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The Effect of Insulin Mimetics on Enzymes of Fatty Acid Biosynthesis

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THE EFFECT OF INSULIN MIMETICS ON ENZYMES OF FATTY ACID BIOSYNTHESIS

by

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Fatty acid biosynthesis, an insulin regulated pathway, is the process of producing fatty acid chains from 2 and 3 carbon units. Two enzymes in this pathway that insulin affects are glucose-6-phosphate dehydrogenase (G6PDH) and fatty acid synthase (FAS). Sodium vanadate and sodium selenate have demonstrated insulin-like effects in several tissue and cell types. These mimetic effects include increasing glucose transport, regulating glycolytic enzymes, normalizing plasma glucose levels and activities of insulin regulated enzymes in diabetic rats. Our goal was to examine effects of sodium vanadate and sodium selenate on fatty acid biosynthesis in two insulin responsive systems: Isolated rat hepatocytes and streptozotocin-induced diabetic rats. We wanted to determine if these agents acted in an insulin-like manner with respect to inducing the enzyme activity of G6PDH and FAS.

In isolated rat hepatocytes incubated in a chemically defined medium, addition of sodium vanadate (10 μM) or sodium selenate (20 μM) showed maximal increases in the activity of both G6PDH and FAS which were comparable to the induction by insulin that we obtained. In stretozotocin-induced diabetic rats, sodium vanadate and sodium selenate increased weight gain, lowered food and water consumption, lowered plasma glucose levels and increased G6PDH and FAS activity in the liver. These results again were comparable to those of insulin. Therefore, these results show that sodium vanadate and sodium selenate mimic insulin with respect to regulation of metabolic parameters and enzyme activities of G6PDH and FAS in diabetic rats.

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INTRODUCTION

Background Information

Metabolism

Metabolism is the summation of chemical reactions which biological systems use to maintain viability. These reactions provide energy, allow for storage, provide means for by-product disposal, and use chemicals provided as building blocks. Some of the metabolic processes include glycolysis, gluconeogenesis, fatty acid oxidation and biosynthesis, amino acid metabolism, and nucleotide metabolism. Many of these processes are controlled by the action of hormones released by various organs. This release of these hormones is tightly regulated and often only very small amounts are adequate for inducing a significant effect. One of these hormones is the peptide insulin which regulates several metabolic pathways including fatty acid biosynthesis.

Fatty Acid Biosynthesis

Fatty acid biosynthesis, the process of making non-polar carbon chains called fatty acids occur in cellular events including membrane synthesis and repair, fat storage, and synthesis of precursors for other macromolecules. Once consumed, food is broken down to fats, proteins and carbohydrates. These molecules are further broken down to fatty acids, glycerol, amino acids, and sugar; and then to two carbon acetyl units. In fatty acid biosynthesis, these acetyl groups are transported to the cell cytosol and attached to a large carrier molecule called Coenzyme A. Some of the acetyl-CoA units are converted to malonyl-CoA, by the enzyme acetyl-CoA carboxylase (Figure 1).

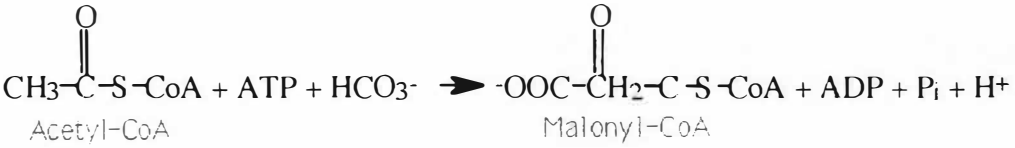


Figure 1. Reaction of Acetyl-CoA Carboxylase.

Acetyl-CoA and malonyl-CoA are used to produce a 16 carbon fatty acid called palmitate. In mammalian cells, this reaction is catalyzed by the enzyme fatty acid synthase or FAS (Figure 2).

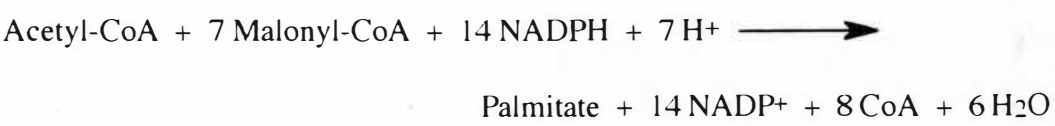


Figure 2. Reaction of Fatty Acid Synthase.

The reaction is dependent on levels of NADPH, which is oxidized in the reaction (Febregat, 1985). The levels of NADPH are primarily controlled by the pentose phosphate pathway, of which, glucose-6-phosphate dehydrogenase is a key enzyme.

Glucose-6-phosphate Dehydrogenase and Fatty Acid Synthase

The pentose phosphate pathway is essential for providing sugars for use in other pathways such as ribose-5-phosphate for nucleotide synthesis and fructose-6-phosphate and glycerol-3-phosphate for energy generation. The pentose phosphate pathway also provides the reducing equivalent, nicotinamide adenine diphosphate (NADPH). This compound is used as an electron donor/acceptor for many biosynthetic reactions. One of the key enzymes in the pentose phosphate pathway is

glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49). G6PDH converts glucose-6-phosphate to pentose-5-phosphate producing NADPH (Figure 3).

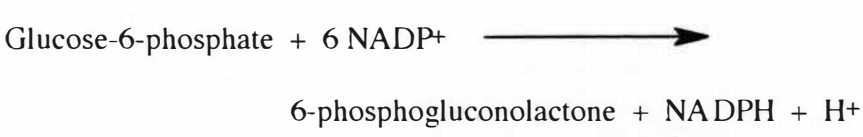


Figure 3. Reaction of Glucose-6-phosphate Dehydrogenase.

G6PDH is expressed in many tissue and is very prevalent in the liver and expression is regulated by insulin (Manos, 1991).

Fatty acid synthase (FAS; 2.3.1.85) is the key enzyme in fatty acid biosynthesis. It is a dimer of two identical subunits with seven catalytic functions in each subunit. It is present in many tissues but is primarily expressed in adipose and liver tissue and expression is regulated by insulin in both tissue types (Alberts and Greenspan, 1984).

The Physiological Role of Insulin

Insulin, a hormone which is important in the regulation of many digestive and storage processes, plays a significant role in the regulation of fatty acid biosynthesis. Insulin is a small peptide which is synthesized in a precursor form in the Beta cells in the Islets of Lagerhorn, processed, and then secreted into the blood. The secretion of insulin is tightly controlled and is dependent on plasma glucose levels. As glucose levels go up, such as after eating, the levels of insulin secretion go up. As glucose levels go down, such as after fasting, levels of insulin go down.

While not completely elucidated, the mechanism behind insulin action is initiated via a specific receptor found on a variety of cell surfaces. When insulin binds to its receptor, the receptor becomes phosphorylated resulting in a variety of events.

These events include: control of transport and metabolism of glucose and control of fatty acid metabolism. This control is achieved by regulating the activity and expression of key proteins (Kahn et al). Insulin binding results in increased synthesis of fatty acid synthase while providing increased amounts of the co-factor NADPH via glucose-6-phosphate dehydrogenase. This hormonal regulation becomes especially important when production or utilization of insulin is hampered as is the case in the disease, diabetes mellitus.

