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Activation of Signal Proteins by Insulin and Selenite in Primary Rat Hepatocytes

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ACTIVATION OF SIGNAL PROTEINS BY INSULIN AND SELENATE IN PRIMARY RAT HEPATOCYTES

by

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Ginny Lynn Garlock

ACTIVATION OF SIGNAL PROTEINS BY INSULIN AND SELENATE IN PRIMARY RAT HEPATOCYTES

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Western Michigan University, 1997

Insulin has a wide range of metabolic and mitogenic effects in cells. The mechanism of insulin action in controlling these events is not well understood, but involves, at least in part, a complex phosphorylation cascade. Selenate has been shown to mimic insulin on several metabolic processes, including the regulation of glucose-6-phosphate dehydrogenase (G6PDH) gene expression. Its mechanism of action, however, is not known. To investigate the mechanisms of insulin and selenate action on the regulation of G6PDH, signal proteins from primary rat hepatocytes were first studied. Insulin and sodium selenate similarly increased tyrosyl phosphorylation of the β-subunit of the insulin receptor and insulin receptor substrate-1, as determined by Western analysis. Insulin and selenate also significantly increased mitogen-activated protein (MAP) kinase activity, as determined by an in-gel activity assay, through a Ras-dependent pathway. Insulin's effects were typically rapid and transient, whereas selenate's effects were delayed in onset and sustained for hours. MAP kinase activation did not appear to be required for insulin-induced G6PDH expression, and expression may not be due to a rapamycin-sensitive pathway. Selenate induction of G6PDH expression was sensitive to the effects of rapamycin.

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INTRODUCTION

Insulin Action

β-cells in the islets of Langerhans synthesize insulin, a pancreatic polypeptide hormone. Insulin is secreted in response to high blood glucose levels, inducing a diverse range of metabolic and mitogenic responses which vary among cell types, dose responses, and time courses of action, among other factors. Insulin is capable of producing generalized effects on all cell types, such as regulation of gene expression, cell growth, and DNA synthesis. Alternatively, insulin can result in more specific effects on the insulin-responsive tissues, muscle, liver, and adipose, such as stimulation of ion transport, carbohydrate metabolism, and fatty acid metabolism (Figure 1).

As mentioned above, insulin effects many aspects of carbohydrate metabolism, including positive regulation of glucose-6-phosphate dehydrogenase (G6PDH) gene expression (Kletzein et al., 1994), one of the enzymes involved in glucose homeostasis. G6PDH is a rate-limiting enzyme in the pentose phosphate pathway, a pathway which is of major importance in carbohydrate metabolism in the liver since one half of the available glucose-6-phosphate can be shuttled through it. This pathway also produces reducing equivalents in the form of NADPH for anabolic processes, such as fatty acid biosynthesis, and for reduction of glutathione.

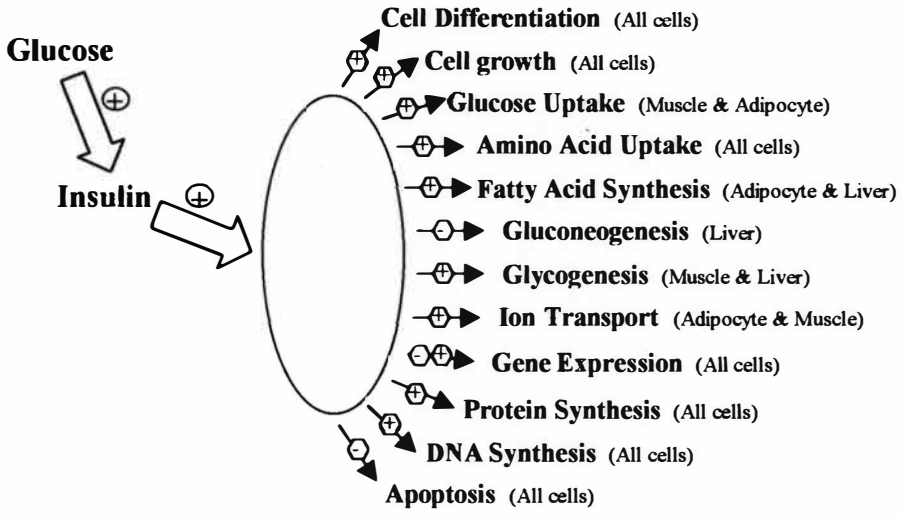

Figure 1. Effects of Insulin on Cellular Processes. (Modified from Myers & White, 1996).

In order to exert its effects on any cellular process, insulin must transmit its message from the extracellular fluid, into the cell, and often to the nucleus. The mechanism of insulin action is not well understood, but involves, at least in part, a complex phosphorylation cascade. The signal transduction pathway of insulin begins with insulin binding to its membrane receptor and activation of its kinase activity (Figure 2). The insulin receptor phosphorylates a number of substrates, including insulin receptor substrate-1 (IRS-1) and Shc. Downstream targets include the mitogen-activated protein (MAP) kinase cascade and the phosphatidylinositol (PI) 3-kinase pathway.

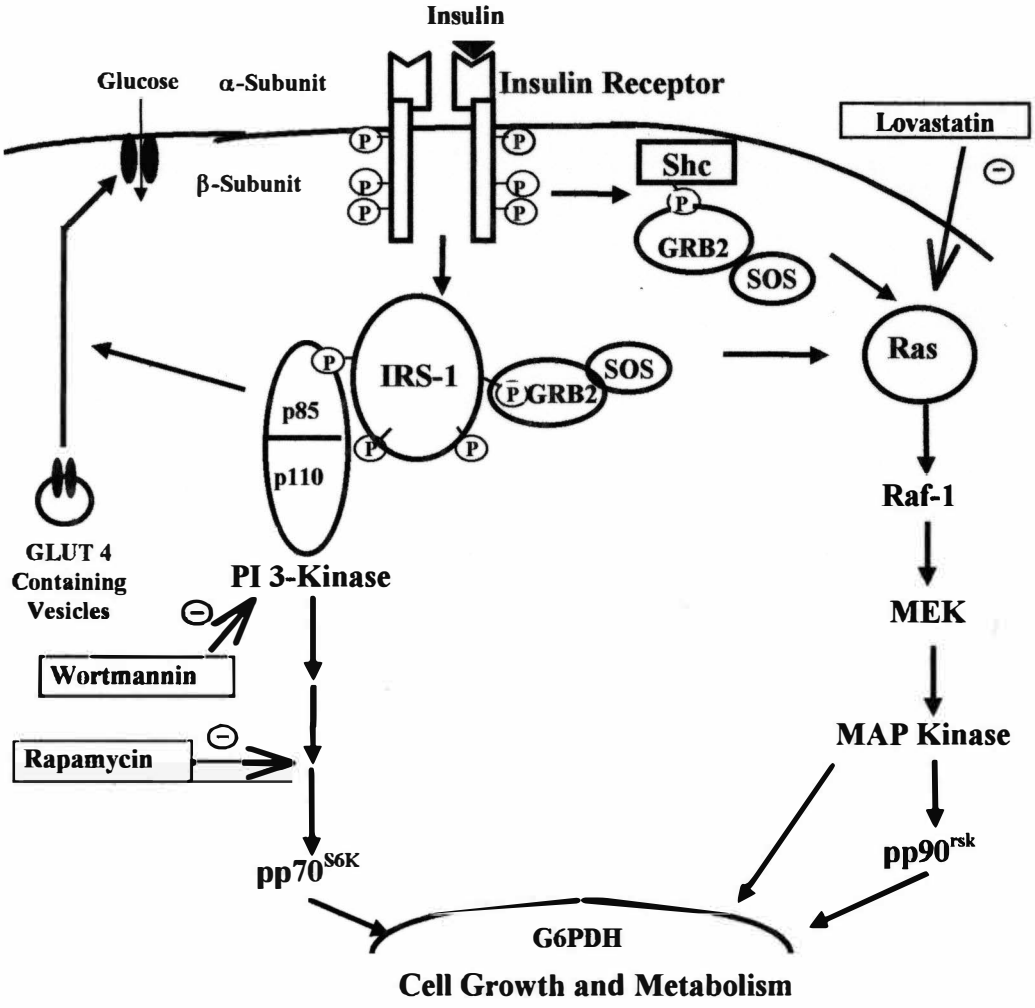

Figure 2. The Insulin Signaling Pathway.

Insulin Signaling

Insulin Receptor

Insulin binds to its plasma membrane receptor to set off the chain of events that leads to insulin action. The insulin receptor is composed of two α and two β

glycosylated protein subunits linked by disulfide bonds to form a heterotetramer (White & Kahn, 1994; Lee & Pilch, 1994; Kahn et al., 1993; Myers & White, 1996). The α-subunit, apparent molecular weight (M_r) of 135,000, is entirely extracellular, and it is responsible for the high affinity binding of insulin (Yip, 1992). The β-subunit, M_r 95,000, is a transmembrane protein specialized for signal transduction (White & Kahn, 1986).

The intracellular portion of the β-subunit is comprised of four distinct functional domains. One domain is an ATP binding domain essential for kinase activity (Chou et al., 1987), whereas the remaining three domains are capable of autophosphorylation upon insulin stimulation and include the juxtamembrane domain, the regulatory domain, and the C-terminal domain. The juxtamembrane domain, containing the residues Tyr^{960}, Tyr^{953}, and Tyr^{972}, is important in receptor substrate binding (White et al., 1988). The regulatory domain, containing the residues Tyr^{1146}, Tyr^{1150}, and Tyr^{1151}, is important in the regulation of the receptor tyrosine kinase activity (Tavare et al., 1988). The function of the C-terminal domain, containing residues Tyr^{1316} and Tyr^{1322} (Tavare et al., 1988), is unknown at this time, but it may play some sort of regulatory role on the tyrosine kinase activity upon phosphorylation (Takata et al., 1991).

