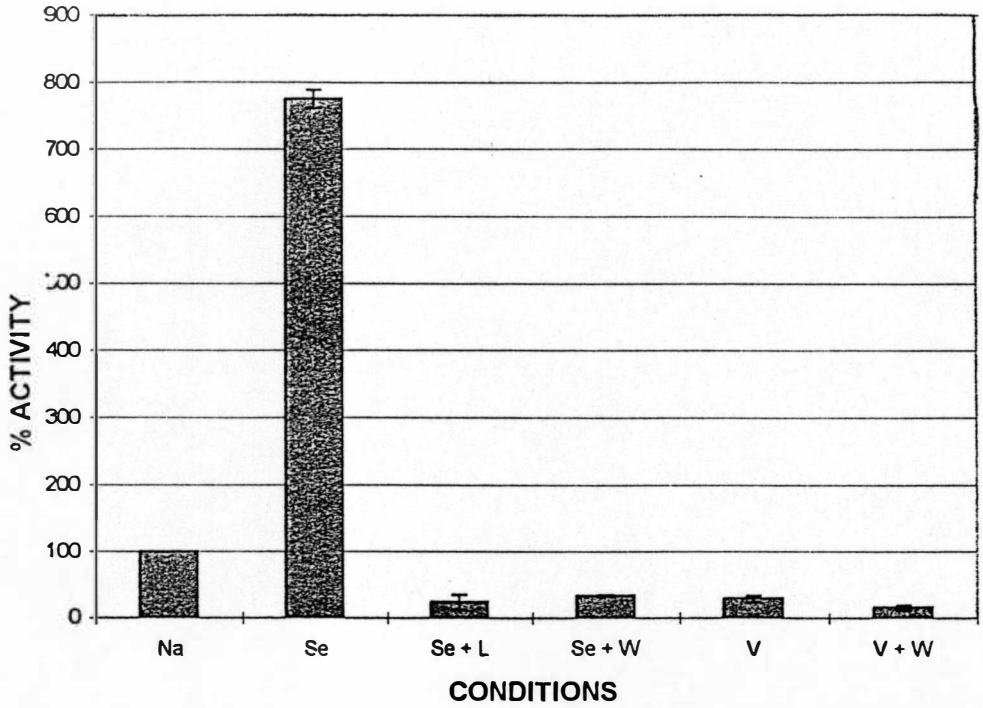
Our laboratory has previously shown that insulin mimetics such as vanadium and seleniuim also induce G6PDH enzyme activity and mRNA levels (Berg et al). To determine whether the mimetics followed the same sequence of events as insulin in the cytoplasm to bring about the final nuclear response in primary rat hepatocytes in culture, we carried out inhibition studies on either selenium or vanadium stimulated cells in a manner similar to the studies done on insulin stimulated cells.

Selenium induces phosphorylation of the insulin receptor and IRS-1 (Stapleton et al). As shown in Figure 8, Se also stimulates PI3K but vanadium does not. To determine if Se stimulation of PI3K requires the association with IRS-1 similar to insulin, we carried out western analysis.



Legend. NA = No addition, I = insulin, Se = selenate, W = wortmannin.