Diabetes

Diabetes mellitus is a disease state in which the production and/or the utilization of insulin is impaired. There are two basic types: Insulin-dependent or IDDM and non-insulin-dependent or NIDDM (Table 1).

Table 1

Comparison of IDDM and NIDDM

Characteristic	IDDM	NIDDM
Level of insulin secretion	None or almost none	May be normal or exceed normal
Typical age of onset	Childhood	Adulthood
Percentage of diabetics	10-20%	80-90%
Basic defect	Destruction of β cells	Reduced sensitivity of insulin's target cells
Associated with obesity?	No	Usually
Genetic and environmental factors inportant in precipitating overt disease?	Yes	Yes

Table 1– Continued

Characteristics	IDDM	NIDDM
Speed of development of symptoms	Rapid	Slow
Development of ketosis	Common if untreated	Rare
Treatment	Insulin injections; dietary management	Dietary control and weight reduction; occasionally oral hypoglycemic drugs

IDDM is typified by the lack of insulin production and very often occurs early in life. NIDDM is characterized as a state in which the insulin produced is not properly utilized. NIDDM usually occurs later in life and often the exact cause is difficult to determine (Sherwood, 1993). Recently, a number of heavy metals have been proposed for potential use in insulin replacement therapy. These metals include vanadium and selenium.

Physiological Role of Vanadium and Selenium

Vanadium has a limited known physiological role. It has been shown to substitute for molybdenum in nitrogenase complexes in same bacteria (Lehninger, 1993). It is reduced in rat liver microsomes although the protein involved and the purpose of this reaction is unknown (Shi and Dalal, 1992). Vanadium, however, has yet to be shown to be used with any regularity in mammalian systems.

Selenium is a metal which has a physiological role in many oxidative and reductive reactions. When it is associated with protein the complexes are called selenoproteins. Selenium is incorporated into most selenoproteins through an amino acid derivative, selenocysteine (Figure 4).

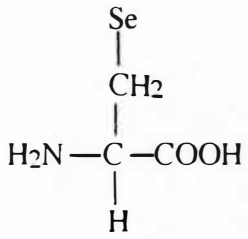


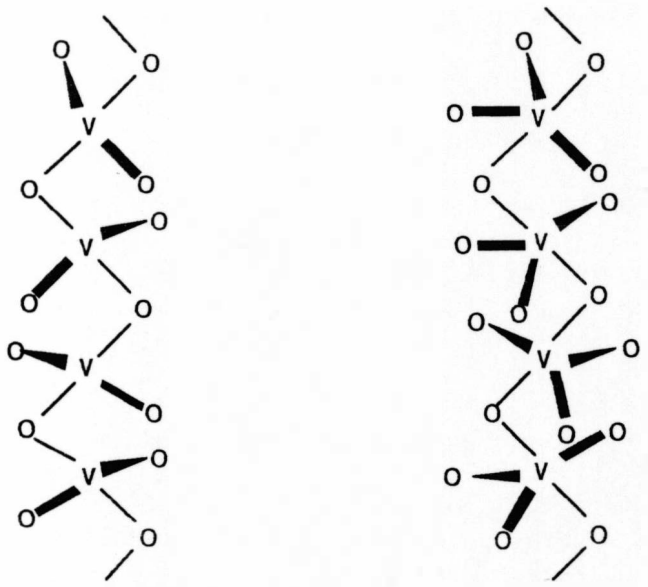
Figure 4. Structure of Selenocysteine.

Some examples of enzymatic selenoproteins include: Formate dehydrogenase, glycine reductase selenoprotein a, and mammalian glutathione peroxidase. The function of these selenoenzymes include energy utilization and chemical oxidation and reduction (Stadtman, 1990).

REVIEW OF LITERATURE

The Insulin-like Action of Vanadate: *In Vitro*

A majority of literature regarding the insulin-like effects of various metals has focused on the vanadium compound, vanadate. There are two different forms that have been primarily used: Orthovanadate and Metavanadate.



Metavanadate Orthovanadate

Figure 5. Structures of Ortho and Meta Vanadate.

These inorganic species form polymers, the size of which are dependent on pH. At pH 2, both vanadates are single ion. As pH increases, larger molecules form becoming as large as 10 vanadium molecules at pH 8.

Vanadate has been examined in a variety of physiological roles and in many

different cell types. Vanadate was first shown to mimic insulin with respect to glucose metabolism. Similar to insulin, vanadate stimulates glucose oxidation and transport in adipocytes. It also stimulates glycogen synthesis in the liver and diaphragm, and inhibits hepatic gluconeogenesis and intestinal glucose transport (Tolman et al, 1979). Vanadate was also shown to mimic insulin in isolated rat hepatocytes by increasing levels of fructose-2,6-bisphosphate, a key regulatory substance in glucose metabolism (Miralpeix, 1989). Vanadate effects glucose output in isolated hepatocytes (Bruck et al, 1991). It also increases insulin binding and sensitivity (Ericksson, 1992). Other vanadate derivatives, such as vanadyl, pervanadate, and Bis(maltolate)-oxovanadium(IV), exhibit insulin-like effects as well (McNeill et al, 1991; Fantus et al, 1989; and Sakurai et al, 1990). 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).

The Insulin-like Action of Vanadate: *In Vivo*

Although, the use of cell culture is an important tool, it can not always yield enough information needed when looking at whole body systems. Use of animal models are therefore also important in determining metabolic effects. Several animal models have been developed to study diabetes. These include chemical, surgical, and genetic models. Chemical models such as, streptozotocin-induced and alloxon-induced diabetic rats, affect beta-cell function in the pancreas. Surgical models, such as pancreatectomized rats eliminate insulin production. Genetic models, such as fa/fa rats and ob/ob mice, can have either impaired insulin production or poor insulin utilization. Vanadate seems to be an effective insulin-mimetic in many of these anima

models.

In streptozotocin-induced diabetic rats, vanadate treatment normalizes plasma glucose (Shechter, 1990; and Pugazhenthi, 1990). It also increases hepatic glycogen levels and raises levels of fructose-2,6-bisphosphate in liver and cardiac tissue (Pugazhenthi and Khandelwal, 1990; and Sachor, 1992). Vanadate also effects some enzyme activities of liver and heart in the streptozotocin model. These include: glycogen synthase a, phosphorylase, phosphorylase kinase, malic enzyme, and glucose-6-phosphate dehydrogenase, hexokinase, pyruvate kinase, and phosphofructokinase I (Saxena, 1992; Sachor, 1992; and Tolman et al, 1979). In alloxan-induced diabetic rats, vanadate was shown to normalize glucose levels. It also normalizes hexokinase, pyruvate kinase, and malic enzyme in both the liver and kidney of alloxan-induced animals (Saxena, 1992).

In the surgical and genetic animals models, vanadate has been demonstrated to also have actions similar to insulin. In pancreatectomized rats, vanadate normalizes glucose levels, increases insulin sensitivity, and normalizes glycogenic rates (Rossetti, 1990). In obese fa/fa rats, an insulin resistant strain, vanadate lowered glucose levels and increased insulin sensitivity (Brichard, 1990). In a diabetic mouse strain (ob/ob), vanadate increased glucose tolerance, glucose clearance, and glycogen stores (Brichard et al,1992).