It is not certain what is happening at the molecular level to activate the insulin receptor following insulin binding (Kahn et al., 1993); however, extensive data suggests the receptor acts as a classic allosteric enzyme as it undergoes conformation

and phosphorylation modifications. In unstimulated cells, the α-subunit suppresses the tyrosine kinase activity of the β-subunit. Upon insulin binding to the α-subunit, conformational changes of the receptor take place, altering the ability of the α-subunit to suppress kinase activity. The subsequent increase in kinase activity stimulates autophosphorylation through a *trans* mechanism. All three of the tyrosine residues in the regulatory domain previously mentioned require phosphorylation for full activation of the kinase activity. This phosphorylation further modifies the conformation enabling tyrosine autophosphorylation in the juxtamembrane and C-terminal domains. With these tyrosine residues phosphorylated, the insulin receptor is now able to phosphorylate its substrates.

Substrates of the Insulin Receptor

Although the insulin receptor tyrosine kinase activity had been fairly well established by 1985, a direct insulin receptor substrate still remained to be identified. The evidence for this substrate came about with the development of anti-phosphotyrosine antibodies (White et al., 1985) which identified a phosphoprotein of 185 kDa known as IRS-1.

IRS-1 is a cytoplasmic protein that associates with the insulin receptor during stimulation (Sun et al., 1992). IRS-1, containing 21 potential tyrosine phosphorylation sites (White & Kahn, 1994; Kahn et al., 1993; Myers & White, 1996), acts as a multi-site "docking" protein for signaling molecules which associate with distinct amino acid

motifs. Of the 21 potential phosphorylation sites, six have a YMXM amino acid motif, and three have a YXXM motif, while the remaining sites are other various hydrophobic motifs. The insulin receptor phosphorylates at least eight tyrosine residues on IRS-1. Four of these residues are found with the YMXM motif. Associations with phosphorylated IRS-1 occur through the *src* homology 2 (SH2) and *src* homology 3 (SH3) domains of the signaling molecules. These noncatalytic domains are conserved among many signaling proteins and function as adapter sites, allowing proteins to transiently associate without covalent attachment (Koch et al., 1991). Both PI 3-kinase (Myers et al., 1992) and growth factor receptor-bound protein 2 (GRB2) (Skolnik et al., 1993) are two proteins which bind to phosphorylated YMXM motifs of IRS-1 through SH2 domains upon insulin stimulation.

In addition to tyrosine phosphorylation sites, IRS-1 also contains over 30 potential serine/threonine phosphorylation sites in the human. IRS-1 is highly serine phosphorylated, but sparsely threonine phosphorylated in its basal state. Insulin stimulation greatly enhances the serine phosphorylation. The significance of this modification is not yet known.

Another family of substrates of the insulin receptor has also been identified, the Shc family. This family of phosphoproteins consists of at least three isoforms ranging from 46 to 52 kDa (Myers & White, 1996). Shc proteins are SH2 domain-containing proteins that have a region homologous with α 1-collagen, from which the name was

derived (Pronk et al., 1993). Upon stimulation with a number of growth factors, including insulin (Kovacina & Roth, 1993), Shc becomes tyrosine phosphorylated.

Both IRS-1 and Shc bind SH2 domains of the adapter protein GRB2. Rat 1 fibroblasts overexpressing the insulin receptor show different kinetics for the associations of GRB2 to Shc or to IRS-1 (Sasaoka et al., 1994). Although the kinetics vary, both Shc and IRS-1 compete for a limited pool of GRB2 in CHO cells overexpressing the insulin receptor (Yamauchi & Pessin, 1994). GRB2 is essential in the transmission of signal between tyrosine kinases and an important downstream target, Ras (Clark et al., 1992). In insulin-stimulated cells it appears that a guanine nucleotide exchange factor, Sos (for Son of sevenless), activates Ras proteins following association of the C-terminal region of with SH2 domains of GRB2 (Chardin et al., 1993).

MAP Kinase Cascade

The MAP kinase cascade is a pathway central to cellular responses to many growth and stress stimuli, including insulin (Seger et al., 1995; Ferrell, 1996). It has been a primary focus of research in recent years. One way MAP kinases become activated is through GTP-bound Ras. Sos exchanges the nucleoside triphosphate GTP for GDP bound to Ras, changing the conformation of the complex. The Ras-GTP conformation is the "active" conformation, being able to activate downstream signaling targets (Maruta & Burgess, et al., 1994). Ras has been found to be

important in signal transduction pathways involving growth stimulation, transformation, proliferation, and differentiation (Satoh et al., 1992).

In addition to nucleotide exchange, the activation process of Ras requires farnesylation in order for Ras to interact with the plasma membrane (Gibbs, 1991). Enzyme inhibitors have been instrumental in determining involvement of Ras, among numerous other signaling molecules, in signal transduction mechanisms. Inhibitors effectively "knock out" pathways or branches of pathways through prevention of downstream events, and therefore, one can determine potential pathways used by stimulatory agents. Lovastatin, a compound used extensively in treatment of cholesterol disorders, inhibits the farnesylation of Ras (McGuire et al., 1993; Xu et al., 1996) by inhibiting isoprenoid synthesis through competitive inhibition of HMG-CoA (Goldstein & Brown, 1990). Without proper Ras activation, downstream signaling events are limited.

Activation of Ras begins a cascade that results in MAP kinase activation. Ras is necessary for Raf-1 activation (Williams et al., 1992), but is not sufficient. Ras recruits Raf-1 to the membrane where it is activated by an unknown mechanism. This activation allows the serine/threonine kinase activity of Raf-1 to phosphorylate and activate MAP kinase kinase (MEK) (Kyriakis et al., 1992). MEK is the preferred substrate of Raf-1. Phosphorylation on serine residues activates MEK, which then phosphorylates MAP kinase on threonine and tyrosine residues, both types of which are required for full enzymatic activity (Anderson et al., 1990). Threonine and

tyrosine phosphorylation of MAP kinase activates the serine/threonine kinase activity of this enzyme (Crews & Erikson, 1992).

There are at least two isoforms of MAP kinase, p42 and p44 (Boulton et al., 1991). A series of immunofluorescence experiments demonstrated that growth factor stimulation causes both isoforms to rapidly translocate to the nucleus while MEK remains in the cytoplasm (Lenormand et al., 1993). The fact that MAP kinase can translocate to the nucleus potentially links MAP kinase to insulin's nuclear events like DNA synthesis and gene expression. Within the nucleus are transcription factors like Elk-1 that are substrates of MAP kinase (Whitmarsh et al., 1995). Besides transcription factors, other kinases and phosphatases are substrates, such as the serine kinases of the 90 kDa kinase family of the S6 kinases, (Sturgill et al., 1988). These substrates give rise to some of the known physiological roles of MAP kinase including cell proliferation, oncogenesis, development and differentiation, and cell cycle involvement.

Phosphatidylinositol 3-Kinase

IRS-1 has been shown to activate the PI 3-kinase pathway. PI 3-kinase is composed of a M_r 110,000 catalytic subunit (p110) (Hiles et al., 1992) and an M_r 85,000 regulatory subunit (p85) (Otsu et al., 1991). p85, containing two SH2 domains flanking a SH3 domain, strongly associates with tyrosyl phosphorylated IRS-1 following insulin stimulation, activating the kinase activity of p110 (Backer et al., 1992). Activation allows PI 3-kinase to phosphorylate a variety of

phosphatidylinositol substrates at the 3 position of the inositol ring (Auger et al., 1989).

At least two significant events have been linked to PI 3-kinase activation. One of these is glucose transport. Upon PI-3 kinase activation, GLUT 4 transporters translocate to the plasma membrane. Some of the evidence for PI-3 kinase involvement in glucose transport came about from the use of wortmannin, a fungal metabolite that inhibits PI 3-kinase (Powis et al., 1994). GLUT 4 is a glucose transporter found in muscle cells and adipocytes that is stimulated by insulin. Wortmannin did inhibit GLUT 4 translocation, suggesting that PI 3-kinase is involved in glucose effects of insulin (Kanai et al., 1993).

In addition to affecting glucose transport, PI-3 kinase activates S6 kinase through a mechanism that is not clear. S6 kinases phosphorylate ribosomal protein S6 following mitogen stimulation (Sturgill & Wu, 1991). PI 3-kinase activates the 70 kDa species of S6 kinases. Rapamycin is an immunosuppressant that has been found to inhibit the activation of S6 kinase (Price et al., 1992) by affecting an intermediate in this cascade upstream of this protein. Rapamycin has been used extensively in studies of insulin signaling to determine involvement of S6 kinase in glycogen synthase activity (Shepard et al., 1995), phosphoenolpyruvate carboxykinase gene expression (Sutherland et al., 1995), and glucose transporter activity (Moule et al., 1995).

Selenium

Selenium is an essential trace element with several biological effects (Bedwal et

al., 1993). It is necessary for growth, as is demonstrated by diseases produced from selenium deficiency, and is an essential component of many metalloenzymes in the form of selenocysteine. Selenium has been studied at length for therapeutic and protective functions. Current research shows selenium as a potential treatment of some forms of cancers (Clark et al., 1996). Selenium also has important antioxidant properties (Rana & Verma, 1997).

