12-1995

Regulation of the Expression of Lipogenic Enzymes by Insulin-Mimetics

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REGULATION OF THE EXPRESSION OF LIPOGENIC ENZYMES BY INSULIN-MIMETICS

by

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A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Arts
Department of Chemistry

Western Michigan University
Kalamazoo, Michigan
December 1995

ACKNOWLEDGMENTS

I would like to extend a special appreciation to my thesis advisor and committee chairperson, Dr. Susan R. Stapleton, for her tremendous and excellent advice, guidance, and financial support in my research and thesis work. Thanks also to Dr. McCarville and Dr.Schreiber for serving as research committee members.

The author is also very grateful and would like to extend his appreciation to his parents in Taiwan for their emotional support during these four years.

Juiyu Wu

REGULATION OF THE EXPRESSION OF LIPOGENIC ENZYMES BY INSULIN-MIMETICS

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Western Michigan University, 1995

Many researchers have shown that selenate (SeO_4^{2-}) and vanadate (VO_4^{2-}) have insulin-mimetic effects in certain biological processes. These mimetic effects include increasing glucose transport, regulating glycolytic enzymes, normalizing plasma glucose levels and activities of insulin regulated enzymes in diabetic rats. To clarify the functions and roles of these insulin-mimetics in different insulin-regulated metabolic pathways, the expression of the genes for glucose-6-phosphate dehydrogenase (G6PDH) and fatty acid synthase (FAS) were investigated. G6PDH is a key enzyme in the pentose phosphate pathway and is also involved in lipogenic metabolism by providing 55-70% of the NADPH utilization in fatty acid biosynthesis. FAS is a multifunctional lipogenic enzyme which utilizes NADPH in the reaction to catalyze the conversion from acetyl-CoA to palmitate. Our goal was to examine the effects of sodium vanadate and sodium selenate on lipogenic metabolism in two insulin responsive systems: isolated rat hepatocytes in culture and streptozotocin-induced diabetic rats. The focus of my study was to elucidate if these agents acted in an insulin-like manner with respect to inducing the mRNA level for both G6PDH and FAS.

Results showed that selenate and vanadate have similar effects to that of insulin on G6PDH and FAS mRNA expression. The effects of the mimetics in combination with insulin were the same suggesting the mimetics may be acting via the same mechanism.

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INTRODUCTION

Metabolism

Metabolism is considered a consecutive series of linked organic reactions that are catalyzed by various enzymes which can be divided into two major categories: (1) anabolism, the processes primarily associated with the assembly of complex organic molecules; and (2) catabolism, the processes related to degradation of complex substances, with concomitant generation of energy.

Some of the major metabolic pathways include glycolysis, the citric acid cycle (TCA cycle), gluconeogenesis, fatty acid biosynthesis and oxidation and amino acid metabolism (Fig. 1). Metabolic pathways are controlled through substrate cycles, compartmentation, and regulation of intracellular enzyme concentration at the level of either enzyme synthesis or degradation. Overlying all of these mechanisms are the actions of hormones, and chemical messengers that act at all levels of regulation. Insulin, one of these important metabolic hormones regulates several metabolic processes including glycolysis, pentose phosphate pathway and fatty acid biosynthesis.

Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PDH), is the rate-limiting enzyme in the pentose phosphate pathway. One of the important roles of this pathway is to generate NADPH (reduced nicotinamide adenine dinucleotide phosphate) in the cytoplasmic fraction of the cell (Fig. 2). Another important role is to provide pentose sugars for lipid and nucleotide synthesis.

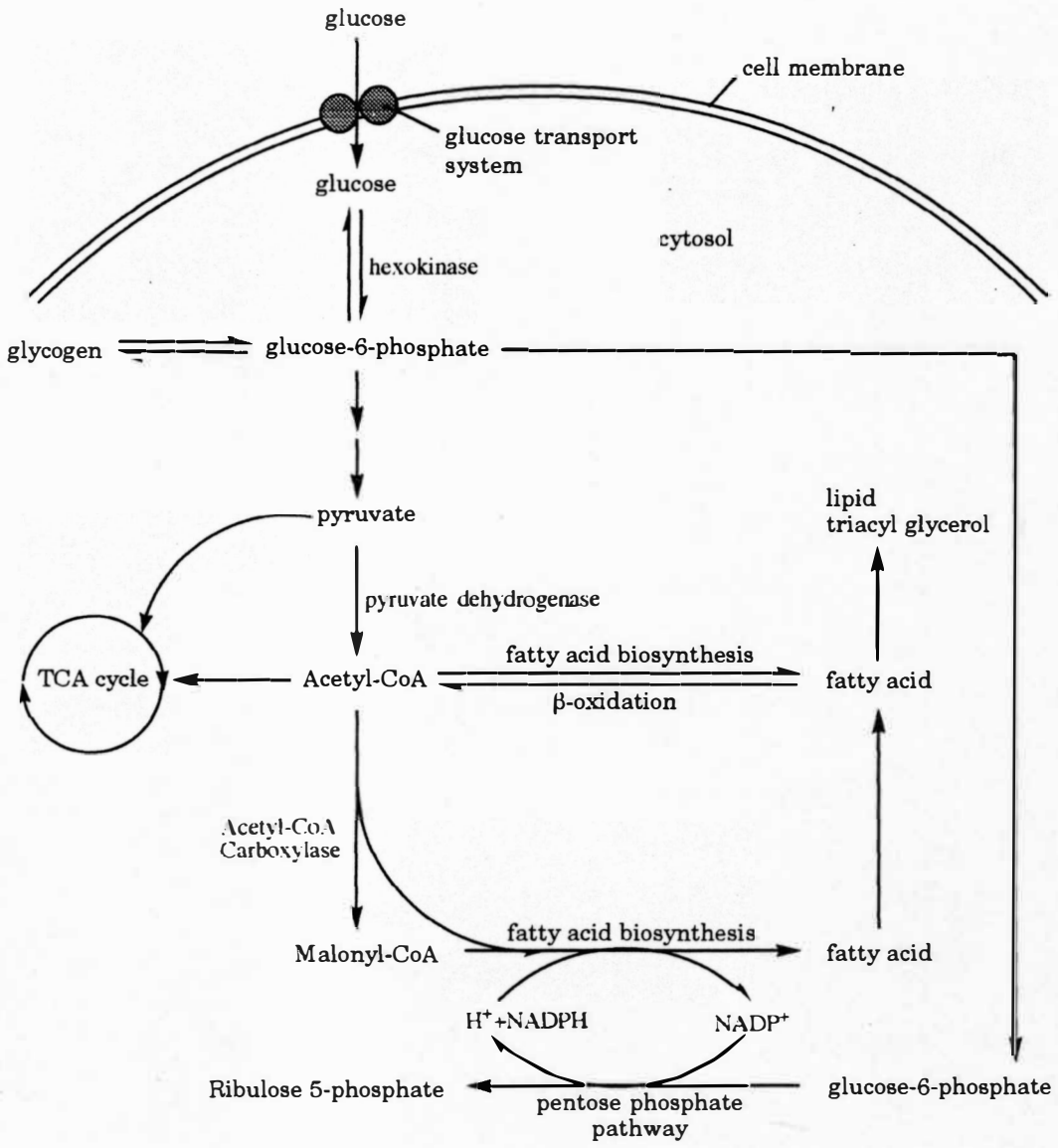


Figure 1. The Primary Pathway of Glucose and Fatty Acid Metabolism.