The Insulin-like Action of Selenate: *In Vitro* and *In Vivo*

Selenium in the form of selenate, a selenium oxide ion, mimics insulin in some metabolic processes. Selenate effects glucose transport, translocation of glucose transporters, and stimulates both cAMP phospho-diesterase and ribosomal S6 phosphorylation in an insulin-like manner in rat adipocytes while also increasing

tyrosyl phosphorylation (Ezaki, 1990). Selenate has also been shown stimulate epidermal growth factor (EGF) receptor phosphorylation and EGF-stimulated phosphorylation in A431 cells, and insulin-stimulated tyrosine phosphorylation in NIH 3T3 HIR3.5 cells (Pillay and Makgoba, 1992). Selenate also mimics insulin in a diabetic animal model. In streptozotocin-induced diabetic rats, selenate normalized plasma glucose levels, reduced food and water intake, and increased weight gain (McNeill, 1991).

Objective of the Study

There have been many studies about the insulin-like effects of vanadate and selenate. Since it has 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 the lipogenic enzymes, G6PDH and FAS. We pursued the following objectives: (a) to determine the effects of vanadate and selenate on the activities of G6PDH and FAS in isolated hepatocytes, and (b) to determine the effects of vanadate and selenate on the activities of G6PDH and FAS in streptozotocin-induced diabetic rats.

Significance of the Study

Insulin performs an important 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 and to determine their mechanism of action. Understanding how these compounds regulate metabolism may provide a greater understanding of hormonal regulation.

MATERIALS AND METHODS

Hepatocyte Isolation and Maintenance of Cells in Culture

Male, Sprague-Dawley rats were obtained from Harlan Sprague Dawley (Kalamazoo, MI). Hepatocytes were isolated using collagenase and hyaluronidase purfusion (Elliget and Kolaja, 1983) and plated on rat collagen coated 60mm Falcon plates in Waymouths MB 752/1 (Gibco BRL, Grand Island, NY) media containing 5% bovine serum albumin (BSA). Cells were then incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2. After 3-4 hours, the media was changed to one without BSA and hormones and (or) metals were added.

Cell Harvest and Enzyme Assays

Cells were incubated for an additional 48 hours. At that time the media is aspirated and cells are scraped in KED buffer (0.1 M potassium phosphate buffer (pH 7.0), 3 mM ethyl-diamine-tetraacetic acid, and 1 mM dithiothreatol) and homogenized 20 times with a Dounce homogenizer. The extracts are then centifuged for 10 minutes at 4°C in a microfuge and the supernatent is saved and used in enzyme assays. Glucose-6-phosphate dehydrogenase and fatty acid synthase are assayed spectrophotometrically by measuring the rate of the change of NADPH absorbance at 340 nm and 25°C. The enzyme activity of glucose-6-phosphate dehydrogenase was assayed in the following method: A 1 ml cuvette containing 867 μl of 0.37 mM $NADP^+$, 6.7 mM MgCl2, and 86.3 mM triethanolamine buffer was mixed with 100 μl of cell supernatent. The reaction was initiated with 33 μl of 1.2 mM glucose-6-phosphate and the change in absorbance at 340 nm was measured for 3 minutes

(Beutler, 1993). The enzyme activity of fatty acid synthase was assayed in the following method: A 1 ml cuvette containing 790 μl of 25 μM acetyl-CoA, 100 μM NADPH in 0.1 M potassium phosphate buffer, 3 mM ethylene-diamine-tetraacetic acid, and 1 mM dithiothreatol was mixed with a 200 μl of cell supernatant. The reaction was initiated with 10 μl of 10 mM malonyl-CoA and the change in absorbance at 340 nm was measured for 3 minutes (Nepokroeff, 1975). Protein concentrations were determined using the Lowry method (Lowry, 1951). Specific activities of the enzymes were determined by calculating change in absorbance per μg protein multiplied by the extinction coefficient of the reaction.

Animals and Protocol Design for *In Vivo* Experiments

Male, Sprague Dawley rats, 150-200 grams, were purchased from Harlan Sprague Dawley (Kalamazoo, MI). Animals 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.

Tissue Isolation and Enzyme Assays

After treatment, animals were anesthetized with 50 mg/kg pentobarbitol and liver was excised. The tissue was then minced and homogenized. The homogenate was then centifuged for 30 minutes at 20,000 xg and 4°C. The supernatent was saved and used for determining enzyme activities. Enzyme activities and protein concentrations are determined in the same manner as isolated hepatocytes.

RESULTS

Effects of Sodium Vanadate and Sodium Selenate *In Vitro*

In isolated rat hepatocytes incubated in a chemically defined medium, the effects of sodium vanadate and sodium selenate were compared to the effects of insulin on the activity of both glucose-6-phosphate dehydrogenase (G6PDH) and fatty acid synthase (FAS). In our system, insulin showed a 2.5 fold increase in G6PDH activity which is comparable to published results (Nakamura, 1982; Spence, 1982). Sodium vanadate stimulated the enzyme activity of G6PDH in a dose dependent manner (Figure 6) with 5 μM sodium vanadate increasing activity slightly higher than the basal condition and 10 μM sodium vanadate maximally increasing activity 2.3 fold. The increase observed with the addition of 10 μM sodium vanadate was shown to be statistically significant compared to basal level. The addition of insulin and sodium vanadate together was comparable to either insulin or vanadate alone.

Sodium vanadate also stimulated FAS activity in an insulin-like manner in primary rat hepatocytes in culture (Figure 7). Insulin produced nearly a 1.8-fold increase in enzyme activity which is similar to results obtained in other studies (Nakamura, 1982; Spence, 1982). Ten μM sodium vanadate maximally stimulated FAS by 1.6-fold which was statistically different from the basal condition. Again, this increase appears to be concentration dependent as a 5 μM addition of sodium vanadate caused only a slight increase in activity. The addition of both insulin and sodium vanadate together produced only the 2-fold increase which was not significantly different than either insulin or sodium vanadate alone.

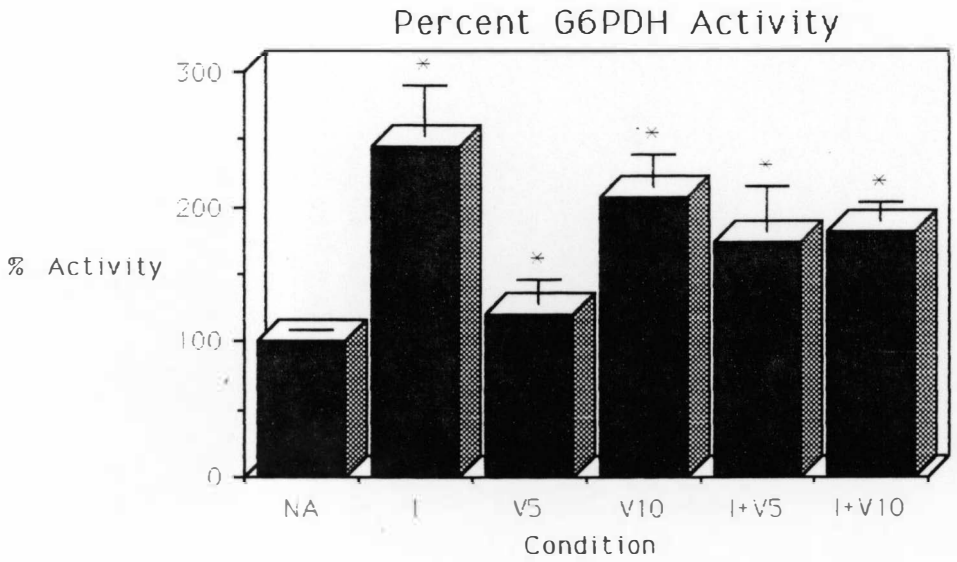


Figure 6. The Effect of Sodium Vanadate on Glucose-6-phosphate Dehydrogenase Activity as a Function of Concentration.