Selenium, in the form of selenate, has been found to mimic insulin on several metabolic processes. Selenate regulates gene expression of two insulin-regulated enzymes, fatty acid synthase and G6PDH in a similar manner to that of insulin (Berg et al., 1995). Selenate lowers blood glucose levels similarly to insulin as seen in streptozocin-induced diabetic rats (Berg et al., 1995; McNeill et al., 1991), as well as stimulates glucose transporter activity, cAMP phosphodiesterase activity, and ribosomal S6 kinase activity in adipocytes (Ezaki, 1990).

Not much is known about how selenium elicits its effects in terms of signaling. Selenium has been found to stimulate tyrosine phosphorylation of several endogenous proteins in adipocytes, without activating the insulin receptor kinase activity (Ezaki, 1990). Selenium, in the form of selenate, also enhanced tyrosine phosphorylation by insulin in NIH 3T3 HIR3.5 cells (Pillay & Makgoba, 1992) and adipocytes (Ezaki, 1990).

Objective of the Study

Selenium mimics insulin on a number of metabolic processes, but not much is

known about how selenium accomplishes its effects. The goal of this study was to determine if selenium elicits its insulin-mimetic effects through a similar signaling cascade as that of insulin. The model system chosen was primary rat hepatocytes, which respond similarly to insulin as whole animals. First, an attempt was made to look at the signaling cascade was by studying tyrosyl phosphorylation of insulin and selenate stimulated hepatocytes, focusing on the β-subunit of the insulin receptor and IRS-1. Next, the activity of MAP kinase was investigated, as well as the effect that inhibition of the upstream signal protein Ras had on this activity. Lastly, the effects of wortmannin, rapamycin and lovastatin on insulin and selenate-induced G6PDH expression were compared.

MATERIALS AND METHODS

All materials, unless specifically noted, were purchased from Sigma Chemical Company (St. Louis, MO) and were of tissue culture grade where applicable.

Hepatocyte Isolation and Cell Culture

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Kalamazoo, MI) weighing 200-300 g were fasted 48 h prior to hepatocyte isolation. Hepatocytes were isolated using collagenase, hyaluronidase perfusion as described (Elliget & Kolaja, 1983) with the following modifications. After collagenase perfusion, the liver was removed and gently forced through four layers of sterile gauze. The cells were washed two times with cold Waymouth's MB 752/1 medium (Gibco BRL, Grand Island, NY) supplemented with 5% bovine serum albumin (BSA) and pelleted for 3 min at 4°C and 50 x g. The cell pellet was resuspended in medium and an aliquot of cells was counted with a hemocytometer. Cell viability was determined by trypan blue dye exclusion. Cells from isolations yielding less than 70% viable hepatocytes were discarded.

Falcon 60 mm tissue culture dishes were collagen coated with 0.2 mg/ml of rat tail collagen (Collaborative Biomedical Products, Bedford, MA) and left overnight under ultraviolet light. Hepatocytes were plated at approximately 3.0×10^6 cells/plate in Waymouth's MB 752/1 medium supplemented with 10 μg/ml gentamicin and 5%

BSA under a humidified atmosphere of 5% CO_2 at 37°C. After 3-4 h of incubation, the cells were washed once with serum-free, BSA-free Waymouth's medium, and fresh serum-free, BSA-free medium was added for further incubation. Cells were treated with insulin (a gift from Eli Lilly Corp., Indianapolis, IN), sodium selenate, or enzyme inhibitors within 24 h of isolation according to the time periods and concentrations indicated in Results. Hepatocytes used in experiments involving inhibitors were treated with the inhibitors prior to insulin or selenate addition. Cells were pretreated with lovastatin (Merck, Rahway, NJ) for 24 h, rapamycin (Calbiochem, La Jolla, CA) for 30 min, wortmannin for 30 min, and okadaic acid for 30 min. These times were chosen based on use of these inhibitors in other documented studies.

Cell Harvest

For G6PDH assays, treated hepatocytes were incubated 42-48 h. Following aspiration of media, two 60 mm plates for each condition were scraped in KED buffer consisting of 0.1 M potassium phosphate buffer, pH 7.0, 3 mM ethylenediamine-tetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT). Hepatocytes were homogenized with 25 strokes in a Dounce homogenizer, and the resulting cellular extracts were centrifuged for 10 min at 4°C and 10,000 x g. Supernatants were removed and stored at -20°C.

One 60 mm plate per condition was utilized for kinase and phosphatase assays as well as Western analysis, while two 60 mm plates were pooled for the MAP kinase

activity assay. Hepatocytes for these assays were then processed as follows. Cells were washed three times in ice-cold phosphate-buffered saline (PBS), pH 7.4, and scraped into a lysis buffer composed of 20 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.3, 100 mM NaCl, 0.5% Triton X-100, 10 mM $MgCl_2$, 10 mM β-glycerophosphate, 5 mM p-nitrophenolphosphate, 1 mM EDTA, 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetracetic acid (EGTA), 1 mM sodium vanadate, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM NaF, 5 μg/ml leupeptin, and 5 μg/ml pepstatin. The total cell extracts were briefly vortexed and centrifuged for 10 min at 4°C and 10,000 x g. The supernatant was removed, quick frozen in a dry ice/isopropanol bath, and stored at -70°C.

Total Protein Determination and Assay of G6PDH Activity

Total protein concentrations were determined by the Lowry method (Lowry, 1951). G6PDH activity was measured spectrophotometrically by the rate of change of NADPH absorbance at 340 nm as described by Berg et al. (1995). Specific G6PDH activity was calculated by change in absorbance per μg protein multiplied by the extinction coefficient of the reaction.

Western Analysis

Western analysis was done with total cellular extracts, immunoprecipitated IRS-1 from cellular extracts, and semi-purified insulin receptors from cellular extracts.

IRS-1 was immunoprecipitated according to manufacturer's instructions. Briefly, 4 μg of anti-rat carboxy-terminal IRS-1 (Upstate Biotechnology Incorporated, Lake Placid, NY) was added to cellular extracts diluted to 1 μg/μl (500-1000 μg total protein) and incubated with rotation overnight at 4°C. The immunocomplex was captured by addition of 100 μl of Protein A agarose (Gibco BRL), followed by a two hour incubation period at 4°C. The agarose beads were collected by brief centrifugation and washed three times with ice-cold PBS, pH 7.4. The beads were resuspended in SDS-PAGE sample buffer.

Insulin receptors were semi-purified by adsorption to wheat germ lectin sepharose beads (Pharmacia Biotech Inc., Piscataway, NJ). Total cellular lysate was added to 200 μl of beads and incubated with rotation at 4° C for 1 h. Beads were briefly pelleted with centrifugation and washed 4-5 times with wash buffer (50 mM HEPES, pH 7.6, 0.15 M NaCl, 0.05% SDS). Insulin receptors were eluted with 50 μl of elution buffer [0.15 mM Tris-HCl, pH 7.6, 4.7% SDS, 24% glycerol, 0.71 M β-mercaptoethanol (β-ME), and 0.3 M N-acetylglucosamine] by incubation with rotation at 4° C for 15 min. Receptors were collected in the supernatant following brief centrifugation.

For Western analysis, either SDS-PAGE sample buffer was added to 5-10 μg of protein from the cellular lysates or equal volumes were taken from the semi-purification of the insulin receptor and IRS-1 immunoprecipitation. The samples were boiled for 5 min, electrophoresed on a 10% polyacrylamide, and transferred to PVDF

membrane (DuPont NEN, Boston, MA) using semi-dry electroblotting techniques as described by the manufacturer, NOVEX (San Diego, CA). Following transfer, the membrane was blocked with 5% BSA in PBS, pH 7.5, containing 0.1% Tween 20. After blocking, the membrane was incubated first with anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology Incorporated) diluted 1:2000 in PBS containing 0.1% Tween 20, and then incubated with anti-mouse IgG conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL) diluted 1:3500 in PBS containing 0.1% Tween 20. Immunoreactive bands were detected on Hyperfilm (Amersham) with an enhanced chemiluminescence Western blotting detection system according to the manufacturer's protocol (Amersham).

Tyrosine Kinase and Serine/Threonine Phosphatase Assays

Tyrosine kinase and serine/threonine phosphatase activities were evaluated with protein tyrosine kinase and protein phosphatase assay systems, respectively, according to manufacturer's instructions (Gibco BRL). The tyrosine kinase assay system contained a 13 amino acid synthetic peptide derived from the phosphorylation site of $pp60^{src}$. Briefly, 10 µl cellular extracts (50-100 µg total protein) were incubated with 0.5-1 µCi of [γ-^{32}P] ATP (Dupont NEN) and the peptide substrate for 30 min at 30°C. The reaction was quenched with ice-cold 10% TCA. After incubation on ice for 10 min, samples were centrifuged in a microcentrifuge for 10 min at 4°C. Supernatants (20 µl) were spotted onto phosphocellulose disks and washed

first with 1% acetic acid then with water. Incorporation of the label was measured by scintillation counting.