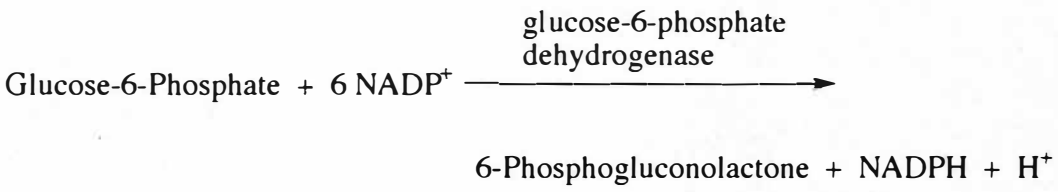


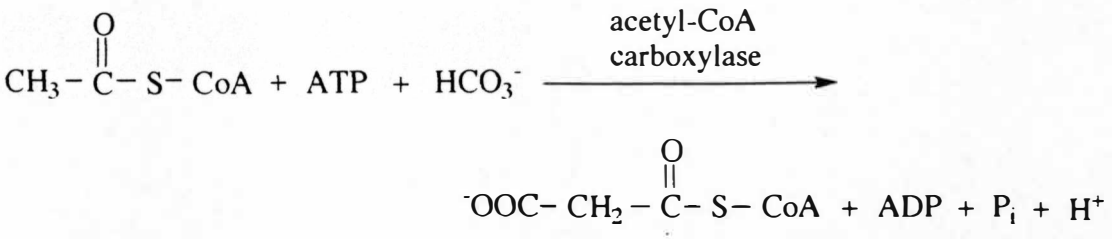
Figure 2. Reaction of Glucose-6-Phosphate Dehydrogenase.

G6PDH is needed in each cell for the production of NADPH and for control of carbon flow through the pentose phosphate pathway. The reducing power of NADPH is utilized for various synthetic processes, especially for synthesis of fatty acids, thus G6PDH participates in the regulation of both lipogenesis and glucose metabolism. G6PDH expression in liver is subject to both nutritional (Kletzien *et al.*, 1985; Prostko *et al.*, 1989) and hormonal (Kletzien *et al.*, 1986; Katsurada *et al.*, 1989) control. Highest levels of expression of this enzyme are also correlated with elevated rates of lipogenesis.

Fatty Acid Metabolism

When food is taken in by the body, large molecules like carbohydrates, proteins and fats are broken down into smaller units: glucose, amino acids, fatty acids and glycerol. These are further degraded into a simpler unit that plays a central role in metabolism. Glucose and other sugars, amino acids, fatty acids and glycerol are converted into the acetyl unit of acetyl-CoA. Acetyl-CoA can be utilized in several metabolic routes. One way is for the acetyl unit to be completely oxidized to CO_2 by the citric acid cycle with the subsequent production of ATP. Alternatively, three molecules of acetyl-CoA can form the six-carbon unit precursor of cholesterol and of ketone bodies. The third possible fate of acetyl-CoA is to be exported to the cytosol in

the form of citrate for the synthesis of fatty acids.



$$CH_3-\overset{\overset{O}{\|}}{C}-S-CoA + ATP + HCO_3^- \xrightarrow{\text{acetyl-CoA carboxylase}} {}^-OOC-CH_2-\overset{\overset{O}{\|}}{C}-S-CoA + ADP + P_i + H^+$$

Figure 3. Reaction of Acetyl-CoA Carboxylase.

In the cytosol, acetyl-CoA carboxylase activates acetyl-CoA into a three-carbon molecule called malonyl-CoA (Fig. 3). More of the acetyl-CoA further reacts with malonyl-CoA in the presence of NADPH to form the 16-carbon fatty acid called palmitic acid (Fig. 4). In higher animals, fatty acid synthesis involves only the formation of palmitate from which other saturated and monounsaturated fatty acids are formed.

$$\text{Acetyl-CoA} + 7\,\text{Malonyl-CoA} + 14\,\text{NADPH} + 7\,H^+ \xrightarrow{\text{fatty acid synthase}} \text{Palmitate} + 14\,NADP^+ + 8\,\text{CoA} + 6\,H_2O$$

Figure 4. Reaction of Fatty Acid Synthase.

Fatty Acid Synthase

In animals, the activities of fatty acid synthase (FAS) are integrated into a single polypeptide chain, catalyzing all the reactions in the conversion of acetyl-CoA and malonyl-CoA to palmitate (Wakil, 1989). The multifunctional FAS is encoded by the FAS gene which is hypothesized to have been evolved by the fusion of several genes

(Amy, Williams-Ahlf, Naggert and Smith, 1992). The dimeric enzyme with a subunit M_r of 250,000 contains distinctive catalytic domains for acetyl transacylase, malonyl transacylase, ß-ketoacyl synthase, ß-ketoacyl reductase, ß-hydroxyacyl dehydratase, enol reductase, acyl carrier protein and thioesterase.

The amount of FAS in liver changes dramatically when animals are subjected to different hormonal, nutritional and environmental states. Numerous studies have shown fatty acid biosynthesis to be controlled by hormonal and nutritional mechanism.

Paulauskis *et al.* found a rapid and dramatic insulin induction of fatty acid synthase mRNA in diabetic mouse liver. Wilson *et al.* showed that, in chicken hepatocytes in culture, thyroid hormone stimulates that accumulation of the mRNA for FAS. Goodridge and co-workers reported that glucagon regulated avian FAS by decreasing its mRNA level. Insulin (Paulauskis *et al.*, 1989; Katsurada *et al.*, 1990), triiodothyronine (Wilson *et al.*, 1986; Stapleton *et al.*, 1990) and glucagon (Goodridge, 1986) have been shown to also affect FAS gene expression.

Ample evidence has accumulated to indicate that fatty acid synthase activity is the rate-limiting step for overall lipogenesis under a variety of physiopathological conditions. Fatty acid synthesis is stimulated when glucose is in abundance and the flow of metabolic intermediates through glycolysis is high. Figure 1 shows the interconnection between glucose metabolism and fatty acid biosynthesis. Effects of insulin on this pathway include: (a) stimulation of glucose entry into cells which increases the flux of glycolysis; (b) stimulation of the activity for pyruvate dehydrogenase, therefore providing the source of acetyl-CoA for fatty acid synthesis.

There are several laboratories that have used rat liver glucose-6-phosphate dehydrogenase as a model to study the mechanisms by which hormones and diet regulate the synthesis of lipogenic enzymes (Coupe *et al.*, 1990; Schaffer, 1985; Iritani

et al., 1993). Evidence exists to show that fatty acid synthase can be controlled by the availability of the reducing equivalents (NADPH), that are generated from G6PDH of the pentose phosphate pathway. Typically, about 55-70% of the NADPH for fatty acid synthesis comes from the pentose phosphate pathway. Therefore, in this study we investigated effects of various agents on the expression of the mRNA levels for both FAS and G6PDH.