Hepatocytes were incubated for 48 h in the presence of either no addition 16 pM Insulin (I), 5, 10, 20 μM sodium vanadate (V), I + 5, or I + 10 μM Van. After harvest, the activity for glucose-6-phosphate dehydrogenase (G6PDH) was determined. The results are expressed as a percentage of the control. Starred conditions are significantly different from control ($p > 0.05$, $n = 6$).

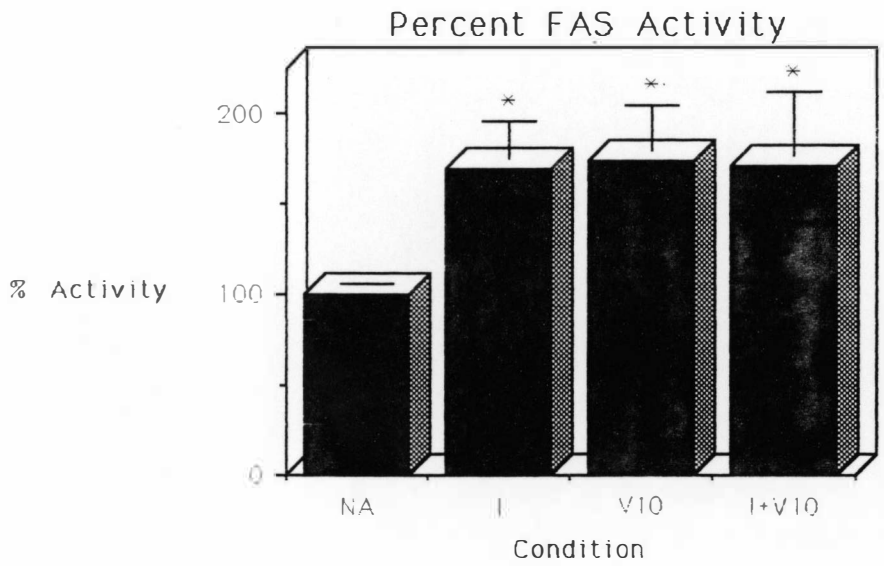


Figure 7. The Effect of Sodium Vanadate on Fatty Acid Synthase Enzyme Activity.

Hepatocytes were incubated for 48 h in the presence of either no addition 16 pM Insulin (I), 10 μM sodium vanadate (V), or I + 10 μM V. After harvest, the activity for fatty acid synthase (FAS) was determined. The results are expressed as a percentage of the control. Starred conditions are significantly different from control ($p > 0.05$, $n = 6$).

Sodium selenate also increased the level of glucose-6-phosphate dehydrogenase (G6PDH) activity comparable to those of insulin (Figure 8). Treatment of cells with insulin shows approximately a 2.5-fold increase in enzyme activity which is within levels demonstrated in other studies (Nakamura, 1982; Spence. 1982). Sodium selenate stimulated G6PDH activity in a dose dependent manner with both the 10 μM and 20 μM concentrations showing significant increases ($p > 0.05$; $n = 6$). Peak stimulation of 2.7-fold was seen with the addition of 20 μM sodium selenate and was not significantly different than the maximum insulin stimulation. Additionally, the exposure of both insulin and sodium selenate together to the cells was not significantly different when compared with either the addition of insulin or sodium selenate alone.

Sodium selenate also showed similar insulin-like effects with respect to the activity of fatty acid synthase (Figure 9). Treatment of the cells with insulin increased enzyme activity comparable to reported results of about 1.7-fold and the increase in activity with 20 μM sodium selenate was also approximately 1.8-fold. The co-addition of insulin and 20 μM sodium selenate did not increase activity higher than either alone. Table 2 summarizes the enzyme activities of all treatments for both enzymes. Statistical analysis was done using Student's t test. These data show overall insulin-like effects of sodium vanadate and sodium selenate at the enzyme activity level for both G6PDH and FAS.

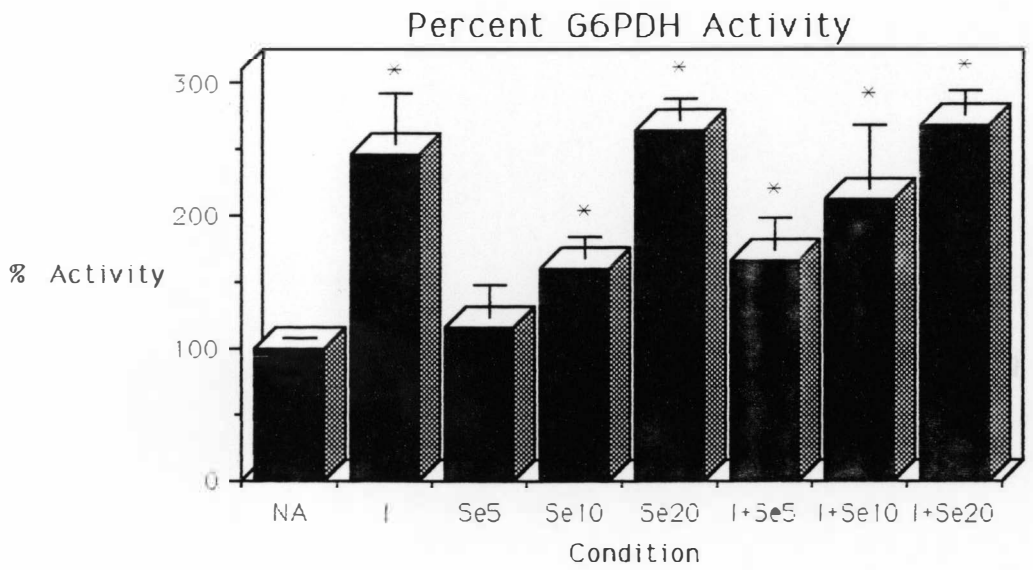


Figure 8. The Effect of Sodium Selenate on Glucose-6-phosphate Dehydrogenase Activity as a Function of Concentration.

Hepatocytes were incubated for 48 h in the presence of either no addition, 16 pM Insulin (I), 5, 10, 20 μM sodium selenate (Se), I + 5, I + 10, or I + 20 μM Se. After harvest, the activity for glucose-6-phosphate dehydrogenase was determined. The results are expressed as a percentage of the control. Starred conditions are significantly different from control ($p > 0.05$, $n = 6$).