The protein phosphatase assay system measured activity of the serine/threonine phosphatases PP-1 and PP-2A on glycogen phosphorylase *a*. Okadaic acid, an inhibitor of these phosphatases, was utilized. Okadaic acid was either added to hepatocytes and allowed to incubate, or, as a control, it was added directly to the reaction tube and incubated for a brief incubation period immediately before assay. Assays were performed as follows. Radiolabeled phosphorylase *a* was generated by the incubation of phosphorylase kinase in the presence of phosphorylase *b* and 0.5 mCi of [γ-^{32}P] ATP for 1 h at 30°C. The reaction was quenched with 90% saturated ammonium sulfate and washed with a 1:2 dilution of the ammonium sulfate solution. The precipitated substrate was resolubilized in a supplied buffer [50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 15 mM caffeine, 0.1% (v/v) β-ME] and concentrated in a protein concentrator (Amicon, Centricon-30) centrifuged at 5000 x g for 20-30 min at 20°C. In short, the assay proceeded as follows. Cellular extracts (20 µl, 100-200 µg total protein) were incubated with the ^{32}P-labeled substrate for 10 min at 30°C. Reactions were quenched with addition of ice-cold 20% TCA and then incubated on ice for 10 min. Following centrifugation in a microcentrifuge for 3 min at 4°C, phosphatase activity was determined by scintillation counting of the liberated ^{32}P-labeled phosphate found within the supernatant.

MAP Kinase Activity Assay

MAP kinase activity was analyzed using an in-gel activity assay with myelin basic protein (MBP) as a substrate (Tobe et al., 1992) with the following modifications. Samples were suspended in SDS-PAGE sample buffer and boiled for 5 min. Equal volumes of samples, containing roughly 60-120 μg total protein, were electrophoresed in a 12% polyacrylamide gel containing 0.55 mg/ml MBP (Gibco BRL). After electrophoresis the gel was washed sequentially in the following solutions: Buffer B (50 mM Tris pH 8.0 and 5 mM β-ME) containing 20% isopropanol, Buffer B only, and then Buffer B containing 6 M guanidine HCl. After denaturation, the gel was washed in Buffer B containing 0.04% Triton X-100 and then in Buffer E (40 mM HEPES pH 8.0, 2 mM DTT, and 10 mM $MgCl_2$). Finally, the gel was incubated with 40 μM [γ-^{32}P] ATP in Buffer E, rinsed in 5% TCA and 1% sodium pyrophosphate, dried, and exposed to X-OMAT AR film (Eastman Kodak Company, Rochester, NY). Densitometric scanning results were equalized for protein amounts.

Densitometric Scanning

Images of Western blots and MAP kinase autoradiographs were captured and quantitated by densitometric scanning on Image-1/MetaMorph, Version 2 (Universal Imaging Corporation). Images were scanned from autoradiographs by a Hewlett Packard Scanjet5p using Paperport (Visioneer, Incorporated), Version v.B, followed by modification in Corel PhotoPaint Select (Corel Corporation), Version 5.00.F4.

Final images were imported into document and printed by Microsoft Word (Microsoft Corporation), Version 97.

Statistical Analysis

The results are expressed as the mean ± S.E.M. Significance was evaluated by one-tailed Student's t-test and tested at $p<0.05$.

RESULTS

Insulin and Selenate Induction of Tyrosine Phosphorylation

Cells from insulin-sensitive tissues exhibit a rapid increase in tyrosine phosphorylated proteins in response to insulin (Myers & White, 1996; Kahn et al., 1993). In order to determine whether this insulin-induced increase occurs in primary rat hepatocytes, Western analysis was performed with whole cell lysates using anti-phosphotyrosine antibody. Figure 3 shows a representative blot of the time course of insulin-stimulated tyrosine phosphorylation. Hepatocytes treated with 80 nM insulin for 1 min, 5 min, 15 min, or 30 min exhibited rapid tyrosyl phosphorylation of a number of endogenous proteins, including protein bands that migrated at the apparent molecular weights of the β-subunit of the insulin receptor and IRS-1. Phosphorylation of the β-subunit of the insulin receptor increased maximally 2-3 fold, while phosphorylation of IRS-1 increased by 3-4 fold. Increases in insulin-induced tyrosyl phosphorylation of the β-subunit of the insulin receptor and IRS-1 have been observed in many cell types including Fao hepatoma cells (White et al., 1985) and CHO cells overexpressing the insulin receptor (Sun et al., 1992). Similar to results observed by others (Kowalski et al., 1983), maximal levels of phosphorylation occurred within five minutes and then diminished to near basal levels within an hour. This transient increase in phosphorylation is most likely due to dephosphorylation by tyrosine

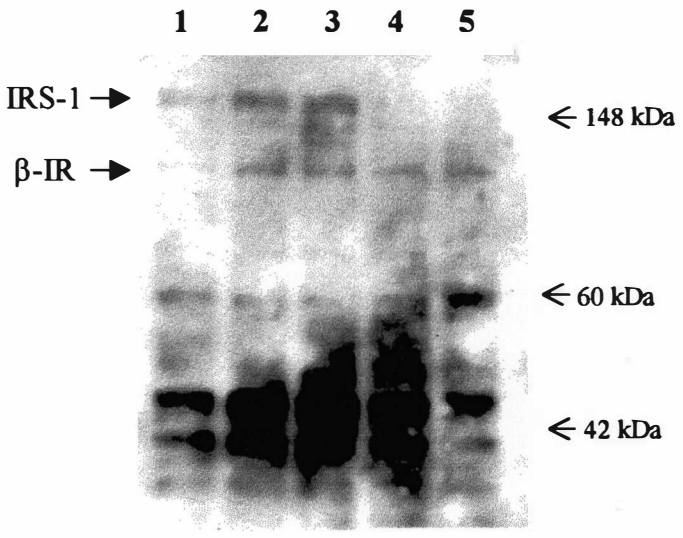

Figure 3. Effect of Insulin on Tyrosyl Phosphorylation.

Hepatocytes were treated with either nothing (Lane 1), or 80 nM insulin for 1 min (Lane 2), 5 min (Lane 3), 15 min (Lane 4), or 30 min (Lane 5). Cellular extracts were subjected to Western analysis with anti-phosphotyrosine antibody. Bands corresponding to IRS-1 and the β-subunit of the insulin receptor (β-IR) are shown on the left. Molecular weight markers are shown on the right.

phosphatases, since known tyrosine phosphatase inhibitors completely abolish the characteristic decrease in tyrosyl phosphorylation (Kowalski et al., 1983).

Selenate mimics insulin action on a number of metabolic processes. To investigate whether selenate mimics insulin action by stimulation of tyrosyl phosphorylation of similar proteins, hepatocytes were treated with either 250 μM sodium selenate for 1 h or 3 h, or 500 μM selenate for 5 min, 30 min, 1 h, or 3 h. Cellular lysates were then analyzed by Western blotting with anti-phosphotyrosine antibody. Figure 4 shows a representative blot of the concentration and time-

dependence of selenate on tyrosine phosphorylation. Selenate increased tyrosyl phosphorylation of many endogenous proteins, including bands migrating at the apparent molecular weights of the β-subunit of the insulin receptor and IRS-1. These results agree with a selenate-induced increase in the phosphorylation of a number of endogenous proteins seen in adipocytes (Ezaki, 1990) and NIH 3T3 HIR3.5 cells (Pillay & Makgoba, 1992). Notable increases in tyrosyl phosphorylation appeared at 3 h with 250 μM selenate or 1 h with 500 μM selenate. Phosphorylation of the β-

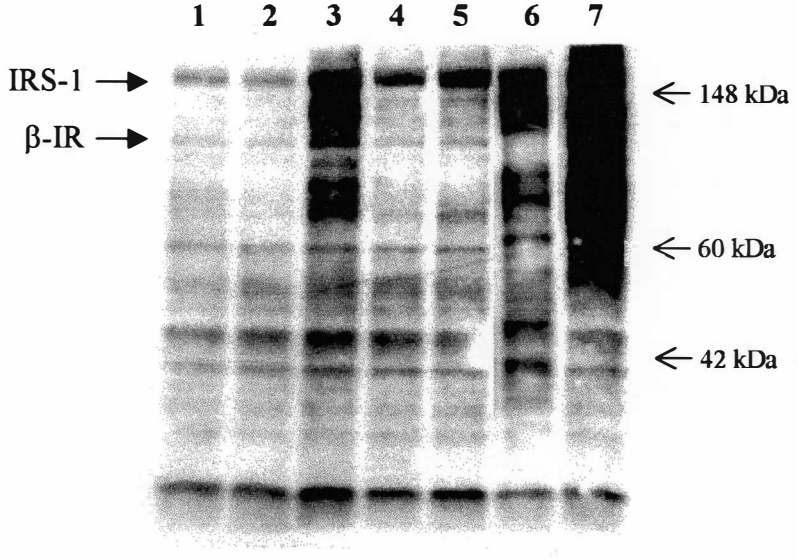

Figure 4. Effect of Selenate on Tyrosyl Phosphorylation.

Hepatocytes were treated with either nothing (Lane 1); 250 μM sodium selenate for 1 h (Lane 2) or 3 h (Lane 3); or 500 μM sodium selenate for 5 min (Lane 4), 30 min (Lane 5), 1 h (Lane 6), or 3 h (Lane 7). Cellular extracts were subjected to western analysis with anti-phosphotyrosine antibody. Bands corresponding to IRS-1 and β-IR are shown on the left. Molecular weight markers are shown on the right.

subunit of the insulin receptor, as compared to control cells, increased from approximately 1.5 fold with 250 μM selenate at 1 h to 3 fold at 3 h; whereas at 500 μM selenate, fold increases changed from 2 fold at 1 h to about 5 fold at 3 h. Phosphorylation of IRS-1, as compared to control cells, increased from approximately 1.5 fold with 250 μM selenate at 1 h to 3 fold at 3 h; while at 500 μM selenate, fold increases changed from 2-3 fold at 1 h to about 6 fold at 3 h. Curiously, the level of tyrosyl phosphorylation in selenate-stimulated cells did not diminish within the time course tested, but in fact escalated within five hours of incubation.