Physiological Effects of Insulin

Insulin is a 5.8-kDa protein that is synthesized in the pancreatic islet, and many actions of insulin are well known including signals that promote the (a) uptake of glucose into a cell, (b) storage of fuels - lipids and glycogen, and (c) biosynthesis of macromolecules -nucleic acid and protein. In addition, the secretion of insulin is tightly controlled and is dependent on plasma glucose level (Fig. 5).

On binding to its receptor, insulin promotes multiple effects on cell metabolism ranging from modulation of enzyme activities, to stimulation of protein synthesis and gene expression. Insulin effects appear to be due to multilevel controls: transcriptional and translational events (modifying, for example, mRNA stability or translatability). Many of the liver-specific regulated genes, including glucose-6-phosphate dehydrogenase and fatty acid synthase, have been shown to be transcriptionally regulated by this hormone (Chu *et al.*, 1988; lynedjian *et al.*, 1989). This hormone regulation becomes very important when production or utilization of insulin is impaired as is the case in the diabetes mellitus.

Despite many efforts in this area, however, the mechanisms by which insulin acts on the transcriptional and translational level of any gene is not fundamentally clear. This is primarily because (a) insulin initiates a multitude of cellular events that do not

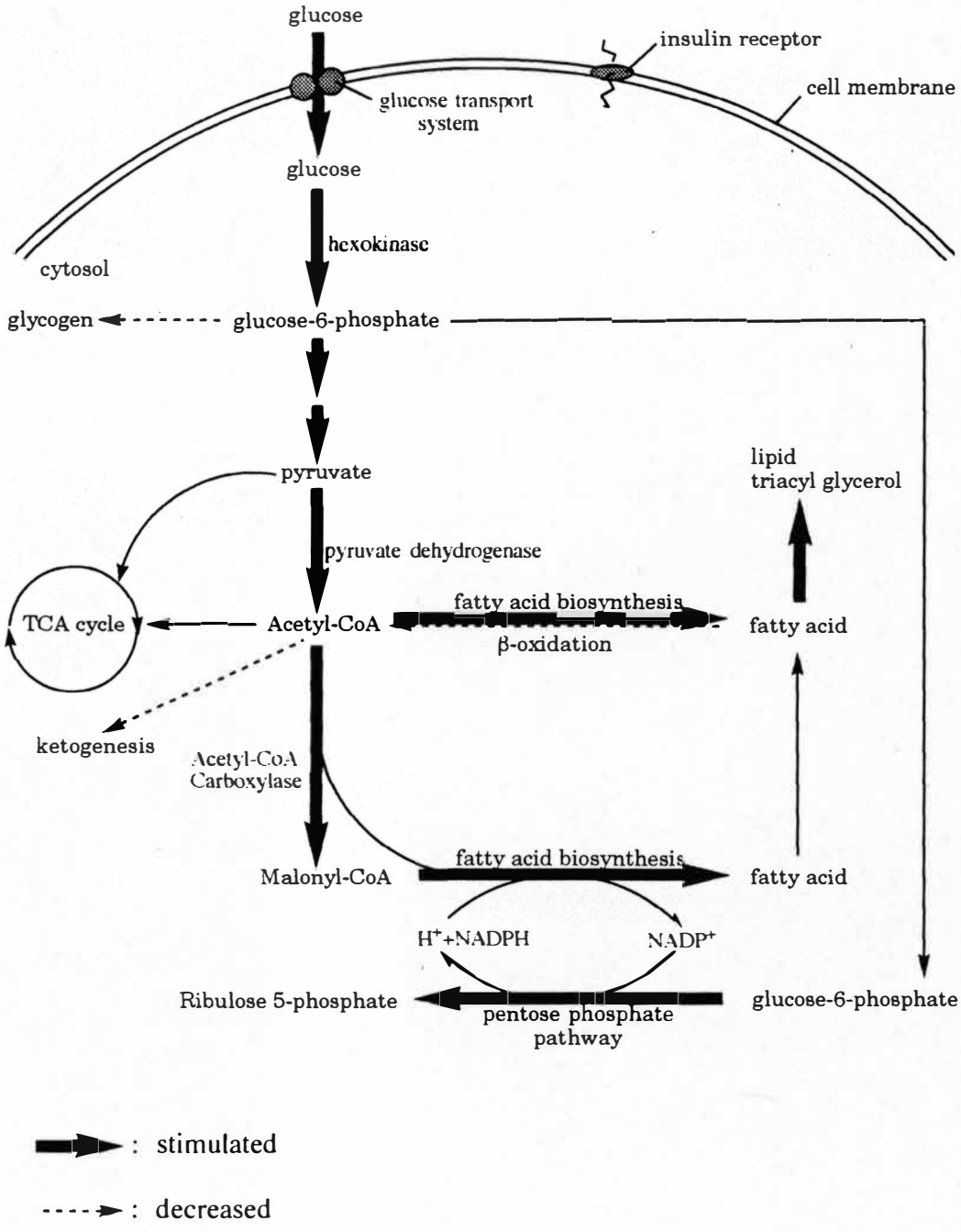


Figure 5. The Physiological Effects of Insulin in Glucose and Fatty Acid Metabolism.

appear to share a common mechanistic pathway, and (b) the cellular machinery involved in insulin action utilizes both integral membrane and cytoplasmic components that further the complexity of this system.

Diabetes

Diabetes mellitus in humans is not a single disease entity, but represents a heterogeneous group of glucose intolerance disorders characterized by expression of fasting hyperglycemia due to an absolute or relative deficiency of the pancreatic β cell hormone, insulin. The two major categories of the disease - noninsulin-dependent diabetes mellitus (NIDDM) and insulin-dependent diabetes mellitus (IDDM) - appear to be separate diseases with different etiopathogenic bases. (Table 1)

Roles of Selenate in Metabolism and Function

Early studies on the biochemical function of selenate focused on its similarity to vitamin E as an antioxidant. In 1973, selenate was discovered as an essential constituent of glutathione peroxidase (EC 1. 11. 1. 9) and thus provided a potential mechanism through which this element could be involved in releasing oxidative stress (Rotruch *et al.*, 1973).