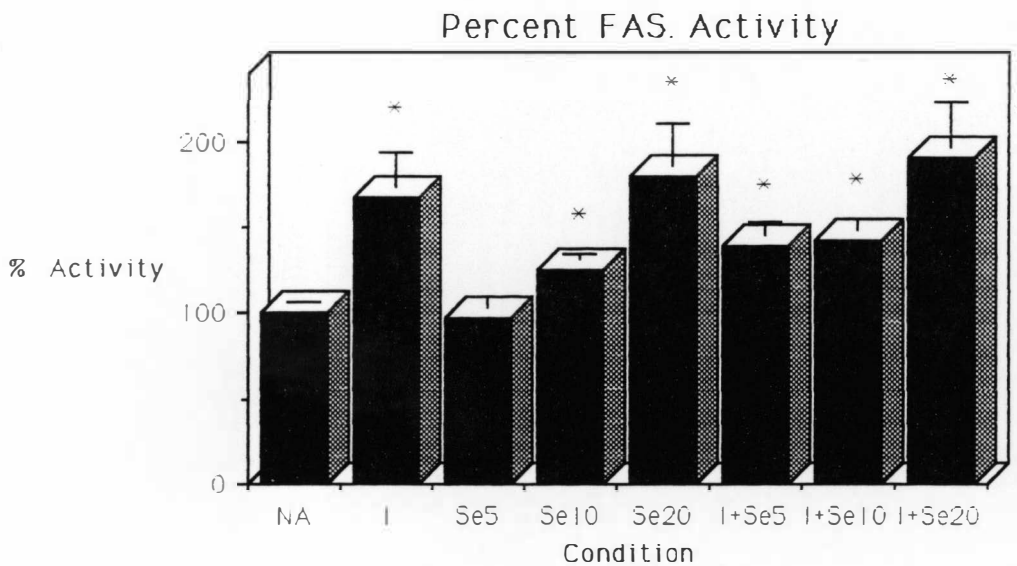


Figure 9. The Effect of Sodium Selenate on Fatty Acid Synthase Activity as a Function of Concentration.

Hepatocytes were incubated for 48 h in the presence of either no addition, 16 pM Insulin (I), 5, 10, 20 μM sodium selenate (Se), I + 5, I + 10, or I + 20 μM Se. After harvest, the activity for fatty acid synthase was determined. The results are expressed as a percentage of the control. Starred conditions are significantly different from control ($p > 0.05$, $n = 6$).

Table 2

Summation of Enzyme Activities

Glucose-6-phosphate Dehydrogenase

Condition	Activity (Units/μg protein x 10^{-5})
None	5.57±0.69
Insulin	13.6±1.34
Sodium vanadate - 5 μM	7.04±0.55
Sodium vanadate - 10 μM	11.5±0.97
Ins. + Sod. van. - 5 μM	12.4±3.79
Ins. + Sod. van. - 5 μM	12.9±1.40
Sodium selenate - 5 μM	6.46±0.67
Sodium selenate - 10 μM	8.91±1.77
Sodium selenate - 20 μM	14.6±1.08
Ins. + Sod. sel. - 5 μM	10.1±1.90
Ins. + Sod. sel. - 10 μM	11.7±2.37
Ins. + Sod. sel. - 20 μM	14.9±1.24

Fatty Acid Synthase

Condition	Activity (Units/μg protein x 10^{-6})
None	1.60±0.50
Insulin	2.70±0.80
Sodium vanadate - 10 μM	2.78±0.82
Ins. + Sod. van. - 10 μM	2.73±1.40
Sodium selenate - 5 μM	1.55±0.94
Sodium selenate - 10 μM	2.00±0.63

Table 2 Continued

Condition	Activity (Units/μg protein x 10^{-6})
Sodium selenate - 20 μM	2.88±0.82
Ins. + Sod. sel. - 5 μM	2.24±0.84
Ins. + Sod. sel. - 10 μM	2.32±0.78
Ins. + Sod. sel. - 20 μM	3.04±1.03

Effect of Sodium Vanadate and Sodium Selenate *In Vivo*

Effects of sodium vanadate and sodium selenate were compared to that of insulin in the streptozotocin-induced diabetic rat model. A streptozotocin-induced diabetic rat colony was produced by injecting 150-200 gram male Sprague-Dawley rats with 65 mg/kg streptozotocin in 0.1M citrate buffered saline. The physical condition of the animals was monitored by observing weight change, food consumption, and water consumption (Table 3). Non-diabetic controls gained weight in a consistent linear manner with increases in weight of approximately 1.5 -fold in 2-3 weeks. Streptozotocin treatment resulted in a slowed weight gain with some rats maintaining or even losing weight. Animals treated with insulin, sodium vanadate, and sodium selenate showed modest increases in average weight gain which were consistent with trends in previous reports (Pugazhenthi and Khandelwal, 1990; McNeill et al, 1992). All animals which were given insulin, sodium vanadate, or sodium selenate appeared to show improvement in weight gain over the diabetic animal.

Food intake in the non-diabetic animal did not significantly change from week to week. In the diabetic animal, however, consumption approximately doubled when compared with the non-diabetic animals. Food consumption lowered to levels

Table 3

Metabolic Parameters of Streptozotocin-induced diabetic rats

	Week 1	Week 2	Week 3
Non-diabetic			
Weight (g)	238±37	318±29	383±26
Food Intake (g)	24±1	31±5	25±1
Water Intake (ml)	135±20	102±20	143±62
Diabetic			
Weight (g)	231±37	264±16	280±10
Food Intake (g)	34±4	37±8	49±1
Water Intake (ml)	132±55	195±39	293±19
Ins treated diabetic			
Weight (g)	244±8	252±6	264±23
Food Intake (g)	28±6	40±4	27±5
Water Intake (ml)	110±14	210±4	150±7
Van treated diabetic			
Weight (g)	207±3	256±8	275±9
Food Intake (g)	26±1	43±4	23±3
Water Intake (ml)	95±14	197±34	79±9
Se treated diabetic			
Weight (g)	193±6	257±10	292±15
Food Intake (g)	30±3	36±3	23±2
Water Intake (ml)	92±10	179±29	73±12

which were comparable to non-diabetic animals after the diabetic animals were given either insulin, sodium vanadate, or sodium selenate. All treatments were equally effective in lowering food intake. Water consumption also stayed at consistant levels in non-diabetic animals with diabetic animals approximately doubling intake. Intake continued to increase throughout the duration of the experiment. Treating animals with insulin, sodium vanadate, or sodium selenate resulted in decreases in water intake which were significantly different than the diabetic control animals. By the third week of treatment, levels of water intake had dropped to levels comparable to the non-diabetic control animals. The Students *t* test

was used to determine statistical significance.

The diabetic state of the animals and the effectiveness of treatment was determined by monitoring glucose levels (Table 4). Non-diabetic control animals had glucose levels of between 100 to 160 mg/dL. Non-diabetic control animals given insulin, sodium vanadate, or sodium selenate did not significantly differ from non-diabetic controls. Animals were not considered diabetic unless glucose levels were at least 450 mg/dL and typically, diabetic subjects had glucose levels ranging between 450-700 mg/dL. Glucose levels usually continued to increase throughout the experiments. All diabetic groups which were given insulin, sodium vanadate, or sodium selenate showed a significant decrease in glucose levels. Animals given sodium vanadate and sodium selenate were not significantly different than those given insulin with all groups showing substantial decreases in each week of the experiment.