To generate additional evidence that increases in tyrosyl phosphorylation of the β-subunit of the insulin receptor and IRS-1 were observed following insulin and selenate stimulation, insulin receptors and IRS-1 were separated from whole cell lysates by wheat germ adsorption and immunoprecipitation, respectively. The resulting proteins were then subjected to Western analysis with anti-phosphotyrosine antibody. Hepatocytes incubated with either 80 nM insulin for 5 min or 500 μM sodium selenate for 1 h had an increase in tyrosyl phosphorylation of the β-subunit similar to that seen in the general tyrosyl phosphorylation of whole cell lysates (data not shown). An increase in tyrosyl phosphorylation of IRS-1 of approximately 3 fold occurred in hepatocytes stimulated with 80 nM insulin for 5 min, while an increase of 2 fold was seen in cells stimulated with 500 μM selenate for 1 h (Figure 5).

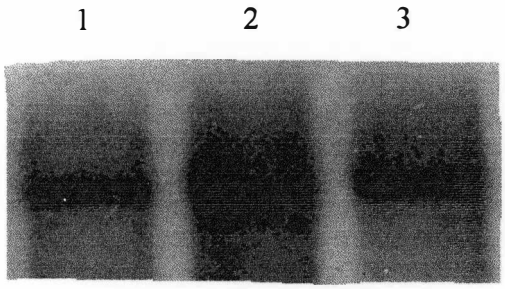

Figure 5. Effect of Insulin and Selenate on Tyrosyl Phosphorylation of IRS-1.

Hepatocytes were dosed with either nothing (Lane 1), 80 nM insulin for 5 min (Lane 2), or 500 μM sodium selenate for 1 h (Lane 3). IRS-1 was immunoprecipitated from cellular extracts and subjected to Western analysis. Tyrosyl phosphorylation was detected by anti-phosphotyrosine antibody.

Activity of Tyrosine Kinases and Serine/Threonine Phosphatases

Incubation of hepatocytes in the presence of sodium selenate for three or more hours resulted in a substantial increase in tyrosyl phosphorylation. This increase could potentially be due to either an increase in tyrosine kinase activity or a decrease in tyrosine phosphatase activity. To explore the effect of selenate on kinase and phosphatase activity, commercially available enzyme activity kits were employed. The tyrosine kinase kit did not prove effective for our line of study due to technical problems. Background incorporation of labeled substrate onto phosphocellulose disks was often higher than that of the samples assayed. Therefore, data was inconclusive concerning the effect of selenate on tyrosine kinase activity.

A tyrosine phosphatase kit was unavailable at the time of these experiments, so a kit analyzing serine/threonine activity of the serine/threonine phosphatases PP-1 and PP-2A was utilized to determine if a decrease in the activity of these phosphatases could potentially affect overall tyrosyl phosphorylation. Incubation of hepatocytes in 500 μM sodium selenate for either 3 or 5 h had no effect on serine/threonine phosphatase activity of PP-1 and PP-2A as compared to activity of control plates (data not shown). In addition, 2 mM selenate added *in vitro* to untreated cells immediately before assay also did not decrease activity. However, 10 nM okadaic acid, a known serine/threonine phosphatase inhibitor, inhibited phosphatase activity by over 60% whether it was incubated with cells or added *in vitro* immediately before assay (data not shown). Thus, selenate does not appear to inhibit the activity of these phosphatases.

MAP Kinase Activation

The MAP kinase cascade is a common pathway utilized by many growth factors, including insulin. To determine if MAP kinase was involved in primary hepatocyte signaling upon insulin and selenate stimulation, an in-gel kinase activity assay was used. Figure 6 shows a representative autoradiograph of insulin and selenate-induced MAP kinase activity. Insulin at 80 nM concentrations incubated for 5 min stimulates an increase in the activity of two isoforms of MAP kinase, p42 and p44, as compared to control. Insulin increased activity of p44 by 2.3 fold at 5 min. Measurable MAP kinase activity was not seen before 5 min and rapidly decreased to

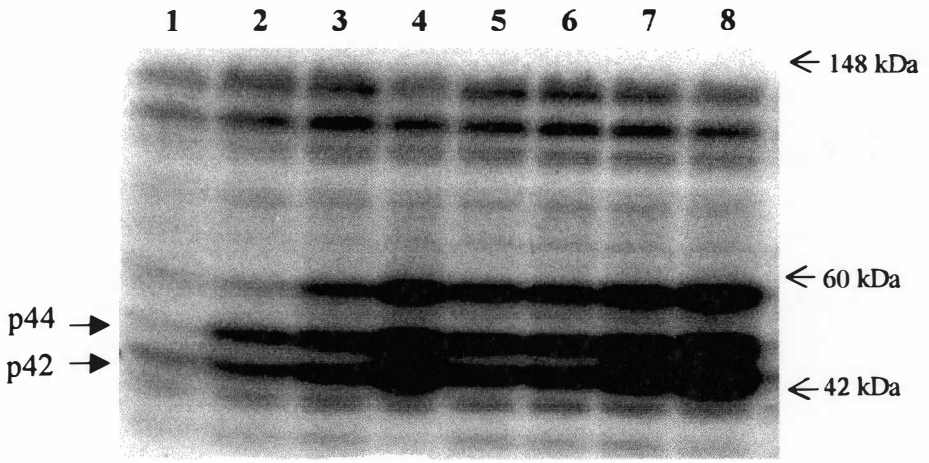

Figure 6. Effect of Insulin and Selenate on MAP Kinase Activity.

Hepatocytes were treated with either nothing (Lane 1); 80 nM insulin for 5 min (Lane 2); 250 μM sodium selenate for 30 min (Lane 3) or 1 h (Lane 4); or 500 μM sodium selenate for 15 min (Lane 5), 30 min (Lane 6), 1 h (Lane 7), or 2 h (Lane 8). Cellular extracts were electrophoresed in a polyacrylamide containing gel containing myelin basic protein as a substrate. The gel was subjected to a kinase reaction using [γ-^{32}P] ATP as a substrate and bands were visualized by autoradiography. Arrows on the left indicate the p42 and p44 isoforms of MAP kinase. Arrows on the right indicate molecular weight markers.

near basal levels within 30 min. Increases in MAP kinase activity upon insulin stimulation have been fairly well-characterized in a variety of cell types (Boulton et al., 1991; Peak et al., 1993; Tobe et al., 1992).

Selenate also stimulates an increase in p42 and p44 MAP kinase activities in a time and concentration-dependent fashion. As shown in Figure 7, 500 μM selenate substantially increased activity of p44 from 5 fold at 30 min to 6.3 fold at 2 h. Since these fold increases were so much higher than that of insulin stimulation, lower

concentrations and shorter time points were explored. Cells stimulated with 250 μM selenate for 15 min increased MAP kinase activity of p44 3.46 fold (mean of n=2) over that of control. In contrast to insulin, selenate continued to increase levels of activity throughout the time periods tested, up to 3 h.

In insulin-stimulated cells, MAP kinase is activated through the Ras/Raf/MEK pathway. Since MAP kinase activation occurred as a result of insulin and selenate stimulation in primary rat hepatocytes, it now became important to determine if the Ras/Raf/MEK pathway was utilized. In order to accomplish this, an inhibitor of Ras

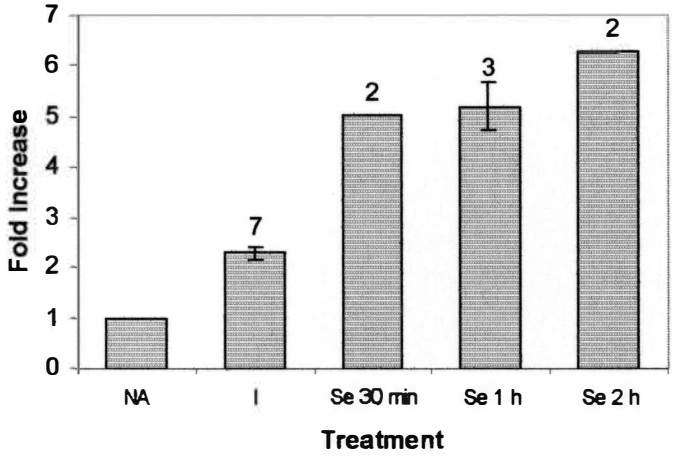

Figure 7. Effect of Insulin and Selenate on p44 MAP Kinase Activity.

Hepatocytes were incubated with either nothing (NA), 80 nM insulin for 5 min, or 500 μM sodium selenate for 30 min, 1 h, or 2 h. MAP kinase activity was determined with an in-gel activity assay as described in Materials and Methods. Autoradiographs of p44 were quantitated by scanning densitometry, equalized for protein amounts, and compared to NA. Data shown is the mean of the fold increases ± S.E. of the number of experiments shown above the bars.

activation, lovastatin, was used to determine its effects on MAP kinase activity. Hepatocytes were pretreated with either 0, 2, or 20 μM lovastatin overnight and then treated with nothing (NA), 80 nM insulin for 5 min, or 500 μM sodium selenate for 1 h. MAP kinase activity was determined by the in-gel activity assay. Autoradiographs of p44 were quantitated by densitometric scanning, equalized for protein concentrations, and compared to control plates (NA) for each of the inhibitor concentrations. The higher the dose of lovastatin, the greater the decrease in p44 MAP kinase activity (Figure 8). Decreases in insulin and selenate stimulated activity were significant at $p<0.05$ for 2 μM and 20 μM lovastatin as compared to unstimulated cells. Xu et al. (1996) also saw a decrease in insulin-stimulated MAP kinase activation in HIRcB fibroblasts in the presence of lovastatin. Lovastatin did not appear to have an effect on MAP kinase activity in unstimulated cells.