In recent years, however, a number of biological and physiological effects of selenate have been described which are not associated with glutathione peroxidase, such as stimulation of glucose transport, translocation of glucose transporters, and stimulation of both cAMP phosphodiesterase and ribosomal S6 phosphorylation in an insulin-like manner in rat adipocytes (Ezaki, 1990). Selenate has also been shown to stimulate epidermal growth factor (EGF) receptor phosphorylation and EGF-stimulated phosphorylation in A431 cells (Pillay and Makgoba, 1992). This information gave a

Table 1

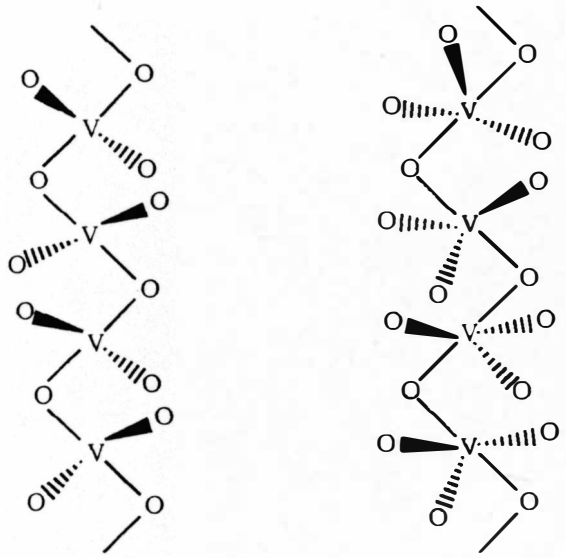
Comparison of NIDDM and IDDM

	NIDDM	IDDM
Level of insulin secretion	may be normal or exceed normal	None or almost none
Typical age of onset	Adulthood	Childhood
Percentage of diabetics	80-90%	10-20%
Basic defect	Reduced sensitivity of insulin's target cells	Impair of β cells
Associated with obesity?	Usually	No
Genetic and environmental factors participating in disease?	Yes	Yes
Speed of development of symptoms	Slow	Rapid
Treatment	Dietary control and weight reduction; occasionally oral hypoglycemic drugs	Insulin injection; dietary management

new role to selenate as an insulin-mimetic. Presently, the role of selenate as an insulin-mimetic on gene expression has not been reported.

Roles of Vanadate in Metabolism and Function

Vanadate ions were shown to mimic all or most of the actions of insulin in intact cell systems, via a post-receptor mechanism. Vanadium is an essential trace element and is an endogenous constituent of all or most mammalian tissue. Most tissues of higher animals contain intracellular vanadium at concentrations varying between 0.1 and 1 μM. There are two different forms that have been primarily used in metabolic studies: Orthovanadate and Metavanadate (Fig. 6). These inorganic species form polymers, the size of which are pH-dependent. At pH 2, both vanadates are single ion. As pH increases, larger molecules form becoming as large as 10 vanadium molecules at pH 8.



Metavanadate Orthovanadate

Figure 6. Structures of Ortho and Meta Vanadate.

Vanadate was first shown to mimic insulin with respect to glucose metabolism (Table 2). Similar to insulin, vanadate stimulates glucose oxidation and transport in adipocytes. It also stimulates glycogen synthesis in the liver and inhibits hepatic gluconeogenesis (Tolman *et al*, 1979). In some instances, vanadate in combination with other compounds, such as vanadate and lithium or vanadate and H_2O_2, is as or more effective in insulin-like action (Heffetz, 1990; Rossetti, 1990; and Zick and Sagi-Eisenberg, 1990).

In vivo, vanadate has been reported to lower and even normalize blood glucose concentrations in various animal models of diabetes, including the severely insulin-deficient state induced by the β-cell toxin streptozotocin (STZ) and certain hyperinsulinemic syndromes corresponding to human non-insulin-dependent diabetes mellitus (NIDDM).

Table 2

Insulin-Like Actions of Vanadate in *In Vitro* Systems

Activity	Direction of activation
Hexose transport	Stimulated
Glucose oxidation	Stimulated
Glycogen synthase	Stimulated
Lipolysis	Inhibited
Lipogenesis	Stimulated

Objective of the Study

When these studies were begun, there were many studies about the insulin-like effects of vanadate and selenate on cellular processes but none on regulation of gene expression. Since it had been established that these metals affect glucose metabolism similar to insulin, we wanted to determine if vanadate and selenate acted in a similar manner with respect to regulation of both a glycolytic and lipogenic enzyme, G6PDH and FAS, respectively. We pursued the following objectives:

1. Determine the effects of vanadate and selenate on the mRNA level of both G6PDH and FAS in isolated hepatocytes and compare to the effects induced by insulin.

2. Determine the effects of vanadate and selenate treatment on the mRNA level of G6PDH and FAS in streptozotocin-induced diabetic rats and compare to the treatment of diabetic animals with insulin.

Significance of the Study

Insulin performs a critical role in the regulation of metabolic processes. Heavy metals, such as selenium and vanadium, have been proposed to replace or be used in conjunction with insulin in treatment of such disease states as diabetes. It is important to establish if these compounds truly mimic insulin in all aspects and to determine their mechanism of action. Understanding how these compounds regulate metabolism may provide a greater understanding of hormonal regulation.

MATERIALS AND METHODS

Materials and Animals

Waymouth's MB 752/1 medium was purchased from Gibco BRL (Grand Island,NY). Collagenase D, restriction endonucleases and RNase (DNase-free) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). [α-^{32}P] dCTP (3000 Ci/mmol) and GeneScreen membrane were obtained from Dupont/NEN (Boston, MA). Guanidinium thiocyanate and phenol (saturated solution) were purchased from Amresco (Solon, OH). Insulin was a gift from Eli Lilly Corp. (Indianapolis, IN). Other reagents were obtained from Sigma (St. Louis, MO). Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Portage, MI), fed Purina rat chow and given water ad libitum until needed.

Hepatocyte Isolation and Tissue Culture

Hepatocytes were isolated using the collagenase and hyaluronidase perfusion method as described by Elliget and Kolaja (1983), with the following modifications. Male rats weighing 150-250 g were fasted 48 hours prior to the isolation procedure. After perfusion with the enzyme preparation, the liver was excised and forced through four layers of sterile gauze. The cells were washed two times with cold Waymouth's MB 752/1 medium supplemented with 0.5% BSA, and pelleted for 2 min at 4 ^{0}C at 50 g. The pellet was gently resuspended in medium and an aliquot of cells counted with a hemocytometer. Cell viability was determined by trypan blue dye exclusion, and cells with a viability greater than 85% were plated. Collagen-coated 60 mm tissue culture

dishes were plated with $3.05|10^6$ cells/plate. The cells were incubated in Waymouth's MB 752/l medium supplemented with 0.5% BSA and gentamicin (10μg/ml) under a humidified atmosphere of 5% CO_2 and 95% air at 37 ^{0}C.

Cell Treatment

Four hours after the initial plating, the cells were washed with Waymouth's MB 752/l without BSA, and fresh medium was applied. This serum-, hormone- and lipid-free, glucose-rich medium is referred to as basal medium. Hepatocytes were exposed to either 1 μM, 5 μM, 10 μM, 20 μM, or 30 μM of either sodium selenate or sodium vanadate, and or 300 ng/ml insulin at times indicated in figure legends. The appropriate volume was then added directly to the medium covering the plated hepatocytes, and swirled to mix.

Animals and Protocol Design for *In Vivo* Experiments

Male, Sprague Dawley rats, 150-200 grams, were fasted for 24 hours, and a blood sample was obtained from the tail vein. The cells were allowed to clot, centrifuged, and plasma was saved. Rats were then injected in the tail vein with 65 mg/kg streptozotocin (a gift from the UpJohn Co.). After 7 days, plasma samples were obtained and glucose concentrations were determined using the Trinder assay (Sigma). Animals were considered diabetic if glucose levels were at least 450-650 mg/dl with 150-200 mg/dl being normal. Animals were then treated with hormones or metals.