Table 4

Glucose Levels of Streptozotocin-induced Diabetic Rats

	Plasma Glucose (mg/dL)			
	Day 0	Day 7	Day 14	Day 21
Control				
Untreated	125±22	140±23	129±5	--
Insulin-treated	133±3	137±6	--	--
Selenate-treated	127±2	--	103±9	--
Vanadate-treated	193±22	144±10	120±13	164±13
Strept.-induced diabetic				
Untreated	160±13	432±13	507±78	602±51
Insulin-treated	156±44	600±82	320±68	--
Selenate-treated	169±1	507±41	411±47	383±44
Vanadate-treated	110±3	487±22	422±57	289±43

Sodium vanadate has been shown to reduce glucose levels in many

diabetic rat models including streptozotocin-induced diabetic rats, alloxan-induced diabetic rats, pancreatectomized rats, and obese fa/fa rats (Shechter, 1990; Pugazhenthi, 1991; Brichard, 1990; Saxena, 1992; and Rossetti, 1990). Sodium selenate, however, has only been shown to reduce glucose levels in streptozotocin-induced diabetic rats (McNeill, 1991). The results of our study are consistent with the trends previously reported. Glucose levels in our study were lowered significantly in all treatment groups.

The enzyme activities of G6PDH and FAS were measured by assaying whole liver homogenates after cellular debris was removed. Non-diabetic controls exhibited glucose-6-phosphate dehydrogenase activity of approximately 16 units x $10^{-5}/\mu g$ protein, was designated as 100% in Figure 10, and fatty acid synthase activity of approximately 27 units x $10^{-6}/\mu g$ protein which is designated as 100% in Figure 11. The effect of insulin, sodium vanadate, and sodium selenate on G6PDH (Figure 10) and FAS (Figure 11) was examined in normal rats. Non-diabetic animals were treated with insulin, sodium vanadate, or sodium selenate exhibited no change in enzyme activities. There was no significant difference in activity between the non -treated rats and the treated control rats.

Diabetic animals treated with insulin, sodium vanadate, and sodium selenate produced an effect on G6PDH and FAS activity in streptozotocin-induced diabetic rats. Diabetic control animals exhibit a markedly lower glucose-6-phosphate dehydrogenase activity approximately 6.6-fold (Figure 12) and fatty acid synthase activity approximately 3-fold (Figure 13). Sodium selenate, sodium vanadate, and insulin treatment increased activities significantly higher than the diabetic group, 3.4 to 3.7-fold for G6PDH and 1.5 to 2.7-fold for FAS. The effects between treatment groups were not significantly different from each other (Figures 12, 13). The

increases observed in enzyme activities, however, were not as high as non-diabetic controls. Table 5 is a summation of *in vivo* enzyme activities.

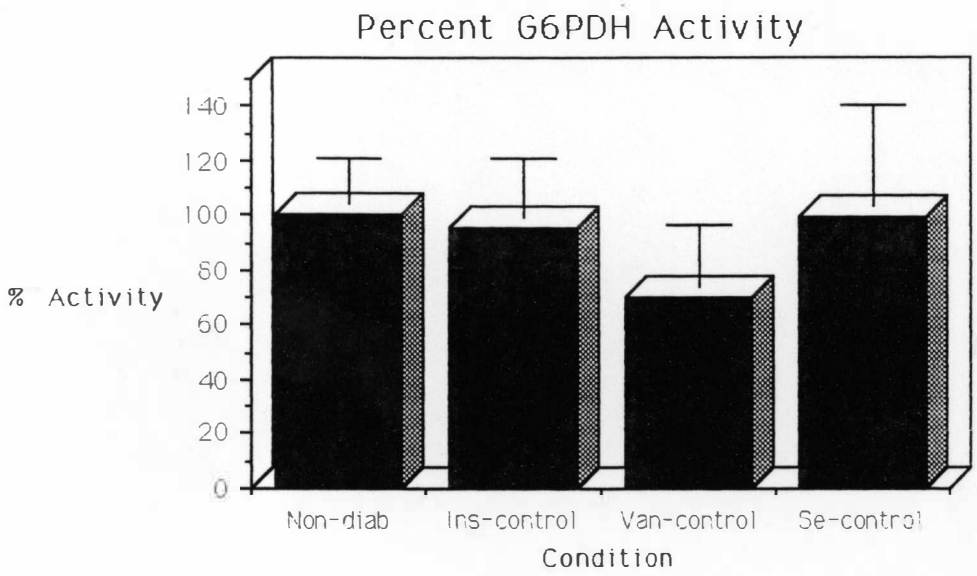


Figure 10. The Effect of Sodium Vanadate and Sodium Selenate on Glucose-6-phosphate Dehydrogenase Activity in Normal Rats.

Male, 150-200 gram, Sprague-Dawley rats were treated for at least two weeks with either nothing (Non-diab), 5 Units insulin (Ins) injected subcutaneous, 0.5 mg/ml sodium vanadate (Van) in drinking water, or 15 μmole/kg sodium selenate (Se) injected I.P. Animals were then euthanized and the activity for glucose-6-phosphate dehydrogenase (G6PDH) was determined from the supernatent of liver homogenates. The results are expressed as percent of non-diabetic control animals ($n = 3$).

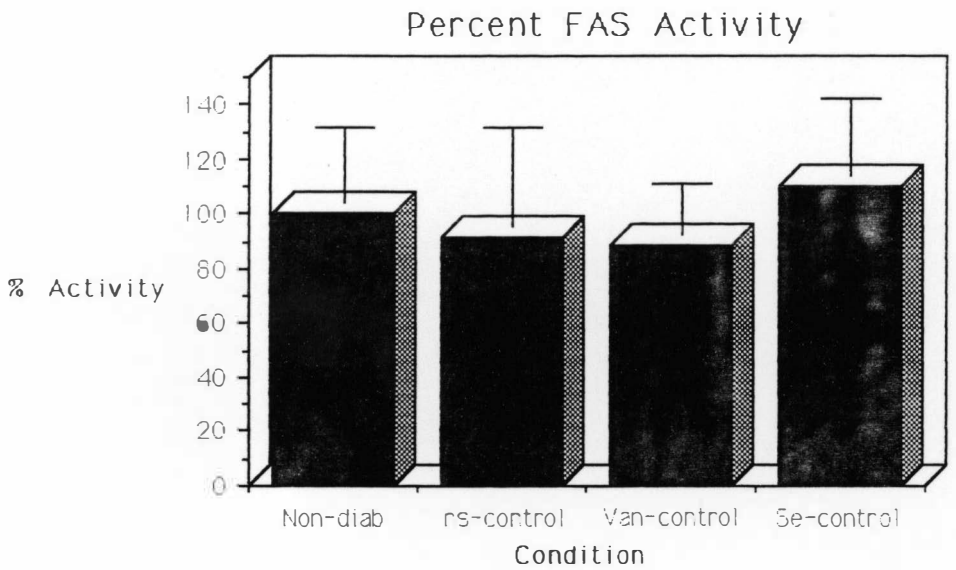


Figure 11. The Effect of Sodium Vanadate and Sodium Selenate on Fatty Acid Synthase Activity in Normal Rats.

Male, 150-200 gram, Sprague-Dawley rats were treated for at least two weeks with either nothing (Non-diab), 5 Units insulin (Ins) injected subcutaneous, 0.5 mg/ml sodium vanadate (Van) in drinking water, or 15 μmole/kg sodium selenate (Se) injected I.P. Animals were then euthanized and the activity for fatty acid synthase (FAS) was determined from the supernatent of liver homogenates. The results are expressed as percent of non-diabetic control animals ($n = 3$).

