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# Glucose Mediated Effects on Glucose-6-Phosphate Dehydrogenase Expression

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# GLUCOSE MEDIATED EFFECTS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE EXPRESSION

by

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# GLUCOSE MEDIATED EFFECTS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE EXPRESSION

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Circulating high glucose levels are believed to cause oxidative stress which is implicated in a number of pathological conditions such as cancer, aging, and diabetes. Oxidative stress regulates the expression of glucose-6-phosphate dehydrogenase (G6PDH), the key enzyme in the pentose phosphate pathway which provides reducing equivalents in the form of NADPH. These reducing equivalents are also required by fatty acid synthase (FAS), the enzyme which catalyzes fatty acid biosynthesis. We examined whether glucose, a known inducer of oxidative stress, could regulate either G6PDH or FAS activity in primary rat hepatocytes. We tested the effects of glucose alone or in combination with insulin or selenate. Insulin and selenate are known to induce G6PDH and FAS expression.

Growth factor and stress mediated regulation of gene expression can utilize the MAP kinase signal pathway, therefore we examined if glucose effects the phosphorylation of the insulin receptor and Shc, two proteins of this pathway.

Our results indicate that G6PDH activity is regulated by glucose concentration, but this does not affect the insulin- or selenate-induced increase in G6PDH activity. The effect of glucose concentration alone or in combination with insulin- and selenate-induced FAS activity was inconclusive. Insulin and selenate caused phosphorylation of the insulin receptor and Shc, but varying the glucose concentration had no effect.

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Daryl Arkwright

TABLE OF CONTENTS

Table of Contents—Continued

Table of Contents—Continued

LIST OF TABLES

LIST OF FIGURES

List of Figures—Continued

# INTRODUCTION

## Free Radicals and Oxidative Stress

Free radicals are highly reactive atoms or molecules that have one or more unpaired electrons in their atomic structure. The most important free radicals in biological systems are radical derivatives of oxygen or reactive oxygen species (ROS). These include oxygen, the superoxide radical, $O_2 \cdot^-$, the hydroxyl radical, $OH\cdot$, and hydrogen peroxide, $H_2O_2$. ROS causes oxidation of important biological molecules such as proteins and membranes and results in tissue damage (Freeman and Crapo, 1982). The toxicity of many chemicals and environmental pollutants is probably due to their production of ROS. The continually growing list of ROS producers includes quinones, bipyridinium herbicides, phorbol esters, pesticides, chemotherapeutic agents, ozone, cigarette smoke and ionizing radiation (Stohs, 1992). Oxidative damage inflicted by ROS is referred to as oxidative stress. Oxidative stress has been associated with a variety of pathological conditions such as cancer, aging, and diabetes mellitus (Cross et al, 1987).

The production of some free radicals is inevitable in aerobic conditions, thus eukaryotes have developed defenses to scavenge and detoxify ROS. This defense includes enzymatic and non-enzymatic antioxidants. The oxidative homeostasis of an organism is maintained through a balance of ROS and antioxidant defenses.

# Antioxidant Defense Mechanisms

## Antioxidant Enzymes

The best characterized antioxidant enzymes are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). These enzymes lower the amount of cellular free radicals or their precursors.

SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen.

$$O_2\cdot^- + O_2\cdot^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Catalase functions to detoxify hydrogen peroxide by converting it to water and oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

GPX, a metalloprotein containing selenocysteine, uses glutathione (GSH) as a substrate to break down hydrogen peroxide to water and reduced glutathione disulfide (GSSG).

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$$

The oxidized GSSG is reduced back to GSH by another enzyme called glutathione reductase (GRED). GPX functions in concert with GRED, which uses NADPH to reduce GSSG to GSH, as shown in Figure 1. The NADPH is generated and supplied

to this cycle by glucose-6-phosphate dehydrogenase (G6PDH), a key enzyme of the pentose phosphate pathway (Ruberfroid and Calderon, 1995). The GPX/GRED cycle is regulated by the intracellular concentrations of GSH, GSSG, NADPH, and the activities of GPX and GRED (Harlan et al, 1984; Schraufstätter et al, 1985). A decrease in any of these substrates or enzyme activities may profoundly impair free radical-scavenging activity.

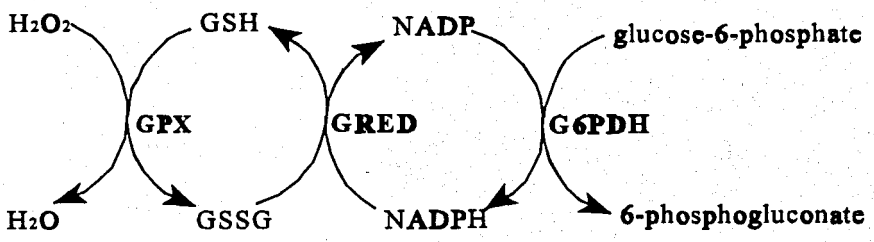



Figure 1. The Glutathione Peroxide/Glutathione Reductase Cycle.

Glucose-6-phosphate dehydrogenase (G6PDH) supplies the NADPH for glutathione reductase (GRED) to reduce GSSG to GSH. Glutathione peroxidase (GPX) uses GSH as a substrate to breakdown hydrogen peroxide.

Non-enzymatic Antioxidants

There are several non-enzymatic compounds known to have antioxidant properties. These compounds function as antioxidants by donating an electron to a free radical and include α-tocopherol (vitamin E), ascorbic acid (vitamin C), ubiquinol and uric acid (Cadenas, 1995).

Additionally, some elements are known to have antioxidant properties. An example of this is selenium (Se). Se is an essential biological trace element. Se

deficiency causes an increase in mitochondrial membrane lipid peroxidation and selenium supplementation can protect membranes from lipid peroxidation, occurring as a result of GPX deficiency (Yang et al, 1984). Se is an intregal part of GPX (Rotruck et al, 1973) and has been shown to stimulate GPX activity (Burk and Lane, 1983). In low concentrations, Se is a potential antioxidant (Burk and Lane, 1983). In higher concentrations, it has been suggested to have anti-cancer effects (Schrauzer, 1987).

The exact mechanism responsible for Se antioxidant effects is unclear. It appears to act indirectly by activating GPX and G6PDH. However, there is evidence of selenium-dependent protection against oxidant injury that is independent of GPX (Burk et al, 1980). This protection could be due to activation of G6PDH or selenoproteins, such as selenoprotein P, that may have antioxidant properties (Hill and Burk, 1994). Se has been shown to increase the activity of G6PDH by increasing mRNA levels (Berg et al, 1995). As indicated above, G6PDH produces reductive potential for the cell in the form of NADPH, which is necessary for the activities of enzymes such as GRED in the antioxidant defense system and for biosynthetic enzymes such as fatty acid synthase (FAS) in lipogenesis, as shown in Figure 2.

## Glucose and Oxidative Stress

High glucose or hyperglycemia has been implicated in the increased production of free radicals. There is strong evidence that glucose, under physiological conditions, may autooxidize generating superoxide and hydroxyl radicals, and hydrogen peroxide (Wolff and Dean, 1987). Peroxides have been shown to be increased in elevated

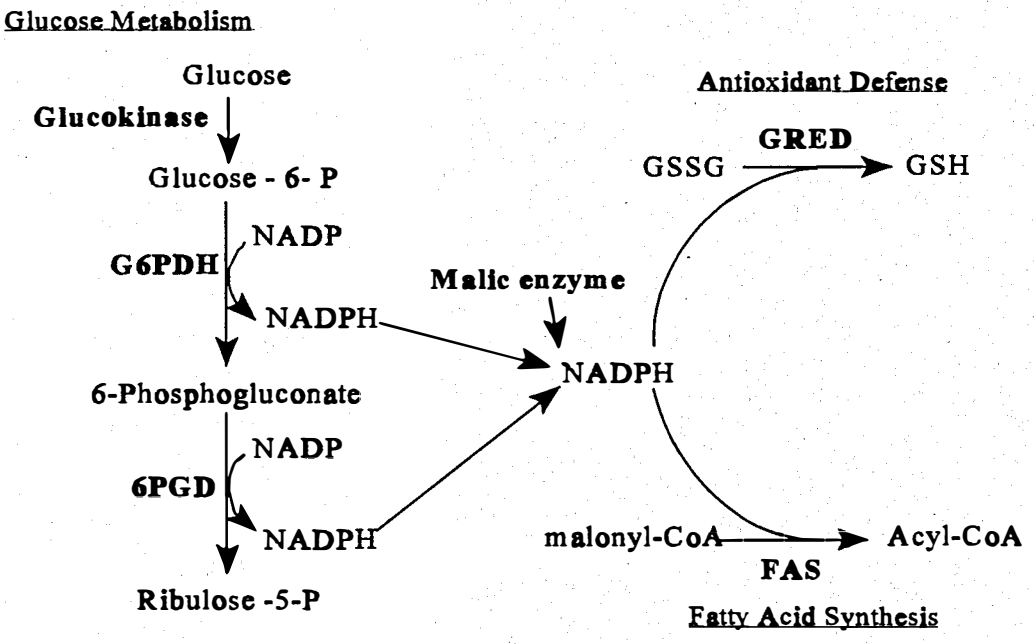



Figure 2. Production of NADPH From Glucose Metabolism in the Liver.

Enzymes involved in glucose metabolism, such as G6PDH and 6PGD produce NADPH which is necessary for the activity of FAS and GRED. ME is an additional source of NADPH. FAS and ME are involved in fatty acid synthesis and GRED is important in antioxidant defense. G6PDH: glucose-6-phosphate dehydrogenase, 6PGD: 6-phosphogluconate dehydrogenase, ME: malic enzyme, FAS: fatty acid synthase, GRED: glutathione reductase.

glucose solution (25 mM) in the presence of transition metals (Hunt et al, 1990).

Even moderately high glucose may become a toxic agent under certain conditions where GSH levels are decreased, such as in diabetes. A moderate rise of glucose in the culture medium (10 mM to 20 mM) of FRTL5 cells in the presence of buthionine-sulfoximine, a GSH inhibitor, increased the free-radical damage marker malondialdehyde. These results suggest that the GSH redox cycle is a key defense in glucose-induced oxidative stress (Donnini et al, 1996).

High glucose has been demonstrated to induce antioxidant enzymes in human

endothelial cell cultures through increased gene expression (Ceriello et al, 1996). Oxidant stress has been shown to induce many other genes as well, such as *c-jun*, *c-fos*, and *c-myc* (Colburn, 1992; Crawford et al, 1988). The protein products of *c-jun* and *c-fos* are members of the AP-1 (Activator Protein-1) family of transcription factors. AP-1 transcription factors bind to TPA-responsive element (TRE) in the promotor regions of several genes, regulating the transcription of those genes. The promotor region of the G6PDH gene contains three putative AP-1 binding sites (Rank et al, 1994), therefore the induction of G6PDH by oxidative stress may be possible through these AP-1 binding sites.

Activation of AP-1 transcription factors is signalled through the MAPK pathway (Figure 3). This pathway is important in growth factor signalling and we believe that it may be the pathway responsible for G6PDH expression in oxidative stress. However, oxidative stress may not be the only regulator of G6PDH expression. Changes in gene expression observed with high glucose can also occur by a direct effect of glucose on gene expression, independent of oxidative stress.

## Glucose Regulation of Gene Expression

Glucose has been shown to directly regulate the expression of several genes and has been implicated in the regulation of others. Some of the genes known to be regulated by glucose are insulin (German, 1990), glucose-regulated proteins (Lee, 1987), acetyl-CoA carboxylase (ACC)( Brun et al, 1993), L-pyruvate kinase (L-PK)(Decaux et al, 1989), malic enzyme (ME)( Dozin et al, 1986), S14 (Shih and

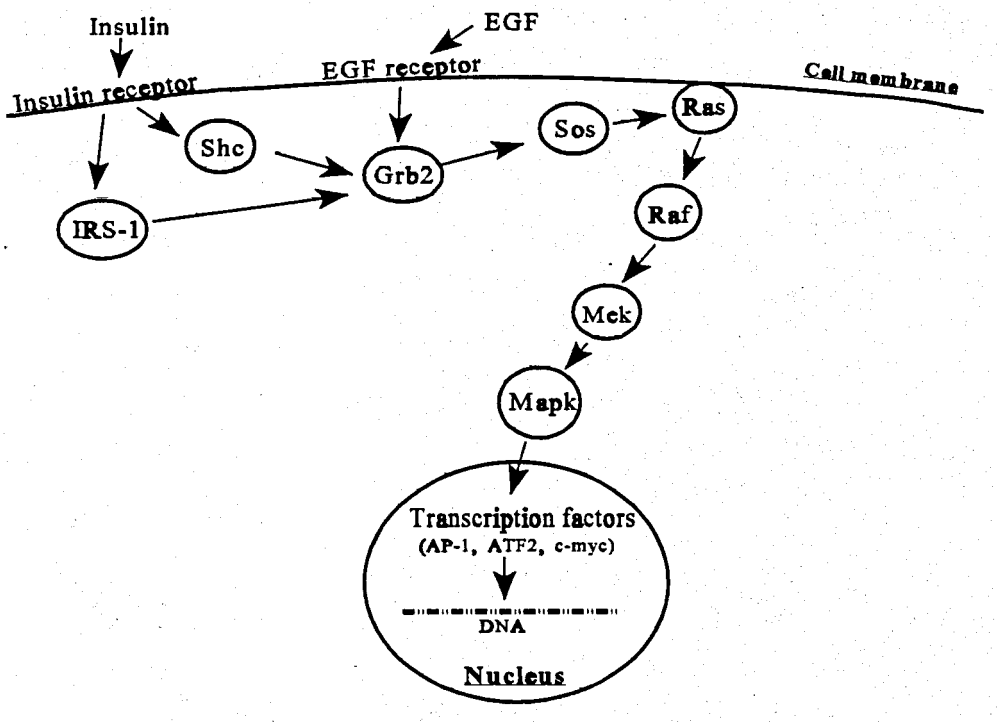



Figure 3. The Mitogen Activated Protein Kinase (MAPK) Pathway.