Treatment and Maintenance of Animals

Nondiabetic (control) and diabetic rats were either not treated or treated with 5-10 units ultra-lenta insulin injected subcutaneously, 0.5 mg/ml sodium orthovanadate

with 1 g/L of sodium chloride in drinking water, or 15 μmoles/kg sodium selenate injected inter-peritoneally. Glucose levels, food intake, and water intake were monitored throughout the experiment.

Total RNA Isolation

For Northern blot analysis of mRNA, two dishes were pooled for each individual determination of RNA at the end of culture time. Cells were lysed and total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). All solutions were prepared with diethylpyrocarbonate (DEPC)-treated water and autoclaved glassware. The lysis solution was 4M guanidinium thiocyanate, 0.025 M sodium acetate, pH 7; 0.5% sarcosyl and 0.1M 2-mercaptoethanol. RNA pellets were dried and dissolved in DEPC-treated sterile water. RNA concentration and purity were determined by UV spectrophotometry at 260 nm and 280 nm, and its integrity was systematically checked by electrophoresis in a 1% agarose gel stained with ethidium bromide and visualized under ultraviolet light.

cDNA Probes

The cDNA probes used in this study were kindly provided to Dr. Susan Stapleton by various laboratories. The pFAS-7 cDNA for rat liver FAS mRNA is 800 bp in length, inserted into *Pst*I site of the plasmid pBR 322 was obtained from Dr. A.G. Goodridge (University of Iowa, Iowa City). The G6PDH cDNA is 1,300 bp in length, inserted into *Eco*RI site of the plasmid pBR 322 was obtained from R. F. Kletzien of the Upjohn Company. The ß-actin cDNA (Cleveland, *et al.*, 1980) was used as a control probe to quantitate total RNA, and amount of G6PDH and FAS

mRNA were normalized to the control probe, ß-actin. FAS, G6PDH and ß-actin cDNAs were purified by gel electrophoresis on a 1% agarose gel in 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA). After restriction digestion, the band was electrophoresed onto and then eluted from Whatman DE81 ion exchange paper.

RNA Electrophoresis and Northern Transfer

Total RNA (10-20 μg) was denatured in a solution containing DEPC-water by heating at 65 ^{0}C for 15 min, then electrophoresed on 1% agarose gel containing 1X MOPS (0.02 M 3-(N-mopholino) propanesulfonic acid pH 7.0, 5 mM sodium acetate and 1 mM EDTA) and 2.2 M formaldehyde. After electrophoresis was complete, the gel was stained with ethidium bromide and photographed using a UV source to determine the positions of 28S and 18S ribosomal RNA bands. This was done to assure the integrity of the RNA as well as the similarity in the RNA loading in each sample. The gel was then placed onto a vacuum-blotting device (VacuGene, Pharmacia LKB, Sweden) for transfer to a gene screen membrane. For transfer of the RNA, the gel was presoaked in 10X SSC (1X SSC is 0.15M NaCl, 0.015M Na_3-citrate, pH 7.0), and transferred at a pressure of 40 mbar for 6-8 h, using 10X SSC as blotting buffer.

The membrane was air-dried briefly and the RNA was fixed by placing the nylon membrane, RNA side down, onto a plastic-wrap-covered UV transilluminator for 5 min.

Northern Blot Hybridization and Autoradiography

The RNA membrane was prehybridized overnight at 42 ^{0}C in a sealed plastic bag with prehybridization solution (50% deionized formamide, 0.25 M $NaHPO_4$ pH

7.2, 0.25 M NaCl, 1 mM EDTA, 100 μg/ml denatured salmon sperm DNA, and 7% SDS). cDNA labeling was carried out with [α-^{32}P] dCTP to a specific activity of 10^9 cpm/μg of DNA by using a multiprimer DNA-labeling system kit (Amersham). Unincorporated nucleotides were separated by gel filtration chromatography on a G50 Sephadex column with NETS (100 mM NaCl, 1 mM EDTA pH 8.0, 10 mM Tris-HCl, 0.1% SDS) solution. After denaturation by boiling for 2 min, the labeled probe (at least 25×10^6 cpm/ml) was added to the prehybridization mixture and hybridization continued for 16-18 hours at 42 ^{0}C. After hybridization, the unbound probe was washed away at 45 ^{0}C twice for 30 min each in 2X SSC/ 0.1% SDS and then at 55 ^{0}C twice for 30 min each in 1X SSC/ 0.1% SDS in shaking water bath.

Autoradiograms were obtained by exposure of radiolabeled GeneScreen^{TM} membranes for varying lengths of time at -70 ^{0}C to Kodak X-Omat AR films with DuPont Lighting-Plus intensifying screens for 1-3 days. Relative densities of the hybridization signals were determined by desitometric scanning of the autoradiograms. Northern blot analysis was performed in duplicate from at least three different cell preparations. All blots within a series of experiments were hybridized, exposed, and stripped in parallel.

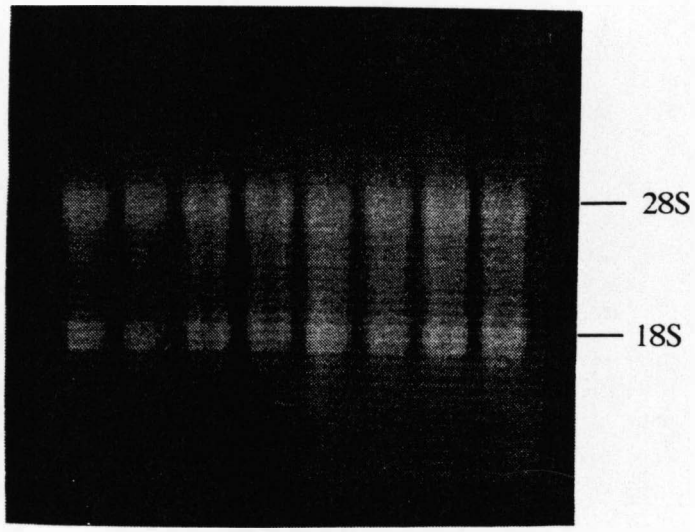
Statistical Analysis

Results are expressed as means ± SE.

RESULTS

Visualization of RNA After Gel Electrophoresis

Visualization of RNA after gel electrophoresis is very important in Northern analysis, because it provides a way of evaluating the integrity of the RNA (Fig. 7). Degradation can be recognized by smearing and disappearance of high-molecular-weight RNA bands. At the same time, staining of the size-separated RNA allows determination of the length of the eventually hybridized mRNA relative to the migration distance of the ribosomal 28S and 18S bands, which serve as internal molecular weight markers (Kroczek *et al.*, 1990).

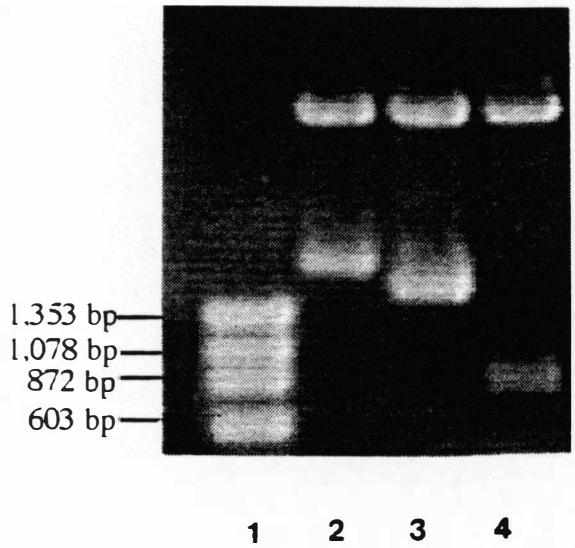


Note: Total RNA(10μg per lane) from rat hepatocytes was size-separated on formaldehyde-agarose (1%) gel as described under Materials and Methods. The gel was stained with ethidium bromide and washed in water two times.