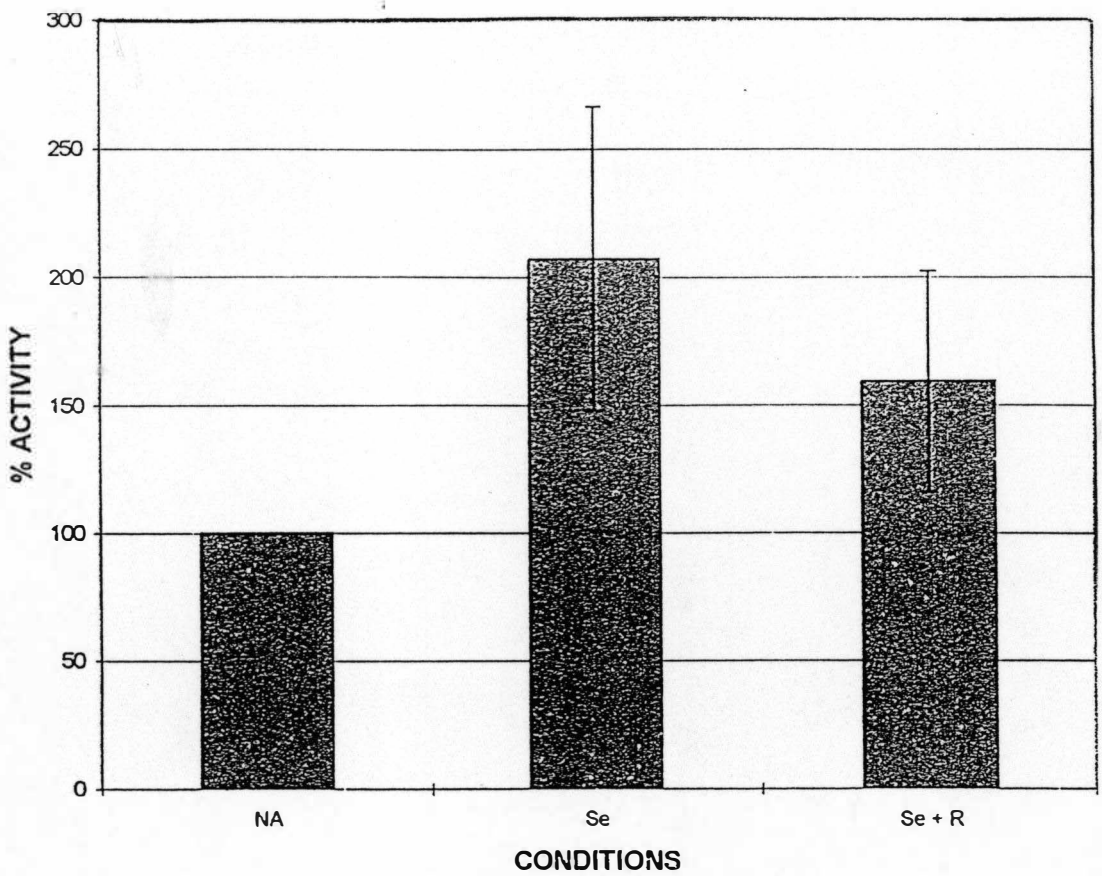
Figure 8. Selenium Does Not Induce Association of IRS-1 and PI 3-K. Cells were treated as described for PI 3-K assay. n = 3. The dark band representing IRS-1 seen with insulin stimulation is absent in Se treated cells.



Legend. NA = No Addition, Se = selenate, V = vanadate, W = wortmannin. LY = LY 294002. * = significant stimulation. ** = significant inhibition at $p < 0.05$.

Figure 9. PI 3-K Activity is Stimulated by Se But Not Vanadate. Cells were pretreated with inhibitors as described in the PI 3-K assay, prior to stimulation with either 500μM selenate or vanadate for 3 hours. n=3.

The actions of Se, downstream of PI3K are also different from insulin since it does not stimulate S^6K activity (Figure 10). Se activated PI3K did associate with Akt significantly more than the control levels but not as strongly as insulin (Figure 6). This association was also blocked similarly to insulin in the presence of wortmannin.