The MAPK pathway is the signaling pathway used by many growth factors, including insulin and epidermal growth factor (EGF). Signaling is initiated by the binding of the ligand to the growth factor receptor, which then phophorylates a substrate protein. The second protein activates the next protein, and so on, initiating a cascade. The final step is phosphorylation and activation of transcription factors.

Towle, 1992) and phosphoenolpyruvate carboxykinase (PEPCK)(Foufelle et al, 1994). In addition, low fructose concentrations have been reported to augment the effects of glucose. Doiron et al (Doiron et al, 1994) reported that 0.2 mM fructose increased the glucose-induced L-pyruvate kinase activity by two to three fold.

G6PDH and fatty acid synthase (FAS) are thought to also be regulated by glucose, but the results are inconsistent. In addition, there is no evidence on the effect of low fructose and glucose on these two enzymes.

If G6PDH or FAS are regulated by glucose, this regulation could be mediated through a signal pathway. A phosphorylation/dephosphorylation signal pathway, ultimately resulting in phosphorylation of a transcription factor, may be responsible for glucose regulated expression of ACC, S14, and L-PK (Foufelle et al, 1996). Transcription factors are often regulated by this mechanism (Jackson, 1992). Therefore, changes in phosphorylation of signal proteins in the MAPK pathway could be responsible for glucose mediated events, especially if glucose regulation of G6PDH or FAS is mediated through oxidative stress.

## Significance of This Study

High glucose is believed to cause oxidative stress which is associated with a variety of pathological conditions. Glucose has been shown to induce antioxidant enzymes and to regulate the expression of several genes. G6PDH is an important enzyme induced in oxidative stress conditions and is thought to be regulated by glucose. Whether glucose regulation of G6PDH expression is a result of oxidative stress or a direct effect of glucose is unknown. Knowledge of the effect of high glucose on the expression of genes, such as G6PDH, will aid our understanding of the underlying mechanisms of diseases such as diabetes.

## Objectives of the Study

There were two objectives in this study. The first objective was to determine if glucose concentration affects G6PDH and FAS activity. Additionally, since both

insulin and selenate are known to increase G6PDH and FAS activity, a combination of these agents with glucose was also tested.

The second objective was to determine if glucose concentration affects the phosphorylation of the insulin receptor and Shc, two components of the MAPK pathway that are known to be activated by insulin and possibly by selenate.

# MATERIALS AND METHODS

## Hepatocyte Isolation and Culture

Male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Indianapolis, IN. Prior to use, rats weighing approximately 180-200 grams were fasted for 48 hours with drinking water ad libitum. Animals were anesthetized with an inter-peritoneal injection of pentobarbital. Hepatocytes were isolated by in situ perfusion (Elliget and Kolaja 1983) using collagenase (Boehringer Mannheim, Indianapolis, IN) and hyaluronidase (Sigma Chemical Co., St. Louis, MO). The liver was excised and forced through four layers of gauze over a beaker containing perfusion solution, and then centrifuged at 4°C in 50 ml centrifuge tubes for 3 minutes at 50 G. The supernatant was aspirated and the cell pellet was suspended, washed, and centrifuged twice with cold Waymouth's MB 752/1 (Gibco, Grand Island, NY) medium containing 0.5% Bovine Serum Albumin (BSA). The cell pellet obtained after the final washing was gently resuspended in the medium and an aliquot was used to determine cell concentration and cell viability on a hemocytometer by trypan blue exclusion. Cells with a viability of greater than 80% were plated to 90% confluency on sterilized Falcon-3002 60 mm, collagen (rat-tail) coated plates. The cells were then incubated in 4 ml Waymouth's MB 752/1 medium with BSA at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air for 4 hours.

## Hepatocyte Treatment

After 4 hours, the BSA supplemented medium was aspirated. Cells were washed once with 2 ml of BSA free Waymouth's medium or carbon source free, BSA free Waymouth's Modified medium. The wash was aspirated and 4 ml of BSA free medium containing various glucose or fructose concentrations was added. Two plates per condition were used. The plates were gently swirled to mix. The cells were then incubated at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air for 24 hours.

## Enzyme Activity

### Cell Harvest

Twenty four hours after isolation and plating, hepatocytes were treated with either nothing, 87 nM insulin, or 20 $\mu$M sodium selenate. Treated hepatocytes were incubated for 48 hours after treatment and processed as follows: medium was aspirated and cells were scraped in 0.5 ml KED buffer [containing 0.1 M potassium phosphate buffer (pH = 7.0), 3.0 mM ethylenediaminetetraacetate (EDTA), and 1.0 mM dithiothreitol (DTT)]; cell-buffer mixture of two plates was combined and transferred to a Dounce homogenizer on ice and homogenized 20 times. The extract was then spun in a microcentrifuge for 10 minutes at 4°C and the supernatant was collected. The samples were either used immediately or stored with 10% (v/v) glycerol at -20°C.

Protein and Enzyme Assays

Total protein concentration of the cell lysate samples was determined by the method of Lowry, et al (Lowry et al, 1951) using BSA as a standard. Protein values for all samples were expressed in $\mu g/\mu l$.

Glucose-6-phosphate dehydrogenase assays were carried out on cell lysates according to Glaser et al (Glaser et al, 1955). The rate of change in absorbance of the reaction mixture with time was measured for 3 minutes at 340 nm on a Beckman DU 7500 spectrophotometer. Specific activity of G6PDH was calculated and expressed as change in absorbance per minute per $\mu g$ of protein.

Fatty acid synthase (FAS) activity of cell lysates was determined using the method of Goodridge (Goodridge, 1972). This method measures the formation of NADP from NADPH in the presence of acetyl- and malonyl- CoA. The rate of change in absorbance with time was measured at 37°C for 3 minutes at 340 nm on a Beckman DU 7500 spectrophotometer. Specific activity of FAS was calculated and expressed as change in absorbance per minute per $\mu g$ of protein.

Lactate dehydrogenase (LDH) activity in the culture media was measured by the method of Jaenicke et al (Jaenicke et al, 1968). This procedure measures the reduction of pyruvate to lactate using NADH. The rate of decrease in absorbance with time was measured for 1 min. at 340 nm on a Beckman DU 7500 spectrophotometer. Specific activity of LDH was calculated and expressed as change in absorbance per minute per $\mu g$ of protein.

Study of Fructose-Treated Cells

Scanning Electron Microscopy

After a seventy two hour incubation in glucose or fructose media, hepatocyte cultures were fixed as in the method of Sabatini et al. (Sabatini et al, 1963), with slight modifications. The cells were fixed directly in the culture plates with approximately 5 ml of 3% glutaraldehyde in 0.1 M phosphate buffer for 15 min. The solution was aspirated from the cells, 0.1 M phosphate buffer was added and the cells were refrigerated overnight. The following day, the phosphate buffer was aspirated from the cells and 1% osmium tetroxide was added to the culture plate and allowed to stand for 15 min. The cultures were then washed three times in phosphate buffer. Then they were dehydrated with a graded (50-100% v/v) ethanol series and a final dehydration with 100% hexamethyldisilazane for 10 min. The culture plates were placed in a 70°C drying oven for 20 min. and then cut into small pieces for gold coating. The pieces were gold coated in a Polaron Autocoating unit E5200. Electron micrographs were taken with a ISI 130 Scanning Electron Microscope.

Lipid Staining

Oil Red O (Sigma Chemical Co., St. Louis, MO) staining was performed on cell cultures as described by Sheehan (Sheehan, 1990) with slight modifications. Hepatocytes were fixed directly in the culture dishes with approximately 5 ml of 0.25% glutaraldehyde for 15 min. Then a series of increasing concentrations of

propylene glycol (50%,75%,85%) were added. The cultures were aspirated, Oil Red O stain added, and then placed in a 60°C oven for 10 min. The cultures were washed twice with 5 ml of 85% propylene glycol and then twice with 5 ml pf deionized water. Approximately 5 ml of Hematoxylin stain (Sigma Chemical Co., St. Louis, MO) was added for 5 min. and then 5 ml of Bluing reagent (Sigma Chemical Co., St. Louis, MO) for 2 min. The cultures were washed twice in water and 50% glycerol was added.

Cell Viability Assays

Cell viability was determined at 24, 48, and 72 hrs. by ethidium bromide staining. Stock solution of ethidium bromide (1$\mu$g/ml in 0.1 M phosphate buffer) was prepared. The media was removed from the hepatocyte cultures and 3 ml of ethidium bromide solution was added. This was allowed to stand for 2 min. at room temperature. The plates were covered to minimize light exposure. The plates were then placed on a microscope stage and exposed to UV light. The red, non-viable cells and the total cells were counted using a calibrated occular. Percent of viable cells were obtained by subtraction of the non-viable cells from the total cells. Three separate counts were made per plate and the average percent viability was determined.

Media pH and Osmolarity

Samples of media from 24 and 48 hr. hepatocyte cultures were taken and pH measured. The osmolarity of the media from the same samples was measured using a

standardized Advanced 3D3 osmometer.

## Protein Identification and Phosphorylation

### Cell Harvest

Twenty four hours after isolation and plating, hepatocytes were treated with either nothing, 87 nM insulin, or 500 $\mu$M sodium selenate. Incubation time for insulin and selenate was 5 min. and 1 hour respectively. After treatment, in a 4°C cold room, the medium was aspirated from the culture plates which were then washed three times with 500 $\mu$l cold PBS buffer. The buffer was removed, 200 $\mu$l of cold lysis buffer containing 20 mM Hepes (pH = 7.3), 0.5% triton X-100, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 mM *p*-nitrophenyl phosphate, 1$\mu$M dithiothreitol, 5 $\mu$g/ml leupeptin, 5$\mu$g/ml pepstatin, 10 mM β-glycerophosphate, and 1 mM phenylmethylsulfonylfluoride was added and the cells were scraped. The lysate was collected and microfuged at 4°C for 10 min. The supernatant of the cell lysate was transferred to a freezing vial and quick frozen in a dry ice and isopropanol bath. The samples were stored at -20°C.

### Wheat Germ Adsorption of Insulin Receptors

Wheat Germ Lectin Sepharose 6MB (WGA)(Pharmacia, Piscataway, NJ) ) was used to adsorb the insulin receptors (IR) from cell lysates. WGA beads (200 $\mu$l) were pelleted and washed twice with 300 $\mu$l of Hepes wash buffer (50 mM Hepes,

0.15 M NaCl, 0.05% SDS, pH 7.3). Cell lysate protein (1 mg) was added to the washed beads and rotated at 4°C for 1 hr. After 1 hr., the beads were pelleted and the supernatant removed. The beads were washed 5 times with 500 $\mu$l of wash buffer. Elution buffer (50 $\mu$l) containing 0.5 mM Tris-HCl (pH 6.8), 20% glycerol, 10% SDS, 0.3 M N-acetylglucosamine, 7 x $10^{-6}$ M pepstatin, 1.2 x $10^{-5}$ M leupeptin, and 5 % β-mercaptoethanol was added to the beads and rotated at 4°C for 15 min. The beads were pelleted by microfugation and the elution containing the IR was collected. The elution was stored at -20°C.