Figure 7. Total RNA in Agarose Gel Electrophoresis.

Visualization of Plasmid DNA After Digestion and Gel Electrophoresis

After treatment with a restriction enzyme and agarose gel electrophoresis, DNA fragments of plasmid can be visualized with ethidium bromide staining prior to elution (Fig. 8). After restriction digest, the band was electrophoresed onto and then eluted from Whatman DE81 ion exchange paper.



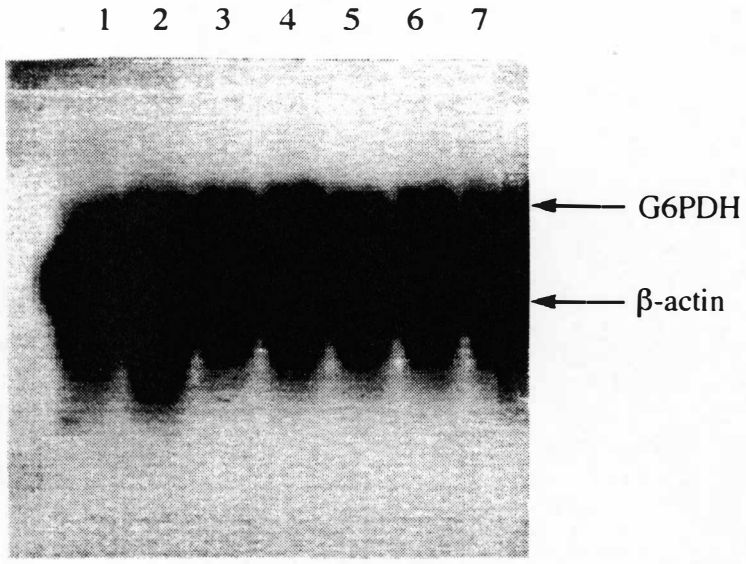
Note: Aliquots of digested plasmid were analyzed by electrophoresis on a 1% agarose gel in TAE buffer. Lane 1: ØX174 molecular weight marker-DNA; 2: ß-actin cDNA in pBR322; 3: G6PDH cDNA in pBR322; 4: FAS cDNA in pBR322.

Figure 8. Analysis of Plasmid DNA by Restriction Digestion in Agarose Gel Electrophoresis.

In Vitro Effect of Insulin on G6PDH mRNA Level

The nutritional condition of an intact animal is communicated to the liver cells through changes in the concentrations of hormones in the blood. On binding to its

receptor, insulin promotes multiple effects on cellular metabolism ranging from modulation of enzyme activities to transcriptional regulation of several liver-specific genes. Insulin is generally considered a positive effector of the lipogenic enzymes including G6PDH and FAS, and is therefore used as a positive control in the following studies. In rat hepatocytes maintained in a chemically defined medium, the addition of insulin alone causes a 1.5-2 fold increase in the G6PDH mRNA level (Fig. 9), the same increase that is exhibited in enzyme activity of G6PDH (data not shown).



Note: Total cellular RNA was harvested from culture as described under "Material and Methods". The RNA samples were blotted and hybridized to radiolabeled cDNA. 10 μg of RNA was load per lane.

Lane: 1. NA 2. insulin 3. Se 10μM 4. insulin + Se 10μM 5. Se 30μM 6. insulin + Se 30μM 7. Va 10μM

Figure 9. Effects of Insulin and Insulin-Mimetics on G6PDH mRNA in Rat Hepatocytes.

In Vitro Effect of Selenate on G6PDH mRNA Level

Early studies on the biochemical function of selenium focused on its role as an antioxidant, and as an essential constituent of glutathione peroxidase (EC 1. 11. 1. 9.). In recent years, selenate (SeO_4^{2-}) was found to have several insulin-like effects in rat adipocytes (Ezaki, 1990). In this study, selenate was found to have an insulin-like effect in rat hepatocytes: stimulation of G6PDH mRNA level. As shown in Table 3, selenate stimulated G6PDH mRNA in a concentration-dependent manner. The stimulation observed at concentrations of 10 μM and 30 μM were about 1.9-fold and 3.3-fold, respectively. The effects of selenate in combination with insulin, insulin+selenate 10μM and insulin+selenate 30 μM showed a stimulation of G6PDH mRNA of 2.4 and 3.4-fold, respectively. The combination of insulin and selenate was not greater than selenate alone, therefore the effect was not additive.

In Vitro Effect of Vanadate on G6PDH mRNA Level

Vanadate treatment of hepatocytes in *vitro* also increased the expression of G6PDH mRNA as shown in Table 4. This results suggest that vanadate has a similar effect as insulin in stimulating G6PDH mRNA level. This is consistent with the action of vanadate on the enzyme activity of G6PDH (Berg and Wu *et al*, 1995).

In Vitro Effect of Insulin on FAS mRNA Level

In our system, to determine whether insulin modulation of enzyme synthesis is at the pre-translational or translational level for FAS, we measured the effect of insulin and the insulin-mimetics on FAS mRNA level (Fig. 10). Two different size mRNAs, 8.2 kb and 9.1 kb are observed for fatty acid synthase.

Table 3

The Effects of Sodium Selenate With and Without Insulin at Different Concentrations on Glucose-6-Phosphate Dehydrogenase mRNA Level in Rat Hepatocytes.

condition	mRNA level as % of control
	Glucose-6-Phosphate Dehydrogenase
control	100
insulin	182±10
Se 10μM	196±15
insulin + Se 10μM	240±16
Se 30μM	333±21
insulin + Se 30μM	338±18

Data are means ± SE; n=6/group

Total cellular RNA was harvested from culture as described under "Material and Methods". The RNA samples were blotted and hybridized to radiolabeled cDNA. 10 μg of RNA was loaded per lane. The control value (NA) is set at 1.0. NA: no addition. I: insulin. Se10: selenate 10 μM. Se 30: selenate 30 μM.

The amount of these mRNA species increased gradually when rat hepatocytes were treated with insulin. The ß-actin mRNA level remained the same during the entire period of insulin treatment and thus served as our control. The fatty acid synthase mRNA level increased 2-fold by 16 h of insulin treatment. Thus, the increase in the enzyme activity for fatty acid synthase observed when rat hepatocytes were treated with insulin, was due to an increase in its mRNA level.