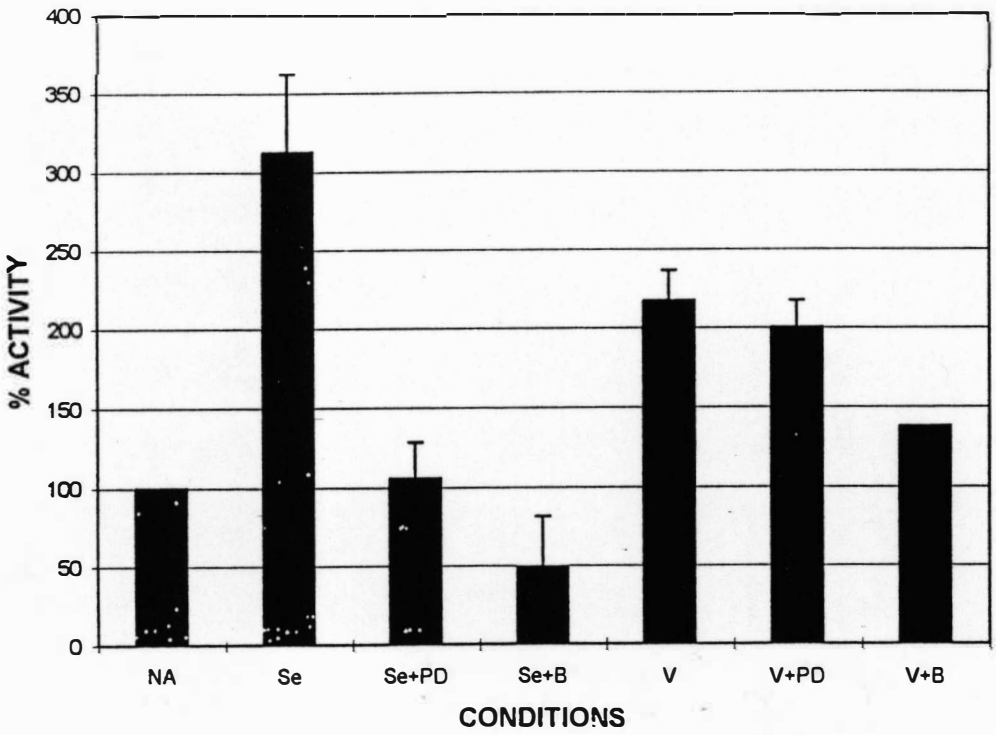


Legend. NA = No Addition, Se = selenate, R = Rapamycin.

Figure 10. Se Does Not Stimulate S^6K Activity. Hepatocytes were pretreated with 100nM Rapamycin for 1 hour prior to stimulation with 500μM selenate for 3 hours. n = 4.

The 3-4 fold stimulation of MAPK activity in response to Se in primary rat hepatocytes as shown in Figure 11, is comparable to that of insulin since the stimulation of MAPK by Se is completely inhibited by B 581 but only partially

inhibited by PD 098059. Contrary to results obtained with either Se or insulin, the vanadium stimulated MAPK activity was neither significantly inhibited by PD 098059, nor was it completely inhibited by B581 suggesting that vanadium stimulates MAPK in a manner different from insulin or Se.



Legend. NA = No Addition, Se = selenate, V = vanadate, PD = PD 098059, B = B 581. * = significant stimulation. ** = significant inhibition at $p < 0.05$.

Figure 11. MAPK Activity is Stimulated by Insullin Mimetics. Cells were pretreated with inhibitors as described for MAPK activation by insulin and subsequently stimulated by 500μM Se on V for 3 hours. n = 3.

CHAPTER IV

DISCUSSION

Insulin is responsible for modulating the activity of many proteins in a variety of cell types. The precise molecular mechanisms responsible for all these changes have not yet been fully identified. The use of inhibitors has gained importance for elucidating the mechanism of insulin signal transduction which brings about the final cellular response. Most studies using inhibitors have focused on their effects in different cell types, some of which are not physiological targets of insulin, like fibroblasts, and others like CHO IR cells which overexpress the insulin receptor. Liver is a primary target of insulin action and hence primary rat hepatocytes in culture is an efficient model to analyse the tissue specific effects of insulin.

Wortmannin, a fungal antibiotic, is well characterized as a PI3K inhibitor in many cell types including adipocytes, myoblasts and CHO IR cells and has been used extensively to analyse the requirement of PI3K for insulin induced metabolic and mitogenic responses. A more specific inhibitor of PI3K, LY 294002 was used to prove the obligatory role of PI3K for the insulin induced stimulation of DNA synthesis, glucose transport and S6K activation in 3T3 L1 adipocytes (Cheatham et al). Our results are consistent with these studies as these compounds are effective inhibitors of the insulin induced stimulation of PI3K in primary rat hepatocytes in culture.

To study the mechanism by which PI3K propagates the stimulus to the nucleus, we examined the role of S^6K as a putative downstream target. S^6K is necessary for the PI3K induced stimulation of DNA synthesis (McIlroy et al) and glyceraldehyde-3-phosphate dehydrogenase (O'Brien & Grammer). S^6K has been