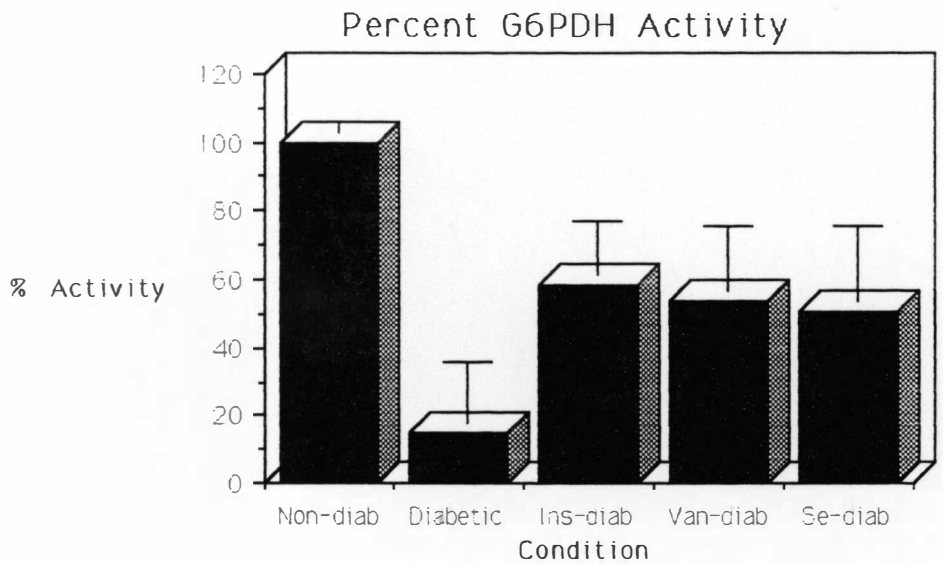


Figure 12. The Effect of Sodium Vanadate and Sodium Selenate on Glucose-6-phosphate Dehydrogenase in Streptozotocin-Induced Diabetic Rats.

Male, 150-200 gram, Sprague-Dawley rats were treated for at least two weeks with either nothing, 5 Units insulin (Ins) injected subcutaneous, 0.5 mg/ml sodium vanadate (Van) in drinking water, or 15 μmole/kg sodium selenate (Se) injected I.P. Animals were then euthanized and the activity for glucose-6-phosphate dehydrogenase (G6PDH) was determined from the supernatent of liver homogenates. The results are expressed as percent of non-diabetic control animals ($n = 4$).

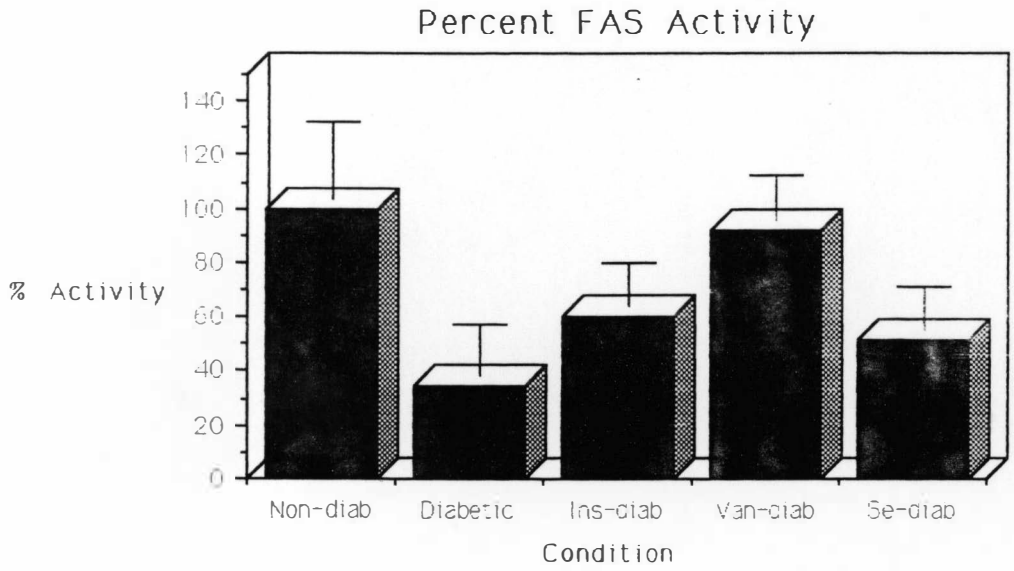


Figure 13. The Effect of Sodium Vanadate and Sodium Selenate on Fatty Acid Synthase in Streptozotocin-Induced Diabetic Rats.

Male, 150-200 gram, Sprague-Dawley rats were treated for at least two weeks with either nothing, 5 Units insulin (Ins) injected subcutaneous, 0.5 mg/ml sodium vanadate (Van) in drinking water, or 15 μmole/kg sodium selenate (Se) injected I.P. Animals were then euthanized and the activity for fatty acid synthase (FAS) was determined from the supernatent of liver homogenates. The results are expressed as percent of non-diabetic control animals ($n = 4$).

Table 5

Summation of Enzyme Activities

Glucose-6-phosphate Dehydrogenase	
Condition	Activity (Units/μg protein x 10^{-5})
Control	16.0±0.43
Diabetic	2.38±0.18
Diabetic + Insulin	9.34±1.43
Diabetic + Vanadate	8.64±1.59
Diabetic + Selenate	8.14±1.78
Fatty Acid Synthase	
Condition	Activity (Units/μg protein x 10^{-6})
Control	27.0±7.61
Diabetic	9.21±0.87
Diabetic + Insulin	16.1±2.64
Diabetic + Vanadate	24.9±4.13
Diabetic + Selenate	15.3±2.33

DISCUSSION

The purpose of this study was to examine the hypothesis that sodium vanadate and sodium selenate mimic insulin with regard to inducing the metabolic pathway of fatty acid biosynthesis (FAB). In order to achieve this, 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 FAB, our goal was to determine if these insulin-mimetics had an effect on the G6PDH or FAS enzyme activity. We also wanted to determine if this effect was comparable to insulin's induction of G6PDH and FAS activity in published studies.

Insulin has been shown to induce the enzyme activity and mRNA levels of G6PDH in hepatocytes. An increase of 2 to 4-fold in G6PDH activity in the presence of insulin in isolated rat hepatocytes was first reported by Nakamura et al (1982) and Spence (1982). In other studies, insulin exposure to isolated hepatocytes resulted in an increase in G6PDH activity that was due to a comparable increase in specific mRNA (Manos, 1987; Yoshimoto, 1983). This increase in activity was shown to be the result of increased G6PDH protein and mRNA synthesis and indicated an effect of insulin on G6PDH transcription (Kletzien, 1986; Manos et al, 1991). In order to match these published results, in our experiments insulin was present in media at a concentration (16 pM) which was considered in excess of the amount needed for maximum G6PDH induction. As illustrated in figures 6 and 8, we were consistently able to demonstrate a significant insulin induction in our cell system, isolated rat hepatocytes, of approximately 2.5-fold. This data is within the range of levels reported.