Table 4

The Effects of Sodium Vanadate With and Without Insulin at Different Concentrations on Glucose-6-Phosphate Dehydrogenase mRNA Level in Rat Hepatocytes

condition	mRNA level as % of control
	Glucose-6-Phosphate Dehydrogenase
control	100
insulin	132±10
Va 10μM	182±15
insulin + Va 10μM	212±12
Va 30μM	230±13
insulin + Va 30μM	253±12

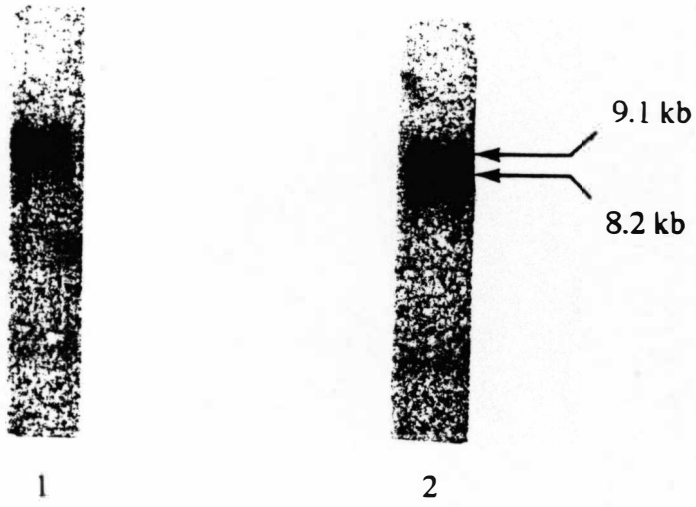
Data are means ± SE; n=6/group

Total cellular RNA was harvested from culture as described under "Material and Methods". The RNA samples were blotted and hybridized to radiolabeled cDNA. 10 μg of RNA was loaded per lane. The control value (NA) is set at 1.0. NA: no addition. I: insulin. Va 10: Vanadate 10 μM. Va 30: Vanadate 30 μM

In Vitro Effect of Selenate on FAS mRNA Level

The effects of selenate on FAS mRNA were observed in a concentration-dependent fashion. The magnitude of induction for FAS mRNA was 3.2- and 3.4-fold when hepatocytes were treated with a concentration of 10 μM and 30 μM, respectively (see Table 5). Again, no differences in induction were observed when insulin and selenate were added together. Selenate-induced changes in FAS mRNA levels were

identical to changes observed in enzyme activity (Berg and Wu *et al*, 1995).



Note: Total cellular RNA was harvested from culture as described under "Material and Methods". The RNA samples were blotted and hybridized to radiolabeled cDNA. 10 μg of RNA was load per lane. β-actin was used as a control to show equal loading of RNA in each lane. The control value (NA) is set at 1.0. Lane: 1. NA and 2. insulin

Figure 10. Effect of Insulin on FAS mRNA in Rat Hepatocytes.

In Vitro Effect of Vanadate on FAS mRNA Level

The results, as shown in Table 6, show that 10μM vanadate treatment doubles the basal expression of FAS mRNA while 30μM yield a 2.5-fold increased. This together with the previous results, strongly suggests that vanadate mimics insulin actions in its ability to regulate enzyme activity (Berg and Wu *et al*, 1995) by modulating gene expression.

Table 5

The Effects of Sodium Selenate With and Without Insulin at Different Concentrations on Fatty Acid Synthase (FAS) mRNA in Rat Hepatocytes

condition	mRNA level as % of control
	Fatty Acid Synthase
control	100
insulin	162±13
Se 10μM	321±25
insulin + Se 10μM	398±27
Se 30μM	340±30
insulin + Se 30μM	410±22

Data are means ± SE; n=6/group

Total cellular RNA was harvested from culture as described under "Material and Methods". The RNA samples were blotted and hybridized to radiolabeled cDNA. 10 μg of RNA was loaded per lane. The control value (NA) is set at 1.0.

In Vivo Effects of Vanadate and Selenate on G6PDH mRNA Level

To determine whether or not vanadate and/or selenate altered the expression of G6PDH in a fashion similar to insulin, the mRNA levels of G6PDH were measured in the non-diabetic(control), diabetic and streptozotocin-induced diabetic rats. Quantification of the autoradiographic signal for G6PDH mRNA revealed an approximate 70% decrease in diabetic rats when compared to non-diabetic condition (Table 7). Treatment of the diabetic rat with either insulin, vanadate or selenate restored the mRNA level for

G6PDH to about 80% of control (Table 7).

Table 6

The Effects of Sodium Vanadate With and Without Insulin at Different Concentrations on Fatty Acid Synthase (FAS) mRNA in Rat Hepatocytes

condition	mRNA level as % of control
	Fatty Acid Synthase
control	100
insulin	162±13
Va 10μM	198±11
insulin + Va 10μM	247±13
Va 30μM	252±18
insulin + Va 30μM	273±25

Data are means ± SE; n=6/group

Total cellular RNA was harvested from culture as described under "Material and Methods". The RNA samples were blotted and hybridized to radiolabeled cDNA. 10 μg of RNA was loaded per lane. The control value (NA) is set at 1.0.

In Vivo Effects of Vanadate and Selenate on FAS mRNA Level

Because of the low molecular weight of vanadate and its similarity to phosphate (Simons, 1979; Crane *et al.*, 1981), suggest that vanadate easily permeates plasma membranes and the intestinal wall. This encouraged Heyliger *et al.* to administrate an oral treatment of vanadate to diabetic rats. Streptozotocin-induced diabetic rats

appeared to be the most suitable animal model. This therapy did not increase the levels of endogenous insulin. *In vivo*, selenate has been reported to lower and even normalize blood glucose concentrations in various animal models. But, the role and mechanism by which selenate acts is still unknown.

As Table 7 shows, the level of FAS mRNA in diabetic rats treated with insulin, vanadate and selenate increased about 1.1-fold, 1.9-fold and 2.1-fold respectively, when compared to the non-diabetic control.

Table 7

The Effects of Sodium Vanadate and Sodium Selenate on Glucose-6-Phosphate Dehydrogenase (G6PDH) and Fatty Acid Synthase (FAS) mRNA in Control and Streptozotocin-Induced Rats

condition	mRNA level as % of control	
	G6PDH	FAS
control (NA) [a]	100 [f]	100
diabetic (Dia) [b]	30±8	15±7
diabetic + insulin [c]	86±11	112±11
diabetic + vanadate [d]	74±7	192±16
diabetic + Selenate [e]	90±12	210±21

[a] Male, 150-200 gram, Sprague-Dawley rats were treated for at least two weeks with either nothing (control) or,
[b] rats were injected in the tail vein with 65 mg/kg streptozotocin
[c] 5 units insulin injected subcutaneous
[d] 0.5 mg/mL sodium vanadate in drinking water
[e] 15 μmole/kg sodium selenate injected I.P.
[f] the results are expressed as percent of non-diabetic control animals (each condition, n=4, $p<0.05$)

DISCUSSION

The purpose of this research was to investigate whether or not sodium vanadate and sodium selenate mimic insulin with regard to inducing the synthesis of enzymes in the metabolic pathways for fatty acid biosynthesis. Lately, the investigation on the effects of insulin-mimetics on cellular processes and regulation of gene expression has increased due to the possible implications in the design of future therapies for diabetic patients. In order to accomplish this work, the effect of these agents were examined in two insulin responsive systems: Isolated rat hepatocytes, as an *in vitro* or cell system, and streptozotocin-induced diabetic rats, as an *in vivo* or whole animal system. Since G6PDH and FAS both have important roles in fatty acid biosynthesis, our goal was to determine if these insulin-mimetics had an effect on the mRNA level of G6PDH or FAS. We also wanted to determine if this effect was comparable to insulin's induction of G6PDH and FAS mRNA in published studies.