Immunoprecipitation of Shc Protein

Cell lysate supernatant (1 mg of protein) was transferred to an eppendorf tube and 10 $\mu$g of rabbit polyclonal anti-Shc IgG antibody (Upstate Biotechnology Incorporated, Lake Placid, NY) was added. The reaction mixture was rotated at 4°C overnight. The immunocomplex was captured by adding it to 50 $\mu$l of packed Protein A agarose beads (Gibco, Grand Island, NY). This reaction mixture was rotated at 4°C overnight. The agarose beads were collected by pulse centrifugation and the supernatant was removed. The beads were washed three times with cold PBS buffer. The wash was removed and beads were resuspended in 50 $\mu$l of 2X Laemmli sample buffer (0.125 M Tris-HCl (pH = 6.8), 20% glycerol, 4% SDS, 0.1% bromophenol blue, and 5% β-mercaptoethanol). The beads, containing the immunoprecipitated Shc, were either stored at -20°C for later use or used immediately.

SDS-PAGE and Immunoblotting

Proteins in the samples containing either partially purified insulin receptor or Shc were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)(Laemmli, 1970) using 8% or 10% Tris-glycine gels (Novex, San Diego, CA). The samples were boiled for 2 mins, pulse centrifuged and 40 $\mu$l of sample was loaded onto the gel. Tris-glycine running buffer was added and electrophoresis was performed at 125 volts for 1½ hours using a Novex XCELL II mini-cell (Novex, San Diego, CA). After electrophoresis, the gel was removed and placed in degassed transfer buffer (39 mM glycine, 48 mM tris base, and 20% methanol, pH 8.3) for 20 min. Polyscreen PVDF transfer membrane (Biotechnology Systems, Boston MA) was prepared by rinsing in methanol, and then equilibrated in degassed transfer buffer for 10 min. The transfer apparatus containing the gel and transfer membrane was assembled and the proteins were transferred at 30 volts for 1 hour using the Novex XCELL II mini-cell blotting module. After transferring, the membrane was blocked in 5% BSA tris buffered saline (TBS) for 1 hour. The membrane was washed 3 times with TBS containing Tween-20 (Sigma Chemical Co., St. Louis, MO)(TBS-T). The appropriate primary antibody, diluted 1:3000 in TBS, was then added. Mouse monoclonal anti-phosphotyrosine IgG antibody (Upstate Biotechnology Incorporated, Lake Placid, NY) was added for studying tyrosine phosphorylation. Rabbit polyclonal anti-Shc IgG antibody (Upstate Biotechnology Incorporated, Lake Placid, NY) was added for detecting Shc isoforms. The membrane was incubated with the antibody at room

temperature with agitation for 45 min. The membrane was washed three times with TBS-T and the last wash decanted. Secondary antibody, a 1:3500 dilution in TBS of horse radish peroxidase (HRP) conjugated anti-mouse IgG antibody, which binds to the rabbit anti-Shc IgG antibody was then added. This secondary antibody was incubated with the membrane for 30 min at room temperature with agitation. The membrane was washed four times with TBS-T. The remainder of the procedure was performed in a dark room. The membrane was incubated with enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, Il) for 1 min. The membrane was then placed with autoradiography Hyperfilm ECL (Amersham, Arlington Heights, Il) and the exposed film was developed. The densities of the protein bands on the exposed film were measured using IMAGE (National Institutes of Health, 1995) for Macintosh.

## Statistical Analysis

All results are expressed as means ± standard error of the mean of two to ten experiments. The differences between the treatment groups were evaluated by one-tailed Student's *t*-test or Analysis of Variance (ANOVA). Significance was tested at $P<0.05$.

# RESULTS

## Effect of Glucose on Glucose-6-phosphate Dehydrogenase Activity

The effect of glucose on G6PDH expression and activity has remained controversial. Previous studies have shown that in rats fed a high carbohydrate diet, hepatic G6PDH gene transcription, mRNA levels, and enzyme activity increased (Prostko et al, 1989). Others have shown that in vitro hepatic G6PDH mRNA level was not greatly increased in the presence of glucose as were the mRNA levels of two other lipogenic enzymes, fatty acid synthase and malic enzyme (Fukuda et al, 1992). Two other studies (Kelly and Kletzien, 1984; Salati et al, 1988 ) demonstrated that glucose enhanced basal and hormone regulated expression of G6PDH with the maximum effect of glucose being a five fold increase in basal expression. In an attempt to help resolve this debate, primary rat hepatocytes were maintained in media containing either 1, 5, 10, or 30 mM glucose. After 72 hrs. in glucose media, the cells were harvested as described in "Materials and Methods" and G6PDH activity was measured. As shown in Figure 4, glucose does have an effect on G6PDH activity. The activity of the enzyme increased with increasing glucose concentrations. The enzyme activity in cells treated with either 1 mM or 5 mM glucose was significantly lower than the 30 mM glucose. There was no significant difference in G6PDH activity in cells treated with 10 mM and 30 mM glucose. These results substantiate those that suggest

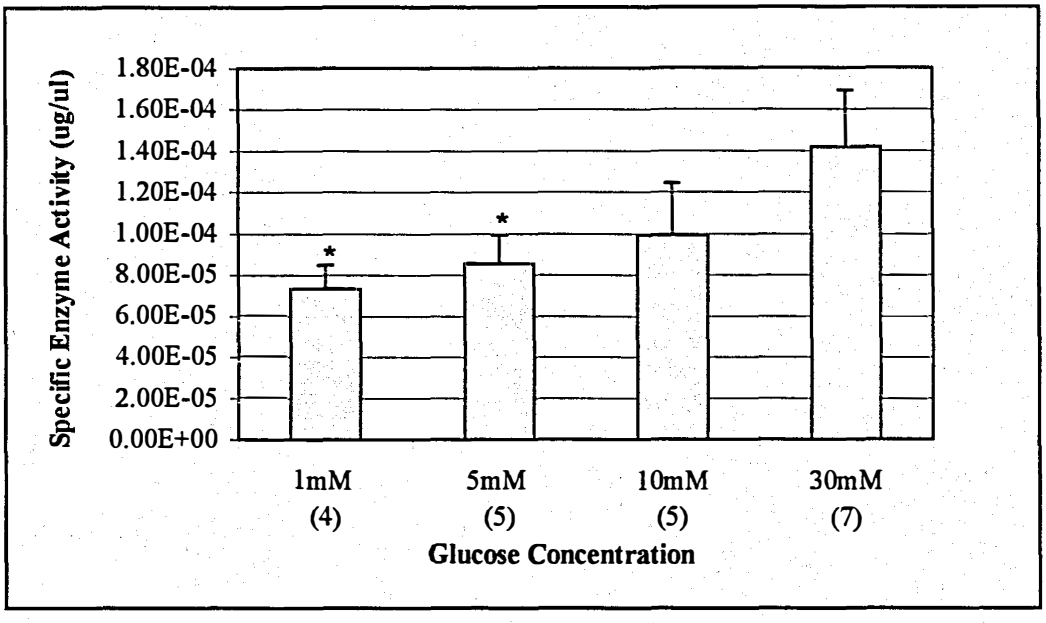



Figure 4. Effect of Glucose on Glucose-6-phosphate Dehydrogenase Activity.

Primary rat hepatocytes were maintained in media containing either 1, 5, 10, or 30 mM glucose. After 72 hours in glucose media, the cells were harvested and the glucose-6-phosphate dehydrogenase activity of the cell lysates was measured spectrophotometrically. Values represent the means ± S.E. Starred treatments were significantly less than the 30 mM glucose control ($p < 0.05$). The number in parentheses represents the N number for each treatment.

G6PDH expression is sensitive to glucose concentration.

Effect of Glucose and Insulin on Glucose-6-phosphate Dehydrogenase Activity

Kurts and Wells (Kurts and Wells, 1981) first demonstrated that insulin could induce G6PDH activity in primary hepatocytes in culture. Subsequent studies showed that the insulin induced increase in activity was due to increased levels of mRNA (Fritz et al, 1986). A study by Fukuda et al (Fukuda et al, 1992) showed that glucose could enhance the insulin-induced expression of all lipogenic enzymes, including G6PDH, in

primary hepatocytes in culture. To test the insulin enhancement of glucose-induced G6PDH activity in our system, primary rat hepatocytes were maintained in media containing either 1, 5, 10, or 30 mM glucose. After 24 hours, the cells were treated with 87 nM insulin. Forty eight hours after insulin treatment, the cells were harvested as described in "Materials and Methods" and the activity of G6PDH was measured. As shown in Figure 5, insulin does increase G6PDH activity but changing the glucose concentration does not have a significant effect on this insulin-induced activity.

Effect of Glucose and Selenate on Glucose-6-phosphate Dehydrogenase Activity

The regulation of G6PDH, FAS, or other lipogenic enzymes by insulin-mimetics, such as selenate, has not been extensively studied. Berg et al (Berg et al, 1995) showed that treatment of streptozotocin-induced diabetic rats with selenate normalized the hepatic G6PDH activity, similar to insulin. This increase in activity was due to increased levels of mRNA expression. Stapleton et al showed that 20 $\mu$m selenate increased the activity of G6PDH in rat hepatocytes in culture (Stapleton et al, 1997). The combined effect of glucose and selenate, however, has not been examined. To look at the effect of selenate on glucose-induced G6PDH activity, primary rat hepatocytes were maintained in media containing either 1, 5, 10, or 30 mM glucose. After 24 hours, the cells were treated with 20 $\mu$m sodium selenate. Forty eight hours after sodium selenate treatment, the cells were harvested and the activity of G6PDH was measured. The results shown in Figure 5 indicate that selenate does increase G6PDH activity, however glucose does not have a notable effect on this selenate-

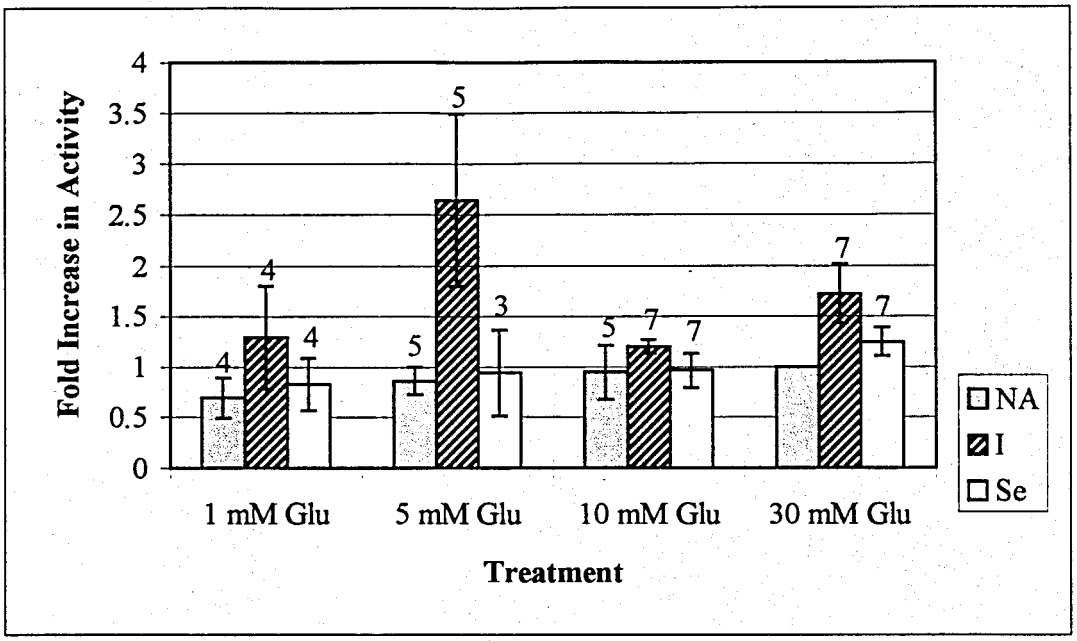



Figure 5. Effect of Insulin and Selenate on Glucose-6-Phosphate Dehydrogenase Activity in Hepatocytes Maintained in Various Glucose Concentrations.

Legend. NA = No Addition, I = Insulin, Se = Selenate

Primary rat hepatocytes were maintained in media containing either 1, 5, 10, or 30 mM glucose. After 24 hours in glucose media, the cells were treated with nothing, 87 nM insulin or 20 $\mu$M sodium selenate. Forty eight hours after treatment with nothing, insulin or sodium selenate, the cells were harvested and the glucose-6-phosphate dehydrogenase activity of the cell lysates was measured spectrophotometrically. Values are given as the mean ± S.E of the fold increase in enzyme activity over the 30 mM No Addition. The number above the data bar represents the N number for each treatment.

induced activity.