Effects of Enzyme Inhibitors on G6PDH Activity

Insulin, as well as selenate, upregulates G6PDH expression. It is not known which pathway is utilized for insulin induction of G6PDH. In addition, it is not known how selenate stimulates G6PDH expression. In order to investigate this, several enzyme inhibitors were employed in an attempt to block certain pathways.

Since an increase in MAP kinase activity had been observed, it was necessary to determine if G6PDH activity was linked to the MAP kinase cascade. Lovastatin was tested at 0.01, 0.1, 1, and 10 μM. Hepatocytes were incubated in the absence or

presence of lovastatin for 24 h and were then either left untreated or treated with 80 nM insulin or 20 μM sodium selenate for 42-48 h. The activities of the control plates for each inhibitor concentration were arbitrarily set at 100 %, and the effects of lovastatin with insulin or selenate were expressed as percent of control. A wide range

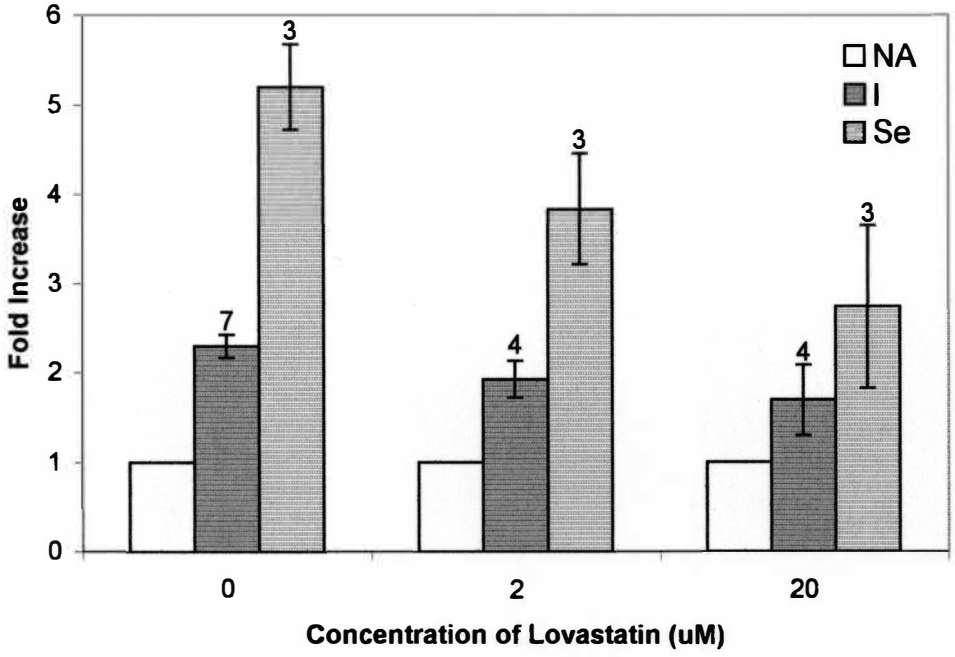

Figure 8. Effect of Lovastatin on p44 MAP Kinase Activity.

Hepatocytes were incubated with either nothing (NA), 80 nM insulin for 5 min, or 500 μM sodium selenate for 1 h after a pretreatment period of 24 h with 0, 2, or 20 μM lovastatin. MAP kinase activity was determined with an in-gel activity assay as described in Materials and Methods. Autoradiographs of p44 were quantitated by scanning densitometry, equalized for protein amounts, and compared to the respective control inhibitor plates. Data shown is the mean of the fold increases ± S.E. of the number of experiments shown above the bars.

of concentrations were tested with few complete experimental runs (at most n=2). General trends were observed, but we did not pursue the effects of lovastatin on G6PDH activity further. Lovastatin did not appear to have any effect on G6PDH activity in insulin-treated cells. For every concentration of lovastatin used, insulin induced increases in G6PDH activity 230 to 260 percent of control, which was no different from cells incubated without inhibitor. It could not be determined if lovastatin affected G6PDH activity in selenate-treated cells. Selenate-induced activity of G6PDH was uncharacteristically variable for these trial runs, and trends could not be identified. The percent increases ranged from 170 to 240 percent of control with no observable pattern.

With evidence against MAP kinase participation in insulin-induced G6PDH expression, attention turned to investigation of the involvement of the PI 3-kinase pathway with this expression. Wortmannin has been a useful tool in determining involvement of PI 3-kinase with metabolic enzymes. Hepatocytes in culture were incubated in the absence or presence of 1, 10, and 100 nM wortmannin for 30 min and were then either left untreated or treated with 80 nM insulin or 20 μM sodium selenate for 42-48 h. The activities of the control plates for each inhibitor concentration were arbitrarily set at 100 %, and the effects of the inhibitors with insulin or selenate were expressed as percent of control. Wortmannin decreased G6PDH activity in insulin-stimulated cells by 45% (Table 1). There were statistically significant decreases ($p < 0.05$) in enzyme activity for each concentration of wortmannin as compared to the control insulin-stimulated cells. In selenate-stimulated cells, wortmannin decreased

Table 1

Effect of Wortmannin on G6PDH Activity in Primary Rat Hepatocytes

Treatment	G6PDH activity as % of control
Ins	243 ± 11 * (3)
Wm 1 + Ins	190 ± 29 □ (3)
Wm 10 + Ins	134 ± 20 □ (3)
Wm 100 + Ins	173 ± 35 □ (3)
Se	186 ± 12 * (3)
Wm 1 + Se	160 ± 46 (3)
Wm 10 + Se	144 ± 18 ◊ (3)
Wm 100 + Se	53 (2)

* Significantly different from NA at $p<0.05$.
□ Significantly different from Ins at $p<0.05$.
◊ Significantly different from Se at $p<0.05$.

Primary rat hepatocytes in culture were incubated in the absence or presence of 1, 10, or 100 nM wortmannin (Wm) for 30 min. Hepatocytes were then either left untreated (NA) or treated with 80 nM insulin (Ins) or 20 μM sodium selenate (Se) for 42-48 h. Cells from two plates were pooled and processed for each experimental condition, and G6PDH activity was determined spectrophotometrically as change in the absorbance of NADPH. The activities of the control plates for each inhibitor concentration were arbitrarily set at 100 %, and the effects of wortmannin with Ins or Se were expressed as percent of control. Data shown is the mean of the specific activities (units/mg protein) ± S.E. of the number of experiments shown in parenthesis.

G6PDH activity by 23% at 10 nM of wortmannin, and this decrease was statistically significant at $p< 0.05$. The activities of the control inhibitor plates were also compared to control plates absent of inhibitor. At concentrations of 10 and 100 nM, percents of

G6PDH activity as compared to control were 74 ± 12 and 59 ± 13, respectively. These values are significantly lower than the control at $p < 0.05$, however, this did not appear to be due to toxicity as seen with isocitrate dehydrogenase (ICDH) and lactate dehydrogenase (LDH) activities. ICDH is a constitutively active enzyme in cellular respiration. Decreases in the activity of this enzyme as compared to control samples would indicate some cellular toxicity, and no appreciable differences were seen in wortmannin-treated samples. Another general indicator of cellular toxicity is LDH leakage into media. We would expect LDH activity to increase in media samples from cells experiencing toxicity. Again, no appreciable differences were seen between control samples and samples from wortmannin-treated samples.

After establishing involvement of PI 3-kinase, metabolic enzymes have been found to signal through at least two different pathways, a rapamycin-sensitive route and a rapamycin-insensitive route. Rapamycin has been used to determine S6 kinase involvement with several insulin-regulated enzymes. To determine if insulin and selenium used a rapamycin-sensitive or rapamycin-insensitive pathway, rapamycin was tested at 0.1, 1, and 10 nM. Primary rat hepatocytes in culture were incubated in the absence or presence of rapamycin for 30 min and were then either left untreated or treated with 80 nM insulin or 20 μM sodium selenate for 42-48 h. The activities of the control plates for each inhibitor concentration were arbitrarily set at 100 %, and the effects of rapamycin with insulin or selenate were expressed as percent of control. Rapamycin did not significantly decrease insulin-induced G6PDH activity at any of the concentrations tested (Table 2). Rapamycin did, however, significantly decrease

selenate-induced G6PDH activity. The activities of the control inhibitor plates were also compared to control plates absent of inhibitor. At 10 nM, the percent of G6PDH activity as compared to control was 70 ± 5, which is a statistically significant decrease from control tested at $p < 0.05$.

Table 2

Effect of Rapamycin on G6PDH Activity in Primary Rat Hepatocytes

Treatment	G6PDH activity as % of control
Ins	254 ± 22 * (4)
Rap 0.1 + Ins	213 ± 19 (4)
Rap 1 + Ins	216 (2)
Rap 10 + Ins	219 ± 23 (4)
Se	209 ± 16 * (4)
Rap 0.1 + Se	151 ± 17 ◊ (4)
Rap 1 + Se	178 (2)
Rap 10 + Se	163 ± 8 ◊ (3)

* Significantly different from NA at $p<0.05$.
◊ Significantly different from Se at $p<0.05$.