Fatty acid synthase activity is also reported to be influenced by the presence of insulin. In isolated rat hepatocytes, Spence and Pitot (1982) have shown that insulin increases the enzyme activity of FAS 2-3 fold. This stimulation of activity apparently is a long term event (Giffhorn-Katz and Katz, 1986). Spence (1982) extended this work by showing 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, was able to show increases in fatty acid synthase activity of approximately 1.7-fold in isolated rat hepatocytes exposed to insulin (figure 7 and 9). These results are comparable to published studies.

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 affecting glucose output, and increasing insulin binding and sensitivity (Miralpeix, 1989; Bruck et al, 1991; Ericksson, 1992). Sodium vanadate also has been shown to stimulate glucose oxidation and transport in rat adipocytes (Tolman et al, 1979). As demonstrated in figures 6 and 7, we were able to show that sodium vanadate induces the enzyme activity of both G6PDH and FAS. Increases in the activity of both G6PDH and FAS resulting from sodium vanadate exposure are comparable to the the increases we observed with insulin. This indicates that sodium vanadate acts in an insulin-like manner in increasing G6PDH and FAS activity. Preliminarily, sodium vanadate has been shown to influence mRNA levels in a similar manner (data not shown) indicating that sodium vanadate may have an effect at a pre-translational level. Insulin and sodium vanadate do not act in an additive manner, suggesting they could be acting on

increasing the activity of these enzymes by s similar mechanism. Insulin acts through a system of highly regulated proteins called a signal transduction pathway (Kahn et al, 1993). Proteins in this system are often regulated by the phosphorylation of certain amino acids, one of which is tyrosine. Since sodium vanadate has been shown to stimulate tyrosine phosphorylation by acting as a phosphatase inhibitor (Zick and Sagi-Eisenberg, 1990), it is possible that sodium vanadate is somehow stimulating the insulin or some other transduction pathway, via a tyrosyl phosphorylation event. More experiments, however, are needed to determine the validity of this hypothesis.

Sodium selenate has also been shown to exhibit insulin-like properties in several cell types. In adipocytes, sodium selenate showed increases in glucose transport and tyrosyl phosphorylation (Ezaki, 1990). In A431 cells, sodium selenate has also shown stimulation in tyrosyl phosphorylation. There has been little, if any, published work to indicate that sodium selenate affects any enzyme activity or mRNA levels. In hepatocytes, we have been able to show insulin-like properties of sodium selenate with respect to the enzyme activities of G6PDH and FAS (figure 8 and 9). Both G6PDH and FAS activities increased approximately 2 to 3-fold which is comparable to levels of insulin induction reported in the literature and reproduced in this study. This indicates that sodium selenate could be an insulin-mimetic in this regard. Sodium selenate also seems to stimulate the mRNA levels for G6PDH and FAS in a similar manner (data not shown). Again, it is possible that there is a correlation between a possible mechanism involving tyrosyl phosphorylation as shown in other cells and the induction of G6PDH and FAS enzyme activity in this study. More experiments need to be performed to examine this possibility.

Many investigations have been done to determine the influence of insulin on the enzyme activity and mRNA levels of G6PDH and FAS *in vivo*. A study done by

Kletzien et al (1989) correlates a high carbohydrate diet to increased levels of G6PDH activity in hepatic tissue. It has also been demonstrated in diabetic animals that hepatic levels of G6PDH enzyme activity and mRNA were decreased and treatment with insulin restored these levels to normal (Katsurada, 1990). Adipose tissue also shows increases in G6PDH activity and mRNA upon treatment with insulin in several studies (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 activity and mRNA in hepatic tissue. Sul (1989) demonstrated insulin effectively increases FAS activity and mRNA levels in diabetic mouse livers.

Sodium vanadate has been reported to demonstrate 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 activity and mRNA levels of some enzymes (Shechter, 1990; Pugazhenthi and Khandelwal, 1990; Rosetti, 1990; Saxena, 1992; Sachor, 1992; Tolman et al, 1979). Sodium selenate, while not having been 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 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, food and water was given ad libitum. As a result, relatively high levels of enzyme activity were maintained thus producing the largest possible effect on G6PDH and FAS activity in diabetic animals compared to normal control animals. 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 increasing weight gain, decreasing food and water intake, lowering plasma glucose levels, and affecting the enzyme activity of G6PDH and FAS in the hepatic tissue of streptozotocin-induced diabetic rats. Both sodium vanadate and sodium selenate treatment yielded similar metabolic trends as treatment with insulin. Sodium vanadate seemed to be the best mimetic with regard to normalization of the metabolic parameters of water and food intake, weight gain, and plasma glucose as well as showing greater increases in enzyme activities. This seems likely due to mode of delivery. Sodium vanadate was present in drinking water and was constantly being ingested while sodium selenate was given as a single injection once a day. Preliminarily, it appears that mRNA levels are similar to the enzyme activity results (data not shown) indicating that both agents may affect transcription and/or translation.

It should be noted that glucose levels and enzyme activities of none of the treated animals reached non-diabetic control levels; this also includes the animals treated with insulin. There could be several plausible explanations for this phenomenon. Among these are variation in the effectiveness of treatment delivery, variation in subjects responsiveness to treatment, a need for tighter control of feeding and sampling schedule, or subjects needing to be treated for a longer period of time. More experiments need to be performed 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 elucidate 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 enzyme activities of glucose-6-phosphate dehydrogenase and fatty acid synthase in a manner similar to insulin in isolated rat hepatocytes. Additionally, we showed that sodium vanadate and sodium selenate can increase weight, decrease food and water intake, and lower plasma glucose levels in streptozotocin-induced diabetic rats. Furthermore, we were able to show the ability of these metals to affect the enzyme activity 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-like with respect to affecting some of the metabolic parameters associated with diabetes as well as the activity of two enzymes, G6PDH and FAS, that are involved in fatty acid biosynthesis.

CONCLUSIONS AND RECOMMENDATIONS

In this study we were able to: (a) demonstrate insulin-like effects of sodium selenate with respect to the enzyme activities of glucose-6-phosphate dehydrogenase and fatty acid synthase in primary rat hepatocytes; (b) demonstrate insulin-like effects of sodium vanadate with respect to the enzyme activities of glucose-6-phosphate dehydrogenase and fatty acid synthase in primary rat hepatocytes; (c) demonstrate insulin-like effects of sodium selenate with respect to the enzyme activities of glucose-6-phosphate dehydrogenase and fatty acid synthase in a streptozotocin-induced diabetic rat model; (d) demonstrate insulin-like effects of sodium vanadate with respect to the enzyme activities of glucose-6-phosphate dehydrogenase and fatty acid synthase in a streptozotocin-induced diabetic rat model.

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

Appendix A

Institutional Animal Care and Use Committee Protocol Approval

WESTERN MICHIGAN UNIVERSITY
INVESTIGATOR IACUC CERTIFICATE

Title of Project: Regulation of Gene Expression by Insulin-Mimetics

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:

III * Injected animal maintained more than 4 hours will be injected through a sterile site

** Examination results in death

IV G Euthanization with be performed by CO2 exposure followed by opening pneumothorax.

Leonard C. Gearing — IACUC Chairperson — 5-13-94 Date

Acceptance of Provisions

Susan O. Stapleton — Signature: Principal Investigator/Instructor — 5/16/94 Date

IACUC Chairperson Final Approval — 5/16/94 Date

Approved IACUC Number 94-04-02

Rev. 3/92

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