## Effect of Glucose on Fatty Acid Synthase Activity

Previous studies have shown rapid induction of FAS mRNA in rats during ingestion of a high glucose meal (Clarke et al, 1990). Other groups have shown that

glucose alone is unable to stimulate FAS expression in cultured hepatocytes from suckling rats (Prip-Buus et al, 1995). In this study, primary rat hepatocytes were maintained in media containing either 1, 5, 10 , or 30 mM glucose. After 72 hrs. in glucose medium, the cells were harvested and the activity of FAS was measured. Results shown in Figure 6 demonstrate that varying glucose concentration alone did not significantly effect FAS activity.

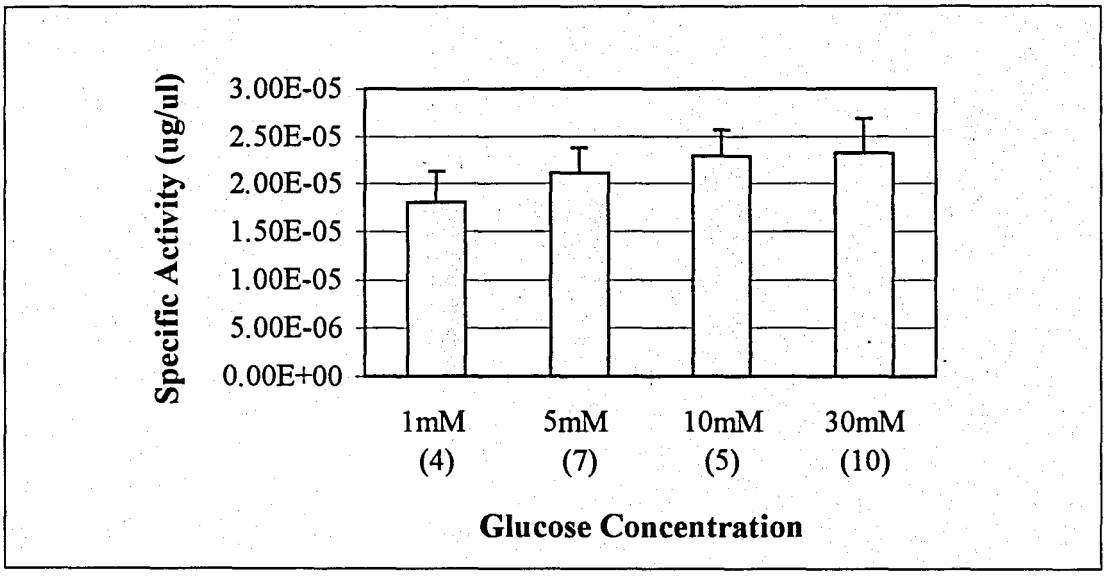



Figure 6. Effect of Glucose on Fatty Acid Synthase Activity.

Primary rat hepatocytes were maintained in media containing 1, 5, 10, or 30 mM glucose. After 72 hours in the glucose media, the cells were harvested and the fatty acid synthase activity of the cell lysates was measured spectrophotometrically. Values represent the means ± S.E. The number in parentheses represents the N number for each treatment.

## Effect of Glucose and Insulin on Fatty Acid Synthase Activity

It has been previously reported that glucose caused a concentration-dependent

increase in the insulin induction of FAS expression in primary rat hepatocyte cultures. (Fukuda et al, 1992). The FAS mRNA reached a maximum 4 fold increase at 20 mM glucose. Other groups have also shown that glucose enhanced the effect of insulin on the activity and mRNA levels of FAS in adult rat hepatocyte cultures (Spence and Pitot, 1982; Giffhorn-Katz and Katz, 1986). Our results did not concur, however, with these previous observations. Primary rat hepatocytes were maintained in media containing either 1, 5, 10, or 30 mM glucose. After 24 hours, the cells were incubated with 87 nM insulin. Forty eight hours after insulin treatment, the cells were harvested and the activity of FAS was measured. As shown in Figure 7, insulin did not increase FAS activity as expected and varying glucose concentration did not alter the results.

## Effect of Glucose and Selenate on Fatty Acid Synthase Activity

As previously mentioned, the regulation of FAS and other lipogenic enzymes by insulin-mimetics, such as selenate, has not been largely studied. Berg et al (Berg et al, 1995) demonstrated that treatment of streptozotocin-induced diabetic rats with selenate normalized the hepatic FAS activity. This increase in activity was due to increased levels of mRNA expression. Results from our laboratory have shown that 20$\mu$m selenate increased the activity of FAS in rat hepatocytes in culture. The combined effect of glucose and selenate has not been examined. To test the effect of glucose and selenate in vitro, primary rat hepatocytes were maintained in media containing either 1, 5, 10, or 30 mM glucose. After 24 hours, the cells were treated with 20 $\mu$m sodium selenate. Forty eight hours after sodium selenate treatment, the

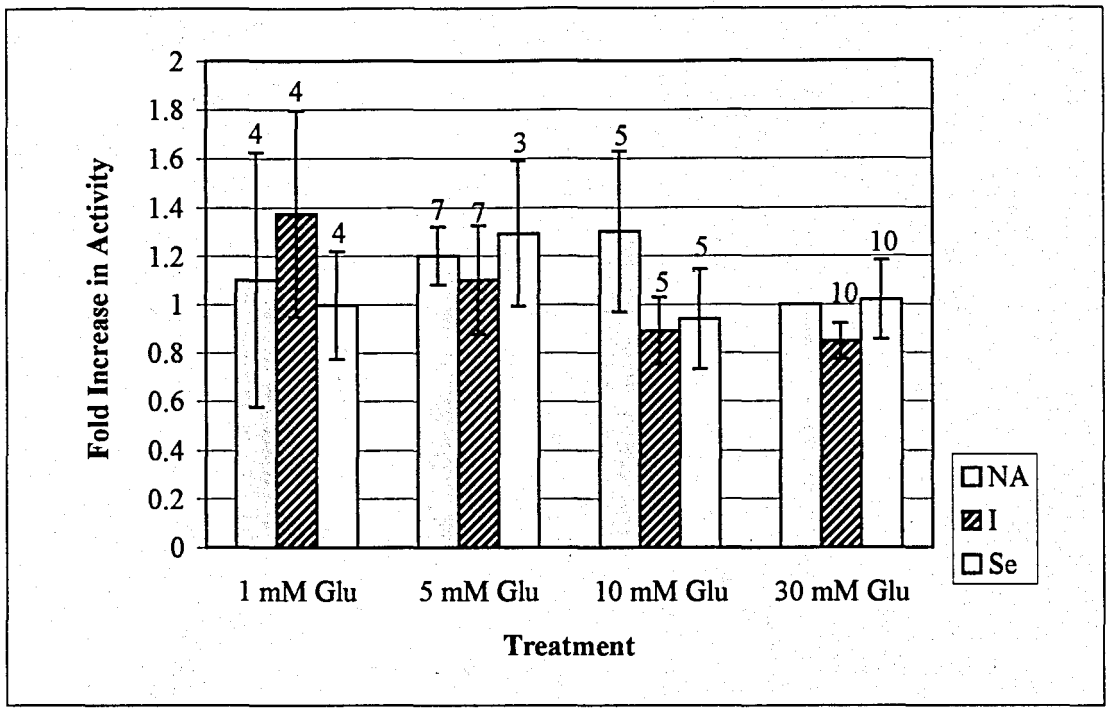



Figure 7. Effect of Insulin and Selenate on Fatty Acid Synthase Activity in Hepatocytes Maintained in Various Glucose Concentrations.

Legend. NA = No Addition, I = Insulin, Se = Selenate

Primary rat hepatocytes were maintained in media containing either 1, 5, 10, or 30 mM glucose. After 24 hours in glucose media, the cells were treated with nothing, 87 nM insulin or 20 $\mu$M sodium selenate. Forty eight hours after treatment with nothing, insulin or sodium selenate, the cells were harvested and the fatty acid synthase activity of the cell lysates was measured spectrophotometrically. Values are given as the mean ± S.E of the fold increase in enzyme activity over the 30 mM No Addition. The number above the data bar represents the N number for each treatment.

cells were harvested and the activity of FAS was measured. As shown in Figure 7, selenate did not increase FAS activity as expected and varying glucose concentration did not influence this non-response.

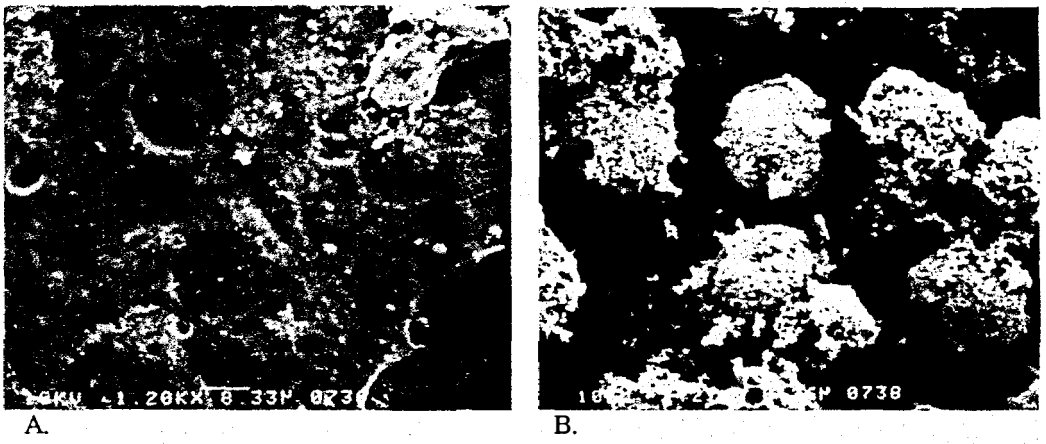
## Effect of Fructose on Glucose-6-phosphate Dehydrogenase and Fatty Acid Synthase Activity

Low fructose concentrations have been reported to enhance the glucose-induced increase in L-pyruvate kinase activity (Doiron et al, 1994). To test the effect of fructose on G6PDH and FAS activities, primary rat hepatocytes were maintained in media containing 0.1, 1, 2, 10, and 30 mM fructose alone and in combination with 5 and 30 mM glucose. After 48 hrs., the cells were harvested and the activities of G6PDH and FAS were measured. The results showed that fructose, at concentrations of 0.1, 1, 2, and 10 mM, did not enhance the 5 or 30 mM glucose effects on G6PDH and FAS activities (data not shown). Fructose alone showed similar results as an equivalent concentration of glucose, except at 30 mM. High fructose (30 mM) showed significantly lower G6PDH activity than 30 mM glucose, however the FAS activity was the same.

## Effect of High Fructose on Cell Morphology

During the initial investigation, it was noted that primary rat hepatocytes incubated in high fructose media (30 mM) undergo drastic morphological changes beginning 24 hours after incubation. This size, shape, and surface modification is complete after 48 hours. This morphological change is not seen in cells exposed to lower fructose concentrations or to an equivalent high glucose concentration. To study this phenomenon further, hepatocytes were maintained in media containing 30 mM glucose or fructose for 72 hours. Cell cultures were fixed and prepared as described in

"Materials and Methods" for scanning electron microscopy (SEM). The electron micrographs (Figure 8) show that the cells incubated in high glucose display the typical flat, extended, relatively smooth morphology of normal rat hepatocytes. Conversely, the cells incubated with high fructose are rough, spheroid, and considerably smaller. In addition, there appears be a secretory process occurring with these cells, based on the visual evidence of extracellular material surrounding the cells. In an attempt to explain this fructose effect, the cells were stained with Oil Red O, a lipid stain, to determine if the rough morphology and "secretion" are due to increased lipid production. The high fructose cells appear to contain more lipid than the glucose cells, based on the amount of red stained lipid seen in the photographs taken (data not shown). This is only a



A. B.

Figure 8. Scanning Electron Micrographs of Primary Rat Hepatocytes in 30 mM Glucose (A) and 30 mM Fructose (B) Media.

Primary rat hepatocytes were maintained in media containing 30 mM glucose or 30 mM fructose for 72 hrs. Cells were fixed and stained directly in the culture plates by the glutaraldehyde-osmium tetroxide method. Cells were then dehydrated with ethanol and hexamethyldisilazane. Culture plates were cut into small pieces, gold coated and electron micrographs were taken.

qualitative study, however, and further investigation is needed to determine quantitatively if this increase in lipid production is real.

## Effect of High Fructose on Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) is an enzyme which is important in anaerobic glycolysis and is found in many different tissues. Injury or death of these tissues causes the release of LDH into the extracellular space. Thus the level of LDH activity in cell culture media is an indication of cell injury. The morphological changes observed in the fructose cells could be due to cell injury or death. Therefore, hepatocytes were maintained in 30 mM glucose or fructose media for 72 hours and the LDH activity of the cell culture media was tested. The results showed no significant difference between the LDH activity of the 30 mM glucose and 30 mM fructose cells (data not shown). However, LDH activity is only an indicator of cell injury or death and is not always reliable. A more direct measure of cell viability, ethidium bromide staining, was then employed.