Primary rat hepatocytes in culture were incubated in the absence or presence of 0.1, 1, or 10 nM rapamycin (Rap) for 30 min. Hepatocytes were then either left untreated (NA) or treated with 80 nM insulin (Ins) or 20 μM sodium selenate (Se) for 42-48 h. Cells from two plates were pooled and processed for each experimental condition, and G6PDH activity was determined spectrophotometrically as change in the absorbance of NADPH. The activities of the control plates for each inhibitor concentration were arbitrarily set at 100 %, and the effects of rapamycin with Ins or Se were expressed as percent of control. Data shown is the mean of the specific activities (units/mg protein) ± S.E. of the number of experiments shown in parenthesis.

DISCUSSION

Insulin has a wide range of metabolic and mitogenic effects in cells. Metabolically, insulin is involved in such cellular processes as glucose uptake, fatty acid synthesis, gluconeogenesis, gene expression, and glycogenesis. Disturbances of any of these processes can have serious metabolic consequences. Deficiencies in synthesis or recognition of insulin by a cell has lead to interest in insulin-mimetic agents that could potentially be used therapeutically to treat metabolic disorders such as diabetes mellitus. To understand whether or not a mimetic truly potentiates properties similar to insulin, one must first understand the mechanism of insulin action. Metabolic and growth-promoting effects of insulin on cells has been well-studied in the past, and recently, much progress has been made on the signaling mechanism of insulin (Myers & White, 1996; Kahn et al., 1993). Insulin's signaling pathway involves, at least in part, a phosphorylation cascade. Insulin binds to its receptor causing tyrosine autophosphorylation and activation of the receptor tyrosine kinase activity in the β-subunit of the insulin receptor. IRS-1 is a substrate of the receptor kinase which transmits the signal in a number of directions, including the MAP kinase cascade and the PI 3–kinase pathway. Further downstream, the insulin signal causes nuclear events such as DNA synthesis, protein synthesis, and gene expression.

Selenate has been shown to mimic insulin on several metabolic processes, including regulation of blood glucose levels in streptozocin-induced diabetic rats (Berg

et al., 1995; McNeill et al., 1991), expression of insulin-regulated enzymes such as G6PDH and fatty acid synthase *in vivo* (Berg et al., 1995) and *in vitro* (Stapleton et al., unpublished data), and glucose transport (Furnsinn et al., 1996). The mechanism of selenate action is not, however, known. We hypothesized that for selenate to mimic G6PDH expression by insulin, the same signaling pathway should be utilized. To test this hypothesis, we looked at the effects of insulin and selenate on signaling proteins in the insulin signal transduction pathway in primary rat hepatocytes. Primary rat hepatocytes are an excellent model for these types of studies, since metabolically they respond similarly to whole animal models. Primary rat hepatocytes have not previously been used to investigate insulin signaling using a metabolic end point such as G6PDH expression. In addition, the effects of selenate on the signaling mechanism of insulin has not yet been studied in this model system.

Insulin stimulates tyrosine phosphorylation in cells from insulin-sensitive tissues (Myers & White, 1996; Kahn et al., 1993), and we chose to focus on two of the most well-characterized tyrosyl phosphorylated proteins, the β-subunit of the insulin receptor and IRS-1. We show that insulin induced immediate increases in the tyrosyl phosphorylation of a number of endogenous proteins, including the β-subunit of the insulin receptor and IRS-1. Insulin signaling of tyrosyl phosphorylation in hepatocytes follows typical signaling mechanisms, that is, rapid initiation followed by fairly rapid inhibition. The results seen here do concur with the transient phosphorylation seen with insulin receptors isolated from insulin-stimulated rat liver

(Kowalski et al., 1983). The transient nature of insulin-induced phosphorylation is most likely due to dephosphorylation by tyrosine phosphatases (Kowalski et al., 1983).

After establishing the increase in phosphorylation of the β-subunit of the insulin receptor and IRS-1, we next examined whether or not selenate could induce tyrosine phosphorylation of these signal proteins. Previous studies have shown selenate-stimulated tyrosyl phosphorylation in NIH 3T3 HIR3.5 cells (Pillay & Makgoba, 1992) and adipocytes (Ezaki, 1990) using concentrations up to 1 mM selenate. We found that 250 and 500 μM selenate initiated phosphorylation changes in the β-subunit of the insulin receptor and IRS-1 in hepatocytes. The selenate-induced changes in tyrosine phosphorylation of the β-subunit phosphorylation and IRS-1 were similar to the increases seen with insulin. Parallel selenate studies were done in collaboration with Adams & Kletzien in a different insulin-sensitive cell-type, 3T3-L1 adipocytes, to show that selenate's effects were not specific to hepatic tissue (Stapleton et al., 1997). In the 3T3-L1 cells, similar results concerning selenate-stimulated tyrosine phosphorylation were obtained. The increase in phosphorylation of IRS-1 from hepatocytes was not due to an increase in expression of the IRS-1 protein as determined by IRS-1 immunoblots (Stapleton et al., 1997). Generally, tyrosine phosphorylation in response to selenate was a concentration and time-dependent phenomenon in primary hepatocytes and in 3T3-L1 cells. There was a characteristic delay in the appearance of phosphorylation. Even at 1 mM selenate, measurable increases in phosphorylation took 30 minutes to appear (Ezaki, 1990). In addition,

phosphorylation was not transient in nature, but in fact increased to extensive proportions with continued incubation. This delay in onset and extensive degree of phosphorylation were effects much different than those seen with insulin.

In experiments looking at tyrosine phosphorylation in our model and in the 3T3-L1 cells (Stapleton et al., 1997), there was consistently a massive tyrosine phosphorylated band detected near 185 kDa. Although we confirmed, using immunoprecipitation, that IRS-1 (M_r 185 kDa) was a component of this signal, questions arose as to whether or not this band could be the epidermal growth factor (EGF) receptor, M_r 170 kDa. EGF receptors are also abundant in hepatocytes, and selenium has been reported to affect this receptor in A431 cells, however, 1 mM selenate was required for phosphorylation (Pillay & Makgoba, 1992). To rule out the possibility of this band being the EGF receptor, we tested the effect of PD 153035, a specific inhibitor of the EGF receptor (Fry et al., 1994), on phosphorylation. Addition of PD 153035 did not appear to have any effect on the selenate-induced tyrosyl phosphorylation (data not shown). Thus, selenate does not appear to initiate its effect on tyrosine phosphorylation through phosphorylation of the EGF receptor in our model system.

Unlike insulin, selenate increased overall tyrosine phosphorylation to an extensive level with continued incubation with cells. A potential cause of this degree of phosphorylation could be cellular toxicity. Using LDH release into media and fluorescent viability studies in which nonviable cells were identified by ethidium bromide stained nuclei (Stapleton et al., unpublished data), we concluded that selenate

was not toxic in primary rat hepatocytes with the concentrations used in these experiments. Thus, the great degree of phosphorylation induced by selenate was not in response to cellular toxicity.

An alternate explanation to the selenate-induced increase in tyrosine phosphorylation involves possibly an increase in tyrosine kinase activity or a decrease in tyrosine phosphatase activity. We used commercially available activity kits to try to determine which activity may be affected. The tyrosine kinase activity kit did not give meaningful results due to technical problems. The substrate supplied with the kit was an amino acid sequence derived from $pp60^{src}$. It is possible that selenate did not affect kinases which were able to adequately phosphorylate the substrate for this kit. However, it was most likely due to something in common to all samples, such as procedure or reagents, since similar problems occurred with insulin-stimulated samples and insulin is reportedly a good stimulator of phosphorylation of the substrate (Pike et al., 1986). We could not draw conclusions concerning selenate's effect on tyrosine kinase activity, however, Ezaki (1990) reports that, in a cell-free system, selenate did not stimulate tyrosine kinase activity.

An inhibition of phosphatase activity seems like a feasible explanation because of the delay in selenate induction of phosphorylation. Inhibition of phosphatase activity would allow phosphate concentrations to build up to the point of detection. There is at least one report that selenate does not inhibit phosphotyrosine phosphatases in a cell-free system (Ezaki, 1990). Although, interestingly, selenium compounds are able to bind thiols (Ganther, 1968). Protein tyrosine phosphatases and

dual-specificity phosphatases, which dephosphorylate tyrosine and serine/threonine residues, contain a consensus sequence in their active site of HCXXGXXRS(T) (Denu & Dixon, 1995). Selenate could potentially bind the thiol of the cysteine residue, rendering these classes of phosphatases inactive. We were unable to test selenate's effects on tyrosine phosphatase activity, however, because this type of activity kit was unavailable at the time of these experiments. We did try a serine/threonine phosphatase activity kit. Our reasoning for attempting this assay was that, since later events in insulin signaling typically involve more serine/threonine phosphorylation, it's possible that inhibition of downstream events could affect upstream tyrosine phosphorylation events in a feedback fashion. Okadaic acid, a specific inhibitor of PP-1 and PP-2A serine/threonine phosphatases, showed substantial decreases in phosphatase activity in treated cells, whereas the activity of these two phosphatases was insensitive to selenate.