shown to be stimulated by phosphorylation which can occur by either of the two mechanisms- either directly by PI3K (Weng et al) or via Akt (Burgering & Coffer). On phosphorylation, S^6K activity is controlled by FRAP, a rapamycin sensitive protein. On binding to FRAP, rapamycin impairs the phosphorylation of S^6K on thr 229 and 389, thus inhibiting S^6K activity (Sugiyama et al). We found that incubation of primary rat hepatocytes with insulin in the presence of rapamycin only partially inhibited S^6K activity. It is possible that there might be a protein involved in insulin signaling which is activated by PI3K and can stimulate S^6K in a rapamycin insensitive manner. Dennis et al have postulated the existence of rapamycin insensitive kinase kinases that differentially regulate the phosphorylation of threonine 229 and 389. Other reports stating that S^6K was not the downstream effector of PI3K for the insulin induced regulation of PEPCK (Sutherland et al), DNA synthesis (Withers et al) and glucose transport (Finger et al) also suggest the presence of a parallel pathway which diverges from PI3K and mediates downstream events. Our results showing insulin activated PI3K directly associates with Akt are striking and indicate that Akt, a protein that has currently gained importance as one of the downstream effectors of PI3K, is also utilized as such in primary rat hepatocytes. Since Akt can stimulate S^6K activity, it is an attractive candidate for explaining the dual control of insulin on S^6K in primary rat hepatocytes. The association between PI3K and Akt is required for insulin induced activation of Akt (Kohn et al). Akt can regulate PI3K mediated effects like DNA synthesis, regulation of glucose transport and activity of glycogen synthase kinase in 3T3 L1 adipocytes (Kohn et al) by a rapamycin insensitive pathway (Cross et al). It would be interesting to study the correlation of Akt kinase activity with S^6Kinase activity to further substantiate the involvement of Akt in insulin signal transduction. Since there are no known inhibitors to date that specifically inhibit the kinase activity

of Akt, it is dificult to state definitely the requirement of Akt for the rapamycin insensitive stimulation of S^6K by insulin.

We also analysed the effect of insulin on the stimulation of another important protein in the signal transduction cascade, MAPK. MAPK is necessary for certain mitogenic effects of insulin such as cellular proliferation and restoration of DNA binding activity (Davis et al). MAPK is activated in response to insulin by the serine/threonine kinase activity of MEK which lies downstream of Ras and Raf. We used the inhibitor of MEK, PD 098059, which is known to completely inhibit MAPK stimulation (Dudley et al). Our results obtained by incubating primary rat hepatocytes with insulin and PD 098059 are not in agreement with previous studies which have reported that insulin induced MAPK stimulation is completely inhibited by PD 098059. Higher concentrations of PD 098059 did not were also ineffective in inhibiting MAPK to basal levels (results not shown). In primary rat hepatocytes, the inhibitor of MEK only partially inhibited the insulin stimulation of MAPK, suggesting that MAPK can be stimulated by insulin via a MEK independent pathway. Insulin induced stimulation of MEK itself is dependent on the activation of RAS. Recent reports have questioned the necessity of RAS for activation of the MEK/MAPK pathway in metabolic cell types such as adipocytes (Whitehurst et al). Using the inhibitor of RAS farnesylation, B 581, we show that, in primary rat hepatocytes which are primarily metabolic, insulin induced MAPK activation is completely RAS dependent since B581 had a striking effect on insulin stimulated MAPK activity. The results presented above indicate that insulin induction of MAPK occurs by two mechanisms, both of which are RAS dependent. Suga et al have postulated the involvement of PI3K in stimulation of MAPK by insulin and EGF in 3T3 L1 adipocytes. We studied the effect of PI3K inhibitors on MAPK. As shown in the results, insulin stimulated activity of MAPK was not inhibited by wortmannin.

There have been other reports of "cross-talk" between the PI3K and MAPK branches of the insulin signal transduction cascade. Rodriguez-Viciana et al reported that PI3K was a direct target of RAS, while Hu et al showed that some processes regulated by PI3K in response to insulin, such as glucose transport, are RAS dependent. In CHO IR cells, PI3K activity is completely distinct from RAS activation (Hara et al). To explore the issue of "cross-talk" and to determine if PI3K was downstream of RAS, we studied the effect of B 581 on PI3K and B581 did not inhibit the insulin stimulated activation of PI3K (results not shown). Thus our results indicate that there is no "cross-talk" between PI3K and RAS, with regards to insulin stimulation.