## Effect of High Fructose on Cell Viability

The viability of hepatocytes incubated in various concentrations of fructose was examined by staining the cells with ethidium bromide. Ethidium bromide is a stain which penetrates non-viable cell membranes and attaches to the chromatin in the cell nucleus. It fluoresces red under ultraviolet light and the red, non-viable cells can

be counted. The results from this study are illustrated in Figure 9. The percent of viable cells (approximately 80%) at 72 hours was equal for all fructose concentrations up to 20 mM, where the viability started to decline. There was a significant drop in viability of cells incubated in 30 mM fructose. Also, the percentage of viable cells incubated in 30 mM fructose declined with increased exposure time. This is shown by the significant decrease of viable cells at 72 hours compared to 24 hours. Thus, high

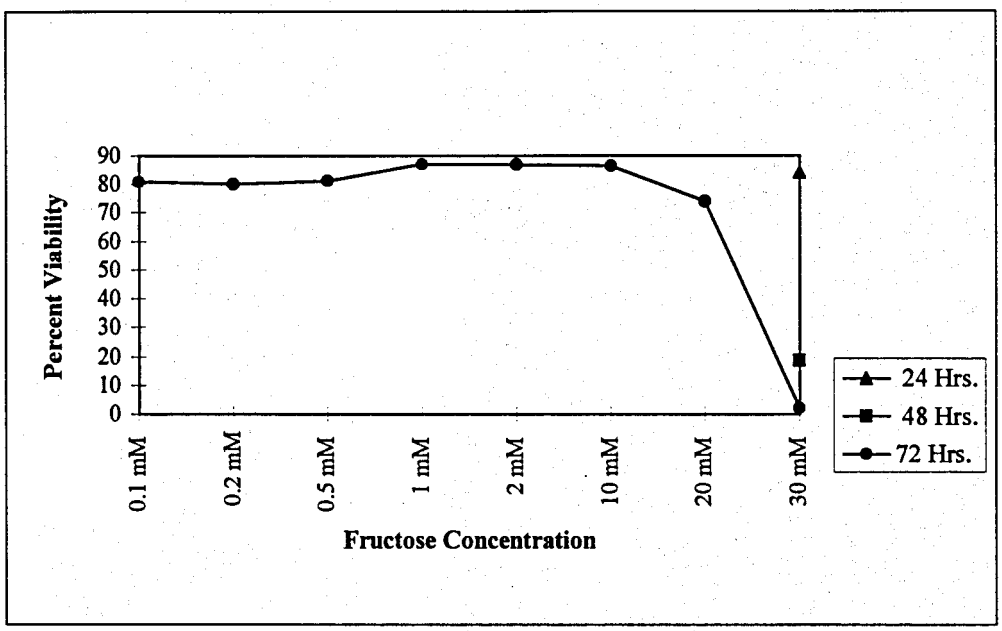



Figure 9. Percent Cell Viability of Primary Rat Hepatocytes Incubated With Fructose.

Primary rat hepatocytes were maintained in media containing various fructose concentrations for 24, 48, and 72 hours. After this time, the culture media was removed and ethidium bromide solution was added. The culture plates were protected from the light and allowed to stand for two minutes. Culture plates were then exposed to UV light and the red, non-viable cells and the total cells were counted using a microscope equipped with a calibrated occular. The percent of viable cells was obtained by subtraction of the non-viable cells from the total cells. Three separate counts were made per plate and the average percent viability was determined.

fructose concentration, above 20 mM, appears to be toxic to hepatocytes. This correlates with the morphological changes seen in cells exposed to this concentration.

## Effect of High Fructose on Culture Media pH and Osmolarity

To determine if the toxicity of high fructose on hepatocytes is due to media pH or osmolarity changes, samples of media from 72 hour hepatocyte cultures were taken and measured for both. There were small differences in the media pH with the various fructose concentrations. Two concentrations, 1 mM and 30 mM fructose, were slightly above normal physiological pH, which is 7.35 - 7.45. This apparently does not effect cell viability though, because the fructose concentration with the highest pH, 1 mm fructose, pH 7.7, has a comparable cell viability to the other fructose concentrations. Thus, media pH does not explain the low viability of cells exposed to high fructose. Under normal physiological conditions, hepatocytes are exposed to blood serum with an osmolarity of 285 to 310 mosmol/kg $H_2O$. The media osmolarity should be near normal serum osmolarity. The osmolarity of 30 mM glucose and fructose media was measured and the results are shown in Table 1. The results are similar for both media. Although the osmolarity of the 30 mM fructose media is slightly below normal, this difference is not enough to cause the marked decrease in viability seen in these cells.

## Selenate Concentration/Time Dependent Phosphorylation of the Insulin Receptor

To study the effects of selenate on insulin receptor phosphorylation, different concentrations and time points were examined and compared to activation of the

Table 1

Osmolarity of Culture Media From Primary Rat Hepatocytes Incubated With Glucose or Fructose

| Treatment | Osmolarity (mosmol/kg $H_2O$) |
|---|---|
| 30 mM Glucose | 290 |
| 30 mM Fructose | 283 |

Normal serum osmolarity is between 285 and 310 mosmol/kg $H_2O$

receptor by insulin. Primary rat hepatocytes were treated with either 87 nM insulin for five min. for maximum IR phosphorylation, or 250 $\mu$M sodium selenate for 30 min and 1 hour, or 500 $\mu$M sodium selenate for 15 min., 30 min., 1 hour and 2 hours. Wheat germ lectin was used to partially purify the IR from the cell lysates. SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody were used to study the phosphorylation state of the insulin receptors. Results of the western blot analysis are shown in Figure 10. Selenate caused a time and concentration dependent increase in phosphorylation of IR, with the greatest degree of phosphorylation seen with 500 $\mu$M selenate at 1 and 2 hours (Figure 11). The selenate treatment most resembling the effect produced by insulin, 500 $\mu$M selenate at 1 hour, was determined to be the treatment of choice for further studies.

## Effect of Glucose on Phosphorylation of the Insulin Receptor

Glucose concentration has been shown to effect protein phosphorylation.

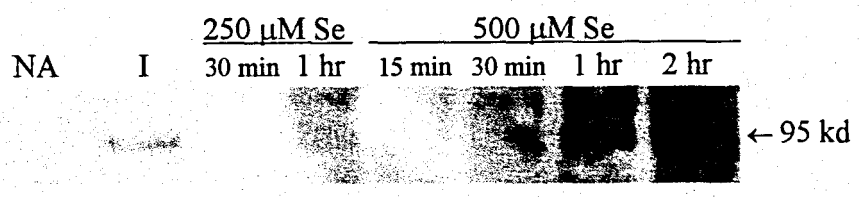



Figure 10. Western Blot Analysis of Insulin Receptor Phosphorylation in Primary Rat Hepatocytes Treated With Nothing, Insulin and Selenate.

Legend. NA = No Addition, I = Insulin, Se = Selenate

Primary rat hepatocytes were maintained in normal Waymouth media (30 mM glucose) for 24 hrs. Cells were then treated with either nothing, 87 nM insulin for 5 min., 250 μM sodium selenate for 30 min. and 1 hr, or 500 μM sodium selenate for 15 min., 30 min., 1 hr. and 2 hrs. The cells were harvested and wheat germ lectin was used to partially purify the insulin receptors from the cell lysates. SDS-PAGE and immunoblotting with mouse monoclonal anti-phosphotyrosine IgG antibody were used to study the phosphorylation state of the insulin receptors. Horse radish peroxidase conjugated anti-mouse IgG, enhanced chemiluminescence, and autoradiography were used to detect the immunocomplexes. This is representative of two separate experiments.

MacFarlane (MacFarlane, 1994) showed that the β-cell specific transcription factor IUF1 binds to the insulin gene enhancer region in a glucose-responsive manner and that changes in the binding activity of the protein occur as a result of an active modulation of its phosphorylation state. Additionally, others have found that prolonged incubation of 3T3-HIR cells with high glucose concentrations in the absence of insulin increases mRNA and kinase activity of the human insulin receptor (Hauguel-DeMouzon et al, 1995). To test the effect of glucose on the insulin receptor phosphorylation in our system, primary rat hepatocytes were maintained in media containing either 5, 10, or 30 mM glucose. After 24 hours, the cells were harvested and wheat germ lectin sepharose beads were used to partially purify the IR.

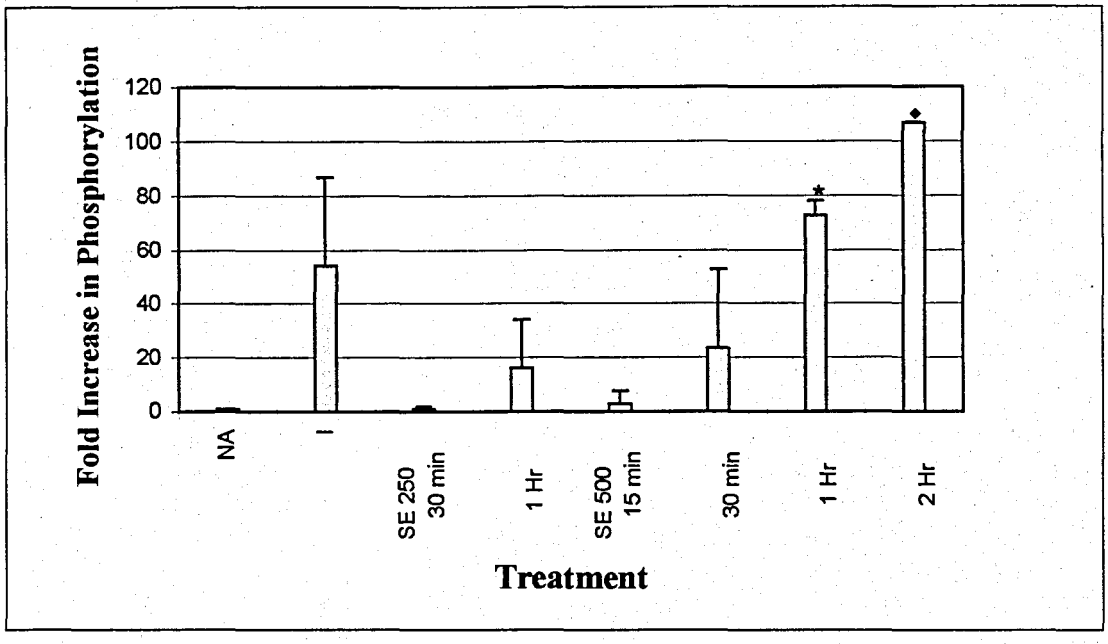



Figure 11. Selenate Concentration/Time Dependent Phosphorylation of the Insulin Receptor.

Primary rat hepatocytes were maintained in normal Waymouth's media (30 mM glucose) for 24 hours. Cells were then treated with either nothing, 87 nM insulin for 5 minutes, 250 $\mu$M sodium selenate for 30 min. and 1 hour, or 500 $\mu$M sodium selenate for 15 min., 30 min., 1 hour and 2 hours. The cells were harvested and wheat germ lectin was used to partially purify the insulin receptors from the cell lysates. SDS-PAGE and immunoblotting with mouse monoclonal anti-phosphotyrosine IgG antibody were used to study the phosphorylation state of the insulin receptors. Horse radish peroxidase conjugated anti-mouse IgG and enhanced chemiluminescence reagents were used to detect the immunocomplexes. Autoradiography was performed and densitometric scans of the autoradiographs were used to determine the values indicated. Values are given as the mean ± S.E. of the fold increase in phosphorylation over No Addition. N = 2. * significant ($p < 0.01$) ♦ significant ($p < 0.001$)

Electrophoresis and immunoblotting with anti-phosphotyrosine antibody were used to study the phosphorylation state of the insulin receptors. Results of the western blot analysis (lane NA) are shown in Figure 12. Changing glucose concentration has no significant effect on the phosphorylation state of insulin receptors in this study, as

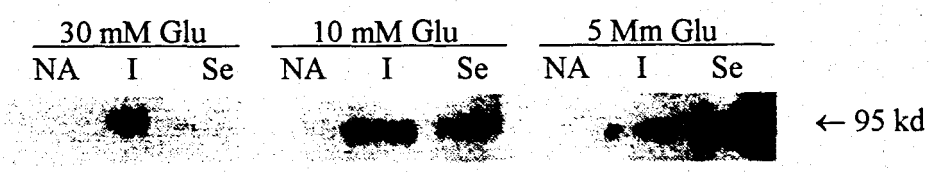



Figure 12. Western Blot Analysis of Insulin Receptor Phosphorylation in Primary Rat Hepatocytes Treated With Glucose, Insulin, and Selenate.