Insulin has been shown to induce the mRNA levels of G6PDH in hepatocytes and this effect is postulated to occur at the level of transcription (Kletzien, 1986; Manos *et al*, 1991). In order to repeat these published results, we included in our experiments insulin at a concentration (16 pM) considered to be in excess of the amount needed for maximum G6PDH induction. As illustrated in figure 9, we were consistently able to demonstrate a significant insulin induction of G6PDH mRNA in isolated rat hepatocytes, of approximately 1.5-2.0 fold.

Fatty acid synthase mRNA level is also reported to be affected by the presence of insulin. In isolated rat hepatocytes, Spence and Pitot (1982) have shown that the effect of insulin on FAS induction is due to increases in specific mRNA levels. Additionally, the effects of insulin on FAS induction have also been demonstrated in

chick hepatocytes (Goodridge, 1986; Goodridge *et al*, 1989; Stapleton, 1990) and 3T3-L1 cells (Sul, 1988; Sul *et al*, 1993). Our study, as mentioned in the results, showed increases in fatty acid synthase mRNA of approximately 1.6-fold in isolated rat hepatocytes incubated with insulin (Table 3).

Sodium vanadate has been shown to produce many insulin-like effects in cultured cells. In isolated rat hepatocytes, these include increasing levels of fructose-2,6-bisphosphate, a glucose metabolite, as well as affecting glucose output, and increasing insulin binding and sensitivity (Miralpeix, 1989; Bruck et al, 1991; Erickson, 1992). Sodium vanadate also has been shown to stimulate glucose oxidation and transport in rat adipocytes (Tolman *et al*, 1979). As demonstrated (Table IV and VI), we were able to show that sodium vanadate induces the mRNA level for both G6PDH and FAS. Increases in the expression of both G6PDH and FAS mRNA resulting from sodium vanadate exposure are comparable to the increases we observed with insulin. This indicates that sodium vanadate acts in an insulin-like manner in increasing the level of G6PDH and FAS mRNA. Sodium vanadate has also been shown to influence the enzyme activities of both G6PDH and FAS in a similar manner (Berg and Wu *et al*, 1995). Insulin and sodium vanadate do not act in an additive manner, suggesting they could be acting on increasing the expression of these mRNA by a similar mechanism.

Sodium selenate has also been shown to exhibit insulin-like properties in several cell types. In adipocytes, sodium selenate increased glucose transport and tyrosyl phosphorylation (Ezaki, 1990). There has been little, if any, published work to indicate that sodium selenate affects any mRNA or enzyme activity levels. In rat hepatocytes culture, we have been able to show insulin-like properties of sodium selenate with respect to the expression of G6PDH and FAS mRNA (Table III and V). Both G6PDH and FAS mRNA levels increased approximately 2.5 and 3.4-fold.

Many investigations have been done to determine the influence of insulin on mRNA levels and enzyme activity of G6PDH and FAS *in vivo*. A study done by Kletzien *et al* (1989) correlates a high carbohydrate diet to increased levels of G6PDH mRNA in hepatic tissue. It has also been demonstrated in diabetic animals that hepatic levels of G6PDH mRNA and enzyme activity were decreased and treatment with insulin restored these levels to normal (Katsurada, 1990). In adipose tissue increases in G6PDH mRNA and enzyme activity were observed upon treatment with insulin (Katrouni, 1984; Carvalho, 1993). *In vivo* induction of FAS by insulin has also been shown. Goodridge (1986) reported that re-feeding fasted chicks increased levels of FAS mRNA in hepatic tissue. Sul (1989) demonstrated insulin effectively increases FAS mRNA levels in diabetic mouse livers.

Sodium vanadate has been reported to have a variety of insulin-mimetic properties in diabetic animal models. Some of these properties include normalizing plasma glucose, increasing hepatic glycogen levels, increasing insulin sensitivity, and affecting the mRNA level and activity of some enzymes (Shechter, 1990; Pugazhenthi and Khandelwal, 1990; Rosetti, 1990; Saxena, 1992; Sachor, 1992; Tolman *et al*, 1979). Sodium selenate, while not as extensively studied as sodium vanadate, also shows insulin-like properties *in vivo*. Sodium selenate normalizes plasma glucose levels, reduces food and water intake, and increases weight gain in streptozotocin-induced diabetic rats (McNeill, 1991). There is presently little or no information to suggest that sodium selenate affects the gene expression or activity of any enzymes.

In the *in vivo* experiments of this project, to simulate a system that was as "normal" metabolically as possible, used animals that were given food and water *ad libitum*. As shown in the results, we have been able to demonstrate in this study that sodium vanadate and sodium selenate mimic insulin with respect to regulate the mRNA level of G6PDH and FAS in the hepatic tissue of streptozotocin-induced diabetic rats.

Enzyme activity increases are similar to the increases of the respective genes mRNA levels (Berg and Wu *et al*, 1995) indicating that both agents may affect transcription and/or translation process.

More experiments are needed to provide insight into the mechanism of sodium vanadate and sodium selenate and to determine the full extent their action. Additionally, it should be determined if either mimetic acts like insulin with respect to interaction with other hormones, such as glucagon or steroid hormones. This could further illustrate the extent of insulin-like action demonstrated by sodium vanadate and sodium selenate.

In conclusion, this study has demonstrated that sodium vanadate and sodium selenate show insulin-mimetic properties by increasing the mRNA level of both glucose-6-phosphate dehydrogenase and fatty acid synthase in a manner similar to insulin in isolated rat hepatocytes. Furthermore, we were able to show the ability of these metals to affect the mRNA level of G6PDH and FAS in an insulin-like manner in streptozotocin-induced diabetic rats. Therefore, it can be concluded that sodium vanadate and sodium selenate are insulin-mimetic with respect to the regulation of two lipogenic genes.

CONCLUSIONS AND RECOMMENDATIONS

In this study we were able to: (a) demonstrate insulin-like effects of sodium selenate with respect to the mRNA of glucose-6-phosphate dehydrogenase and fatty acid synthase *in vitro*; (b) demonstrate insulin-like effects of sodium vanadate with respect to the mRNA of glucose-6-phosphate dehydrogenase and fatty acid synthase *in vitro*; (c) demonstrate insulin-like effects of sodium selenate with respect to the mRNA of glucose-6-phosphate dehydrogenase and fatty acid synthase *in vivo*; and (d) demonstrate insulin-like effects of sodium vanadate with respect to the mRNA of glucose-6-phosphate dehydrogenase and fatty acid synthase *in vivo*.

The following are recommended for further study: (a) determine if sodium vanadate and sodium selenate interact with any other hormones on mRNA levels; (b) attempt to determine the level of toxicity of sodium vanadate and sodium selenate; and (c) examine other potential insulin-mimetics such as zinc and chromium.

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