The induction of tyrosine phosphorylation by selenate illustrated differences in kinetics as compared to insulin induction, specifically, a delay in the onset of phosphorylation. Selenate does not appear to be binding to the insulin receptor, since the rapid effects of autophosphorylation and initiation of the cascade were not seen. The chemical configuration of selenate is a tetrahedral arrangement, which is similar to the shape of vanadate. It has been suggested that vanadate could act through a post-receptor mechanism (Blondel et al., 1990; Green, 1986), gaining entry into cells through phosphate-like transport (Heinz et al., 1982). Ezaki (1990) has reported that selenate does not activate the insulin receptor kinase activity in a cell-free system,

although tyrosine phosphorylation of a 95 kDa protein was seen. Phosphorylation of the insulin receptor does not necessarily mean activation, but is correlated with activation. In the present studies and in the parallel studies with 3T3-L1 adipocytes (Stapleton et al., 1997), we examined only phosphorylation of the receptor. The possibility remains that activation of the receptor is not critical to initiate selenate's mimetic effects. This phosphorylation may be a by-product of some other mechanism of selenate action, or it may play some role in the sustained/amplified selenate responses observed.

The MAP kinase family was a prominent family of kinases studied at the time of these investigations. MAP kinases are conserved among many species and are triggered by a variety of growth and stress stimuli (Seger et al., 1995; Ferrell, 1996). These proteins were a logical choice for our study, since reports indicated that members of the MAP kinase family are activated in response to insulin (Tobe et al., 1992; Boulton et al., 1991). One well-defined cascade which activates MAP kinase is the Ras/Raf/MEK cascade (Nishida & Gotoh, 1993). Inhibitors have been used extensively in characterizing this and other signaling pathways. One caution to keep in mind when utilizing inhibitors is that "specific" inhibitors are not usually specific, in that they often are found later to affect other proteins. However, inhibitors do give us initial results on which to design further experiments and correlate subsequent results. Lovastatin, an inhibitor of Ras farnesylation (Hohl & Lewis, 1995), was one of the earliest inhibitors used to investigate Ras participation in activation of the MAP kinase cascade.

In agreement with studies done with 3T3-L1 cells (Stapleton et al., 1997), insulin increased activity of MAP kinase over 2 fold that of control. The activation was transient, increasing maximally with five minutes of stimulation and quickly decreasing to basal. In primary rat heptocytes, insulin-induction of MAP kinase activation seems to be Ras-dependent, since the highest concentration of lovastatin significantly decreased activity. This decrease in MAP kinase activity by lovastatin is probably not due to a decrease in MAP kinase expression, but is most likely due to a decrease in activation resulting from reduced tyrosine phosphorylation (Xu et al., 1996). Subsequent studies with B581, a Ras inhibitor distinct from that of lovastatin, confirm the dependence of MAP kinase activity on Ras activation (Stapleton et al., unpublished data).

Now that we had established MAP kinase activation through a Ras-dependent pathway, we investigated the effect of selenate on the activation of this protein. MAP kinase activation occurs with selenate in the present study in hepatocytes and in the parallel study in 3T3-L1 adipocytes (Stapleton et al., 1997), although adipocytes responded to a greater degree than hepatocytes. Selenate induction of MAP kinase activity exhibited similar characteristics in both hepatocytes and 3T3-L1 cells (Stapleton et al., 1997) as those seen with tyrosine phosphorylation in terms of the delay of onset of activity and sustained activity through later time points.

Selenate-induction of MAP kinase activation was significantly inhibited by lovastatin, and these results have been subsequently confirmed with B581 (Stapleton et al., unpublished data). Thus, MAP kinase activation by selenate, like insulin, appears

to be Ras-dependent. There have been no reports to date concerning the pathway by which selenate signals MAP kinase activation, however, vanadate has been shown to activate MAP kinase in HeLa cells through Ras (Zhao et al., 1996). Vanadate, a tyrosine phosphatase inhibitor, increased MAP kinase activity, and this activity was sustained in a manner to that seen in our current study with selenate. Inhibition of tyrosine phosphatase activity was sufficient to initiate the complete MAP kinase cascade (Zhao et al., 1996). Although Ezaki (1990) reported no inhibition of phosphotyrosine phosphatase activity by selenate in a cell-free system, selenate could potentially inactivate tyrosine phosphatases *in vivo* by binding to the active site thiol, thereby activating MAP kinase.

Since we had established the phosphorylation of signal proteins in response to insulin and selenate, initial experiments were carried out to determine whether or not the increase in phosphorylation could mediate a downstream metabolic event such as regulation of G6PDH expression. G6PDH expression is upregulated by insulin (Berg et al., 1995), but the signaling pathway that gives rise to increased expression is not known. In order to characterize a potential signaling pathway for expression of G6PDH, inhibitors were again employed. Inhibitors used in this set of experiments were lovastatin, wortmannin (a PI 3-kinase inhibitor), and rapamycin (an S6 kinase inhibitor). We utilized these inhibitors in an attempt to block the known pathways of insulin signaling to determine the effect on G6PDH expression. Inhibitors are not the best approach for these types of experiments because of their lack of specificity,

however, these were the tools that were available at the time of this study, and they were some of the few tools that can be utilized with our primary cell cultures.

MAP kinase activity was elevated in response to insulin, and this potentially has importance in mediating many of insulin's effects. In order to determine if the increase in MAP kinase activity was linked to G6PDH expression, the inhibitor lovastatin was used. Lovastatin did not have any effect on insulin-stimulated G6PDH activity. Thus, MAP kinase appears to not be involved in G6PDH expression by insulin. Subsequently, both B581 and PD 098059, a specific inhibitor of MEK (Alessi et al., 1995), have been used (Stapleton et al., unpublished data), and the results confirm that the MAP kinase pathway is not involved. These results are in agreement with other studies attempting to link the MAP kinase pathway to metabolic events. Recently, it has been shown that inhibition of the MAP kinase pathway had no effect on PEPCK expression in H4IIE cells (Gabbay et al., 1996), as well as no effect on glucose uptake in 3T3-L1 cells or glycogen synthase in 3T3-L1 cells and M6 myocytes (Lazar et al., 1995).

With evidence against MAP kinase participation in G6PDH expression, our attention turned to the PI 3-kinase pathway as a potential link to expression. The PI 3-kinase pathway has been associated with some of the metabolic effects of insulin. Wortmannin and rapamycin have been used to study glycogen synthase activity (Shepard et al., 1995), PEPCK gene expression (Sutherland et al., 1995), and glucose transporter activity (Moule et al., 1995). The use of these inhibitors has helped in the discovery of at least two signaling branches from PI 3-kinase in insulin stimulation, a

rapamycin-sensitive pathway and a rapamycin-insensitive pathway. Many of the metabolic enzymes are now being characterized in accordance to these pathways.

Wortmannin significantly decreased G6PDH activity induced by insulin. Thus, it appears that PI 3-kinase participates in insulin-induced G6PDH expression. The two highest concentrations of wortmannin did, however, significantly lower endogenous G6PDH activity in unstimulated cells. This did not appear to be due to toxicity. After experiments had been concluded, reports indicated a stability problem with wortmannin when incubated for lengths of time (Kimura et al., 1994), and this problem is successfully circumvented by replacing wortmannin in media every two hours (Gabbay et al., 1996). Keeping these results in mind, it is difficult to draw substantial conclusions from the experiments with wortmannin.

Rapamycin did not significantly decrease insulin induction of G6PDH expression. Thus, these results indicate that insulin regulation of G6PDH expression may not be through a rapamycin-sensitive pathway. Subsequent studies in the lab, however, show regulation of G6PDH expression to be rapamycin-sensitive (Stapleton et al., unpublished data). Explanations for this discrepancy are not clear, however, it could be due to times of incubation with rapamycin. Further investigations are necessary to determine rapamycin's effects on insulin-induced G6PDH expression.

Parallel inhibitor studies were done to test which signal proteins might be involved in selenate induction of G6PDH expression. Results show that both wortmannin and rapamycin significantly decreased selenate-stimulated G6PDH expression. Again, involvement of PI 3-kinase in G6PDH expression in selenate-

stimulated cells is uncertain because of instability of wortmannin under our experimental conditions, however, selenate appears to signal increases in G6PDH expression through a rapamycin-sensitive pathway.

In summary, selenate mimics insulin action on a number of metabolic processes, however, the mechanism of action is unknown. It appears as though selenate and insulin signaling share some similarities. Tyrosine phosphorylation increases in two of the early signaling proteins in the insulin phosphorylation cascade, the β-subunit of the insulin receptor and IRS-1. MAP kinase is substantially activated through a Ras-dependent mechanism. Initial evidence suggests increases in G6PDH expression are a result of signaling events transmitted through the PI 3-kinase pathway and possibly through a rapamycin-sensitive pathway, however, more studies are needed for signal pathway characterization of this metabolic event. Insulin and selenate signaling mechanisms also have differences. Effects of selenate are first seen at much later time points than that of insulin, and the effects are sustained over long periods of time. Several signaling proteins are affected by selenate, and selenate signaling is somewhat cascade-like because of the fact that inhibitors affect downstream targets. However, selenate acts with less specificity than insulin and with less regulation as compared to the tightly regulated, transient effects seen in response to insulin. Further studies will help fully define the role selenate plays in insulin-mimicking actions on metabolic events.

Appendix A

Protocol Clearance From the Institutional Animal Care and Use Committee

WESTERN MICHIGAN UNIVERSITY
INVESTIGATOR IACUC CERTIFICATE

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

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 copies of database searched (e.g. Medline) to determine non-duplication

② specific room number where animals will be housed

IACUC Chairperson _____ Date 8/24/97

Acceptance of Provisions

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

IACUC Chairperson Final Approval _____ Date 9/5/97

Approved IACUC Number 97-07-03

Rev. 3/92

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