Legend. NA = No Addition, I = Insulin, Se = Selenate

Primary rat hepatocytes were maintained in media containing either 5, 10, or 30 mM glucose for 24 hrs. After 24 hrs, the cells were harvested and wheat germ lectin was used to partially purify the insulin receptors from the cell lysates. SDS-PAGE and immunoblotting with mouse monoclonal anti-phosphotyrosine IgG antibody were used to study the phosphorylation state of the insulin receptors. Horse radish peroxidase conjugated anti-mouse IgG, enhanced chemiluminescence, and autoradiography were used to detect the immunocomplexes. This is representative of four separate experiments.

illustrated in Figure 13.

Effect of Glucose and Insulin on Phosphorylation of the Insulin Receptor

Like the receptors for many other growth factors, the insulin receptor is a tyrosine kinase that undergoes hormone-dependent autophosphorylation (Kasuga et al, 1982). To test whether changes in glucose concentration have an effect on insulin-induced insulin receptor phosphorylation, primary rat hepatocytes were maintained in media containing either 5, 10, or 30 mM glucose. After 24 hours, the cells were treated with 87 nM insulin. Five minutes after insulin treatment, the cells were harvested and the insulin receptors isolated. Anti-phosphotyrosine antibody was used to study the phosphorylation state of the insulin receptors. Results of a typical western blot analysis (lane I) are shown in Figure 12. Insulin caused a 5 to 17 fold increase in

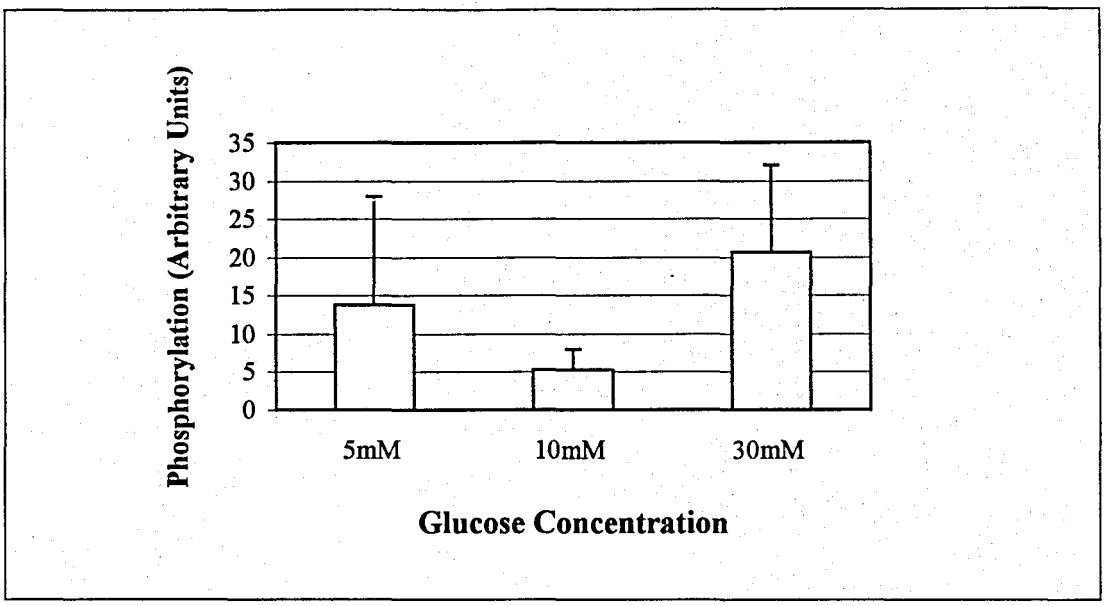



Figure 13. Effect of Glucose Concentration on Insulin Receptor Phosphorylation.

Primary rat hepatocytes were maintained in media containing either 5, 10, or 30 mM glucose for 24 hours. The cells were harvested and wheat germ lectin was used to partially purify the insulin receptors from the cell lysates. SDS-PAGE and immunoblotting with mouse monoclonal anti-phosphotyrosine IgG antibody were used to study the phosphorylation state of the insulin receptors. Horse radish peroxidase conjugated anti-mouse IgG and enhanced chemiluminescence reagents were used to detect the immunocomplexes. Autoradiography was performed and densitometric scans of the autoradiographs were used to determine the values indicated. Values represent the means ± S.E N = 4.

insulin receptor phosphorylation and a change in glucose concentration did not effect the amount of phosphorylation (Figure 14).

## Effect of Glucose and Selenate on Phosphorylation of the Insulin Receptor

Pillay and Makgoba (Pillay and Makgoba, 1992) demonstrated that in intact NIH 3T3 3.5 cells, 1 mM selenate stimulated tyrosine phosphorylation of the insulin receptor β-subunit by approximately 2-fold in the absence of insulin. In the presence

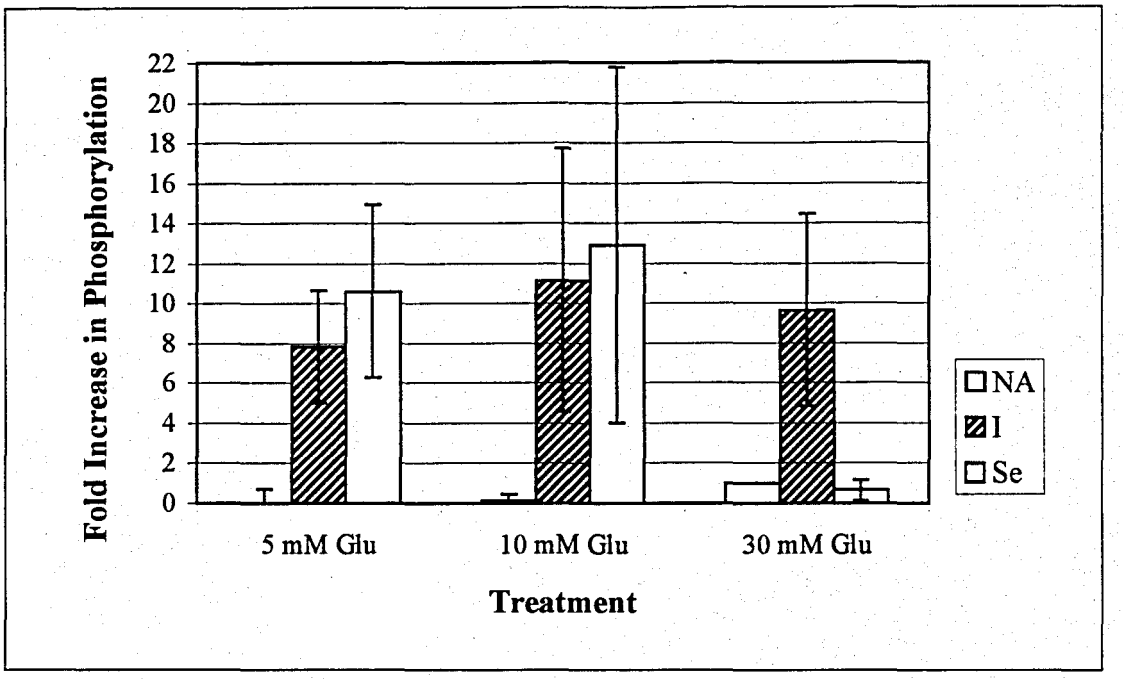



Figure 14. Insulin, Selenate and Glucose Effects on Insulin Receptor Phosphorylation.

Legend. NA = No Addition, I = Insulin, Se = Selenate

Primary rat hepatocytes were maintained in media containing either 5, 10, or 30 mM glucose for 24 hours. After 24 hours, the cells were treated with nothing, 87 nM insulin for 5 mins., or 500 $\mu$M sodium selenate for one hour. The cells were harvested and wheat germ lectin was used to partially purify the insulin receptors from the cell lysates. SDS-PAGE and immunoblotting with mouse monoclonal anti-phosphotyrosine IgG antibody were used to study the phosphorylation state of the insulin receptors. Horse radish peroxidase conjugated anti-mouse IgG and enhanced chemiluminescence reagents were used to detect the immunocomplexes. Autoradiography was performed and densitometric scans of the autoradiographs were used to determine the values indicated. Values are given as the mean ± S.E of the fold increase in phosphorylation over the 30 mM No Addition. N = 4.

of insulin, the effect of selenate on this phosphorylation was potentiated. In rat adipocytes, however, selenate did not stimulate insulin receptor phosphorylation, but clearly enhanced insulin-stimulated kinase activity towards endogenous substrates (Ezaki,1990). The effect of selenate on insulin receptor phosphorylation was tested in

our model system as well as whether or not glucose could influence selenate's effects. Primary rat hepatocytes were maintained in media containing either 5, 10, or 30 mM glucose. After 24 hours, the cells were treated with 500 $\mu$M sodium selenate. One hour after selenate treatment, the cells were harvested and the insulin receptors isolated. The phosphorylation state of the insulin receptors was determined with anti-phosphotyrosine antibody. Results of a typical western blot (lane Se) are shown in Figure 12. Selenate increased phosphorylation of the IR up to 22 fold (Figure 14). Varying the glucose concentration had no effect on this phosphorylation as demonstrated by the similar selenate responses in the 5 mM and 10 mM glucose treated cells. The cause of the reduced selenate response in the 30 mM glucose treated cells is unknown and not in agreement with other results from our laboratory. These results demonstrate the phosphorylation of the IR by selenate which is in agreement with previous results from our laboratory.

## Shc Isoforms in Primary Rat Hepatocytes

Shc has been shown to exist in three isoforms, 66, 52, and 46 kD (Pelicci et al, 1992). Not all of these isoforms are present in all tissues. The 66 kD isoform is absent in some hematopoietic cells. The 52 and 46 kD isoforms are believed to be ubiquitously expressed. We first needed to establish whether or not all three isoforms are present in primary rat hepatocytes. Hepatocytes were maintained in 30 mM glucose for 24 hours. After 24 hours, the cells were treated with nothing, 87 nM insulin for five min. or 500 $\mu$M sodium selenate for one hour. The cells were harvested

and rabbit polyclonal anti-Shc IgG antibody was used to immunoprecipitate the Shc proteins from the cell lysates. Electrophoresis and immunoblotting with anti-Shc antibody were used to determine which Shc isoforms were present. Results of a typical western blot analysis are shown in Figure 15. As demonstrated in the immunoblot, all three isoforms are present in primary rat hepatocytes, but the expression of each isoform varies with the treatment. Only the 52 kD isoform is present in unstimulated cells. Insulin and selenate induced the expression of all three Shc isoforms, but in varying amounts.

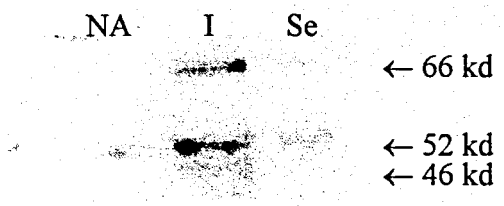



Figure 15. Western Blot Analysis of Shc Isoforms in Primary Rat Hepatocytes Treated with Insulin and Selenate.

Legend. NA = No Addition, I = Insulin, Se = Selenate

Primary rat hepatocytes were maintained in normal Waymouth media (30 mM glucose) for 24 hrs. After 24 hrs., the cells were treated with nothing, 87 nM insulin for 5 min., or 500 μM sodium selenate for 1 hr. The cells were harvested and rabbit polyclonal anti-Shc IgG antibody was used to immunoprecipitate the Shc proteins from the cell lysates. SDS-PAGE and immunoblotting with anti-Shc IgG antibody were used to determine which Shc isoforms were present. Horse radish peroxidase conjugated anti-mouse IgG antibody, enhanced chemiluminescence, and autoradiography were used to detect the immunocomplexes. This is representive of two separate experiments.

Previous investigators have shown isoform-specific phosphorylation of Shc when activated by different stimuli. In CHO cells expressing the human insulin and

EGF receptors, insulin induces phosphorylation of the 52 kD isoform, whereas EGF causes phosphorylation of both the 52 and 46 kD isoforms (Okada et al, 1995). We wanted to test whether insulin and selenate cause isoform-specific phosphorylation in primary rat hepatocytes and whether glucose has an effect on this phosphorylation.