The role of insulin induced phosphorylation events has been studied in detail with regards to the regulation of key metabolic enzymes that control glucose homeostasis, with PEPCK, the key enzyme in gluconeogenesis, being the most extensively characterized. Insulin effects on lipid metabolism have been analysed with respect to the regulatory enzyme, fatty acid synthase (FAS), which is exquisitely controlled by insulin in adipocytes. However, few studies have been done on insulin regulation of the pentose phosphate pathway. The regulatory enzyme of the pathway, G6PDH is modulated by insulin in the liver (Kletzein et al) but the sequential pathway of insulin signal transduction required for G6PDH gene induction has not been outlined. Using well characterized inhibitors of insulin induced signaling proteins, as described above, we have outlined some of the phosphorylation events in primary rat hepatocytes that are necessary for the insulin induction of G6PDH.

These effects of inhibitors on insulin stimulation of G6PDH gene expression in primary rat hepatocytes were independently characterized in our laboratory (Stapleton et al, unpublished results). Insulin stimulation of G6PDH gene expression was completely inhibited by wortmannin and LY 294002 and significantly, by rapamycin.

B581 and PD 098059, however, did not have any effect on the insulin induced G6PDH gene expression. Insulin induced stimulation of the endogenous G6PDH activity was also insensitive to B 581 and PD 098059 but was completely inhibited by wortmannin, LY 294002 and rapamycin. We therefore conclude, that insulin induced stimulation of G6PDH gene expression requires the activation of PI3K and is also dependent on the rapamycin sensitive induction of S^6K. Our results are interesting with regards to the involvement of S^6K in gene transcription since other metabolic processes such as regulation of PEPCK (Sutherland et al), glycogen synthesis and glucose transport (Withers et al) have been shown to be S^6K independent.

Our laboratory has previously shown that insulin mimetics mimic the actions of insulin on the induction of G6PDH enzyme activity. Selenium and vanadate both upregulate the endogenous activity of G6PDH as well as stimulated the expression of the G6PDH gene as measured by increases in endogenous mRNA levels and G6PDH promoter driven luciferase reporter activity. We have analysed the signal propagation mechanism of selenium and vanadate to determine if they follow the same signal transduction pathway as insulin. Results presented here show that selenium does, to a certain extent, induce the proteins in a manner similar to insulin. Selenium stimulates the phosphorylation of insulin receptor, IRS-1 and activates MAPK as shown by Stapleton et al. It is interesting that unlike insulin, we did not observe the association between PI3K and IRS-1 in selenium stimulated cells, although selenium does stimulate PI3K activity. One possibility is that, selenium being a potent stimulator of tyrosine phosphorylation (Stapleton et al), it also phosphorylates other cell surface recepters like the receptor for PDGF and EGF which can lead to the direct activation of PI3K (Kapellar & Cantley). Selenium differed from insulin in activating the proteins downstream of PI3K as it failed to induce activation of S^6K. Selenium induced association of PI3K and Akt is also much weaker than that induced by insulin,

although stimulation of PI3K is similar. The mechanism by which selenium stimulates MAPK may be similar to insulin since PD 098059 and B 581 both inhibit the selenium stimulated MAPK activity.

Vanadium did not appear to be a true insulin mimetic with regard to activation of signal proteins. In our model, vanadate did not induce the activation of PI3K or S6K. Vanadate induced stimulation of MAPK is also MEK independent in primary rat hepatocytes and is in agreement with previous studies by Band et al using bisperoxovanadium 1,10-phenanthroline. Vanadate, a potent phosphatase inhibitor may activate MAPK by inhibiting a specific tyrosine, threonine phosphatase that has the potential to downregulate MAPK. Unlike MAPK, the activity of PI3K is not modulated by its phosphorylation status, and this may explain the reason for the inability of vanadate to stimulate PI3K.

From the results presented here and the results obtained with insulin induction of G6PDH gene expression, as studied by Sanjay Jivraj, we conclude that insulin stimulation of the G6PDH gene expression requires the IRS-1/PI3K/S6K pathway and not the RAS/RAF/MAPK pathway and the mimetics, selenium and vanadate mediate their signals differently than insulin.

Appendix A

Investigator IACUC Certificate

WESTERN MICHIGAN UNIVERSITY
INVESTIGATOR IACUC CERTIFICATE

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

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 (eg Medline) to determine non-duplication
② specific room number where animals will be housed

IACUC Chairperson — 8/26/97 Date

Acceptance of Provisions

Signature: Principal Investigator/Instructor — 9/4/97 Date

IACUC Chairperson Final Approval — 9/5/97 Date

Approved IACUC Number 97-07-03

Rev. 3/97

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