## Effect of Glucose on Phosphorylation of Shc

The effect of glucose on Shc phosphorylation has not been previously documented. It was of interest to look at the possible effect of glucose on Shc phosphorylation because Shc is a substrate on the insulin receptor. Primary rat hepatocytes were maintained in media containing either 5, 10, or 30 mM glucose. After 24 hours, the cells were harvested and rabbit polyclonal anti-Shc IgG antibody was used to immunoprecipitate the Shc proteins. Electrophoresis and immunoblotting with anti-phosphotyrosine antibody were used to study the phosphorylation state of the Shc proteins. Results of a representative western blot analysis (lane NA) are shown in Figure 16. Note that the migration of the Shc proteins is somewhat distorted by the IgG heavy chains of the Shc antiserum used in the immunoprecipitation (Pronk et al, 1993). The IgG heavy chains migrate in the same molecular weight range (50-55 kD)(Rozakis-Adcock et al, 1992) and interact with the secondary antibody (Upstate Biotechnology Incorporated, 1997). Varying glucose concentration appeared to have no effect on the phosphorylation of Shc, as illustrated in Figure 17.

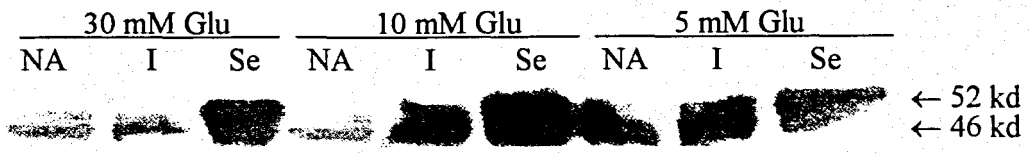



Figure 16. Western Blot Analysis of 52 kD Shc Phosphorylation in Primary Rat Hepatocytes Treated with Glucose, Insulin, and Selenate.

Legend. NA = No Addition, I = Insulin, Se = Selenate

Primary rat hepatocytes were maintained in media containing either 5, 10, or 30 mM glucose for 24 hours. After 24 hours, the cells were treated with nothing, 87 nM insulin for 5 min., or 500 μM sodium selenate for 1 hour. The cells were harvested and rabbit polyclonal anti-Shc antibody was used to immunoprecipitate the Shc proteins from the cell lysates. SDS-PAGE and immunoblotting with mouse monoclonal anti-phosphotyrosine IgG antibody were used to study the phosphorylation state of the Shc proteins. Horse radish peroxidase conjugated anti-mouse IgG, enchanced chemiluminescence, and autoradiography were used to detect the immunocomplexes. This is representative of three separate experiments.

## Effect of Glucose and Insulin on Phosphorylation of Shc

It has been reported that upon insulin treatment of fibroblasts expressing elevated levels of the human insulin receptor, Shc proteins are rapidly phosphorylated (Pronk et al, 1993). This insulin-induced phosphorylation of Shc is isoform specific (Okaka et al, 1995). There have been no published reports on the dual effect of glucose and insulin on Shc phosphorylation. To study this, primary rat hepatocytes were maintained in media containing the glucose concentrations discussed previously. Twenty four hours after plating, the cells were treated with 87 nM insulin. Five minutes after insulin treatment, the cells were harvested and the Shc proteins were isolated and studied. Results of a typical western blot analysis (lane I) is shown in Figure 16. Incubation of the cells with insulin increased phosphorylation of both the 46

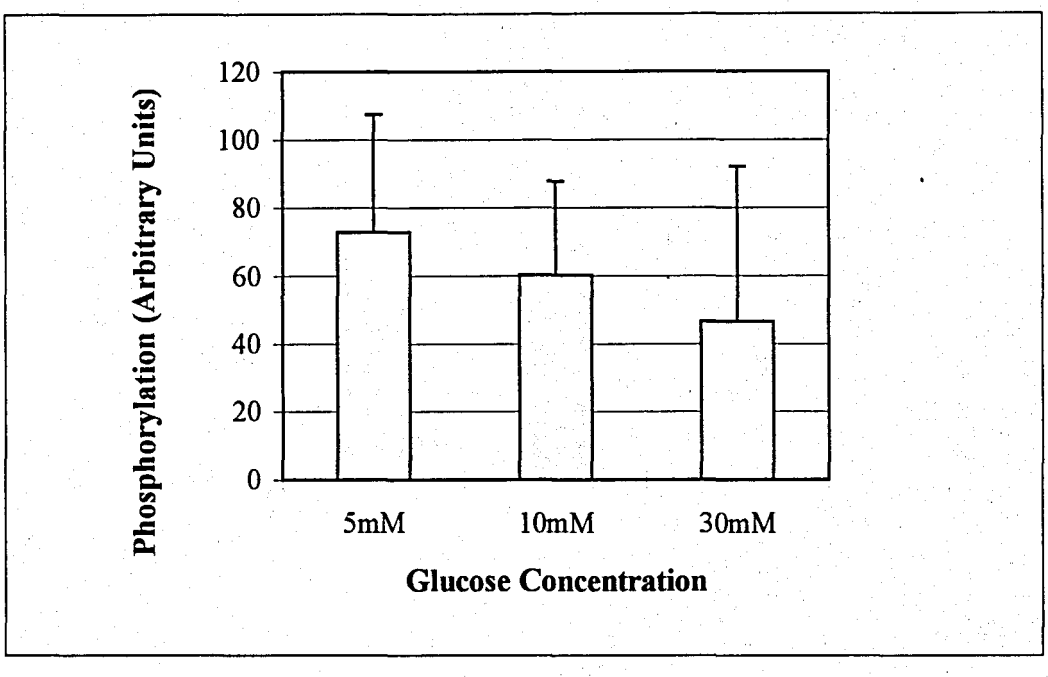



Figure 17. Effect of Glucose Concentration on 52 kD Shc Phosphorylation.

Primary rat hepatocytes were maintained in media containing either 5, 10, or 30 mM glucose for 24 hours. The cells were harvested and rabbit polyclonal anti-Shc antibody was used to immunoprecipitate the Shc proteins from the cell lysates. SDS-PAGE and immunoblotting with mouse monoclonal anti-phosphotyrosine IgG antibody were used to study the phosphorylation state of the Shc proteins. Horse radish peroxidase conjugated anti-mouse IgG and enhanced chemiluminescence reagents were used to detect the immunocomplexes. Autoradiography was performed and densitometric scans of the autoradiographs were used to determine the values indicated. Values represent the means ± S.E. N = 3.

and 52 kD Shc isoforms, but not the 66 kD form. A 5 to 20 fold insulin-stimulated increase in phosphorylation of 52 kD isoform was observed compared to the 30 mM glucose NA, as illustrated in Figure 18. This result is in agreement with previous studies (Pronk et al, 1993; Okada et al, 1995). Glucose concentration, however, had no significant effect on this insulin-induced Shc phosphorylation.

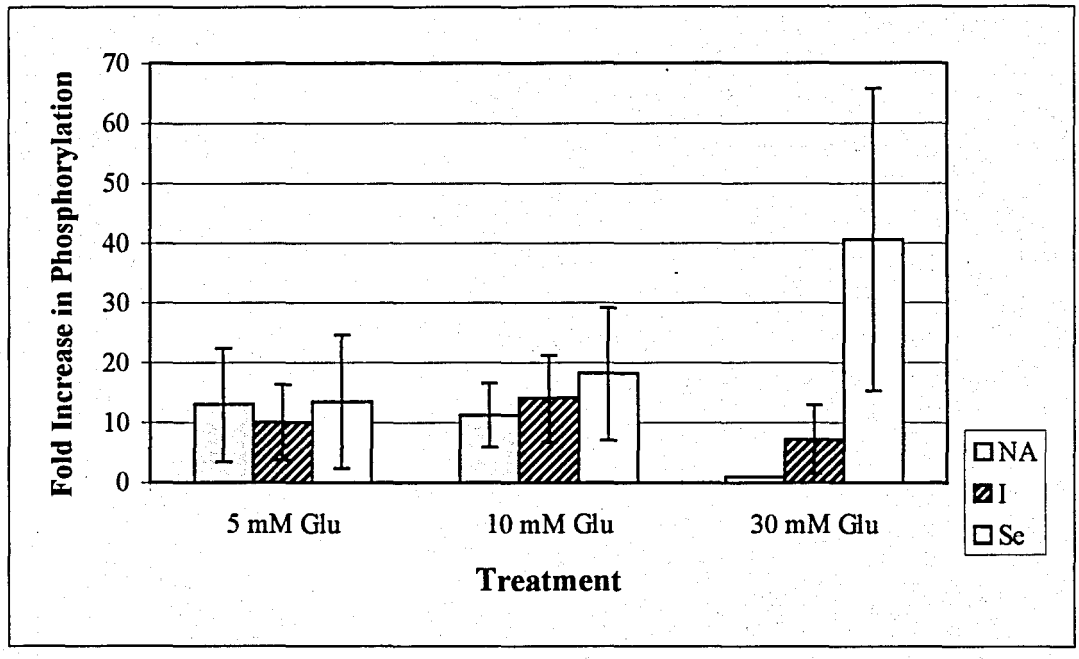



Figure 18. Insulin, Selenate and Glucose Effects on 52 kD Shc Phosphorylation.

Legend. NA = No Addition, I = Insulin, Se = Selenate

Primary rat hepatocytes were maintained in media containing either 5, 10, or 30 mM glucose for 24 hours. After 24 hours, the cells were treated with nothing, 87 nM insulin for 5 mins., or 500 $\mu$M sodium selenate for one hour. The cells were harvested and rabbit polyclonal anti-Shc antibody was used to immunoprecipitate the Shc proteins from the cell lysates. SDS-PAGE and immunoblotting with mouse monoclonal anti-phosphotyrosine IgG antibody were used to study the phosphorylation state of the Shc proteins. Horse radish peroxidase conjugated anti-mouse IgG and enhanced chemiluminescence reagents were used to detect the immunocomplexes. Autoradiography was performed and densitometric scans of the autoradiographs were used to determine the values indicated. Values are given as the mean ± S.E of the fold increase in phosphorylation over the 30 mM No Addition. N = 3.

## Effect of Glucose and Selenate on Phosphorylation of Shc

To date, there are no published studies on the effect of selenate, or selenate in combination with glucose, on Shc phosphorylation. Therefore it was of interest to

examine these effects. Primary rat hepatocytes were maintained in media containing the glucose concentrations discussed previously. Twenty fours hours after plating, the cells were treated with 500 $\mu$M sodium selenate. One hour after selenate treatment, the cells were harvested and the Shc proteins were studied. Figure 16 (lane Se) shows the results of a representative western blot analysis. Selenate did not induce the phosphorylation of the 66 kD Shc isoform, but induced phosphorylation of both the 46 and 52 kD isoforms, the latter one most significantly. As shown in Figure 18, selenate produced up to a 65 fold increase in phosphorylation of 52 kD Shc in 30 mM glucose, however it did not significantly increase Shc phosphorylation in 10 or 5 mM glucose. Although there appears to be a large difference in Shc phosphorylation between the various glucose concentrations, this difference is not statistically significant due to the low N number and large variability of the data. Therefore, we can not establish that glucose concentration has an effect on Shc phosphorylation.

# DISCUSSION

G6PDH expression and activity may be regulated by the glucose concentration in the cell. Previous studies on glucose regulation of G6PDH expression or activity have yielded conflicting results. In this study, we found that glucose does have a significant effect on G6PDH activity. The enzyme activity increased with increasing concentrations of glucose. These results are in agreement with Salati et al (Salati et al, 1988) who demonstrated a five fold increase in G6PDH activity when glucose concentration was increased from 0 to 25 mM in rat hepatocyte cultures.

The mechanism responsible for the glucose-induced increase in G6PDH activity in primary rat hepatocytes is unknown. Glucose has been implicated in the increase in G6PDH transcription and mRNA stability, demonstrated for rat hepatic G6PDH after ingestion of a high-carbohydrate diet (Prostko, et al, 1989). This stimulus for glucose induction of the G6PDH gene may be related to a glucose response element (GIRE). Other genes such as L-PK (Thompson and Towle, 1991) and S14 (Shih and Towle, 1994) have been shown to contain a GIRE. A metabolite of glucose, probably glucose-6-phosphate, is thought to be necessary for the induction of these genes (Foufelle at el, 1996). The rat G6PDH promoter contains a putative GIRE and reporter constructs driven by the G6PDH promoter do respond to glucose (unpublished results from our laboratory).

Glucose induction of gene expression may also occur through glucose-induced oxidative stress. Although it was not demonstrated whether oxidative stress was indeed produced by glucose in this study, other studies have shown that glucose at the same concentrations used in this study, does induce free radical production and peroxidation of liposomes and human low-density lipoprotein (Hunt et al, 1990). In addition, primary cultures of hepatocytes maintained in high glucose Waymouth medium produce excessive quantities of lactic acid (Kletzien and Berdanier, 1993) which has been shown to cause oxidative stress in yeast (Knight, 1995). Moreover, hepatic G6PDH has been shown to be induced by diquat (Cramer et al, 1993), ethanol (Kletzien et al, 1994), cadmium (Cook et al, 1997), $H_2O_2$, phenazine methosulphate and menadione (Ursini et al, 1997), all of which cause oxidative stress. It therefore remains unclear if G6PDH activity is increased due to direct effects of glucose on mRNA stability and/or transcription rates through a putative GIRE, or if it is increased due to oxidative stress induced by high glucose. Further studies are needed to clarify this issue.

Since glucose in our system affects G6PDH activity, it was of interest to determine if insulin, a known inducer of G6PDH activity, could enhance the glucose-induced G6PDH activity. Insulin was shown to increase G6PDH activity over no addition, however, changing the glucose concentration did not have a significant effect on the insulin-induced G6PDH activity. This is in agreement with Fukuda et al who demonstrated that in rat hepatocytes treated with 0.1 $\mu$M insulin, glucose, and 1 $\mu$M dexamethasone, increasing the glucose concentration (0 - 30 mM) had little effect on

G6PDH mRNA levels (Fukuda et al, 1992). These results also correlate with Salati et al who showed in whole animal that the carbohydrate-induced G6PDH activity was due to the insulin secretion of the animal (Salati et al, 1988).

Similarly when we tested selenate, an insulin mimetic, in the presence of varying glucose concentrations, we found that changing the glucose concentration had no effect on the selenate-induced G6PDH activity.

Since G6PDH provides the reducing equivalents for fatty acid biosynthesis, it was of interest to investigate whether FAS activity is regulated by glucose concentration. Previously, 25 mM glucose for 12 hours was shown to be sufficient to induce FAS expression in HepG2 cells (Semenkovich et al, 1993). FAS expression was also induced in rat adipocytes maintained in 20 mM glucose for 24 hours (Foufelle et al, 1992). In both studies, FAS expression was increased predominately by increasing mRNA stability. In rat hepatocytes however, it has been reported that 5 mM glucose for 24 hours alone is not sufficient to induce FAS activity (Spence and Pitot, 1982) and 10 mM glucose for 0 to 48 hours did not increase mRNA levels (Fukuda et al, 1992 ). Contradicting results have also been reported, showing that in rat hepatocytes maintained for 48 hours in 0 to 25 mM glucose, the FAS activity increased with increasing glucose (Gifforn-Katz and Katz, 1986). We found that glucose alone did not significantly affect FAS activity, although these results may not be reliable, as insulin did not increase FAS activity in this study. These insulin-stimulated FAS activity results do not concur with previous reports from our lab (Stapleton et al, 1997) or others (Spence and Pitot, 1982; Gifforn-Katz and Katz,

1986; Fukuda et al, 1992 ). We also did not find glucose concentration to have an effect on the insulin non-response. Others have shown that 5 to 20 mM glucose potentiates the effects of insulin and dexamethosone on FAS mRNA in rat hepatocytes (Fukuda et al, 1992 ). In addition, glucose (5 mM for 24 hours and 0 to 25 mM for 48 hours) increased insulin-induced FAS activity in rat hepatocytes (Spence and Pitot, 1982; Giffom-Katz and Katz, 1986).

The insulin mimetic selenate also did not increase the FAS activity. This again does not agree with previous reports from our lab (Stapleton et al, 1997). Glucose concentration had no effect on this selenate non-response. Thus, selenate displayed the same lack of effect on FAS activity as insulin in this study. Typically, results from our lab show a two to three-fold increase in basal FAS activity in response to insulin and selenate in rat hepatocytes maintained in normal Waymouth media (30 mM glucose). The basis for the lack of induction by insulin and selenate on FAS activity in this study is unknown. Given the lack of a positive insulin control, a conclusion pertaining to the effects of glucose alone or in combination with insulin or selenate on FAS activity can not be made from these results.

Since others have hypothesized the need for an intermediate, such as glucose-6-phosphate, in the regulation of glucose mediated events, there is support that an increase in glucokinase activity is also necessary. Low fructose concentrations (0.2 mM) have been reported to stimulate glucokinase in rat hepatocytes (Van Schaftingen and Vandercammen, 1989) This fructose-induced increase in glucokinase activity was shown to augment the glucose-induced increase in L-pyruvate kinase activity in rat

hepatocytes (Doiron et al , 1994). To test if fructose was capable of exerting regulation on G6PDH and FAS activities, various concentrations of fructose were used in combination with, and in place of, glucose. We found that fructose did not potentiate the effects of glucose on either G6PDH or FAS activity. This may be due to the longer incubation time used in this study. G6PDH and FAS activities were measured 72 hours after addition of fructose and glucose. Van Schaftingen and Vandercammen (Van Schaftingen and Vandercammen, 1989) found that maximum stimulation of glucokinase occurred within the first 20 min of incubation and rapidly declined after 60 min. These results were confirmed by Wals and Katz (Wals and Katz, 1994) who also showed that in rat hepatocytes, the fructose effect on glucokinase is transient and the cells quickly become refractory to fructose. Therefore, in our study, the stimulatory effects of low fructose probably had elapsed before the enzyme activities were measured.

We found that fructose alone showed similar results on G6PDH and FAS activities as an equivalent concentration of glucose. These results are not inconsistent with the hypothesis that glucose-6-phosphate may be the signalling intermediate. In isolated liver cells, fructose is one of the best gluconeogenic substrates, with the maximum rate of glucose production near twice that from lactate (Van den Berghe, 1978). Therefore, fructose could potentially be converted to glucose-6-phosphate, which could provide the signal for regulating gene expression. The possibility that the signalling molecule could be another intermediate such as glucose-1-phosphate, fructose-6-phosphate, or mannose-6-phosphate can not be ruled out at this time

(Girard, 1997).

While studying the effects of fructose on G6PDH and FAS activity, we also found that incubating the cells in high fructose medium (30 mM) caused astounding morphological changes which began as early as 24 hrs after addition of high fructose media. The cells became small, spheroid, and had a rough surface texture. The viability of these cells dropped the longer the cells were exposed to the high fructose medium, plunging to 20% after 48 hrs and cell death at 72 hrs. These phenomena are not caused by medium pH or osmolarity changes.

There are several processes which may be responsible for these drastic cellular changes. Fructose feeding increases hepatic glycogen deposition through the activation of glycogen synthase (Wals and Katz, 1994) and rat hepatocytes maintained in 20 mM fructose for 12 min. have increased glycogen synthase activity (Ciudad et al, 1988). Therefore, in this study, the cells incubated in high fructose may be producing more glycogen than the high glucose cells. Fructose also stimulates lipogenesis more than glucose (Noguchi & Tanaka, 1993), thus the high fructose cells could be producing more fat, although a conclusion regarding this can not be drawn because an increase in FAS activity was not seen. Either increased glycogen or fat production may explain the "bumpy" surface texture of the high fructose cells. Glycogen deposition or fat levels were not determined in these cells.

Death of the high fructose cells could be due to increased lactate production. Lactate formation from fructose is severalfold faster than from glucose (Van den Berghe, 1978). In isolated liver cells, the glycolytic rate of 20 mM fructose was shown

to be 10 times faster than that of an equal amount of glucose (Seglen, 1974). Thus lactate levels could increase more quickly in the high fructose cultures than the high glucose cultures. This increased lactate could cause oxidative stress which would kill the cells. Although, one would expect an increase in lactate levels to cause a change in the media pH which was not seen. Lactate levels or measurements of oxidative stress, such as glutathione peroxidase activity or malondialdehyde, were not determined.

Since we were able to show G6PDH regulation by glucose, the next question was whether this regulation was mediated through a signal pathway. Growth factor and stress mediated regulation of gene expression has been shown to utilize the MAPK signal transduction pathway. In this pathway the signal proteins are regulated by phosphorylation and dephosphorylation, therefore it is possible that glucose may regulate this phosphorylation. The phosphorylation cascade is initiated by the binding of the ligand to the receptor, causing phosphorylation of the receptor. This induces the phosphorylation and activation of the receptor substrate protein, which then activates the next protein in the cascade and so on, until MAPK is activated. Members of this cascade include tyrosine kinase receptors such as EGF and IR, Shc, insulin receptor substrate 1 (IRS-1), Grb2, Sos, Ras, Raf, Mek, and MAPK. MAPK can activate transcription factors which regulate gene expression. We began our studies by examining the phosphorylation of the IR, a requirement of all insulin mediated processes, as well as the insulin-induced activation of MAPK. We found that insulin caused a 5 to 17 fold increase in IR phosphorylation which was not altered by changes in glucose concentration. This differs from Hauguel-De-Mouzon et al who found that

high glucose (25 mM for 48 hours) potentiates the insulin-induced phosphorylation of the IR in mouse fibroblasts (Hauguel-De-Mouzon et al, 1995). To my knowledge, glucose effects on insulin-induced IR phosphorylation has not been studied in hepatocytes.

The insulin mimetic selenate, at 1 mM for 5 mins., has also been shown to stimulate IR phosphorylation 2-fold in the absence of insulin in NIH 3T3 3.5 cells (Pillay and Makgoba, 1992). However in rat adipocytes, selenate (1 mM for 30 min.) did not stimulate IR phosphorylation (Ezaki, 1990). This study found that selenate increased phosphorylation of IR 22-fold in rat hepatocytes, which agrees with previous results from our lab (Stapleton et al, 1997). Varying the glucose concentration had no effect on this selenate-induced phosphorylation, again similar to insulin. This is believed to be the first time the effects of glucose and selenate have been studied.

Another signal protein investigated due to its relationship in the MAPK pathway was Shc. All three isoforms of Shc, 66, 52, and 46 kD, were found to be expressed in varying amounts in rat hepatocytes. Insulin caused a 5 to 20 fold increase in phosphorylation of the 52 kD Shc isoform. Insulin also induced phosphorylation of the 46 kD isoform, but to a lesser degree. This agrees with a previous study which showed that 100 nM insulin for 5 min. induces the phosphorylation of the 52 kD Shc isoform in CHO cells expressing human IR (Okada et al, 1995). It also agrees with Pronk et al who showed that in NIH3T3 and CHO cells overexpressing the human insulin receptor, insulin induces both the 46 and 52 kD Shc isoforms, with the latter being the most significant (Pronk et al, 1993). Selenate was found to induce

phosphorylation of both the 46 and 52 kD Shc isoforms, the later one as much as 65 fold. This is the first time the selenate-induced Shc phosphorylation has been demonstrated. Glucose concentration was found to have no effect on basal, insulin- or selenate-induced Shc phosphorylation.

Therefore, we found that the glucose-induced increase in G6PDH activity was not due to increased signalling through IR or Shc phosphorylation. IR and Shc phosphorylation under oxidative stress conditions has not been examined. In addition, it is unknown if glucose increases the phosphorylation of other MAPK signal proteins.

Our lab has recently demonstrated glucose responsiveness in reporter constructs containing the G6PDH promoter, suggesting that the glucose-induced increase in G6PDH activity is likely due to increased transcription of the G6PDH gene, possibly through a putative GIRE. The mechanisms responsible for this GIRE mediated response is unknown.

Recently published studies on G6PDH gene expression show that it may be regulated differently when induced by various signals. Hodge and Salati (Hodge and Salati, 1997) showed that in liver tissue of mice starved and refed a high carbohydrate diet, the regulation of G6PDH expression was mediated by a nuclear posttranscriptional mechanism. Ursini et al demonstrated that oxidative stress induced G6PDH expression in HepG2, Hep3b and Jurkat T-cells was mainly due to increased transcription, with a minor contribution from posttranscriptional mechanisms (Ursini et al , 1997).

To further complicate the situation, the regulation of G6PDH expression may

also be cell specific and involve accessory factors. Therefore, there may be several regulatory steps and/or factors which are modified by glucose. Clearly, we are only beginning to understand the mechanisms which are responsible for glucose-mediated events. With the list of glucose-regulated genes continuously growing, there is an even greater need for research in this area.

# Appendix A

## Protocol Clearance From the Institutional Animal Care and Use Committee

IACUC Number 97-07-03

Date of Receipt

Date of Approval 9/5/97

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

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

## Application to use Vertebrate Animals for Research or Teaching

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

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

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

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

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

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

Check One: Teaching ____ Research X Other ____

I. ANIMAL USE CATEGORIES (check ONLY one category)

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

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

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

# WESTERN MICHIGAN UNIVERSITY
# INVESTIGATOR IACUC CERTIFICATE



Title of Project: Regulation of gene expression by insulin, insulin-mimetics and other 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 (e.g. Medline) to determine non-duplication

② specific room number where animals will be housed

IACUC Chairperson — Date: 6/24/97

Acceptance of Provisions

Signature: Principal Investigator/Instructor — Date: 9/11/97

IACUC Chairperson Final Approval — Date: 9/15/97

Approved IACUC Number 97-07-03

Rev. 3/92

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